## HELEN L. HENRY

University of California, Riverside

HELEN L. HENRY received her Ph.D. in 1970 from Washington University, St. Louis, and did postdoctoral work in animal reproduction at Ohio State University. Following further postdoctoral work at the University of California, Riverside, she joined the faculty and is currently a professor of biochemistry. From 1990 to 1996, she served as Associate Dean of Biological Sciences in the College of Natural and Agricultural Sciences.

Dr. Henry's laboratory has made major contributions to the understanding of vitamin D metabolism, particularly regulation of the production of the active vitamin D hormone by the kidney. She pioneered the use of cell culture systems to study renal vitamin D metabolism. A related area of research focus is the regulation of gene expression in the kidney by 1,25-dihydroxyvitamin $D_3$. In addition to peer-reviewed research articles, Dr. Henry has authored chapters for the books *Vitamin D* and *Handbook of Physiology*, as well as the article "Vitamin D Metabolism" for this encyclopedia.

Dr. Henry was awarded a National Institutes of Health (NIH) Research Career Development Award in 1977. She received the Fuller Albright Award from the American Society for Bone and Mineral Research in 1984 and has served this society as a member of the council and on several scientific program committees. Dr. Henry has been a member of the NIH General Medicine B Study Section (1989–1993) and the National Science Foundation Panel on Integrative Biology (1994–1996). She has served on the editorial boards of several scientific journals, including *Endocrinology*, *American Journal of Physiology*, and *Journal of Bone and Mineral Research*. She is currently a member of the Endocrine Society, the American Society of Biochemistry and Molecular Biology, the American Society for Bone and Mineral Research, and the American Institute of Nutritional Sciences.

## ANTHONY W. NORMAN

University of California, Riverside

ANTHONY W. NORMAN received an A.B. from Oberlin College in 1959, and an M.S. and Ph.D. in biochemistry in 1961 and 1963, respectively, from the University of Wisconsin, Madison. Following postdoctoral work in Paul D. Boyer's group at UCLA, in 1964 he joined the Department of Biochemistry at the University of California, Riverside, as an assistant professor. From 1976 to 1981, he served as chair of the department and currently holds a Presidential Chair and is a Distinguished Professor of Biochemistry and Biomedical Sciences. Dr. Norman has also been active for some 25 years in medical education on the UC Riverside campus and at UCLA through participation in the UC Riverside/UCLA Program in Biomedical Sciences, of which he was Dean and Director from 1986 to 1991.

Dr. Norman's biomedical research career has focused on the mechanism of action of the vitamin D family of steroids. His chief contributions to these areas of cellular and molecular endocrinology have played a pivotal role in defining the boundaries of this research domain via discoveries that have opened new areas of investigation. The first of these was the discovery in 1968, and chemical characterization in 1971, of the hormonally active form of vitamin D, $1\alpha,25(OH)_2$-vitamin $D_3$. Subsequent achievements include the discovery and characterization of the nuclear receptor for $1\alpha,25(OH)_2D_3$, the clinical evaluation of $1\alpha,25(OH)_2D_3$ in renal osteodystrophy, articulation of the concept of the vitamin D endocrine system, the importance of $1\alpha,25(OH)_2D_3$ to insulin secretion, and the discovery of a new rapid, nongenomic, signal transduction process for $1\alpha,25(OH)_2D_3$.

Dr. Norman has been the recipient of awards that include a Fulbright Fellowship, 1970; Public Health Service Career Development Award, 1970; Mead Johnson Award, American Institute of Nutrition, 1977; Ernst Oppenheimer Award, Endocrine Society, 1977; Visiting Lecturer, Australian Society of Endocrinology, 1978; Visiting Faculty Member, Mayo Clinic, 1981; Prix Andre Lichtwitz (INSERM, Paris, France), 1981; Faculty Research Lecturer, UC Riverside, 1982; MERIT Award from National Institutes of Health, 1986; David Curnow Plenary Lecturer, Australian Society for Clinical Biochemistry, 1989; Osborne and Mendel Award, American Institute of Nutrition, 1990; Visiting Professor, Catholic University of Leuven, Belgium, 1992; William F. Neuman Award, American Society for Bone and Mineral Research, 1995; Fellow of American Association for the Advancement of Science, 1995; and Visiting Professor, Department of Biochemistry, UC San Francisco/Presidential Chair in Biochemistry, UC Riverside, 1999.

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## ANTONY W. BURGESS

Ludwig Institute for Cancer Research, Melbourne, Australia

ANTONY W. BURGESS has had an interest in protein chemistry since his early studies on the conformational determinants of peptides and proteins. He received a Ph.D. at the University of Melbourne; after postdoctoral studies at Cornell University and the Weizmann Institute between 1972 and 1974, Dr. Burgess returned to Australia, to the Walter and Eliza Hall Institute, to study the growth factors that stimulate blood cell formation. He and his colleagues purified the first colony-stimulating factor (CSF) and discovered growth and differentiation factors controlling the development of blood cells. This work led to the molecular cloning of a CSF and the initial clinical studies with recombinant forms of these factors.

Dr. Burgess was involved in the detection and biology of the cell surface receptors for the CSFs before taking up studies on the epidermal growth factor (EGF)/receptor system. These studies have contributed to the understanding of the three-dimensional structure of EGF and its receptor, as well as the mechanisms associated with signal transduction from the different EGF receptor complexes. At present, Dr. Burgess is attempting to develop improved approaches to cancer therapy through the use of EGF receptor inhibitors. He has an active research program on the molecular basis of colon cancer, in particular the biology and molecular biology of the apc protein.

Presently on staff at the Ludwig Institute for Cancer Research in Melbourne, Dr. Burgess is a committee member of the ARC Selection Committee for Centres of Excellence, a past president of the Australian Society for Biochemistry and Molecular Biology, a former chairperson of the Board of the Biomolecular Research Institute, a former board member of the International Society of Differentiation, and a former World Committee member of the Society for Research into Comparative Leukemic and Associated Diseases. He has been awarded honors that include the Australian Academy of Science Gottschalk Medal, 1981; Australian Academy of Science Fellow, 1993; the Amgen Prize, 1996; Companion of the Order of Australia, 1998; and the MOG/AMRAD Cancer Achievement Award, 1999.

## P. MICHAEL CONN

Oregon National Primate Research Center,
Beaverton, Oregon

P. MICHAEL CONN is Associate Director and Senior Scientist of the Oregon National Primate Research Center and Special Assistant to the President and Professor of Physiology and Pharmacology at Oregon Health and Science University. After receiving a B.S. and teaching certification from the University of Michigan in 1971, an M.S. from North Carolina State University in 1973, and a Ph.D. from Baylor College of Medicine in 1976, Dr. Conn did a fellowship at the NIH/National Institute of Child Health and Human Development. He then joined the faculty in the Department of Pharmacology at Duke University Medical Center, where he was promoted to Associate Professor in 1982. In 1984, he became Professor and Head of Pharmacology at University of Iowa College of Medicine, a position he held for 11 years.

Dr. Conn is presently Editor-in-Chief of *Endocrine, Methods*, *Contemporary Endocrinology,* and *Contemporary Drug Therapy*; prior Editor-in-Chief of *Endocrinology*, *Molecular and Cellular Neurosciences*, *Methods in Neuroscience,* and *Recent Progress in Hormone Research*; and prior Editor of *Journal of Clinical Endocrinology and Metabolism*. He has edited texts in the fields of pharmacology (*Essentials of Pharmacology*), neuroscience (*Neuroscience in Medicine*), neuroendocrinology (*Neuroendocrinology in Physiology and Medicine*), endocrinology (*Endocrinology: Basic and Clinical Principles*), and molecular endocrinology (*Principles of Molecular Regulation),* as well as more than 100 volumes in endocrinology and neuroscience.

Best known for his research in the area of neuroendocrinology, Dr. Conn has focused on the cellular basis of action of gonadotropin-releasing hormone action in the pituitary and central nervous system. He has authored or coauthored nearly 300 publications in this area. The work of his laboratory has been recognized with the MERIT Award from the NIH, the J.J. Abel Award of the American Society for Pharmacology and Experimental Therapeutics, the Weitzman and Oppenheimer awards of the Endocrine Society, the National Science Medal of Mexico (the Miguel Aleman Prize), and the Stevenson Award of Canada. Dr. Conn has served on the National Board of Medical Examiners, including two years as

Chairman of the Reproduction and Endocrinology Committee, and is a previous member of council for the American Society for Cell Biology and a past president of the Endocrine Society. Conn is a member of the Mexican Institute of Medicine and an honorary investigator of the Mexican Institute of Social Security.

## GEORGE H. GREELEY, JR.

University of Texas Medical Branch, Galveston, Texas

GEORGE H. GREELEY, Jr. earned his Ph.D. in endocrinology at the Medical College of Georgia in 1974 and was recently recognized by the school as alumni of the year. Dr. Greeley did postdoctoral training in neuroendocrinology at the University of North Carolina in Chapel Hill. Presently, he directs an internationally recognized research program in physiology of gastrointestinal hormones in the Department of Surgery at the University of Texas Medical Branch in Galveston.

Dr. Greeley has authored or coauthored more than 190 peer-reviewed journal articles and serves on the editorial boards of *Endocrinology*, *American Journal of Physiology*, and *Regulatory Peptides*. His primary areas of research include feedback mechanisms underlying regulation of gut hormone secretion, a new stomach hormone called ghrelin, and luminal regulation of cholecystokinin (CCK) secretion. Honors awarded Dr. Greeley include the NATO Collaborative Research Grant, 1989 to present; Invited Participant, Second Galveston International Symposium, Galveston, Texas, 1989; Invited Participant, International Conference of Gut Hormones, Shizuoka, Japan, 1993; Mentor in the APS–NIDDK Travel Fellowship Program for minority students, Experimental Biology Meetings, 1995, 1996; and Mentor, American Gastroenterological Association, Endocrine Society, Summer Student Research Fellowship Recipients, 1995–1998.

## MARTIN J. KELLY

Oregon Health and Science University, Portland, Oregon

MARTIN J. KELLY is a professor of physiology and pharmacology at Oregon Health and Sciences University (OHSU). He obtained his Ph.D. in 1976 from the University of Texas Southwestern Medical School in Dallas and did his postdoctoral training at the Max Planck Institute for Biophysical Chemistry (1976–1979). In 1980, Dr. Kelly joined the Department of Physiology at the University of Pittsburgh School of Medicine and moved to OHSU in 1982. His area of research is the cellular neurophysiology and neuropharmacology of hypothalamic neurons that control neuroendocrine functions, motivation, and reward in the female. Dr. Kelly has been the recipient of two research scientist development awards from the NIH, the Research Career Development Award from the National Institute of Child Health and Human Development (1987–1992), and the Research Scientist Development Award from the National Institute on Drug Abuse (1994–1999).

Dr. Kelly was the first to demonstrate rapid signaling of estrogen in the central nervous system and to show how it alters reproductive function in the female. In particular, he found that 17β-estradiol can directly alter the excitability of gonadotropin hormone-releasing hormone (GnRH) neurons, which are critical for the control of female reproduction. He also found that the rapid activation by estrogens of protein kinase activity in hypothalamic opioid and dopamine neurons alters the coupling of neurotransmitter receptors to their effector systems (e.g., channels) in the female. These results have significant ramifications in terms of stress responses, appetite control, fluid balance, and motivated behaviors, and may explain many of the gender differences in these functions.

Dr. Kelly and colleagues are also interested in characterizing the membrane properties of proopiomelanocortin (POMC), dopamine, and GnRH neurons in males and females, and the effects of neurotransmitters on these neurons. Using guinea pigs and transgenic mice as models, they are trying to identify the phosphorylated target proteins that are altered by neurotransmitters and steroids in hypothalamic neurons that are critical for the control of neuroendocrine functions, motivation, and reward in the female. Dr. Kelly's recent manuscripts include "Rapid Actions of Plasma Membrane Estrogen Receptors" with E. R. Levin in *Trends in Endocrinology and Metabolism* and "Rapid Membrane Effects of Estrogen in the CNS" with O. K. Rønnekleiv in the Academic Press book *Hormones, Brain and Behavior.*

## PAUL B. LARSEN

University of California, Riverside

PAUL B. LARSEN received his Ph.D. from Purdue University in 1994, did postdoctoral work at Cornell University (1994–1997) and at the University of

Maryland (1997–2000), where he was also a U.S. Department of Agriculture Fellow. Dr. Larsen is presently an assistant professor of biochemistry in the College of Natural and Agricultural Sciences at the University of California, Riverside.

Dr. Larsen's laboratory focuses on two topics of plant biology. The first deals with the elucidation of the mechanism responsible for the signal transduction of ethylene, a plant hormone that regulates many physiological processes throughout plant growth and developement. Using *Arabidopsis thaliana* as a model genetic system, he has been responsible for identification of novel components of the ethylene signaling pathway, an approach that may ultimately give the means to better control such ethylene-regulated processes as fruit ripening, tissue senescence, and induction of pathogen defenses. Additionally, Dr. Larsen's group is exploring the mechanisms that plants utilize to cope with abiotic stress, particularly that of aluminum toxicity in acid soil. Aluminum toxicity in acid soils is a global problem limiting crop productivity for more than 30% of agriculturally available land. As for ethylene signaling, Dr. Larsen is also using *Arabidopsis* as a model system for identification of genes that are required for plant growth in aluminum toxic environments.

Dr. Larsen has presented papers in his areas of expertise at prestigious seminars at Cambridge University and at the American Society of Plant Physiologists and has authored or coauthored a number of articles published in scientific journals such as *Plant Physiology*, *Plant Molecular Biology*, and the *Proceedings of the National Academy of Science*. He also authored the article, "Ethylene" in this *Encyclopedia of Hormones*.

## WARREN J. LEONARD

National Institutes of Health, Bethesda, Maryland

WARREN J. LEONARD is Chief, Laboratory of Molecular Immunology, at the National Heart, Lung, and Blood Institute of the National Institutes of Health. Dr. Leonard was a pioneer in the interleukin-2 field, having cloned the IL-2 receptor $\alpha$ chain in 1983. Dr. Leonard has published more than 200 articles and reviews, most of which are related to the IL-2 family of cytokines. His main research focus relates to IL-2 and other cytokines with receptors that contain the common cytokine receptor $\gamma$ chain, $\gamma c$, which Dr. Leonard's group demonstrated is the protein that is mutated in X-linked severe combined immunodeficiency. He demonstrated that this is a disease of defective cytokine signaling and that mutations in the Janus family tyrosine kinase, Jak3, which associates with $\gamma c$, cause a similar clinical phenotype. Most of his research focuses on signal transduction and gene regulation related to $\gamma c$-dependent cytokines.

Dr. Leonard is currently Vice President and President-elect of the International Cytokine Society, a member of the American Association of Immunologists, American Society for Clinical Investigation, and the American Association of Physicians, and a fellow of the American Association for the Advancement of Science. He is a current or past member of major editorial boards, including *Immunity*, *Journal of Immunology*, *Journal of Biological Chemistry*, and *Cytokine*. He has helped organize major international meetings in the cytokine field, including a Keystone Symposium and an annual meeting of the International Cytokine Society. Dr. Leonard has received a number of major awards, including the Outstanding Investigator Award of the American Federation for Clinical Research Foundation. (Note that Dr. Leonard's contributions to this book were performed in his private capacity, and the contents of this book do not necessarily reflect the views of NIH.)

## GERALD LITWACK

Thomas Jefferson University College of Medicine, Philadelphia, Pennsylvania

GERALD LITWACK obtained his Ph.D. in biochemistry from the University of Wisconsin in 1953. After a postdoctoral year at the Biochemical Laboratories of the Sorbonne in Paris, he spent the early part of his academic career at Rutgers University and the University of Pennsylvania, where he was the recipient of a National Institutes of Health Career Development award. In 1964, Dr. Litwack became Professor of Biochemistry at the Fels Institute for Cancer Research and Molecular Biology at Temple University School of Medicine. In the 1980s, he became Deputy Director of the Institute and Laura H. Carnell Professor of Biochemistry and received the Faculty Research Award of Temple University.

In 1991, Dr. Litwack moved to the Jefferson Medical College as Chairman of the Department of Pharmacology and Deputy Director of the Jefferson Cancer Institute (now the Kimmel Cancer Institute), an appointment he continued when, in 1996, he became Chair of the newly fused Department of Biochemistry and Molecular Pharmacology and Associate Dean for Scientific Affairs. In 2000, he was appointed Vice Dean for Research.

Dr. Litwack's research has centered on regulation, particularly by hormones, and, during the past 15 years, the mechanism and regulation of apoptosis. The Litwack laboratory group characterized the mammalian glucocorticoid receptor and co-discovered "ligandin," subsequently found to be the glutathione *S*-transferase family of enzymes. Dr. Litwack has published more than 300 papers in these areas and is a co-discoverer on several patents covering many of the caspases in the apoptotic cascade. His service includes participation on scientific advisory boards (the Diabetes Center of the University of Pennsylvania) and on grant review panels of the National Science Foundation, National Institutes of Health, U.S. Army, Israel Cancer Research Fund, and American Cancer Society. His editorial board service includes *Endocrinology*, *Cancer Research*, *Anticancer Research*, *Oncology Research*, *Proceedings of Experimental Biology and Medicine*, *Journal of Nutrition, Growth and Cancer*, *ISI Atlas of Science*, *Cancer Communications*, *Chemtracts*, *Oncology Reports*, *Critical Reviews in Eukaryotic Gene Expression,* and *Apoptosis*.

The books and publications Dr. Litwack has authored, coauthored, or edited include *Experimental Biochemistry* (1960, John Wiley & Sons), *Biochemical Actions of Hormones* (1970–1987, Academic Press, a serial in 14 volumes), *Actions of Hormones on Molecular Processes* (1964, Academic Press), *Receptor Purification* (1989–1990, Humana Press), *Receptor* (renamed *Receptors & Signal Transduction*; 1990–1998, a journal founded by G. Litwack and Editor-in-Chief, Humana Press), *Hormones* (1987, 1997, Academic Press), and *Vitamins and Hormones* (Academic Press, the publisher's longest running serial).

## Alexander S. Raikhel

University of California, Riverside

Alexander S. Raikhel is Professor at the University of California, Riverside. He received his Ph.D. in 1975 from the Zoological Institute of the Academy of Science in St. Petersburg, Russia. Dr. Raikhel is the leading authority in molecular endocrinology of insects. His research focuses on endocrine control of insect reproduction. In particular, he and his collaborators have elucidated a complex network of nuclear receptors that mediate the action of a steroid hormone ecdysone in gene activation and repression during egg maturation in mosquitoes.

Dr. Raikhel is the author of more than 100 research papers and reviews on molecular aspects of insect reproduction. He has served as a member of the World Health Organization Advisory Committee and has organized several symposia on the topic of insect reproduction and endocrinology, including several Keystone Symposia in Molecular Insect Science. He is the recipient of numerous awards for his research achievements. Dr. Raikhel is Editor-in-Chief of *Insect Biochemistry and Molecular Biology* and is a member of the editorial board of *Annual Reviews of Entomology.*

## R. Paul Robertson

University of Washington, Seattle, Washington

R. Paul Robertson, M.D. is President, Scientific Director, and CEO of the Pacific Northwest Research Institute. He became Professor of Medicine and Pharmacology at the University of Washington in 1980, where he received most of his postgraduate training. He has been elected into membership of the American Society for Clinical Investigation and the Association of American Physicians.

Dr. Robertson has been Editor-in-Chief of *Diabetes*, the research journal of the American Diabetes Association, and is currently on the editorial board of the *Journal of Biological Chemistry.* Honors received by Dr. Robertson include the endowed Pennock Chair for Diabetes Research at the University of Minnesota, Banting and Best Lecturer at the Joslin Clinic, the Moses Barron Award of the Minnesota Affiliate of the American Diabetes Association, and the Albert Renold Award of the American Diabetes Association. He has published more than 260 manuscripts with primary emphasis on beta cell function in humans, animals, and clonal cell lines. His most recent research activities are centered on studies of glucose toxicity of the beta cell as seen in type 2 diabetic patients. He is also very active in studies of the metabolic consequences of successful pancreas and islet transplantation in patients with type 1 diabetes mellitus.

## Charles Eugene Roselli

Oregon Health and Science University, Portland, Oregon

Charles Eugene Roselli is a professor in the Department of Physiology and Pharmacology at the Oregon Health and Science University, where he has been on the faculty since 1985. He received his Ph.D. in 1981 from Hahnemann University in Philadelphia, Pennsylvania, and did his postdoctoral training at the Oregon Regional Primate Research Center (1981–1984). Dr. Roselli's research focuses on the neurobiological activity of androgens. His work has contributed to an integrated understanding of the subcellular signaling pathways, the steroid-sensitive brain circuitry, and the neurochemical mechanisms that are responsible for the behavioral and neuroendocrine actions of androgens.

A major emphasis of Dr. Roselli's research has been directed at the characterization of the aromatase (CYP19, or estrogen synthetase) signaling pathway in neural tissue, characterizing the distribution and regulation of CYP19, and defining its role in neural development, adult reproductive behavior, and gonadotropin secretion. Dr. Roselli and his colleague Dr. Resko were the first to demonstrate that androgens regulate their own efficacy in the mammalian brain through androgen receptor-dependent positive feedback of the aromatization pathway. His research also demonstrated for the first time that gender differences in neural responsiveness to androgens are expressed at the subcellular level through the differential expression of androgen receptors and aromatase. Dr. Roselli and collaborators recently identified a sexually dimorphic preoptic nucleus in the ovine brain and found that the volume of this nucleus correlates with sexual partner preference in rams. They are currently studying the neuroendocrine and neuroanatomical basis of naturally occurring variations in sexual partner preference.

## EVAN R. SIMPSON

Prince Henry's Institute of Medical Research, Clayton, Australia

EVAN R. SIMPSON began his career in 1964 and is presently Director of Prince Henry's Institute of Medical Research of Monash University, Australia. Historically, Dr. Simpson's research has been in three major fields: the regulation of steroid hormone biosynthesis in the adrenal cortex and in the ovary, the role of lipoprotein cholesterol as precursor for steroid hormone biosynthesis, and the study of estrogen biosynthesis, in particular the regulation of biosynthesis of the enzyme aromatase, responsible for the biosynthesis of estrogens. Most recently, Dr. Simpson's research group developed the aromatase knockout mouse as a model of estrogen insufficiency. This has provided insights into the role of estrogens in the physiology and pathophysiology of both males and females, revealing many unexpected and nonsexually dimorphic roles for estrogens unrelated to sexual differentiation or reproduction.

Dr. Simpson has authored more than 300 peer-reviewed articles and some 70 book chapters and nonreviewed publications. He has been a featured speaker at events ranging from closed workshops such as the Wyeth Ayerst sponsored symposium on Frontiers in Estrogen Action to international and national society meetings. Currently, he is Chairman of the International Organizing Committee of the International Congress of Hormonal Steroids and Hormones and Cancer, and until last year was Chairman of the International Aromatase Conference. In 1998, he was Chairman of the Program Organizing Committee of the U.S. Endocrine Society Annual Meeting. He is a member of the council of the Endocrine Society of Australia, Editor-in-Chief of the *Journal of Molecular Endocrinology*, and Associate Editor of *Endocrine Reviews*.

Dr. Simpson's honors and awards include the Transatlantic Medal Lecturer for the U.K. Society for Endocrinology in 1990, and in 2003 he is the society's Asia and Oceania Medal Lecturer; he received the President's Scientific Achievement Award from the Society for Gynecological Investigation (United States). He has been a guest lecturer at several institutions, including the University of Western Ontario, the University of Kansas, and Johns Hopkins University, and at Princess Takematsu's Annual Symposium on Breast Cancer in Tokyo.

## GUIDO VERHOEVEN

Catholic University of Leuven, Leuven, Belgium

GUIDO VERHOEVEN was born in Antwerpen, Belgium, on March 26, 1945. In 1970, he completed medical studies at the Catholic University of Leuven, Belgium, and in 1974 obtained a Ph.D. from the same institution. He is a Registered Specialist in Clinical Chemistry with authorization for nuclear medicine *in vitro*. In 1974, he received a Biomedical Fellowship of the Population Council in New York and worked for one year in the laboratory of Professor Jean D. Wilson at the University of Texas Southwestern Medical School in Dallas on the problem of androgen insensitivity syndromes. Professor Verhoeven received several postdoctoral fellowships from the National Fund for Scientific Research from Belgium and became Full Professor at the Catholic University of Leuven in 1986, where he teaches pathophysiology and general medicine both at the Medical School and at the School for Pharmacy.

Professor Verhoeven has been Chairman of the Department of Developmental Biology for 14 years. He is Full Member of the Belgian Royal Academy of Medicine and has served in a variety of capacities within the scientific community. These include Secretary of the Belgian Contact Group on Steroid Hormones and Secretary and Vice President of the Belgian Society for Endocrinology. He is a member of the Endocrine Society, an academician of the European Academy of Andrology, and a member of the Permanent Scientific Committee of the European Testis Workshops. In 1984, he organized the 8th European Workshop on the molecular and cellular endocrinology of the testis in De Panne, Belgium. Professor Verhoeven's main research interests are cell–cell interactions and androgen action in the testis, and androgens and the control of proliferation and differentiation in the normal prostate and in prostate tumor cells. In recent years, his research has increasingly focused on prostate cancer. Professor Verhoeven has published nearly 200 papers in international peer-reviewed journals.

## NANCY L. WEIGEL

Baylor College of Medicine, Houston, Texas

NANCY L. WEIGEL earned undergraduate degrees at Cornell University and a Ph.D. from Johns Hopkins University. Dr. Weigel was named a National Institutes of Health Postdoctoral Fellow (1979–1981) and a Searle Scholar (1983–1986). Following postdoctoral work in steroid receptors, she joined the faculty of Baylor College of Medicine, where she is presently a professor in the Department of Molecular and Cellular Biology.

Dr. Weigel is engaged in a variety of research projects, including the regulation of human steroid receptors by phosphorylation, the role of androgen receptors in prostate cancer, and the effects of vitamin D on both bone loss and prostate cancer. She has authored or coauthored more than 100 scientific journal publications. Her manuscripts are also included in a number of books: *Gene Regulation by Steroid Hormones* (1982, Springer-Verlag), *Mechanisms of Steroid Action* (1981, Macmillan Press Ltd.), *Methods in Enzymology* (1982, Academic Press), *Steroid Hormone Receptors, Structure and Function* (1983, Elsevier), *Laboratory Methods Manual for Hormone Action and Molecular Endocrinology*, (1989, Houston Biological Associates), *Receptor Purification* (1990, Humana Press), *Endocrinology*, 4th Edition (2000, Saunders Company), and *Encyclopedia of Molecular Medicine* (2002, Wiley and Sons).

Dr. Weigel has served on a number of review panels and editorial boards and has participated in many invited lectures around the United States and Europe, most recently for the fifth consecutive year at Frontiers in Reproduction in Woods Hole, Massachusetts. She has chaired the American Cancer Society Tumor Biochemistry and Endocrinology Study Section, is an editorial board member for *Steroids*, *Endocrinology*, and *Journal of Biological Chemistry*, and is a member of the Endocrine Society, the American Association for Cancer Research, Women in Cancer Research, the American Society for Bone and Mineral Research, the American Society for Biochemistry and Molecular Biology, the American Association for the Advancement of Science, and Women in Endocrinology, serving as Secretary–Treasurer from 1998 until 2001.

## ROY E. WEISS

University of Chicago, Chicago, Illinois

ROY E. WEISS is Professor of Medicine at the University of Chicago and is Associate Director of the Clinical Research Center. Since the mid-1980s, Dr. Weiss has been involved in efforts to understand the molecular basis of thyroid hormone action. As an active clinician, educator, and molecular biologist, he has studied the clinical and physiological abnormalities in patients with the syndrome of resistance to thyroid hormone, to understand mutant thyroid hormone receptor isoform and cofactor interaction. Dr. Weiss was the first to demonstrate that this syndrome could be diagnosed at birth and the effect of treatment on outcome. Dr. Weiss has also demonstrated the importance of nuclear coactivators in thyroid hormone action *in vivo*. Currently, he is working on understanding the basis for resistance to thyroid hormone in patients with normal thyroid hormone receptors.

# FOREWORD

The discipline of endocrinology was born with the discovery of hormones, but the concept of endocrinology has been substantially expanded by the more recent discovery of paracrine and autocrine regulators. The field of hormone action was formed to understand the molecular mechanisms by which hormones act in cells, and continues to expand explosively. In the late 1960s, the prevailing view of hormone action ranged from effects on membrane transport of nutrients and precursors for RNA and protein synthesis to effects on the translation of mRNA at the level of ribosomes. Nevertheless, a cadre of voices predicted a possible nuclear action on mRNA synthesis. These voices were correct in that steroid hormones, acting via their receptors, indeed were proved to regulate gene transcription. To the best of my knowledge, the first paper to be presented at the national endocrine meetings in a new field of hormone action was in 1967, and it dealt with hormonal stimulation of oviductal protein synthesis. It was about this time that a small group of scientists interested in hormone effects in cells attended a Gordon Conference in New Hampshire; this was one of the first conferences to focus an entire program on hormone action and mechanisms. The attendees were primarily involved in aspects of steroid hormone and thyroid hormone actions; peptide hormone action was yet to experience its own birth and a similar expansive growth. Only a short time previously, Elwood Jensen had discovered the estrogen-binding protein that eventually became the "estrogen receptor," thus it was logical that the conference dealt mainly with steroid receptors; there were also a few papers presenting data that steroid hormones could induce specific enzyme/protein synthesis in target cells. The mechanisms of these effects were the subject of great debate at this first conference on steroid hormone action, a meeting that persists to this day in New England each summer.

Following the monumental discovery of peptide immunoassays, workers in the peptide field were immersed in the work of measuring hormones, ranging from insulin, to luteinizing hormone, to follicle-stimulating hormone, to thrytropin-releasing hormone, to growth hormone, to name a few. For over a decade, little attention was given to the more difficult task of understanding the functions of their receptors and intracellular signaling pathways. Nevertheless, the advent of this assay methodology, including the ability to synthesize radiolabeled peptide hormones, eventually allowed the identification and quantification of cell surface receptors for peptide and amine-containing hormones. The time of this application was about 1970. Researchers demonstrated that cAMP levels were induced in concert with ligand occupation of certain membrane receptors, and the second messenger cAMP was postulated to initiate intracellular phosphorylation of unknown targets. At this point, the field of peptide hormone action also was born.

The two distinct but related fields, steroid hormone action and peptide hormone action, developed together for much of the next decade. Hormone action conferences invariably contained talks on both types of receptors and progress was rapid and in concert with the development of molecular biology. In the steroid field, progress was more rapid initially, but by the mid 1980s, the peptide field attained equal mechanistic status.

Investigators of steroid hormone action concentrated on first understanding the "pathway of action" for their hormones. Scientists looked for model systems showing large responses to steroids. One approach was to assess changes in enzyme levels in cultured cells. The intact chicken oviduct was another of the more notable systems, in that regard because of the ability of sex steroids (estrogen, progesterone) to induce large increases both in certain egg-white proteins and in their respective mRNAs. Viral proteins also were shown to be induced by glucocorticoids. The finding that purified steroid receptors could bind to DNA directly led to a new understanding of the pathway of steroid, to intracellular receptor, to DNA, to mRNA, to protein, and to function. Still, many complexities remained to be sorted out when the receptor cDNAs were cloned in the 1980s.

Our concept of hormones expanded considerably with the advent of growth factors and cytokines. Arguably, the myriads of growth factors only represent an additional list of peptidelike hormones that often act within the tissue of their origin; they have a strong predilection for growth and cell cycle control. Cytokines have both local and distal actions and are particularly oriented to processes such as smooth muscle function and inflammation and apoptosis.

For a decade, it seems as if the peptide action researchers were unduly fixated on cAMP induction and protein kinase A activation. The complexity of signaling pathways emanating from membrane receptors increased logarithmically with the discovery of the numerous protein kinases that phosphorylated serine and tyrosine, the kinase-kinases, the phosphatases, the calcium and diacylgycerol regulators, and the regulators of all of the phosphorylation intermediates. The types of receptors that proliferated ranged from seven membrane (protein kinase A), growth factors (tyrosine kinase, protein kinase C), cytokine, and eventually even chemokine in nature. The appreciation of G-proteins as upstream targets of the cAMP pathway was key to eventual solutions of the signaling cascade. The discoveries of the ras/raf pathway for mediating the effects of mitogens, and the JAK/Stat pathway as mediator for cytokines and certain peptide hormone effects, were also important milestones in unraveling signal transduction in eukaryotic cells. The realization that CREB and Stat proteins were regulatable transcription factors that eventually acted on DNA united the steroid–peptide fields, in part, at the level of the nucleus. That order was made out of apparent chaos is a striking tribute to the intellectual prowess and perseverance of the workers in this field. Most importantly, the signaling pathways emanating from the membrane have brought new insights to pathologies such as cancer, and have led to an explosive development of new pharmaceutical stimulators and inhibitors, with good promise for therapies of neuropsychiatric disorders, cancer, and other human disorders.

After a consolidation period in the 1970s, the steroid action field heated up again with the cloning of the steroid, thyroid, vitamin D, and retinoic receptors. Certain unpredictable events occurred. Investigators began to clone (by cross-screening) numerous molecules that were similar to steroid receptors, but that were not known to be activated by an existing ligand. The term “orphan receptors” was born and the deduction was made that the steroid receptors were part of a giant superfamily of nuclear receptor transcription factors, numbering 48 in humans. The availability of cloned cDNAs and other reagents allowed mutational analyses of receptors, followed by their reintroduction into cells to monitor effects on synthetic reporter genes; structure–function relationships proved the existence of receptor domains for transcriptional activation, nuclear translocation, and DNA binding. Now at a frenetic pace, information on dimeric DNA binding and heterodimeric partners (retinoic acid X receptor), receptor crosstalk with peptide pathways, ligand-independent receptor activation, and receptor phosphorylation was accumulated. More definitive appreciation of the biology of classical and orphan receptors was accomplished by the emerging transgenic technologies and gene knockout strategies. The ability to screen for new ligands for orphan receptors extended the range of hormones to lipids (peroxisome proliferator-activated receptors) and other previously unsuspected metabolic regulators. The yearly stream of publications on the physiology of orphan receptors and their novel ligands continued to bring excitement and more expansion to the field. Pharmaceutical companies salivated at the possibilities of new drugs acting at the nuclear level. The tamoxifen paradox (it acts in one tissue as an agonist and in another as an antagonist) provided encouragement for the generation of a successful search for selective receptor modulators that contain tissue- and function-specific profiles.

Still, the field clamored for a greater molecular understanding of how the nuclear receptors worked at the level of DNA. The discovery of receptor-associated regulatory proteins provided this missing link and changed the field of hormone action further. We moved from a situation wherein many believed that intracellular receptors carried out the transcriptional regulation inherent to the actions of steroid/thyroid hormones and vitamins, to an understanding that the receptor-associated “coregulators” are the primary mediators of this genetic response. We now know that the receptor co-activators act as powerful transducers of hormone action, either through inherent enzyme activities or by serving as a scaffold for recruitment of additional co-activator proteins. The coregulators can be divided loosely into two camps: co-activators and co-repressors. Taken together, these molecules mediate the two main tasks of receptors: stimulation and repression of gene expression.

It is perhaps fitting that a burst of recent attention has focused again on the membrane, where both steroids and their receptors have been postulated to have biologically important effects. Actions of nuclear receptors, and also of free hormones binding to traditional membrane receptors or ion channels, are likely to be of increasing future interest to workers investigating hormonal function. In this respect, we have traveled full circle. I have no doubt that the future will see many additional examples of membrane reinforcement of nuclear gene regulation, of important ligand-independent activities of receptors initiated at the membrane, and of pathway crosstalk among the varieties of hormones and their intracellular pathways.

These volumes of the *Encyclopedia of Hormones* represent one of the most ambitious projects completed to date in the field of hormones and their actions. The Editors have assembled articles that address a full spectrum of the biology and cellular physiology of numerous hormones and their actions in many species. This effort allows the reader the opportunity to survey the state of both membrane receptor-initiated signaling and nuclear receptor-initiated signaling from the viewpoints of a wide variety of leading investigators. The history and breadth of this field are evident within the articles of these volumes. The ambitious project will stand as a major reference source for the field, and I predict that readers will have no problem savoring the current rapid progress and heightened excitement that exists in this vast field of molecular endocrinology.

BERT W. O'MALLEY
*Thompson Professor and Chair*
*Department of Molecular and Cellular Biology,*
*Baylor College of Medicine Houston, Texas*

# PREFACE

The publication of the *Encyclopedia of Hormones* is intended to provide a comprehensive reference work on all known hormones in vertebrate animals, insects, and plants. The list of classical hormones that had been discovered and characterized over the interval from 1914 to 1985 numbered approximately 55; however, as a consequence of the application of modern chemical characterization techniques and molecular biology methodology, this list now exceeds 150 and is still expanding. In fact, during the production interval for the *Encyclopedia,* several new hormones were discovered and new activities for existing hormones were clearly defined. In addition, enormous strides have been made in our understanding of the detailed actions of hormones at the molecular and cellular levels. There have been dramatic applications of this new knowledge in the medical arena with respect to both diagnosis and treatment of diseases; for the plant and insect hormones, new applications have arisen in the realms of agricultural biotechnology and biological control.

Some comment is appropriate concerning the definition of a hormone that has been utilized in compiling entries in the *Encyclopedia*. Of course, the classical definition of a hormone is that it is a chemical messenger in the body: it is secreted by an endocrine gland and is delivered through the circulatory system to target cells that possess receptors specific for the hormone. Occupancy of the receptor by its cognate hormone leads to the initiation of signal transduction processes that result in generation of specific biological responses. But in this post-human-genome era, and with the rich array of technologies used to study and define at the cellular and molecular levels the enormous array of signal transduction pathways employed by cells, the Editors have adopted a broader definition of hormone. Hormones can now be considered to include not only chemical messengers in the classical sense, but also local paracrine and autocrine signals. Thus, the *Encyclopedia* includes articles on many growth factors, interleukins, and intracellular mediators of signal transduction.

The *Encyclopedia of Hormones* is intended to serve as a useful and comprehensive source of information spanning all aspects of the general subject of hormones. It consists of nearly 300 articles that collectively describe hormones from several key perspectives: (1) the cellular and subcellular sites of functioning of the hormone, (2) the major physiological system(s) in which it is operative (e.g., reproductive, immune, neuroendocrine, digestive, and developmental), (3) the nature of the receptor and signal transduction pathway(s) used by the hormone (e.g., nuclear or membrane signal transduction), and (4) for the vertebrate hormones, the important diseases of deficiency or excess or other instances for which there is unusual molecular insight available. We expect that the *Encyclopedia of Hormones* will be as useful to the scientific expert concerned with cutting-edge questions as it will be to students and interested nonscientists.

Given the broad scope of such a major reference work, it was essential to assemble a team of Associate Editors. Each of these 14 individuals has dedicated his or her professional career to researching scholarly endeavors in a specific domain of hormones and, as a consequence of the breadth and depth of achievement in this area, is an acknowledged leader in their field. These interests include the hormone domains of adrenal cortex, calcium-regulating hormones, cytokines, female reproduction, male reproduction, gastrointestinal hormones, growth factors, thyroid, membrane signal transduction, neuroendocrinology, nuclear signal transduction, pancreas, plant hormones, and insect hormones.

The *Encyclopedia* was launched at a two-day meeting of the Editors, Associate Editors, and Elsevier–Academic Press representatives in La Jolla, California, in April, 2001. Here the preliminary list of article titles prepared by the Editors was refined and a list of potential authors created. Each Associate Editor was then responsible for the crucial process of recruiting authors for the individual entries. As the manuscripts were received by Academic Press, they were critically reviewed by the Associate Editors and Editors, as well as by the editorial staff at Academic Press. The final total of 296 articles entered production in only 16 months.

All of the articles are formatted according to the same blueprint and each is intended to be a self-contained presentation. Each article begins with a brief topical content outline that provides the reader with a listing of the major topics presented in the article. The article body begins with an introductory paragraph that defines the topic under discussion and summarizes the content of the article. Following the article are reference citations to provide the reader with access to further in-depth consideration of the topic at hand and a cross-reference to related entries in the *Encyclopedia*. A glossary list defines key terms that may be unfamiliar to the reader and are important to an understanding of the article. A compilation of all glossary terms appearing in the complete multivolume *Encyclopedia* is presented in the final volume as a dictionary of subject matter relevant to hormones.

If the *Encyclopedia* has merit, it is due largely to the contributions of the authors of all the articles, and as well to the dedication of the Associate Editors. Shortcomings are, of course, the responsibility of the Editors and we would appreciate having them brought to our attention. The completion of this large project in the relatively short time, from launch meeting to the actual printing of the *Encyclopedia* (only 23 months), is the result of much hard work and dedication. Certainly the primary credit must go to the some 500 authors who prepared their contributions in a timely fashion. The board of Associate Editors also provided exceptional leadership and service. The Editors thank them all.

Finally, thanks are due to the staff of Elsevier–Academic Press, including Tari Paschall, Judy Meyer, Chris Morris, and Carolan Gladden, who each provided skillful and friendly ongoing management of the project.

Helen L. Henry
Anthony W. Norman

# A

## Abscisic Acid

Sébastien Mongrand, Peter D. Hare, and
Nam-Hai Chua
*Rockefeller University*

I. INTRODUCTION
II. STRUCTURE AND OCCURRENCE
III. SYNTHESIS
IV. DEGRADATION
V. TRANSPORT
VI. ROLES OF ABA
VII. THE ABA SIGNAL TRANSDUCTION PATHWAY
VIII. BIOTECHNOLOGICAL FEATURES
IX. CONCLUSIONS

**The development of strategies that enable growth without excessive consumption of water was vital to the evolution of terrestrial plants. It is now well established that phytohormones, in particular, abscisic acid, regulate plant water status.**

### I. INTRODUCTION

During the 1960s, two independent groups identified a compound active in the initiation of bud dormancy in sycamore and cotton boll abscission, naming it dormin and abscisin II, respectively. Following its purification from cotton fruits, the chemical structure of this compound was determined in 1965 and it was renamed abscisic acid (ABA). Shortly after this, it was discovered that ABA levels increase considerably when plants wilt and that ABA causes stomatal closure. These two discoveries highlighted the importance of ABA in mediating responses of vegetative tissues to environmental stresses such as drought, high salinity, and low temperature. ABA is also required for the accumulation of seed nutrient reserves, the acquisition of desiccation tolerance, and the arrest of embryonic development during seed maturation. Despite its name, ABA is not a major regulator of absicission, which is primarily controlled by ethylene.

### II. STRUCTURE AND OCCURRENCE

Like all hormones, ABA responses depend not only on the sensitivity of the tissue to ABA, but also on local ABA concentration. This is regulated by the biosynthesis, degradation, inactivation, transport, and subcellular compartmentation of the hormone.

#### A. Structure

The 15 carbon atoms of the sesquiterpene ABA configure an aliphatic ring with one double bond, three methyl groups, and an unsaturated chain containing the carboxyl group (Fig. 1). The *cis* and *trans* isomers differ in the orientation of the carboxyl group, and the asymmetric carbon at the $1'$ position of the ring distinguishes between the $S(+)$ and $R(-)$ enantiomers. The different forms of ABA occur in different proportions in plants and can have different activities. The *S*-*cis* form is the most abundant naturally occurring form and is the active form in fast responses such as stomatal closure. Both enantiomers are active in long-term responses such as changes in gene expression and protein synthesis. In contrast to the *cis*-*trans* isomers, the *S* and *R* forms cannot be interconverted *in planta*. The exact ABA chemical structure is essential for its physiological activity, and the loss of a carboxyl group, a tertiary hydroxyl group, a 2-*cis* 4-*trans*-pentadienoic side chain, a $4'$-ketone, or a double bond in the cyclohexane ring greatly reduces activity.

#### B. Occurrence

ABA is widespread in vascular plants, occurring in mosses, ferns, liverworts (where a similar compound, lunaric acid, plays a similar role), and all algal classes, including photosynthetic prokaryotes such as cyanobacteria. Some pathogenic fungi make ABA, but the biosynthetic pathway appears to be quite different from that of higher plants. ABA is also reported to occur in the mammalian brain, although its role there is not known.

### III. SYNTHESIS

The endogenous ABA concentration can rise and fall dramatically in response to environmental or developmental cues. It appears that ABA is synthesized in almost all cells containing chloroplasts or amyloplasts (i.e., plastids), but the regulatory controls appear to differ between tissues. Not only do absolute ABA concentrations increase dramatically during embryogenesis, but the ABA content of leaves and roots increases 10- to 50-fold when water potentials fall below $-1.0$ MPa (approximately $-10.0$ bar).

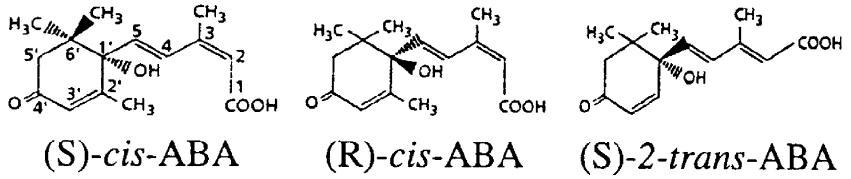
(S)-*cis*-ABA
(R)-*cis*-ABA
(S)-2-*trans*-ABA

**FIGURE 1** Chemical structures of the *S* and *R* forms of *cis*-ABA and the (*S*)-2-*trans* form of ABA.

The concentration of ABA in the xylem sap of well-watered plants is 1.0–15.0 nM and can increase to 3.0 μM after water stress. The main rise in ABA caused by water loss occurs some 2–3 h after the onset of wilting. The ability of cycloheximide to block this process indicates a requirement for *de novo* protein synthesis and thus implicates an up-regulation of ABA biosynthesis in stressed tissues.

The plant ABA biosynthetic pathway represents a minor branch of the carotenoid pathway and begins in plastids. In contrast with isoprene biosynthesis in animal cells, the main precursor of ABA, isopentenyl diphosphate (IPP), is generated by the methyl erythritol phosphate pathway and not from mevalonic acid. Eight IPP residues are combined to form geranylgeranyl diphosphate, the precursor for the biosynthesis of the C40 compound β-carotene. Both rings of β-carotene are hydroxylated to form the xanthophyll zeaxanthin, which can be regarded as the first intermediate in ABA synthesis (Fig. 2). The role of xanthophylls as intermediates in ABA biosynthesis is supported by the reduced ABA content of maize *vp* (*vivi*p*arous) mutants that are blocked in early steps of carotenoid synthesis. Zeaxanthin is then oxidized to antheraxanthine and to all-*trans*-violaxanthin by zeaxanthin epoxidase (ZEP), which is absent in the *Nicotiana plumbaginifolia aba2* mutant. ZEP contains a putative N-terminal transit sequence for targeting to chloroplasts. *ABA2/ZEP* expression is detected in stems, leaves, roots, and seeds and it is strongly induced by drought stress in roots but not leaves.

The oxidative cleavage of the 9-*cis*-epoxycarotenoid precursor generates the 15C skeleton of ABA. The maize *vp14* mutant is deficient in the chloroplastic 9-*cis*-epoxycarotenoid dioxygenase (NCED) responsible for the cleavage of the 9-*cis*-isomers, but not the all-*trans*-isomers, to xanthoxin. Therefore, the precise order of isomerization-type reactions remains uncertain. The gene is expressed constitutively in embryos and roots and in contrast with *ABA2/ZEP* transcripts, *NCED* transcripts accumulate to high levels in water-stressed leaves. Thus, ABA accumulation in wilted leaves is primarily regulated at the level of plastidic xanthoxin production, which appears to be rate-limiting in ABA biosynthesis. Transgenic experiments indicated that ZEP is primarily involved in the regulation of ABA synthesis during seed development.

The final steps are not yet completely defined. Xanthoxin is converted to ABA in the cytosol via either AB-aldehyde or xanthoxic acid. Genetic evidence suggests that xanthoxin is first oxidized to AB-aldehyde, although the involvement of xanthoxic acid as a precursor has not been completely eliminated. *Arabidopsis thaliana aba3* and tomato *flacca* and *sitiens* mutants are defective in the last oxidation step and are thus unable to oxidize AB-aldehyde to

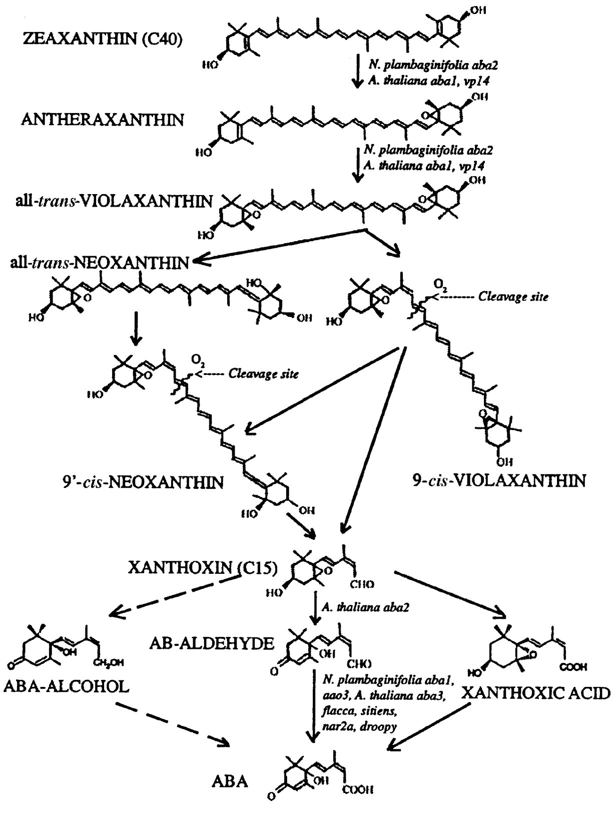
ZEAXANTHIN (C40)
*N. plambaginifolia aba2*
*A. thaliana aba1, vp14*
ANTHERAXANTHIN
*N. plambaginifolia aba2*
*A. thaliana aba1, vp14*
all-*trans*-VIOLAXANTHIN
all-*trans*-NEOXANTHIN
Cleavage site
Cleavage site
9'-*cis*-NEOXANTHIN
9-*cis*-VIOLAXANTHIN
XANTHOXIN (C15)
*A. thaliana aba2*
AB-ALDEHYDE
ABA-ALCOHOL
*N. plambaginifolia aba1, aao3, A. thaliana aba3, flacca, sitiens, nar2a, droopy*
XANTHOXIC ACID
ABA

**FIGURE 2** The ABA biosynthetic pathway. The metabolic blocks in various ABA-deficient mutants are indicated in italics. Adapted from Taylor *et al.* (2000).

ABA. This last step involves an enzyme that requires a molybdenum cofactor. *Arabidopsis aba3* and *N. plumbaginifolia aba1* mutants cannot produce the functional molybdate cofactor required by AB-aldehyde oxidase (AO). This last enzyme of ABA synthesis is not highly substrate-specific and interestingly, AO can also catalyze synthesis of another plant growth regulator, the auxin indole-3-acetic acid. In *Arabidopsis*, a multigene family comprising at least four members encodes AO, only one of which appears to act specifically in ABA synthesis.

### A. Alternative Pathways

Although *ABA2* is a single-copy gene, the ABA content of the *N. plumbaginifolia aba2* null mutant is 23–48% that of the wildtype (WT). Moreover, ABA is present in tomato *flacca* and *sitiens* mutants that lack an effective AO. These results suggest either that there is more than one biosynthetic pathway or that there is some redundancy in part of the primary biosynthetic pathway. The 2-*trans*-ABA-alcohol accumulated during water stress in *flacca* and *sitiens* mutants could be formed via a P450 mono-oxygenase able to add a second oxygen atom to the C1 position and can be further slowly converted to ABA. This reaction may also occur to a small extent in WT plants. Unlike plants, fungi are able to synthesize ABA directly from the 15C compound farnesyl pyrophosphate.

## IV. DEGRADATION

After wilted leaves regain turgor, ABA can be inactivated by oxidation to phaseic acid and dihydrophaseic acid or by conjugation to glucose to form a glucose ester. In the first case, catabolic inactivation proceeds via hydroxylation at the $8'$ position to form an unstable intermediate that subsequently forms phaseic acid. The ABA-$8'$-hydroxylase considered as the pivotal enzyme in ABA degradation is a membrane-associated cytochrome P450 mono-oxygenase. It is expressed at high levels in plant tissues recovering from hyperosmotic stresses. Although phaseic acid is still able to trigger stomatal closure in some species, its activity is much weaker than that of ABA. In contrast, dihydrophaseic acid, which is the reduced form of phaseic acid, has no detectable activity. Conjugation of ABA to glucose not only renders ABA inactive but also changes its distribution in the cell. Whereas free ABA is mainly cytosolic, ABA-β-D-glycosyl ester accumulates in vacuoles and could be a storage form of the hormone. Until now, neither the enzymes involved in ABA catabolism nor the genes that encode them have been isolated.

## V. TRANSPORT

ABA is secreted by cells into the apoplast (i.e., intercellular space) and is easily transported in both xylem and phloem sap to most plant parts, especially stems, leaves, roots, and ripening fruits. Since roots are the primary sites of perception of water deficit, ABA synthesized in roots can be transported to shoot tissues via the transpiration stream, where it triggers stomatal closure to reduce water loss from leaves.

Within leaves, ABA is redistributed as a function of pH. In a well-watered plant, the xylem sap is more acidic (approximately pH 6.3) and ABA occurs in its protonated form (ABAH). During drought stress, the sap becomes slightly alkaline (approximately pH 7.2), favoring the deprotonation of ABAH to ABA. As a result, less ABA is taken up by mesophyll cells and more is diverted to guard cells. Therefore, even though absolute ABA levels may not change, the pH-dependent redistribution of root-derived ABA to guard cells can induce stomatal closure.

A similar redistribution may exist within cells. When photosynthesis is active, the pH of the chloroplast stroma increases as protons are pumped into the thylakoid lumen. A prevalence of deprotonated ABA limits its ability to cross the chloroplast membrane, causing the accumulation of ABA in the stroma. During drought stress, photosynthetic rates decrease. The resulting drop in stromal pH increases levels of ABAH, which can traverse membranes and be released for transport to guard cells.

## VI. ROLES OF ABA

ABA is unquestionably involved in a plant's response to stress and in the initiation and maintenance of seed dormancy. However, it also influences many other aspects of plant physiology, often by interacting synergistically/antagonistically with hormones such as ethylene, gibberellins, cytokinins, auxin, jasmonic acid, and brassinosteroids or by modulating metabolic sensing pathways such as those monitoring cellular sugar status.

### A. ABA Triggers Stomatal Closure During Water Stress

Stomata are pores, found on the aerial surfaces of plants, that allow $CO_2$ uptake for photosynthesis and at the same time the loss of water, which drives the transpiration stream. Stomatal pore diameter is regulated by turgor changes of the two surrounding guard cells. Unlike most other cells in higher-plant tissues, the absence of plasmodesmata in mature guard cells renders them independent of surrounding cells and enables them to respond autonomously to stimuli such as $CO_2$, water status, temperature, red/blue light, and plant pathogens. Applied ABA inhibits the opening and promotes the closure of stomata. The increased transpiration rates observed in ABA-biosynthetic mutants and the accumulation of ABA in stressed leaves with reduced transpiration are consistent with the view that endogenous ABA normally plays an important role in the reduction of water loss by transpiration. Expression of an anti-ABA antibody in transgenic tobacco plants retained ABA in the endoplasmic reticulum and caused leaves to wilt by impairing stomatal closure.

The molecular mechanism by which ABA induces stomatal closure has been studied using genetic, biochemical, single-cell, and electrophysiological approaches. Opening and closing of stomata is thought to provoke turgor and volume changes in guard cells. During water stress, the increase in cellular ABA or in apoplastic ABA at guard cell surfaces mediates guard cell closure by triggering a net efflux of $K^+$ and $Cl^-$ from the vacuole to the cytoplasm and from the cytoplasm to the apoplast. Additionally, sucrose and malate are metabolized to osmotically inactive starch, all of which function to reduce the osmolarity in the guard cells. During stomatal opening, guard cells swell following the accumulation of $K^+$, anions, and sucrose. The resulting out-bowing of the guard cell pair increases pore aperture and allows reestablishment of transpiration.

## B. ABA Promotes Seed Maturation and Dormancy

One of the clearest effects of ABA is to prepare the seed for desiccation and to impose embryo dormancy to prevent premature germination. Seeds of ABA-deficient mutants or transgenic plants depleted of endogenous ABA by expression of an ABA-specific antibody fail to mature fully and acquire dormancy.

Seed development can be divided into two phases of equal duration. The first includes growth and development of the embryo and the endosperm. The second phase begins with the arrest of cell division and the accumulation of storage reserves and is followed by preparation for desiccation, which occurs in the last stages of seed maturation. Seeds prepare for desiccation by accumulating nutritive reserves and proteins that allow the cell to survive the ensuing loss of up to 90% of its water content. As a consequence of dehydration, seeds become dormant. The ABA content of seeds increases during the first half of seed development and decreases during the second phase involving seed maturation.

ABA strongly induces genes that encode abundantly expressed seed storage proteins (e.g., zein, conglycinin, and lectin proteins) as well as proteins involved in desiccation tolerance. The highly conserved water-soluble and basic late-embryogenesis abundant (LEA) proteins are rich in glycine/lysine and low in hydrophobic residues and are thought to stabilize other proteins when the cell is dehydrated. They are related to members of the DHN (dehydrin) and RAB (Responsive to ABA) protein families.

## C. ABA Inhibits Germination and Seedling Growth

Seed germination can be defined as the resumption of growth of the embryo following dormancy. As possibly the most critical developmental transition in the plant life cycle, germination is contingent on suitable environmental conditions. However, dormant seeds will not germinate even under normally permissive conditions of temperature or water, light, and oxygen availability. Seed dormancy introduces a delay in germination that provides additional time for geographical dispersal and also maximizes seedling survival by preventing germination under adverse conditions.

ABA appears to be the most important mediator of seed dormancy. The ability of exogenous ABA to prevent seed germination in many species has been used to isolate several *abi* (ABA-*i*nsensitive) *Arabidopsis* mutants (Table 1). Exogenous ABA can also inhibit the precocious germination of immature embryos in culture. ABA-deficient *Arabidopsis* (*aba*) mutants are nondormant at maturity, and embryos of maize *vp* mutants germinate directly on the cob while still attached to the mother plant. This precocious germination, named vivipary, suggests that ABA normally constrains developing embryos in an early developmental stage. In contrast with maize, ABA deficiency in *Arabidopsis* does not cause vivipary because the rigid seed coat prevents embryo growth while the seed is in the seed pod. Nevertheless, vivipary occurs when *Arabidopsis abi3* mutant embryos are dissected out of the seed coat before complete desiccation.

TABLE I **Mutations Affecting ABA Biosynthesis and Signal Transduction Pathway in Plants**

| Plant species | Mutations[a] | Gene product/function |
|---|---|---|
| **ABA-deficient mutants** | | |
| *Zea mays* | *vp2,5,7–9* | *Carotenoid biosynthesis* |
| *Z. mays* | *vp14* | 9-*cis*-Epoxycarotenoid dioxygenase |
| *Chlamydomnas reinhardtii* | *M526* | ABA xanthophyll biosynthesis |
| *Arabidopsis thaliana* | *aba1* | Zeaxanthin epoxidase |
| *A. thaliana* | *aba2* | Xanthoxin oxidase |
| *A. thaliana* | *aba3* | Molybdenum cofactor biosynthesis |
| *A. thaliana* | *aao3* | Aldehyde oxidase |
| *Nicotiana plumbaginifolia* | *aba2* | Zeaxanthin epoxidase |
| *N. plumbaginifolia* | *aba1/ckr1* | Molybdenum cofactor biosynthesis |
| *N. plumbaginifolia* | *aba2* | ABA xanthophyll biosynthesis |
| *N. plumbaginifolia* | *cnxA* | Molybdenum cofactor biosynthesis |
| *Lycopersicum esculentum* | *notabilis* | 9-*cis*-Epoxycarotenoid dioxygenase |
| *L. esculentum* | *flacca* | Molybdenum cofactor biosynthesis |
| *L. esculentum* | *sitiens* | Aldehyde oxidase |
| *Solanum phureja* | *droopy* | Aldehyde oxidase |
| *Hordeum vulgare* | *nar2a* | Molybdenum cofactor biosynthesis |
| *Pisum sativum* | *wilty* | ND |
| **ABA-insensitive mutants** | | |
| *A. thaliana* | *abi1* (SD) | Type 2C protein phosphatase |
| *A. thaliana* | *abi2* (SD) | Type 2C protein phosphatase |
| *A. thaliana* | *abi3* | Seed-specific putative transcription factor |
| *A. thaliana* | *abi4* | Transcription factor |
| *A. thaliana* | *abi5* | bZIP transcription factor |
| *A. thaliana* | *axr2 (D)* | Auxin response factor (allelic to the auxin mutant *iaa7*) |
| *A. thaliana* | *gca1–8* | ND |
| *A. thaliana* | *gpa1* | Heterotrimeric G-protein α-subunit |
| *Z. mays* | *vp1* | Seed-specific bZIP transcription factor |
| *Z. mays* | *rea* | ND |
| *Hordeum vulgare* | *cool* (ND) | ND |
| *Craterostigma plantagineum* | *cdt-1* (D) | Regulatory RNA or short peptide |
| **ABA-hypersensitive mutants** | | |
| *A. thaliana* | *abh1* | Subunit of a nuclear RNA cap-binding complex |
| *A. thaliana* | *bri1* | Steroid receptor kinase |
| *A. thaliana* | *era1* | Farnesyltransferase β-subunit |
| *A. thaliana* | *era2* | ND |
| *A. thaliana* | *era3* | Novel transmembrane protein (allelic to the ethylene mutant *ein2*) |
| *A. thaliana* | *fiery1* | Inositol polyphosphate 1-phosphate |
| *A. thaliana* | *jar1* | ND |
| *A. thaliana* | *jin4* | ND |
| *A. thaliana* | *hyl1* | Double-stranded RNA-binding protein |
| *A. thaliana* | *sax* | ND |

[a] Unless indicated, all mutations are recessive (SD, semidominant; D, dominant; ND, not determined).

Germination is regulated by an antagonism between ABA, which promotes dormancy, and gibberellic acid (GA), which counteracts the effects of ABA by promoting growth and the mobilization of storage reserves. An elegant demonstration of this antagonism is the recovery of mutants defective in ABA synthesis in a screen for revertants of GA-deficient mutants.

## D. ABA Controls Root and Shoot Growth

ABA shows different effects on root and shoot growth depending on plant water status. Under water stress, ABA depresses both shoot and root growth, but the overall effect is a dramatic increase in the root:shoot ratio, which facilitates water conservation.

## E. ABA Mediates Wound Responses

After mechanical wounding, a specific set of defense-related proteins, such as protease inhibitors I and II, cathepsin D inhibitor, and threonine deaminase, accumulate both at the site of injury and systemically throughout the plant. ABA, together with jasmonic acid, appears to play a role in the induction of these genes.

## VII. THE ABA SIGNAL TRANSDUCTION PATHWAY

Clues as to how the ABA signal is transduced to mediate its physiological and developmental processes are now beginning to emerge. It should be emphasized that although many individual components have been identified mainly by molecular genetic approaches, the complete network has not yet been elucidated.

### A. Receptor(s)

ABA is thought to initiate its effects by binding to a receptor(s) that triggers the signal transduction cascade. Currently, the identity of the receptor(s) is unknown. Cells may possess at least two sites of ABA perception, one of which is located at the plasma membrane and is triggered by extracellular ABA. Biophysical studies indicate that ABA effects in stomatal guard cells also involve intracellular receptors accessible to the protonated form, ABAH, which readily permeates membranes.

### B. Downstream Signaling Events

Recently, considerable insights have been gained into the identities of molecular components of the complex signaling network that mediates the actions of ABA. In particular, ion channels and fairly ubiquitous small second messengers have been implicated in ABA action.

#### 1. *Ion Channels Regulated by ABA Control Stomatal Aperture*

Electrophysiological studies, either by whole cell impalement or by patch clamping of the plasma membrane of guard cell protoplasts or isolated vacuoles, have identified a number of membrane ion channels. The sequence of events in ABA-induced stomatal closure is thought to be the following: (1) ABA induces release of $Ca^{2+}$ into the cytosol from an internal store, e.g., the vacuole. (2) The resultant increase in cytosolic $Ca^{2+}$ inhibits plasma membrane $H^+$ pumps and inward $K^+$ ($K^+_{in}$) channels, but activates two types of plasma membrane anion-efflux channels. One of these shows voltage-dependent slow activation (S-type), whereas the other shows rapid transient activation (R-type). The two types may reflect different states of a single channel. (3) The conjugate actions of these channels lead to a transient or sustained depolarization and the alkalinization of the guard cell cytoplasm, which (4) deactivates ($K^+_{in}$) channels and also contributes to the opening of voltage-gated $K^+_{out}$ channels. The ensuing long-term efflux of both anions and $K^+$ from guard cells contributes to loss of turgor and to stomatal closure.

#### 2. *$Ca^{2+}$ Channels*

Considerable evidence indicates that ABA produces repetitive, transient increases or oscillations in intracellular $Ca^{2+}$ levels. These encode information required for stomatal closure. $Ca^{2+}$-induced $[Ca^{2+}]_{cyt}$ oscillations include a repetitive $Ca^{2+}$ influx across the plasma membrane coupled to $Ca^{2+}$ release from an intracellular compartment. Stomatal closure is abolished in guard cells when a nonoscillating $Ca^{2+}$ plateau is imposed experimentally. Many other stimuli responsible for stomatal closure (cold shock, oxidative stress, and increases in $CO_2$) also cause $[Ca^{2+}]_{cyt}$ elevations. Anion channel regulation and stomatal movement phenotypes of *Arabidopsis abi1-1* or *abi2-1* mutants are suppressed by experimentally elevating $[Ca^{2+}]_{cyt}$.

The mechanisms by which ABA activates guard cell plasma membrane $Ca^{2+}$ channels remain unknown. In *Arabidopsis* guard cells, ABA causes a rapid increase of reactive oxygen species (ROS) that activate hyperpolarization-activated $Ca^{2+}$-permeable channels. ROS-induced stomatal closure and $Ca^{2+}$ activation are abolished in the ABA-insensitive mutant *gca2*. The origin of the $Ca^{2+}$ required to elevate $[Ca^{2+}]_{cyt}$ in response to ABA is unclear, but is probably mediated by inositol 1,4,5-trisphosphate ($InsP_3$) and/or cyclic ADP-ribose (cADPR). *RAB18* expression in *Arabidopsis* suspension culture cells requires rapid ABA-induced $Ca^{2+}$ influx and S-type anion channel activation. ABA-induced membrane depolarization in radish seedlings and tobacco epidermal and mesophyll cells indicates that these mechanisms are of general importance for ABA signaling in different cell types. A $Ca^{2+}$-independent pathway also appears to exist.

#### 3. *$H^+$ Channels*

Inhibition of the plasma membrane $H^+$-ATPase mediated by both cytosolic alkalinization and the increase in cytosolic $Ca^{2+}$ may also contribute to membrane depolarization. The origin of ABA-induced cytosolic alkalinization is unknown.

### 4. Cyclic Nucleotides (cAMP, CGMP, CADPR)

Cyclic ADP-ribose (cADPR) plays a central role in ABA responses. Microinjection of hypocotyl cells of the tomato *aurea* mutant with both potential intermediates in the ABA signaling cascade and fusions of the *Arabidopsis RD29A* and *KIN2* promoters to a reporter gene suggested that ABA triggers a transient accumulation of cADPR, which induces a release of $Ca^{2+}$ from internal stores such as vacuoles and the endoplasmic reticulum. Microinjection of mutant *abi1-1* protein inhibited ABA-, cADPR-, and $Ca^{2+}$-induced gene expression, and these effects were reversed by an excess of WT ABI1 protein.

Other cyclic nucleotides may also act in a $Ca^{2+}$-dependent stomatal opening pathway. For example, cAMP or the membrane-permeable cyclic GMP analog 8-Br-cGMP stimulates stomatal opening. cGMP-induced stomatal opening is inhibited by chelation of external $Ca^{2+}$ or by inhibitors of intracellular $Ca^{2+}$ release.

### 5. Lipid-Derived Second Messengers

Various lines of evidence suggest that ABA stimulates phosphoinositide metabolism. ABA-treated guard cell protoplasts showed a slight increase in $InsP_3$. The release of caged $InsP_3$ into the cytosol of guard cells caused $[Ca^{2+}]_{cyt}$ increases, inhibition of $K^+_{in}$ channels, and stomatal closure. The *Arabidopsis fry1* (*fiery1*) mutant, which is defective in an inositol polyphosphate 1-phosphatase, accumulates more $InsP_3$ than WT plants after ABA treatment and is hypersensitive to ABA in germination and gene expression assays. Similarly, overexpression of a different $InsP_3$ phosphatase blocked the inhibition of germination and seedling growth in *Arabidopsis*. Overexpression of a stress- and ABA-inducible phosphatidylinositol-specific phospholipase C (PI-PLC) in *Arabidopsis* suggests that although increased $InsP_3$ levels are necessary for maximal ABA-induced gene expression in vegetative tissues, the AtPLC1 isoform is normally latent and probably participates in secondary ABA responses. A reduction in $InsP_3$ levels in transgenic lines expressing antisense *AtPLC1* correlated with their insensitivity to ABA in germination and seedling growth assays.

ABA also stimulates production of myo-inositol-hexakisphosphate ($InsP_6$) in guard cells to a greater extent than $InsP_3$. $InsP_6$ inhibits $K^+_{in}$ channels in a $Ca^{2+}$-dependent manner with greater efficiency than $InsP_3$. Whether $InsP_6$ causes $[Ca^{2+}]_{cyt}$ elevations and whether both messengers function in the same or separate signaling branches is unknown.

Phosphatidic acid (PtdOH) generated from phospholipase D (PLD) increases transiently following ABA treatment of *Vicia faba* guard cells. PtdOH promotes stomatal closure and inactivates $K^+_{in}$ channel currents but does not elicit a $[Ca^{2+}]_{cyt}$ increase, suggesting that PLD acts either in a parallel $Ca^{2+}$-independent pathway or downstream of $Ca^{2+}$ release. Both U-73122 (a PI-PLC inhibitor) and 1-butanol (a PLD inhibitor) only partially inhibit ABA-dependent stomatal closure, and simultaneous application of both inhibitors does not have additive effects. Thus, PLC and PLD appear to act in the same pathway that requires the cooperation of an additional pathway(s) to attain the complete effects of ABA. This may be mediated by cADPR, since simultaneous application of 1-butanol with the cADPR antagonist nicotinamide increases the extent to which ABA-induced stomatal closure is reduced.

Sphingosine-1-phosphate (S1P) is another lipid-derived $Ca^{2+}$-mobilizing agent capable of inducing stomatal closure. An increase in S1P levels occurs in leaves following drought stress, but disruption of S1P production causes only partial inhibition of ABA-induced stomatal closure.

### 6. Protein Kinases and Protein Phosphatases

Phosphorylation/dephosphorylation events are central mediators in ABA signaling. In guard cells, the $Ca^{2+}$ signal is possibly relayed by specific protein kinases and phosphatases. Protein kinase inhibitors abolish the activation of S-type anion channels and thus block ABA-induced stomatal closure. Reciprocally, the protein phosphatase inhibitor okadaic acid (OKA) maintains guard cell S-type channels in the active state. In-gel phosphorylation assays demonstrated that ABA rapidly activates a $Ca^{2+}$-independent 48 kDa Ser/Thr protein kinase in *V. faba* guard cells. ABA fails to activate anion channels or induce stomatal closure in guard cells that express a dominant loss-of-function allele of this kinase.

Several stress- and ABA-inducible protein kinases have been identified. In epidermal peels of *Pisum sativum*, the ABA-induced accumulation of a *DHN* transcript was reduced by K-252a (an inhibitor of Ser/Thr protein kinases) and also by OKA or cyclosporin A (an inhibitor of Ser/Thr protein phosphatases type 2B). In barley aleurone protoplasts, the stimulation of a MAP kinase activity appeared to be correlated with the induction of *RAB16* transcript. OKA inhibited the induction of *HVA1* and *RAB16* transcripts by ABA, and phenylarsine oxide (an inhibitor of Tyr protein phosphatases) blocked *RAB16* induction.

The analysis of *abi1* and *abi2* has shed new light on the involvement of phosphorylation events in ABA signaling. These two dominant ABA-insensitive mutants, originally isolated in a screen for mutants able to germinate and grow in nonpermissive ABA concentrations, have phenotypes reminiscent of ABA deficiency viz. reduced seed dormancy, improper regulation of stomatal aperture, and decreased expression of various ABA-inducible genes. *ABI1* and *ABI2* encode Ser/Thr protein phosphatases type 2C (PP2C). The dominant mutant alleles *abi1-1* and *abi2-1* have point mutations that substitute a conserved Gly with Asp, probably disrupting the conformation of a site required for $Mg^{2+}$-binding or phosphatase activity. Several downstream responses to ABA are impaired in *abi1-1* and *abi2-1*, including $K^+_{out}$ and $K^+_{in}$ channel regulation, anion channel activation, and increases in $[Ca^{2+}]_{cyt}$. Because the mutations are dominant, it remains unclear whether the ABI1 and ABI2 are positive or negative regulators of ABA signaling or, indeed, whether they affect ABA signaling at all in WT plants. However, because intragenic revertants of *abi1-1* and *abi2-1* have reduced or no phosphatase activity *in vitro* and a double mutant of both revertants is hypersensitive to ABA, ABI1 and ABI2 are probably negative regulators of ABA signaling. Accordingly, overexpression of WT ABI1 in maize mesophyll protoplasts blocks ABA regulation of gene expression. The precise roles of kinases and phosphatases in ABA signaling and the identities of their protein substrates have not been clearly established.

### 7. *Farnesylation*

Although researchers have focused mainly on positively acting components of the ABA signaling pathway, inactivation of negative regulators of ABA signaling should result in an enhanced response to ABA. An *Arabidopsis* mutant *era1* (*e*nhanced *r*esponse to *A*BA) was isolated based on its inability to germinate in the presence of low concentrations of ABA (0.3 μM) that do not inhibit germination of WT seeds. The *era1* mutation markedly increases seed dormancy and ABA hypersensitive activation of S-type anion currents in this mutant increases stomatal closure, reducing water loss during drought. The *ERA1* gene encodes the β-subunit of a heterodimeric farnesyltransferase. Farnesyltransferases catalyze the attachment of a 15-carbon farnesyl lipid to C-terminal target sequences, which localizes specific soluble signaling proteins to membranes. In addition to enhancing ABA signaling, loss of *ERA1* function affects several other signaling pathways and developmental programs, including meristem development. Thus, although *ERA1* targets are not restricted to ABA action, a factor that normally suppresses ABA responses requires farnesylation. The exact relationship between *ERA1* and the *ABI* loci remains unknown.

### 8. *RNA Binding and ABA*

*Arabidopsis abh1* mutants are hypersensitive to ABA-mediated inhibition of germination as well as induction of stomatal closure and increases in $[Ca^{2+}]_{cyt}$. *ABH1* is expressed in stomata and encodes a nuclear transcript cap-binding protein that apparently functions in a heterodimeric complex. *ABH1*, by analogy to yeast and mammalian RNA cap-binding proteins, is proposed to regulate the strength of ABA signaling by transcript modification of early signaling components.

### 9. *Heterotrimeric G-Protein Action*

Heterotrimeric G-proteins are central to many signaling processes. *Arabidopsis* contains only a single gene (*GPA1*) encoding a prototypic Gα subunit. ABA-mediated inhibition of stomatal opening, but not ABA-controlled promotion of stomatal closure, is impaired in *gpa1* null mutants. *GPA1* is required for negative regulation of $K^+_{in}$ channels and the pH-independent activation of anion channels.

### 10. *Role of the Actin Cytoskeleton in Stomatal Movements*

A reorganization of the actin cytoskeleton of guard cells has been observed after ABA treatment. Cytochalasin D (an actin filament-depolymerizing agent) activates $K^+_{in}$ channels, while phalloidin (an actin filament stabilizer) inhibits $K^+$ channel currents. ABA treatments reorganize actin cytoskeleton architecture from a radial arrangement to a randomly oriented and fragmented pattern. A small *Arabidopsis* GTP-binding protein, AtRac1, is a negative regulator in ABA-induced actin reorganization. The inactivation of AtRac1 by ABA is impaired in *abi1-1*.

## C. Regulation of Gene Expression by ABA

ABA regulates the expression of numerous genes during embryogenesis and seed maturation as well as under stress conditions such as heat shock, low temperature, drought, and high salinity.

### 1. *ABA-Inducible Genes*

The use of ABA-deficient and ABA-insensitive mutants has demonstrated that ABA contributes to the regulation of numerous genes involved in seed maturation and/or the response of vegetative tissues to hyperosmotic stress. Characterization of the promoters of ABA-responsive genes has enabled identification of the *cis*- and *trans*-acting elements that act at the termini of branches in the ABA signaling cascade. Considerable evidence indicates the existence of ABA-independent dehydration and cold-induced signaling pathways.

### 2. *Cis-Acting Elements*

Gene activation is mediated by the binding of transcription factors to ABA-responsive elements (ABREs) located in the promoters of ABA-induced genes. To date, more than 20 functional ABREs have been found in ABA-inducible genes that are abundantly expressed in desiccating seeds and/or are responsive to drought stress and ABA in vegetative tissues.

The first type of ABRE defined was a sequence of 8–10 bp that shares a conserved ACGT core motif, named the G-box. The sequence flanking the ACGT core is important for *in vivo* and *in vitro* function. Some ACGT elements confer developmental and tissue-specific expression on a minimal promoter. In a natural promoter context, an ABRE functions with a coupling element (CE). ABA-responsive complexes comprising an ABRE and a CE can confer ABA-inducible transcription upon a minimal promoter. The sph element, first identified in the promoter of the *C1* gene involved in anthocyanin synthesis in maize endosperm, is a second category of *cis*-acting element distinct from the G-box.

### 3. *Trans-Acting Factors*

Yeast one-hybrid assays to identify ABRE-binding proteins (AREBs) have enabled cloning of several homologous transcription factors of the basic leucine zipper (bZIP) family. ABA-regulated transcription factors of the homeodomain leucine zipper, basic helix-loop-helix leucine zipper, and MYB classes have also been identified. AREBS are capable of activating reporter genes fused to ABREs and their induction by ABA at the transcript level frequently precedes the induction of other ABA-responsive genes. *Arabidopsis* ABI5, the only bZIP AREB recovered in a genetic screen, is also subject to posttranscriptional modification by ABA. Maize VP1 and *Arabidopsis* ABI3 appear to be orthologous seed-specific transcriptional activators, the loss of which affects several aspects of seed maturation, including the expression of storage proteins and *LEA* genes. VP1/ABI3-like proteins appear to activate transcription by distinct mechanisms depending on the target *cis* elements. VP1 interacts directly with the sph element of the C1 promoter and acts on ABREs via association with a distinct *trans*-acting factor(s).

## D. Novel Genetic Screens

Quantitative and mechanistic characterization of new signaling mutants is necessary for a complete molecular understanding of the ABA signaling cascade. Several mutants have been isolated in screens for deregulated ABA control of ABREs fused to reporter genes. Eight *gca* (growth control by ABA) mutants are characterized by reduced sensitivity to the inhibition of seedling growth by exogenous ABA and aberrant stomatal regulation. An elegant screen that uses small differences in leaf temperature to distinguish transpiration rates in mutants and WT plants is likely to identify new elements which mediate ABA action in guard cells. Screens for enhancer or suppressor mutations offer one approach to identify genes which interact genetically with known participants in ABA signaling.

## VIII. BIOTECHNOLOGICAL FEATURES

Fresh water scarcity is currently one of the principal threats to global food security. Plants account for approximately 65% of global fresh water use. Losses in agricultural yields resulting from the desiccation of crops and horticultural plants during periods of drought have severe social and economic repercussions. Unfortunately, because of the high cost of synthesis and its instability in UV light, there are no practical uses of ABA. However, synthetic ABA analogs such as the acetyleneacetal-type compounds LAB 173 711 and LAB 144143 reduce crop water use and increase cold-hardiness. Engineering the ABA signal transduction network in guard cells to control $CO_2$ intake and water loss could contribute substantially to more sustainable water use under adverse environmental conditions. The manipulation of seed maturation and dormancy in certain species by modification of ABA-regulated developmental programs may also be of considerable agricultural significance.

## IX. CONCLUSIONS

Recent advances have filled in many gaps concerning the biochemistry and subcellular localization of ABA synthesis as well as demonstrating the unquestionable involvement of ion channels, cytosolic pH, protein (de)phosphorylation, and cADPR- and phosphoinositide-mediated increases in $[Ca^{2+}]_{cyt}$ in transducing the ABA signal. Substantial progress has been made in characterization of the terminal signaling elements involved in ABA-mediated transcriptional regulation. Nonetheless, the mechanism(s) of ABA perception and early signaling events that result in cADPR synthesis or $InsP_3$ release remain to be resolved. Considering the multitude of physiological responses modulated by ABA, it will be interesting to assess the extent of overlap in the signaling events involved in well-characterized ABA effects such as the regulation of stomatal closure, the inhibition of seed germination, and the induction of stress-responsive gene expression. The striking degree of phenotypic pleiotropy observed in many mutants recovered in screens for altered sensitivity to ABA indicates extensive overlap between ABA action and other signaling pathways. Better insight into the regulation of ABA concentrations and the cellular capacity for response to ABA will provide a more complete picture of its importance to growth and development throughout the plant life cycle.

## Glossary

| | |
|---|---|
| **abscission** | The rejection of plant organs at an abscission zone where hydrolytic enzymes reduce cell adhesion. |
| **dormancy** | A resting condition with reduced metabolic rate found in ungerminated seeds and nongrowing buds. |
| **stomata** | Small openings located in the epidermal layers of plants allowing uptake of $CO_2$ and loss of water. Stomata are surrounded by two guard cells that control the pore size. |
| **stress** | The consequence of suboptimal environmental conditions that significantly decrease plant growth and/or reproductive capacity below potential. |
| **vivipary** | The ability of a plant embryo to bypass dormancy and proceed directly from embryogenesis to germination if rescued from the normal dehydration that occurs during seed maturation. |

## See Also the Following Articles

**Auxin • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Jasmonates • Salicylic Acid**

## Further Reading

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# ACTH

See *Adrenocorticotropic Hormone*

# Activating and Inactivating Receptor Mutations

Ilpo T. Huhtaniemi

*University of Turku, Finland and Imperial College London*

I. INTRODUCTION
II. ACTIVATING MUTATIONS
III. INACTIVATING MUTATIONS
IV. DOMINANT NEGATIVE MUTATIONS
V. SUMMARY

**Human hereditary diseases can be divided according to the Mendelian inheritance pattern into three categories: autosomal dominant, autosomal recessive, and X-linked recessive. The same division applies to the mutations that are known today in hormone receptor genes. The autosomal dominant mutations of receptor genes cause *activating* or *gain-of-function***

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Recent advances have filled in many gaps concerning the biochemistry and subcellular localization of ABA synthesis as well as demonstrating the unquestionable involvement of ion channels, cytosolic pH, protein (de)phosphorylation, and cADPR- and phosphoinositide-mediated increases in $[Ca^{2+}]_{cyt}$ in transducing the ABA signal. Substantial progress has been made in characterization of the terminal signaling elements involved in ABA-mediated transcriptional regulation. Nonetheless, the mechanism(s) of ABA perception and early signaling events that result in cADPR synthesis or $InsP_3$ release remain to be resolved. Considering the multitude of physiological responses modulated by ABA, it will be interesting to assess the extent of overlap in the signaling events involved in well-characterized ABA effects such as the regulation of stomatal closure, the inhibition of seed germination, and the induction of stress-responsive gene expression. The striking degree of phenotypic pleiotropy observed in many mutants recovered in screens for altered sensitivity to ABA indicates extensive overlap between ABA action and other signaling pathways. Better insight into the regulation of ABA concentrations and the cellular capacity for response to ABA will provide a more complete picture of its importance to growth and development throughout the plant life cycle.

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**mutations. In these cases, the structure of the receptor protein is usually altered in such a fashion that its signal transduction is constitutively activated in the absence of ligand hormone, and one altered allele is sufficient to cause the phenotype. The autosomal recessive mutations are usually of the *inactivating* or *loss-of-function* type; i.e., the mutant allele encodes a functionally inactive receptor protein. In most cases, the normal allele is sufficient to maintain normal hormonal function, but in some situations the heterozygous carrier has a phenotype, usually in the form of mild hormone resistance. X-linked recessive mutations cause a phenotype in all males and in homozygous females, and in some cases heterozygous females have a milder phenotype. A special case of inactivating receptor mutations is the *dominant negative* mutations, where the mutated receptor inhibits the function of that encoded by the wild-type allele. This is the only situation where an inactivating mutation causes the phenotype in dominant fashion. Besides the above hereditary (germ line) mutations, somatic mutations are also known in hormone receptors. They are invariably found in tumors, are of the activating type, and play a role in tumor formation or growth. In addition, numerous polymorphisms are known in receptor genes; they either cause no phenotype or may alter susceptibility to certain diseases.**

## I. INTRODUCTION

A large number of hormone receptor mutations are known today, and this article presents appropriate examples of the different mechanisms by which the mutated receptor brings about either receptor inactivation or constitutive activation. Hormone receptors can be subdivided into two functional categories on the basis of their cellular location, i.e., those present on the plasma membrane and those present inside the cell, in the nucleus and/or cytoplasm. Because of differences in the function of these two receptor types, they are presented separately, under the headings of activating and inactivating mutations. Table 1 summarizes the phenotypes, types of functional alteration, and modes of inheritance of most of the currently known hormone receptor mutations.

## II. ACTIVATING MUTATIONS

Activating or gain-of-function mutations can be classified, according to the functional alteration, into four categories: (1) constitutive receptor activation in the absence of ligand hormone, (2) increased sensitivity of the receptor to its normal ligand, (3) relaxed specificity of the receptor to ligands, and (4) acquired novel functions of the mutated receptor. Category 1 is most common among the activating receptor mutations. Some of these mutations are inherited via the germ line, but some, in particular those encountered in tumors, are somatic (Table 1).

### A. Nuclear Receptors

Only a few cases of gain-of-function mutations of nuclear receptors are currently known, although it is possible to produce constitutively activated nuclear receptors through site-directed mutagenesis *in vitro*. The reason for their paucity may be the fundamental role that many of the nuclear receptors play in cellular functions and because their inappropriate activation may be embryo-lethal. In fact, almost the only germ-line mutations known in nuclear receptors are those of the androgen receptor (AR), not necessary for life. The extensions of the polyglutamine (CAG) repeat in the N-terminal part of AR (see below) in Kennedy's disease apparently bring about novel neurotoxic functions for the mutated receptor protein. Conversely, if this repeat is shortened, as is found in somatic mutations in advanced forms of prostatic cancer, the mutated AR either is constitutively activated or has relaxed ligand specificity. Activating mutations of retinoic acid receptors may play a role in the pathogenesis of certain leukemias and hepatomas, due to chromosomal rearrangements within the retinoic acid receptor genes, resulting in fusion proteins with novel functions. The only other piece of information on constitutively activated nuclear receptors concerns estrogen receptors that may contribute to the formation of estrogen-independent cell clones in breast cancer.

### B. Cell Membrane Receptors

The receptors for the glycoprotein hormones, i.e., luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), constitute a good example of gain-of-function mutations of cell membrane receptors. They all belong to the family of G-protein-associated seven-transmembrane domain receptors. Normal receptor activation entails binding of the ligand to the extracellular receptor domain, whereby a conformational change occurs in the transmembrane domain, allowing activation of the intracellular second-messenger generation and ultimately the functional response of the target cell to hormonal stimulation. Accordingly, most of the activating mutations in these

**TABLE 1 Currently Known Mutations in Hormone Receptor Genes**

| Receptor | Type of mutation | Type of inheritance | Phenotype |
|---|---|---|---|
| Nuclear receptors | | | |
| Thyroid hormone | Dominant negative | D | Thyroid hormone resistance |
| | Inactivating | R | Thyroid hormone resistance |
| Estrogen-α | Inactivating | R | Male: unfused epiphyses, poor sperm, osteoporosis, insulin resistance |
| | Activating | S | Receptor positive, hormone-resistant breast cancer |
| Androgen | Inactivating | XR | Lack of male sexual differentiation (testicular feminization) |
| | Activating | XR | Kennedy's disease |
| | | S | Advanced prostate cancer |
| Glucocorticoid | Inactivating | R | Variable symptoms of glucocorticoid resistance |
| | | S | Nelson's syndrome (pituitary adenoma) |
| Mineralocorticoid | Inactivating | D | Autosomal dominant pseudohypoaldosteronism I |
| Vitamin D | Inactivating | R | Hypocalcemic vitamin D-resistant rickets |
| Retinoic acid | Activating | S | Various malignant tumors |
| | Dominant negative | D | |
| Plasma membrane receptors | | | |
| TSH | Activating | S | Toxic thyroid adenomas |
| | | D | Toxic thyroid hyperplasia (hereditary) |
| | Inactivating | R | Euthyroid, elevated TSH (mild) |
| | | R | Hypothyroidism (severe) |
| LH | Activating | D | Males: gonadotropin-independent precocious puberty<br>Females: no phenotype |
| | | S | Leydig cell tumors |
| | Inactivating | R | Males: lack of male sexual differentiation<br>Females: anovulatory infertility |
| FSH | Activating | D | Normal spermatogenesis in the absence of gonadotropins |
| | Inactivating | R | Females: infertility with arrest of follicular maturation<br>Males: suppressed spermatogenesis |
| ACTH | Inactivating | R | Familial glucocorticoid deficiency |
| | Activating | D | Normal cortisol, low ACTH |
| MSH | Inactivating | R | Red hair and light skin |
| Vasopressin | Inactivating | XR | Nephrogenic diabetes insipidus |
| GnRH | Inactivating | R | Hypogonadotropic hypogonadism |
| $Ca^{2+}$-sensing | Inactivating | R | Familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism |
| | Activating | D | Hypoparathyroidism, hypocalcemia |
| PTH/PTHrP | Activating | D | Jansen's metaphyseal chondrodysplasia |
| GH | Inactivating | R | Dwarfism |
| | Dominant negative | D | Dwarfism |
| GHRH | Inactivating | R | Dwarfism |
| Insulin | Inactivating | R | Leprechaunism, Rabson–Mendenhall syndrome |
| | Dominant negative | D | Type A insulin resistance |
| Erythropoietin | Activating | D | Primary polycythemia |
| Leptin | Inactivating | R | Obesity, pituitary dysfunction |

D, dominant; R, recessive; S, somatic; X, X-linked; TSH, thyroid-stimulating hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; GnRH, gonadotropin-releasing hormone; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; GH, growth hormone; GHRH, growth hormone-releasing hormone.

receptors have been discovered in the third intracellular loop or the sixth transmembrane region of the receptor, assumed to be the hot spot for activating mutations. The mutation apparently brings about a conformational change in the transmembrane receptor domain, allowing activation of signal transduction without preceding ligand binding. The functional alteration can be demonstrated by transfecting cDNA that encodes the mutated receptor into a cell line, which then displays the cellular response, for instance, cAMP production, in the absence of ligand hormone (Fig. 1).

Both activating germ-line and somatic mutations have been observed in the TSH receptor (R), and the latter mutations explain the molecular pathogenesis of a large proportion of toxic thyroid adenomas; they have also been implicated in some follicular thyroid carcinomas. Tens of this type of mutations are currently known in the TSHR, but why this receptor in particular is prone to somatic mutations remains unclear. Activating germ-line mutations of the TSHR are found in hereditary and sporadic toxic thyroid hyperplasia. The hereditary form is characterized by autosomal dominant transmission, hyperthyroidism with variable age of onset, hyperplastic steadily growing goiter, and absence of stigmata of autoimmunity. The sporadic cases present with congenital hyperthyroidism, due to neomutations in the TSHR gene. The mutations found in toxic adenomas and neomutations overlap, but another set of mutations are found in hereditary hyperthyroidism. The latter present with milder phenotypes and, hence, the autonomous receptor activation is milder than in the other two cases with more severe phenotypes. Natural selection may explain this difference. The sporadic cases display such strong TSHR activation that carriers of the same mutations in the germ line would apparently not have survived until fertile age in the past.

The other examples of activating mutations in cell membrane receptors are those of the LHR. Approximately 15 activating mutations of the LHR are currently known, but interestingly, not a single unequivocally documented case of activating FSHR mutation is known. The latter may be due to the fact that activating FSHR mutations do not cause a clear phenotype or to the fact that the phenotype is unexpected and the right types of patients have not been investigated. Gain-of-function mutations of the LHR cause in males gonadotropin-independent early onset precocious puberty (testotoxicosis), but in women, for reasons not clearly understood, there is no phenotype. Most of the activating LHR mutations bring about a clear, approximately 10-fold increase in basal cAMP production in cell lines expressing the mutated receptor. This explains why testosterone production in affected boys is activated well before the normal age of puberty. An interesting special case is an activating somatic LHR mutation that was recently discovered in Leydig cell tumors. This mutation activated, in addition to cAMP, the inositol phosphate signaling pathway, providing an example of an activating mutation with acquired novel function.

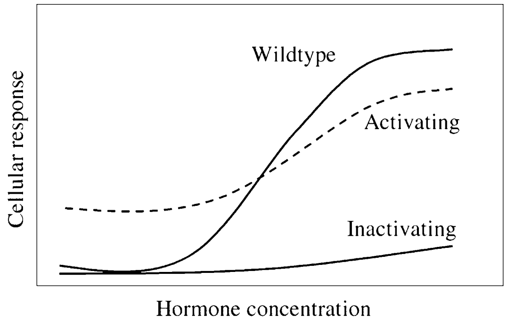

**FIGURE 1** Schematic presentation of hormone-stimulated response, for instance. cAMP production, in cells transfected with cDNA encoding the wild-type receptor and one carrying an activating or inactivating mutation. The elevated response in the absence of hormonal stimulation is typical for activating mutation. In inactivating mutation the response is totally or nearly completely missing.

## III. INACTIVATING MUTATIONS

An inactivating mutation can cause the following functional aberrations in the receptor protein: (1) decreased synthesis, (2) aberrant intracellular processing, (3) impaired or missing ligand-binding activity, (4) impairment or lack of signal transduction, (5) inability to anchor to the plasma membrane, (6) inability to dimerize, if needed for signal transduction, or (7) increased degradation.

### A. Nuclear Receptors

A good example of a nuclear receptor with inactivating mutations is the AR, a member of the steroid hormone/thyroid hormone/retinoic acid receptor family. It is extremely polymorphic and more than 250 AR mutations are known today. Because the AR gene is located on the X chromosome, mutational AR defects exist only in men (X-linked dominant inheritance). A homozygous female is impossible due to obligatory infertility of men carrying a mutated AR gene. The AR protein is a single polypeptide chain, containing, like all nuclear receptors, three functional domains, i.e., the transactivation domain, DNA-binding domain, and ligand-binding domain. The phenotypes with androgen insensitivity vary from mild defects of virilization (Reifenstein's syndrome) to complete female phenotype (testicular feminization), and the extent and location of the AR mutation often predict the phenotype. Splicing defects, frameshifts, immature termination codons, and partial or complete gene deletions invariably result in total androgen insensitivity. Single amino acid substitutions, in contrast, result in a wide variety of phenotypes, with severity depending on location of the mutation. Substitutions in the DNA-binding domain are a relatively homogenous group in which ligand binding is not altered, but the capability of the AR to modulate androgen-responsive genes is impaired. Mutations in the hormone-binding domain have more varying effects. The mutated receptor is seldom totally unable to bind hormone, whereas the ligand binding may be qualitatively altered or has low affinity. The phenotype of these mutations is often difficult to predict. Mutations in the transactivation domain are relatively rare. This part of the AR is the least conserved and it possibly allows greater variability in structure without hampering receptor function. In addition, no abnormalities in ligand binding can be detected in a large proportion of the patients despite their clear phenotype of androgen insensitivity. Some other genes involved in androgen action, e.g., those of AR co-activators, may be mutated in these cases.

AR has been found to have an intriguing type of structural polymorphism, a polyglutamine repeat (CAG at the DNA level), in exon 1 of its N-terminus. Short repeats (20 residues) are detected in advanced forms of prostatic cancer, apparently as somatic mutations, in which case the ligand specificity of the AR is relaxed and various steroidal and nonsteroidal ligands can activate it. The normal length of the repeat is 23–27 residues. Slight androgen insensitivity is apparent when the repeat length is 28, as can occur in men with oligo-azoospermia. Finally, clearly extended polyglutamine repeats (40 repeats) exist in spinal and bulbar muscular atrophy due to degeneration of motor neurons (Kennedy's disease) where the receptor function is more drastically altered, and it may have acquired novel toxic effects on motor neurons.

Inactivating mutations are also known in other steroid receptors. Only one case of estrogen receptor-α mutations has been described. This was detected in an adult male with tall stature, incomplete closure of epiphyses, osteoporosis, and insulin resistance. The receptor inactivation was due to a point mutation that induced a premature stop codon and hence formation of nonfunctional receptor protein. No progesterone receptor mutations have been described, possibly since they would be effectively eliminated from the genetic pool due to their adverse effect on reproduction. Glucocorticoid receptor (GR) mutations present a variable range of phenotypes from asymptomatic to isolated chronic fatigue and hypertension with or without hypokalemic alkalosis and/or hyperandrogenism. All GR mutations detected cause partial hormone resistance, since complete inactivation of glucocorticoid function would probably be lethal. Differences in impact of the various mutations on glucocorticoid sensitivity of the different target organs probably explain the variability of phenotypes encountered in this syndrome. A mild form of autosomal dominant hypoaldosteronism (type I) has been found to be due to inactivating mutations in the mineralocorticoid receptor. Inactivating mutations of thyroid hormone receptors cause, as expected, thyroid hormone resistance. The vitamin D receptor mutations cause hypocalcemic vitamin D-resistant rickets. In this case, the mechanism of receptor inactivation falls into three categories: (1) suppressed DNA binding/nuclear localization, (2) suppressed hormone binding, and (3) inhibition of heterodimerization with retinoic acid X receptor (RXR). The latter effect is incompatible with the mechanism of vitamin D action, where the vitamin D-receptor complex must heterodimerize with RXR to evoke a biological response.

## B. Cell Membrane Receptors

An inactivating mutation of a cell membrane-associated receptor causes hormone resistance usually in the recessive mode of inheritance. Large deletions of the gene often predict the inactivation mechanism of the receptor, but especially if a point mutation is discovered, it is difficult to decipher from the small structural alteration the type of functional impact of the mutation. Functional analysis of synthesis, intracellular processing, and function of the mutated receptor protein in cell culture transfections is therefore needed to prove its functional significance and to solve the molecular basis of the inactivation mechanism. A typical finding with an inactivating receptor in functional analysis is schematically presented in Fig. 1. Similar functional tests are available for nuclear receptors.

Mutations of the growth hormone receptor (GHR) provide good examples of the various mechanisms of receptor inactivation. More than 30 GHR mutations are currently known, mostly in the area encoding the extracellular receptor domain, including exonic deletions and nonsense, frameshift, splice-site, and missense mutations. As is well known, the phenotype of this condition is GH resistance (Laron dwarfism), which is usually inherited in an autosomal recessive manner. The various categories of GHR inactivation in this syndrome can be grouped according to their effects on circulating GH-binding protein (GHBP), which is a proteolytic cleavage product of the plasma membrane GHR. In some of the cases, the GHBP level is suppressed, as a sign of failure of the mutated GHR protein to bind GH or to be expressed at the cell membrane. In other types of this syndrome, the GHBP levels are either normal or elevated. One of them, with normal GHBP levels and a point mutation in the extracellular domain, was found to be caused by inactivated GHR due to impairment of expression and signaling. The third type of GHR mutation has increased GHBP levels, and in this case a point mutation resulted in alternative splicing and a premature stop codon, with the resulting receptor protein lacking the transmembrane domain and most of the intracellular domain. The truncated receptor was unable to anchor to the cell membrane, but was instead secreted in large amounts into the circulation, thus causing the elevated GHBP levels and lack of GH response. In addition, there are cases of GH insensitivity with normal GHR function, explained by abnormalities in postreceptor signaling mechanisms. A fourth mechanism of GHR inactivation, through a dominant negative mutation, will be discussed below.

Another example of inactivating receptor mutations is that of the TSHR. A number of mutations are known, and depending on the extent of receptor inactivation, their phenotypes vary greatly. The mildest cases represent euthyroidism with elevated TSH levels; the mutation lowers only the affinity of the TSHR-ligand complex, and higher TSH levels can maintain euthyroidism, since otherwise the receptor function is normal. In more severe cases, the affected individual is hypothyroid, and the mutation causes sequestration of the mutated receptor inside the cells and near total abolition of its function.

A third example of inactivating receptor mutations concerns those of the gonadotropins LH and FSH. The inactivating LHR mutation in the male causes, depending on the extent of receptor inactivation, an array of phenotypes ranging from micropenis and hypospadias to total lack of male sexual differentiation (XY, pseudohermaphroditism). The female phenotype is much milder, entailing only anovulatory infertility. In functional analyses of the LHR mutations there is a good correlation between the severity of phenotype and the completeness of receptor inactivation, as demonstrated by the variably suppressed functional responses to gonadotropin stimulation in cells expressing the various forms of mutated receptors. In most cases, the real mechanism of receptor inactivation, whether a defect in synthesis, intracellular processing, transport to the plasma membrane, ligand binding, or signal transduction, has not been studied.

As with the LHR, inactivating mutations of the FSHR show correlation between the severity of phenotype and the completeness of receptor activation. Complete receptor inactivation in women causes primary hypergonadotropic amenorrhea with impaired pubertal development, lack of follicular maturation, and infertility, whereas the phenotype of incomplete receptor activation is secondary amenorrhea with arrest of follicular maturation. In men, a surprisingly mild phenotype with variable derangement of spermatogenesis, but no obligatory infertility, is observed with complete FSHR inactivation. The impairment of function of the mutated FSHR is primarily due to sequestration of the synthesized receptor protein inside the cell. The few mutated receptors expressed at the plasma membrane of transfected cells seem to display a normal cAMP response to hormonal stimulation.

## IV. DOMINANT NEGATIVE MUTATIONS

The concept of dominant negative mutation means that a heterozygously inherited mutated receptor has the capability of blocking the action of functional receptors encoded by the wild-type allele. Abolition of hormone action occurs thus even though only one allele is mutated, and therefore, the mode of inheritance is dominant despite a loss-of-function mutation. This type of inactivation mechanism is encountered with receptors that need to be homo- or heterodimerized in order to achieve their biological effects. The different modes in which a dominant negative mutation brings about inactivation of the wild-type receptor are presented in Fig. 2.

In thyroid hormone resistance, typical loss-of-function mutations with recessive inheritance are rare. Usually, the mutation causes receptor inactivation in a dominant negative fashion. In most cases, the patients have an inactivating mutation in one of their thyroid hormone receptor (TR) β alleles. The mutated receptor interferes negatively with functional receptor proteins encoded by the two normal TRα alleles and one TRβ allele. Unliganded TR is bound as a homodimer, or as a heterodimer with RXR, to the thyroid hormone response element of a target gene and is associated with a complex of corepressor proteins, being transcriptionally inactive. Mutations usually abolish ligand-binding activity

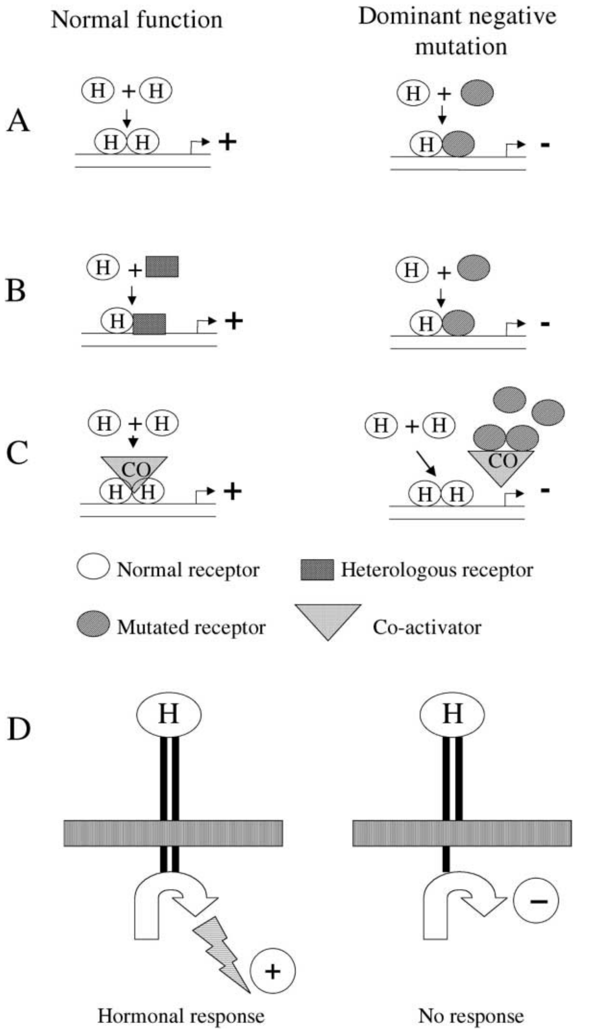

FIGURE 2 Models for mechanisms of receptor inactivation by dominant negative mutations of nuclear (A–C) and cell membrane (D) receptors. In each case, this type of receptor inactivation requires that the wild-type receptor forms homo- or heterodimers in order to exert its biological action.

of the TR. Normally, ligand binding promotes replacement of co-repressor with co-activators, which results in receptor activation. Since the mutated receptor dimerizes with wild-type TR, it keeps the complexes transcriptionally inactive, explaining the dominant negative effect. Mutated TR may also make stable dimers with RXR at the TR recognition site, thus blocking the binding of wild-type TR, needed for activation of the heterodimer.

In retinoic acid receptors, chromosomal rearrangements and other types of mutations have been described in connection with various malignancies; some of the mutated genes function in dominant negative fashion and some in gain-of-function fashion.

Dominant negative mutations can explain hormone resistance in the molecular pathogenesis of a rare syndrome with insulin resistance, acanthosis nigricans and polycystic ovary syndrome (type A insulin resistance). Another example of a dominant negative mutation in cell membrane receptors concerns GHR. In this case, a particular single base substitution can cause skipping of exon 9 of the GHR upon transcription with consequent formation of a receptor without the intracellular domain, which is an essential part for signal transduction. The mutated receptor was unable to transmit signal, but it effectively heterodimerized with a wild-type receptor to form dimers devoid of signal transduction capability (Fig. 2D).

## V. SUMMARY

The hormone receptor mutations can be divided in two categories: (1) the constitutively activating mutations that cause inappropriate activation of a hormonal effect in the absence of ligand hormone and (2) the inactivating mutations that are the most common cause of hormone resistance syndromes. Mutations have already been detected in a number of hormone receptor genes, and new mutations are expected to be found. However, it should be kept in mind that the receptor mutations may represent only the tip of the iceberg. All genes encoding the various components of a hormonal regulatory cascade, from synthesis of ligand to specific cellular responses downstream of receptor activation, are prone to mutations. Conditions with a clear phenotype of activating or inactivating receptor mutation, but intact receptor function, are candidates for mutations of the other genes involved in hormone action. Due to the large number of such candidate genes it will be a long time before all aspects of the molecular pathogenesis of inappropriate hormone action and hormone resistance are fully understood.

## Glossary

**activating (gain-of-function) mutation** Structural alteration of the receptor makes it constitutively activated, i.e., in the absence of ligand hormone. Activating mutation can also increase the receptor's affinity for the ligand hormone, alter its binding specificity, or permit new functions.

**cell membrane receptor** A hormone-binding transmembrane protein. Binding of the ligand hormone triggers a cascade of responses: conformational change of the receptor → activation of the intracellular second-messenger response(s) → secondary intracellular response(s) → functional target cell response(s) to hormone stimulation.

**dominant negative mutation** A mutation in a receptor that must be dimerized to evoke the functional response. When the mutated receptor dimerizes with wild-type receptor, the heterodimer remains functionally inactive.

**hormone resistance** Disease condition in which a specific hormone is unable to exert its biological actions; it is usually caused by an inactivating receptor mutation.

**inactivating (loss-of-function) mutation** A mutation that inactivates the function of a receptor by one of the following mechanisms: decreased synthesis, aberrant intracellular processing, impaired or missing ligand binding, impaired or missing signal transduction, inability to anchor to plasma membrane (cell membrane receptors), inability to dimerize (if needed for action), or increased degradation.

**nuclear receptor** A hormone receptor present inside the cell, in either the cytoplasm or the nucleus, binding hormones that are able to pass through the cell membrane (e.g., steroid and thyroid hormones). The hormone-receptor complex binds in dimerized form to specific promoter sequences of hormone-responsive genes, thus functioning as a transcription factor.

## See Also the Following Articles

**Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Resistance • Luteinizing Hormone Receptor Signaling • Membrane Receptor Signaling in Health and Disease • Receptor–Receptor Interactions • Signaling Pathways, Interaction of • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations**

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# Activin Receptor Signaling

Peter C. K. Leung[*] and Chun Peng[†]

[*]*University of British Columbia, Vancouver* • [†]*York University, Toronto*

I. ACTIVIN RECEPTORS
II. SMADS AS INTRACELLULAR MEDIATORS
III. MODULATION OF ACTIVIN SIGNALING
IV. SUMMARY

**Activins are dimeric proteins consisting of two inhibin β-subunits. Homo- and heterodimerization of two isoforms of β subunits, βA and βB, result in three forms of activins, activin-A, -B, and -AB. It is now recognized that activins function mainly as autocrine/paracrine factors to regulate the proliferation, differentiation, and apoptosis of many types of cells and are involved in a variety of physiological processes. Activins also play important roles during embryonic development. Activins elicit their biological effects through interaction with a receptor complex that contains two types of structurally related transmembrane serine/threonine kinases referred to as type I and type II receptors, respectively. The signal is then propagated from the receptors at the cell surface to target genes in the nucleus by intracellular mediators, the Smad family of proteins. This signaling cascade can be modulated at the cell surface by interfering with the interaction between activins and their receptors and in the cytoplasm by facilitating or blocking the activation**

response(s) → functional target cell response(s) to hormone stimulation.

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**of Smads. Finally, activin signaling can be terminated through ubiquitin-mediated degradation of Smads.**

Activins share structural similarities with transforming growth factor-β (TGF-β) and are recognized as members of the TGF-β superfamily. In addition to activins and TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors, inhibins, Müllerian inhibiting hormone, glial cell line-derived neurotrophic factors, and Nodal are also members of this superfamily.

Originally, activin-A was isolated from porcine follicular fluids and identified as a stimulator of pituitary follicle-stimulating hormone (FSH). This molecule was found to be identical to a protein that exhibited potent differentiating effects on erythroleukemia cells and was designated erythroid differentiation factor.

## I. ACTIVIN RECEPTORS

Initial affinity labeling and cross-linking studies using activin-A as a ligand revealed the presence of two activin receptor complexes with molecular weights of approximately 65 and 85 kDa, designated type I and type II activin receptor complexes, respectively. Subsequently, cDNAs and genes for type I and type II activin receptors were cloned. Receptors that can bind to activin in transfection assays include activin type I (ActR-I, also known as ActR-IA or activin receptor-like kinase 2, ALK-2), ActR-IB (or ALK-4), ActR-II (also named ActR-IIA), and ActR-IIB. However, as discussed below, these receptors can also bind to and mediate the actions of other ligands of the TGF-β superfamily.

### A. Type II Receptors

Using an expression cloning strategy, in 1991 Mathwes and Vale cloned the first activin receptor from AtT20 mouse corticotropic cells. The cDNA encodes a protein of 513 amino acids. The mature peptide has 494 amino acids and contains an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular region containing a juxtamembrane region, a kinase domain predicted to be a serine/threonine kinase, and a C-terminal tail (Fig. 1). Based on its ability to bind activin and its size, the receptor was identified as the type II activin receptor and is now referred to as ActR-IIA. Another activin receptor that showed a structure similar to that of ActR-IIA was subsequently cloned and designated ActR-IIB. The human ActRII-A gene is mapped to 2q22.2–q23.3 while the ActR-IIB gene is located in 3p22. When type II receptors are expressed alone in mammalian cells, they are able to bind to activins with high affinity. The $K_d$ value of activin type II receptors ranged from 0.1 to 0.7 nM.

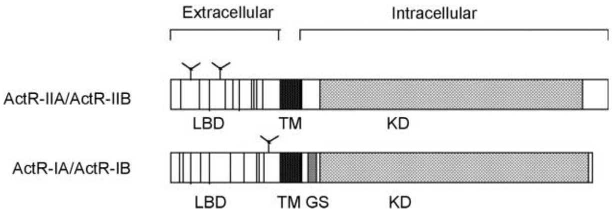

FIGURE 1 Schematic representation of activin receptors. Both type I and type II receptors have an extracellular region where the ligand-binding domain (LBD) is located, a transmembrane (TM) region, and an intracellular region containing a juxtamembrane region, a serine/threonine kinase domain (KD), and a C-terminal tail. The LBD has 10 cysteine residues (indicated by vertical lines) and one or two glycosylation sites (Y). Type I receptors have a GS domain in the juxtamembrane region and a shorter C-terminal tail.

The extracellular domain of activin type II receptors has two N-glycosylation sites and 10 cysteine residues (Fig. 1). The spacing between each cysteine is identical between ActR-IIA and ActR-IIB and is conserved in different groups of vertebrates. Studies on the ActR-IIA have shown that deglycosylation of the receptor resulted in only a moderate decrease in receptor affinity, suggesting that it is not critical for receptor binding. On the other hand, disulfide bonds formed by cysteine residues are important in maintaining the proper confirmation required for activin binding. In ActR-IIA, five disulfide bonds are formed from the cysteines in the following arrangement: C1–C3, C2–C4, C5–C8, C6–C7, and C9–C10. The crystal structure of the extracellular ligand-binding domain has been solved. This region exhibits a three-finger toxin-like fold formed by seven disulfide-cross-linked β sheets. Further analyses have revealed that three conserved hydrophobic residues (Phe42, Trp60, and Phe83) interact with one another to form a binding site on ActR-IIA for activin-A.

The kinase domain of ActR-IIA and ActR-IIB contains sequence similar to that of serine/threonine protein kinases. Studies have shown that they indeed have kinase activities. Substrates of type II receptor kinases include the receptors themselves and type I activin receptors. Type II receptors are constitutively phosphorylated, even in the absence of ligands. The phosphorylation of type II receptors is, at least in part, the result of autophosphorylation. The phosphorylation occurs predominantly on serine residues and to some extent on threonine.

Gene knock-out studies in mice have shown that deletion of the ActR-IIA gene resulted in severe suppression of FSH secretion and impaired reproductive functions. Female mice showed defects in folliculogenesis and were infertile. Male mice showed decreased sperm production and delayed fertility. These findings demonstrate that ActR-IIA is a functional receptor that mediates the action of activin on FSH secretion. On the other hand, the ActR-IIA-deficient mice had several developmental defects that are quite distinct from those found in activin-deficient mice, suggesting that other ligands, rather than activins, signal through ActR-IIA to regulate embryonic development. Targeted disruption of the mouse ActR-IIB gene caused abnormal left–right axis formation and lateral asymmetry. Mice deficient in the ActR-IIB gene died shortly after birth due to complicated cardiac defects. The developmental abnormality of axial formation observed in ActR-IIB knockout mice is a characteristic nodal activity, suggesting that ActR-IIB mediates Nodal signaling during embryonic development.

Type II activin receptors have a broad specificity. In addition to binding to activins, they have been shown to bind to BMPs, such as BMP2 and BMP7, and Nodal when co-expressed with appropriate type I receptors. Studies using truncated ActR-IIA and IIB have shown that these receptors mediate both activin and BMP signaling. As discussed above, gene knock-out experiments also support the notion that other ligands signal through type II activin receptors to regulate vertebrate embryonic development.

### B. Type I Receptors

Following the cloning of type II receptors, the sequences of type I activin receptors were also obtained. Similar to type II receptors, the type I receptors also have an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain. The extracellular domains of type I receptors also have 10 cysteine residues; however, the spacing among these residues is different between ActR-IA and ActR-IB and also differs from that in the type II activin receptors. A unique feature of the type I receptors is that they contain a segment of about 30 residues that is rich in serine, threonine, and glycine, located between the transmembrane and kinase domains. This segment has a characteristic sequence of SGSGSG and therefore was named the GS domain. The GS domain is present in all type I receptors of the TGF-β superfamily. The C-terminal tail in the type I receptors is shorter than that in the type II receptors. The human ActR-IA is mapped to 2q23–q24 and the ActR-IB gene is located at 12q13.

Unlike type II receptors, type I receptors do not bind to activins on their own. They can bind to ligands only in the presence of activin type II receptors. On the other hand, type I receptors are essential for receptor signaling to downstream mediators. It is well established now that activins first bind to type II receptors, which then recruit type I receptors to form a complex that contains two or more of each of the type I and type II receptors. Type II receptors subsequently phosphorylate type I receptors, primarily, but not exclusively, at the GS domain, which then in turn propagate the signals to downstream targets, namely, Smads.

Although initial studies demonstrated that ActR-IA, when co-expressed with type II activin receptors, can bind to activins, increasing evidence has indicated that the physiological ligand for this receptor is not an activin. In *Xenopus laevis* embryos, ActR-IA mimics the role of BMP-4, but not activins. Many other reports have documented that ActR-IA binds to and/or mediates the signaling of other members of the TGF-β superfamily, including Müllerian inhibiting substance, BMP-2, BMP-4, and BMP-7. Also, ActR-IA activates Smad1, which is known to mediate BMP, but not activin, signaling. On the other hand, the role of ActR-IB in activin signaling has been confirmed. ActR-IB mediates the growth-inhibitory effects of activin in several cell lines. Also, the regulatory effects of activins on target gene expression, such as plasminogen activator inhibitor-1, are conferred by ActR-IB.

## II. SMADS AS INTRACELLULAR MEDIATORS

Once type I receptors are phosphorylated by type II receptors, they become active kinases and can phosphorylate downstream signaling molecules. Smad proteins are intracellular mediators of the TGF-β superfamily. They function as effector molecules to transduce the signals of the TGF-β superfamily from the cell surface to the nucleus. The first member of the Smad family was discovered in *Drosophila melanogaster* through genetic screenings and was named Mad (mother against *dpp*). A related protein was also identified in *Caenorhabditis elegans* and is referred to as Sma. Subsequently, homologues of Mad and Sma were found in various vertebrates and are now called Smads.

## A. Structural Features of Smads

To date, eight Smads have been identified in vertebrates and they are classified into three subfamilies based on their structural and functional characteristics: receptor-regulated Smads (R-Smads), common Smads (Co-Smads), and inhibitory Smads (I-Smads). The R-Smads, Smad1, 2, 3, 5, and 8, are activated by specific type I receptors of the TGF-β superfamily through phosphorylation. Smad4, also referred to as "deleted in pancreatic carcinoma" (DPC4), is the only Co-Smad in vertebrates and can form a complex with phosphorylated R-Smads. The I-Smads (Smad6 and 7) function as inhibitors of the receptor-regulated Smads. Figure 2 shows the major structural features of Smads. In R-Smads and Co-Smads, there are two highly conserved regions, MH1 and MH2. These two domains, located at the N-and C-terminals, respectively, are linked by a less conserved linker region. The MH1 domain is much shorter in the inhibitory Smads than in the R-Smads and Smad4. R-Smads also have a characteristic phosphorylation site, SSXS, at their C-termini. All Smads lack intrinsic enzyme activity. They function primarily through regulation of protein–protein and protein–DNA interactions.

Smads that have been identified as being involved in activin receptor signaling include Smad2, 3, 4, and 7. This set of Smads also mediates TGF-β signal transduction. In human, genes for Smad2, Smad4, and Smad7 are all mapped to the region 18q21.1, and the Smad3 gene is found at 15q21–q22. The MH1 domain of Smad3 can bind to DNA directly, but the MH1 domain of Smad2 lacks DNA-binding activity. This is apparently due to an insertion of 30 residues in the MH1 domain of the Smad2. This insertion is thought to alter the conformation of the MH1 domain, thereby preventing the direct interaction of Smad2 with DNA. The MH1 domain of Smad4 also has DNA-binding activity. The MH2 domain of Smad2, Smad3, Smad4, and Smad7 is mainly responsible for protein–protein interactions. Proteins that are found to associate with this region include Smad themselves to form oligomers and many transcription factors. In the case of Smad2 and Smad3, this domain also contains phosphorylation sites for ActR-IB and a binding site for a Smad anchor for receptor activation (SARA), which recruits Smad2 and 3 to activin and TGF-β receptors. The linker region, although poorly conserved, also has important functions. This region is known to contain the phosphorylation sites for mitogen-activated protein kinases. The linker region of Smad4 has a Smad activation domain that is important for transcriptional activation.

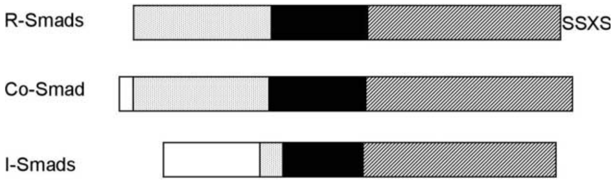

**FIGURE 2** Structural features of Smads. R-Smads and Co-Smad (Smad4) have two conserved domains, MH1 (gray) and MH2 (vertical rules), connected by a linker region (black). I-Smads have a much shorter MH1 domain.

## B. Signal Transduction via Smads

Upon activin binding, Smad2 and (or) Smad3 are transiently associated with activin receptor complexes. Both Smad2 and Smad3 can be phosphorylated by ActR-IB. Subsequently, the activated Smad2 and (or) Smad3 form a complex with Smad4 and are translocated into the nucleus. This Smad complex then interacts with DNA to regulate target gene expression. Although the Smad complex can bind to DNA directly, the affinity and specificity of such binding are low and therefore DNA-binding partners are required for regulation of specific target gene expression. The best-characterized transcription factor that interacts with Smads to regulate activin-induced transcription is the forkhead activin signal transducer (FAST), a winged helix forkhead transcription factor. Activin induces the transcription of homeobox genes *Mix*. 2 in *Xenopus* and *goodecoid* in mouse during embryonic development. Activation of these genes by activin involves the formation of a transcription complex comprising Smad2, Smad4, and FAST. The protein complex binds to specific elements in the promoter regions of these genes through both FAST- and Smad-binding sites. Following the binding of Smad and DNA-binding partners to the DNA, Smads can further recruit transcription co-activators or co-repressors to positively or negatively regulate gene expression.

# III. MODULATION OF ACTIVIN SIGNALING

## A. Inhibition of Signaling at the Cell Surface

Activin signaling can be blocked at the receptor level by two types of molecules, follistatins and inhibins. Follistatins are binding proteins of activins that neutralize activin actions in many biological systems by controlling the accessibility of activins to their receptors. There are two major forms of follistatins, FS-288 and FS-315, generated from alternative splicing of the same gene. Each β-subunit can bind to one follistatin and therefore, the activin–follistatin complex consists of one activin molecule and two follistatin molecules. The affinity of follistatin binding to activins is similar to that between activins and their receptors. Follistatins neutralize activin bioactivity by inhibiting the binding of activins to their type II receptors. In addition, FS-288 inhibits activin action by increasing the endocytotic degradation of activin through the association with cell surface heparan sulfate.

Inhibins are structurally related to activins by sharing the same β-subunit. It is well documented that inhibins block many actions of activins and function as activin antagonists. Several studies have shown that inhibins can bind to ActR-IIA and, therefore, antagonize activin actions. The binding affinity of inhibins to ActR-IIA is 10-fold lower than that between activins and ActR-IIA; however, it has been recently shown that betaglycan, a proposed TGF-β type III receptor, can bind to inhibins with high affinity and that the binding of inhibins to betaglycan results in an increased affinity of inhibin binding to ActR-IIA. It was therefore suggested that inhibins bind to betaglycan and ActR-IIA and block activin signaling by competing with activins for their type II receptors.

## B. Modulation in Cytoplasm

The interaction between activin receptors and Smads in the cytoplasm is facilitated by some proteins with a FYVE (Fab1p, YOTB, VAC1p, EEAI) domain, such as SARA and Hgs. It has been shown that SARA and Hgs (Hrs) cooperate to recruit Samd2 and Smad3 to

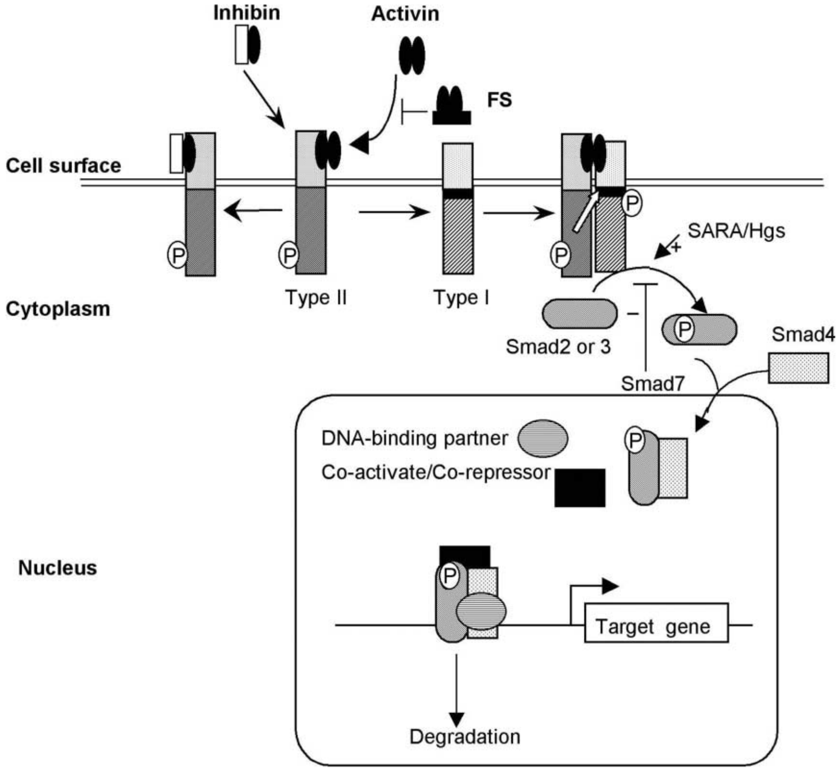

**FIGURE 3** Activin signaling via the Smad pathway. Activin first binds to the type II receptors, which in turn recruit and phosphorylate type I receptors. The type I receptors then phosphorylate Smad2 and/or Smad3. The phosphorylated R-Smad forms a complex with Smad4 and is then translocated to the nucleus. The R-Smad–Smad4 complex interacts with other DNA-binding co-factors to regulate gene expression. Follistatin inhibits activin signaling by binding to activin and making it unavailable to its receptor. Inhibin blocks activin signaling by competing with activin for type II receptors. SARA and Hgs enhance activin signaling by recruiting R-Smads to the activin receptors while Smad7 inhibits activin signaling by preventing phosphorylation of R-Smads by the activin receptor. Finally, Smad signaling may be turned off via ubiquitin-mediated degradation.

the activin receptor complex. Overexpression of SARA and Hgs enhances activin signaling by increasing the phosphorylation and activation of Smad2 and Smad3 by ActR-IB. On the other hand, overexpression of a nonfunctional mutant of SARA or Hag decreases activin signaling.

Smad7 inhibits activin signaling by binding stably to the activated receptor complex and thereby blocking the association of Smad2 and/or Smad3 with the receptors and preventing the phosphorylation and activation of R-Smads. Smad7 may also facilitate the degradation of the active receptor complex. Interestingly, the Smad7 gene is induced by activin. Therefore, Smad7 likely plays a role in the negative feedback control of activin signaling. Smad7 has been shown to attenuate the actions of activin on cell growth, apoptosis, and *Xenopus* embryonic development.

### C. Termination of Signaling in the Nucleus

The activated Smad2 has been shown to be degraded through the ubiquitin-dependent 26S proteasome pathway. Although the precise involvement of this pathway in activin signaling requires further studies, degradation of activated Smads in the nucleus could provide a mechanism to switch off activin signaling.

## IV. SUMMARY

Although activin receptors were discovered almost a decade ago, the signaling mechanisms underlying activin actions have been uncovered only in recent years. As shown in Fig. 3, activins bind to cell surface serine/threonine kinase receptors via two activation steps. First, activins bind to constitutively active type II receptors and this complex then recruits type I receptors. Second, type II receptors phosphorylate type I receptors at the GS domain. Following receptor activation, the activin signal is transmitted into the nucleus via intracellular mediators, Smads. The type I receptor, ActR-IB, phosphorylates Smad 2 and/or Smad 3. The phosphorylated Smad2 and (or) Smad3 then form(s) a complex with Smad4, and the complex enters the nucleus. The Smad complex then interacts with other transcription factors, such as FAST-1, and recruits transcription co-activators or co-repressors to regulate the expression of target genes. Activin signaling can be blocked at the receptor level by their binding proteins, follistatins, and by their antagonists, inhibins. In the cytoplasm, SARA and Hgs facilitate activin signaling by recruiting R-Smads to activin receptors while Smad7 inhibits activin signaling by blocking the interaction between R-Smads and receptors. Finally, Smad signaling can be terminated through the proteolytic degradation of Smads via the ubiquitin-dependent pathway.

## Glossary

**activins** Protein hormones belonging to the transforming growth factor-β superfamily that regulate many developmental and physiological processes, particularly reproduction. They are either homo- or heterodimers of two related inhibin β-subunits, βA and βB.

**receptor serine/threonine kinase** A membrane receptor that phosphorylates serines and/or threonines on target proteins. The receptor has an extracellular domain, a transmembrane region, and an intracellular kinase domain.

**Smad proteins** A group of intracellular proteins that mediate signaling by members of the TGF-β superfamily. They are vertebrate homologues of *Drosophila* Mad (mother against *dpp*) and *Caenorhabditis elegans* Sma. They include receptor-mediated Smads that are phosphorylated and activated by type I receptors, common Smads that form complexes with receptor-mediated Smads, and inhibitory Smads that antagonize signaling by members of the TGF-β superfamily.

**type I activin receptor** A component of the activin receptor complex. It is a serine/threonine kinase that phosphorylates intracellular mediators after being phosphorylated by a type II activin receptor.

**type II activin receptor** A component of the activin receptor complex. It binds to activins and in turn phosphorylates type I receptors via its intracellular serine/threonine kinase domain.

## See Also the Following Articles

**Activins • Angiotensin II Receptor Signaling • Follicle Stimulating Hormone (FSH) • Inhibin Receptor Signaling • Inhibins, Activins, and Follistatins • Luteinizing Hormone Receptor Signaling • Protein Kinases**

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# Activins

Gail P. Risbridger
*Monash University, Melbourne*

I. INTRODUCTION
II. EXPRESSION AND BIOSYNTHESIS OF ACTIVINS
III. SIGNALING OF ACTIVIN LIGANDS
IV. ACTIVINS IN PHYSIOLOGY
V. ACTIVINS IN PATHOLOGY AND DISEASE
VI. CLINICAL APPLICATIONS
VII. SUMMARY

**Activins, acting in opposition to inhibins, are involved in diverse physiological activities, including stimulating pituitary, hypothalamic, and gonadal hormones, insulin secretion, germ cell development and differentiation, erythroid differentiation, nerve cell survival, and embryonic development. Activin β-subunit types A–E form homodimers and heterodimers that play different roles in health and disease in different tissues throughout the body. Assays that allow comparisons of specific subunit activities are necessary to understand the complex roles of each of the activin subunits.**

## I. INTRODUCTION

Activin subunits are present in numerous human tissues of both endocrine and nonendocrine organs. Homo- or heterodimers of activin $\beta_A$ or $\beta_B$ subunits are members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors. Since these proteins were first described as regulators of the release of follicle-stimulating hormone (FSH) from the pituitary, multiple actions have been assigned to them in a variety of tissues. An additional three activin β-subunit proteins have been described, i.e., $\beta_C$, $\beta_D$, and $\beta_E$, and there is a growing family of recognized activin- and inhibin-binding proteins, receptors, and signaling molecules (Table 1).

## II. EXPRESSION AND BIOSYNTHESIS OF ACTIVINS

The TGF-β superfamily of growth factors includes bone morphogenetic proteins (BMPs) and Müllerian inhibitory substance (MIS), and currently over 45 members of this family have been identified. Structural similarities between activins and other members of the TGF-β superfamily are based on the conservation of the number and spacing of the cysteines within each subunit and the disulfide linkages between the two subunits that form the characteristic cysteine knots. Other similarities relate to dimer formation, the location of the bioactive peptide in the carboxy-terminal region of the precursor molecule, and the similarities in intracellular signaling mechanisms.

The biosynthesis of activins requires the formation of homo- or heterodimers of disulfide-linked $\beta_A$- or $\beta_B$-subunit proteins that share 63% amino acid identity. The assembly of different combinations of activin subunits $\beta_A$ and $\beta_B$ is reflected in their nomenclature; thus, activin A is a dimer composed of $\beta_A\beta_A$, activin B is composed of $\beta_B\beta_B$, and activin AB is composed of $\beta_A\beta_B$. There is an independent pattern of synthesis of the two subunit genes, which are located on different chromosomes; the activin $\beta_A$-subunit gene is located on 7p15–7p14 and the activin $\beta_B$-subunit gene on 2qcen–2q13.

Another subset of activin β-subunits ($\beta_C$, $\beta_D$, and $\beta_E$) was identified more recently, based on their homology to subunits $\beta_A$ and $\beta_B$; this subset of subunits shares 62% amino acid identity with each other. The activin $\beta_C$-subunit shows close similarity to the activin $\beta_E$-subunit in terms of genomic organization and chromosomal localization on 12q13.1, and subunits $\beta_C$, $\beta_D$, and $\beta_E$ are considered to be a separate subset of activins. The $\beta_C$-subunit dimerizes with itself *in vitro* and with subunits $\beta_A$ and $\beta_B$ to form the putative activins, activin C, AC, and BC. Activin subunits $\beta_D$ and $\beta_E$ have been isolated from *Xenopus* and mouse cDNA libraries, respectively, but their capacity to form activin heterodimers

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TABLE 1 Activin Ligands: Purified Homo- or Heterodimers of Activin β-Subunit Proteins

| Subgroups of activin β-subunits | Purified proteins | Evidence of putative dimeric proteins |
|---|---|---|
| $\beta_A$ | Activin A[a] | — |
| $\beta_B$ | Activin B[a], activin AB[a] | — |
| $\beta_C$ | Activin C[b] | Activin AC[c], activin BC[c] |
| $\beta_D$ | ? | — |
| $\beta_E$ | Monomeric activin $\beta_E$ | — |

[a] Bioactive and detected in biological fluids.
[b] No known bioactivity.
[c] Heterodimeric proteins formed *in vitro*; currently there is no evidence for their existence *in vivo*.

with subunits $\beta_A$ and $\beta_B$ or between themselves is unknown.

Homo- and heterodimers of activin subunits $\beta_A$ and $\beta_B$ were originally isolated from follicular fluid as inactive full-length precursor proteins. Similar to other growth and differentiation factors, the precursor activins require intracellular processing for full bioactivity. Precursor activins are 110-kDa dimers and consist of a signal peptide, a glycosylated prodomain (Pro-β), and a mature C-terminal domain (β) that is bioactive. Proteolytic cleavage releases the pro and signal peptides, resulting in mature, unglycosylated activin subunits.

Dimers of the activin β-subunits form activin ligands, but heterodimers of activin β-subunits with the inhibin α-subunit result in the formation of inhibin proteins (inhibin A or B). The inhibin α-subunit gene is located on 2q33–q36 and the pattern of expression of the inhibin α-subunit is independent of the activin β-subunits. As is the case for activins, the inhibins are synthesized as precursor proteins and proteolytic cleavage occurs intracellularly as well as in serum.

## III. SIGNALING OF ACTIVIN LIGANDS

The high degree of homology between the ligands extends to the elements in their signaling pathways. At the cell surface, activin ligands interact with a dual receptor system involving a family of transmembrane serine/threonine kinase receptors classified as type I or type II receptors. Activin binding to the type II receptor (ActRII) leads to the recruitment of the type I receptor (ActRI) and formation of a heteromeric complex. Formation of this complex induces phosphorylation of the type I receptor, leading to activation of the receptor-regulated Smad (R-Smad). R-Smads are ligand specific, with Smad2 and Smad3 mediating activin and TGF-β signaling and Smad1, Smad5, and Smad8 mediating BMP signaling. The interaction between the R-Smads and the receptor complex involves a membrane-bound protein named Smad anchor for receptor activation (SARA). After phosphorylation, the R-Smads are released and form heteromeric complexes with Smad4, a common mediator (Co-Smad). The R-Smad and Co-Smad complexes then translocate to the nucleus to regulate gene expression. A third class of Smads, the inhibitory Smads (I-Smads; Smad6 and Smad7), antagonize the signaling events just described and prevent access and phosphorylation of the R-Smads or interfere with the formation of the R-Smad/ Co-Smad complexes.

In the nucleus, Smads target specific gene promoters with low binding affinity for DNA, and Smad binding alone is insufficient for gene activation. Smads use members of the forkhead activin signal transducer (FAST; also called FoxH1) family as DNA-binding partners to regulate gene transcription. Other transcription factors likely to be involved in the Smad pathway include *fos*, *jun*, and the vitamin D receptor. However, in contrast to FAST/FoxH1, many of the other DNA binding partners can function independently of Smads, whereas FAST/FoxH1 target genes require Smad function for transcriptional activation. Thus, the binding of activin ligands to the membrane-bound receptor initiates a cascade of protein–protein interactions that controls gene expression and specific biological responses.

## IV. ACTIVINS IN PHYSIOLOGY

### A. Biological Actions of Activins

Activins were originally isolated based on the stimulation of FSH production and secretion by rat pituitary cells *in vitro*. It is now recognized that activins have a range of biological activities that include, but are not limited to, mesoderm induction in *Xenopus laevis* embryos, reproduction through the regulation of pituitary FSH production, bone growth, nerve cell survival, wound healing, and tissue differentiation in pancreas, kidney, and heart.

In some instances, the actions of activins are antagonized by inhibins. The most well-known opposing actions of activin and inhibin relate to effects on the reproductive system, including the regulation of pituitary FSH and gonadal steroidogenesis. Both ligands also oppose each other during chondrogenesis in chick limb bud mesoderm and in T-cell proliferation. However, there are other instances when the effects of activins are not opposed by inhibin, such as during mesoderm induction, in neuronal cell survival, and in some developmental processes. In these circumstances, other factors, such as activin-binding proteins (e.g., follistatins) are powerful antagonists of activins. In general, inhibins are regarded as endocrine factors that function primarily in the regulation of FSH, whereas activins are local paracrine and/or autocrine growth factors.

As predicted by their wide pattern of expression throughout the body and during all stages of life, the bioactivities of activins have been recorded in many cells and tissues (Table 2). *In vitro* model systems of cell growth (using cell lines and organ cultures) are common means to demonstrate significant effects that involve proliferative or antiproliferative actions, as well as those that alter cell differentiation. Most studies have been performed with activin A, less with activin B, and even fewer have reported the actions of activin AB; there are no reports of bioactivity of other putative activin dimers or heterodimers. Many of the effects of activins *in vivo* are inferred by expression of subunit mRNA or protein; due to the limited supply of purified ligands, very few studies have directly demonstrated *in vivo* actions of dimeric ligands. However, significant progress in understanding the roles for activins has been made using other approaches. Knockout and knock-in mouse models have been critical in the evaluation of the functional effects of activins, and many of these were developed in the Matzuk laboratory. As well, the development of specific immunoassays for the different forms of activins, follistatins, and inhibins has been essential in evaluating the expression and roles for specific ligands or binding proteins in physiology and in disease.

## B. Knockout and Knock-in Mouse Models

A number of activin β-subunit knockout mice that exhibit unique phenotypes have been generated (Table 3). These studies confirm an essential role for activins in normal development of reproductive organs as well as other tissues. Activins are mesoderm-inducing factors, but mice deficient in subunits $\beta_A$ or $\beta_B$ develop to term. Activin $\beta_A$-subunit-deficient mice lack whiskers and lower incisors, have defects in their secondary palates, and die within 24 h of birth. Mice deficient in activin $\beta_B$ are viable and fertile but have defects in eyelid development. The defects are additive in mice deficient in both subunits $\beta_A$ and $\beta_B$ and suggest that the subunits do not have overlapping functions up to this age of development. To determine if the unique phenotypes of these mice are related to differences in the temporal and spatial expression of the two subunits or to specific ligand–receptor signaling interactions, mice were generated in which activin $\beta_B$ was expressed in the spatial and temporal pattern of activin $\beta_A$ (i.e., activin $\beta_B$ knock-in to the activin $\beta_A$ locus). The craniofacial malformations

**TABLE 2 Examples of Biological Fluids and Tissues in Which Activins A or AB or Follistatins Have Been Detected or Measured**

| Protein | Fluid or tissue |
|---|---|
| Activin A | Sera, e.g., from pubertal boys and girls, women with pre-eclampsia, or patients with chronic hepatitis<br>Extracts, e.g., from gonadal, placental, choriodecidual, and allantoic tissues or fluids<br>Media from cell lines, e.g., human prostate tumor cells, gonadotropin-releasing hormone-secreting neurons, and pituitary-derived folliculo-stellate cells<br>Fluids, e.g., follicular fluid, seminal plasma, and amniotic fluid in normal and Down syndrome pregnancies |
| Activin AB | Follicular fluid from human, bovine, ovine, and porcine tissues<br>Media from granulosa cells |
| Follistatin | Sera from pubertal girls, pre-eclamptic pregnancies, hypertensive pregnant women, postmenopausal women with epithelial ovarian cancer, and patients with chronic hepatitis<br>Extracts from amnion, choriodecidual, and placental tissues<br>Supernatants from prostate tumor cell line cells<br>Amniotic fluid in normal and Down syndrome pregnancies |

**TABLE 3 Summary of Some of the Transgenic Mouse Model Phenotypes Bearing Modifications to Activin Subunits or Intermediates in Activin Signaling**

| Genetic modification | Phenotype |
|---|---|
| $\beta_A$ knockout | Craniofacial defects, e.g., lack whiskers and lower incisors; cleft palate; die $<$ 24 h after birth |
| Overexpression of $\beta_A$ | Sterility in male and females; testicular degeneration, but no testicular tumors |
| $\beta_B$ knockout | Viable and fertile but defects in eyelid development |
| $\beta_A$ and $\beta_B$ combined knockout | Additive defects; mice develop to term with defects in whiskers, incisors, and secondary palate formation |
| $\beta_B$ knock-in to $\beta_A$ locus | Rescues craniofacial malformations and neonatal lethality of $\beta_A$ deficiency, but defects in hair, gonads, and external genitalia |
| Activin type II receptor knockout | Gonadal defects and altered fertility; some mice die due to mandible defects |
| Follistatin knockout | Craniofacial defects, growth retardation, and skin defects; neonatal lethal |
| Follistatin overexpression | Defects in testes, ovary, and hair |
| Inhibin $\alpha$ knockout | Gonadal and adrenal tumors develop, followed by cachexia-like wasting syndrome |
| Inhibin $\alpha$ + activin type II receptor knockout | Tumors develop, but without cachexia |
| Inhibin $\alpha$ + overexpression of follistatin | Reduced rate of tumor development and reduced symptoms of cachexia |

and neonatal lethality were rescued and the results implied that activin subunits signaled through the same receptors in these tissues; further analyses revealed a greater level of complexity. In older mice, during testicular growth, $_B$-subunit does not replace the $_A$-subunit function and the knock-in mice show delayed testicular maturation and onset of fertility. Similarly, there are other defects in hair, ovarian growth, development of external genitalia, and somatic growth. Thus, although some actions of the $_A$ subunit are replaced by $_B$, others are not and the importance of the spatial and temporal patterns of expression of the subunits has been confirmed. The success of the approach of generating null mice for functional analyses of the activin subunits is less informative for the other activin subunits, $_C$ and $_E$. Mice lacking these subunits alone or in combination are viable and have no obvious abnormalities. Unequivocal evidence for a functional role for activin homo- or heterodimers of $_C$ or $_E$ or combinations with the $_A$ or $_B$ subunits has yet to be revealed.

## C. Assays for Activins

The development of specific assays to measure the activin ligands in biological fluids and tissues, including serum, has significantly increased our understanding of the role of these ligands. Many, but not all, of the assays were developed in the Groome laboratory. The activin A assay measures total activin A, i.e., bound and unbound forms of the ligand, and is used to measure activin A in sera and other biological fluids (e.g., follicular fluid) in a number of species, including humans. A specific assay for activin B is unavailable, but an enzyme-linked immunosorbent assay (ELISA) for activin AB detects the homodimer in some biological fluids. A specific antibody for subunit $\beta_C$ has been developed by Mellor *et al.*, who have also shown that $\beta_C$ dimerizes with subunit $\beta_A$ or $\beta_B$. The field awaits the development of new activin assays to expand our understanding of the role and expression of the different activin ligands in diverse tissues and systems. By analogy, the use of specific assays for inhibin A and B has been fundamental to understanding the roles of the inhibins in men and women, particularly in the field of reproductive physiology.

## D. Overlapping Functions of Activins and Other Ligands in the TGF-β Superfamily

The relative redundancy between the ligands relates to the demonstration that $\beta_B$ can replace $\beta_A$ in some circumstances. The degree of overlap in functions of subunits that are less similar to subunits $\beta_A$ and $\beta_B$ is unknown, although activin subunit $\beta_C$ can dimerize with $\beta_A$ and $\beta_B$ *in vitro*. The degree of overlap in the functions of activins and other members of the

TGF-β family is also unclear. TGF-β and activins signal through type I and type II receptors and utilize Smad2 and Smad3, but it is uncertain if the biological responses to the ligands are the same or different. Apparently, not all of the actions of activins are mimicked exactly by TGF-β or vice versa. For example, separate studies have shown that TGF-β inhibits cell proliferation in human prostate tumor cell lines PC3 and DU145, whereas the same cell lines fail to respond to activin A. Conversely, the LNCaP androgen-dependent human prostate tumor cells are growth inhibited by activin A, but are unresponsive to TGF-β. Relatively few studies have directly compared the ligands in the same biological systems, and the question of the redundancy between activins and TGF-β remains unexplored.

### E. Follistatin-Binding Proteins

Prior to any signaling event, activins can be bound to binding proteins such as follistatins (FSs). In many tissues, the interplay between activins and FSs provides a potent mechanism to regulate the access of the ligands to their receptors. Follistatins are encoded from a single gene located on chromosome 5q11.2 and alternative splicing of the mRNA generates different molecular weight isoforms. The FS proteins include an "FS domain" of 10-cysteine structures and an extracellular calcium-binding domain that makes them structurally homologous with epidermal growth factor (EGF), a group of enzyme inhibitors of the Kazal family, and other proteins such as SPARC, agrin, testican, and follistatin-related protein (FSRP). The function of the FS domains is not known but binding to activins differentiates follistatins from other members.

The biological activity of FS is mediated via its high-affinity binding to activins. The binding is nearly irreversible and prevents ligand access to receptors on the cell surface, making FS binding a critical regulator of activin bioactivity in cells in which FS is expressed. FS also contains a heparin-binding domain that permits high-affinity association with cell surface heparin-sulfated proteoglycans (HSPGs). When activins are bound to FS–HSPG complexes at the cell surface or in the extracellular matrix, access to the receptors is restricted and hence the paracrine actions of locally or systemically derived ligands are inhibited. As well as activins, FS binds and neutralizes the mesoderm-inducing actions of other members of the TGF-β superfamily, such as BMP-2, -4, and -7, although the affinity of FS for the BMPs is significantly lower than that for the activins.

## V. ACTIVINS IN PATHOLOGY AND DISEASE

The role and regulation of activin ligands in disease have received little attention in comparison to almost a decade of research into the use of inhibins for the detection of ovarian cancer or Down syndrome. Almost exclusively, the investigations center on activin A.

### A. Pregnancy

In pregnancy, activin A (as well as inhibin A) is a product of the healthy placenta, but changes that have been reported to occur in pathological conditions might lead to diagnostic applications. Follistatins are also consistently higher at all stages of pregnancy, suggesting that activin A is present in bound form during a healthy pregnancy. Higher levels of activin A have been reported in women with pre-eclampsia, gestational hypertension, and chronic hypertension. In established cases of pre-eclampsia, several groups have shown that activin A (and inhibin A) levels are ~10 times higher, but it is not clear that changes in activin A are useful to predict the onset of pre-eclampsia in asymptomatic women. Research continues to assess if activin A, in combination with other markers, may be useful. The normal elevation in activin A in late pregnancy is related to the onset of parturition, but might also be used for the detection of preterm labor. There is no evidence of the usefulness of any activins as markers for Down syndrome, similar to that reported for inhibin A.

### B. Inflammation

Activin A and FS have been implicated in inflammatory processes. In synovial fluid from patients with the inflammatory condition of rheumatoid arthritis, levels of activin A are elevated compared to those patients with degenerative osteoarthritis. An increased expression of activins related to change in $\beta_A$-subunit expression is implied in patients with inflammatory bowel diseases such as Crohn's disease and in ulcerative colitis. Indirect evidence for a role of activins has emerged from the detection of elevated levels of FS in patients with sepsis and meningitis, although activin levels in these conditions are unknown.

### C. Kidney/Renal Ischemia

Evidence for a role of activin A in renal regeneration after ischemic injury has emerged from animal studies. Activin A, produced in the tubules, delays tubular regeneration by inducing apoptosis, and FS antagonizes this effect. However, the interplay between activins and FS in the pathophysiology of renal diseases such as chronic glomerulonephritis remains unknown.

### D. Bone

The osteogenic actions of activin A in bone have been demonstrated *in vivo* and *in vitro*. In animal models, activin A promotes fracture healing, and the detection of significant levels of activin A in bone matrix indicates an involvement in bone resorption and formation. Thus, activins (as well as FS) have potential roles to play in osteoporosis.

### E. Tissue and Wound Repair

Mechanisms involved in tissue repair often closely resemble those involved in embryonic development. A number of studies have implicated activins in repair or regeneration of kidney, skin, and hair. During cutaneous wound repair in mice, activin A regulates formation of granulation tissue and extracellular matrix deposition. In animal models of acute brain injury, basic fibroblast growth factor (bFGF) is neuroprotective and neurotropic and induction of activin A appears to be a part of the signaling cascade for bFGF in this process. A potential role for activins in the prevention of neuronal loss during ischemic and traumatic brain injury in humans remains unknown.

### F. Cancer

Activin, like TGF-β, can inhibit or stimulate tumor cell growth. For example, activins have growth inhibitory effects on breast, liver, and prostate cancer cells and pituitary adenomas, whereas growth stimulatory actions are described in ovary and testis. The role of activins in cancers is unclear and may be different in different organs and tissues or may vary even within the same tissue type. In tissues in which the effects are growth inhibitory, it is relevant to ask if the tumors become resistant to activins due to mutations in the signaling pathway, but very little is known about mutations in activin receptors or signaling factors specific for activin in cancer, compared to TGF-β. In tissues in which growth-promoting effects of activins have been documented, the sustained synthesis of the ligands in malignancy may contribute to tumorigenesis due to other effects of activins, e.g., on immune suppression, tissue remodeling, and angiogenesis. Apart from local effects on tumor tissues, activins are implicated in the promotion of cachexia. Mutant mice bearing deletions of both the inhibin α-subunit and the ActRII receptor develop gonadal tumors, but cachexia is reduced relative to animals with a single gene deletion.

## VI. CLINICAL APPLICATIONS

In contrast to the assays for inhibins or the α-subunit, assays for activins remain to be validated for use as new diagnostic tools in clinical medicine.

## VII. SUMMARY

There is substantial evidence for a role for activins in normal physiological systems and in disease. However, much of the information about these ligands relates to activin A (not activin B or activin AB), and even less is known about the biological roles of the other subset of activins (C and E) or the putative ligands formed between the groups of subunits. The overlap or redundancy between activins and other members of the superfamily is largely unexplored and few studies have made direct comparisons of the different members of the superfamily. For example, the bioactivities of TGF-β and activin A or B are seldom compared in the same models, and the regulatory actions of binding proteins such as follistatins, which bind both activins and BMPs, are unknown. Development of new assays and purified ligands to study these ligands and direct comparisons of activin and other TGF-β family members using new mouse models and new systems will facilitate and promote our understanding of the wide and diverse roles for activins in biology and disease.

## Glossary

**bone morphogenetic protein (BMP)** Member of the transforming growth factor-β superfamily.

**follistatins (FS)** Activin-binding proteins.

**Müllerian inhibitory substance (MIS)** Member of the transforming growth factor-β superfamily.

**transforming growth factor-β (TGFβ)** Superfamily of growth and differentiation factors.

## See Also the Following Articles

**Activin Receptor Signaling • Anti-Müllerian Hormone • Bone Morphogenetic Proteins • Inhibins • Inhibins, Activins, and Follistatins**

## Further Reading

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# Adipokinetic Hormones and Carbohydrate Metabolism

Wil J. A. Van Marrewijk
*Utrecht University, The Netherlands*

I. CARBOHYDRATE MOBILIZATION
II. HORMONAL ACTIVATION OF GLYCOGEN PHOSPHORYLASE
III. HORMONAL SIGNAL TRANSDUCTION

**Insect adipokinetic hormones (AKHs) belong to a large AKH/red pigment-concentrating hormone family, whose members are involved in the mobilization of energy substrates for flight activity. This article provides a survey of the way in which adipokinetic and other related hormones play a key role in the mobilization of carbohydrate reserves as flight fuels. Special emphasis is put on the hormonal control of the enzymes involved and on the mechanism of intracellular transduction of the hormonal signals.**

## I. CARBOHYDRATE MOBILIZATION

Carbohydrate is the major fuel in insects that fly only short distances, such as flies and bees, and it also provides most of the energy for the initial period of flight in long-term flying insects, such as locusts. Reserves of carbohydrate are stored in the fat body in the form of glycogen, which upon flight is degraded by the action of the enzyme glycogen phosphorylase. Unlike glucose in vertebrates and most invertebrates, the carbohydrate transport form in insects is trehalose, a nonreducing disaccharide that contains two D-glucose residues. The mobilization of fat body glycogen and the subsequent release of trehalose into the hemolymph is controlled by adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) peptides, which are released by the corpus cardiacum upon the onset of flight. The involvement of these hormones in carbohydrate metabolism was demonstrated *in vivo* by the activation of fat body glycogen phosphorylase or by increases of trehalose in hemolymph after hormone injection and in fat body *in vitro* by activation of the enzyme or by increased trehalose release into the medium when isolated fat body tissue was incubated in the presence of hormone. The hormonal stimulation of carbohydrate mobilization in an insect does not necessarily lead to increased trehalose concentrations in the hemolymph under the appropriate physiological conditions. In the locust, for instance, in which the trehalose utilization rate is increased during prolonged flight, the enhanced rate of trehalose release does not lead to hypertrehalosemia.

## II. HORMONAL ACTIVATION OF GLYCOGEN PHOSPHORYLASE

The rate of glycogenolysis in the fat body depends on the activity of glycogen phosphorylase. Hormonal stimulation of glycogenolysis leading to an increased

## Further Reading

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## II. HORMONAL ACTIVATION OF GLYCOGEN PHOSPHORYLASE

The rate of glycogenolysis in the fat body depends on the activity of glycogen phosphorylase. Hormonal stimulation of glycogenolysis leading to an increased biosynthesis and release of trehalose has been investigated extensively in cockroaches, which rely mainly on carbohydrate as a fuel for flight activity. Hypertrehalosemic hormones (HTHs), representatives of the AKH/RPCH family, were shown to activate fat body glycogen phosphorylase and to stimulate trehalose synthesis in *Periplaneta americana* and *Blaberus discoidalis*. Activation of fat body phosphorylase by AKH peptides has been demonstrated in locusts, the moth *Manduca sexta*, and the beetle *Pachnoda sinuata*. In the locust *Locusta migratoria*, all three known AKHs (AKH-I, -II, and -III) are able to activate glycogen phosphorylase in a bioassay, although with different potencies. In vertebrates, phosphorylase is regulated by reversible phosphorylation/dephosphorylation between nonphosphorylated inactive

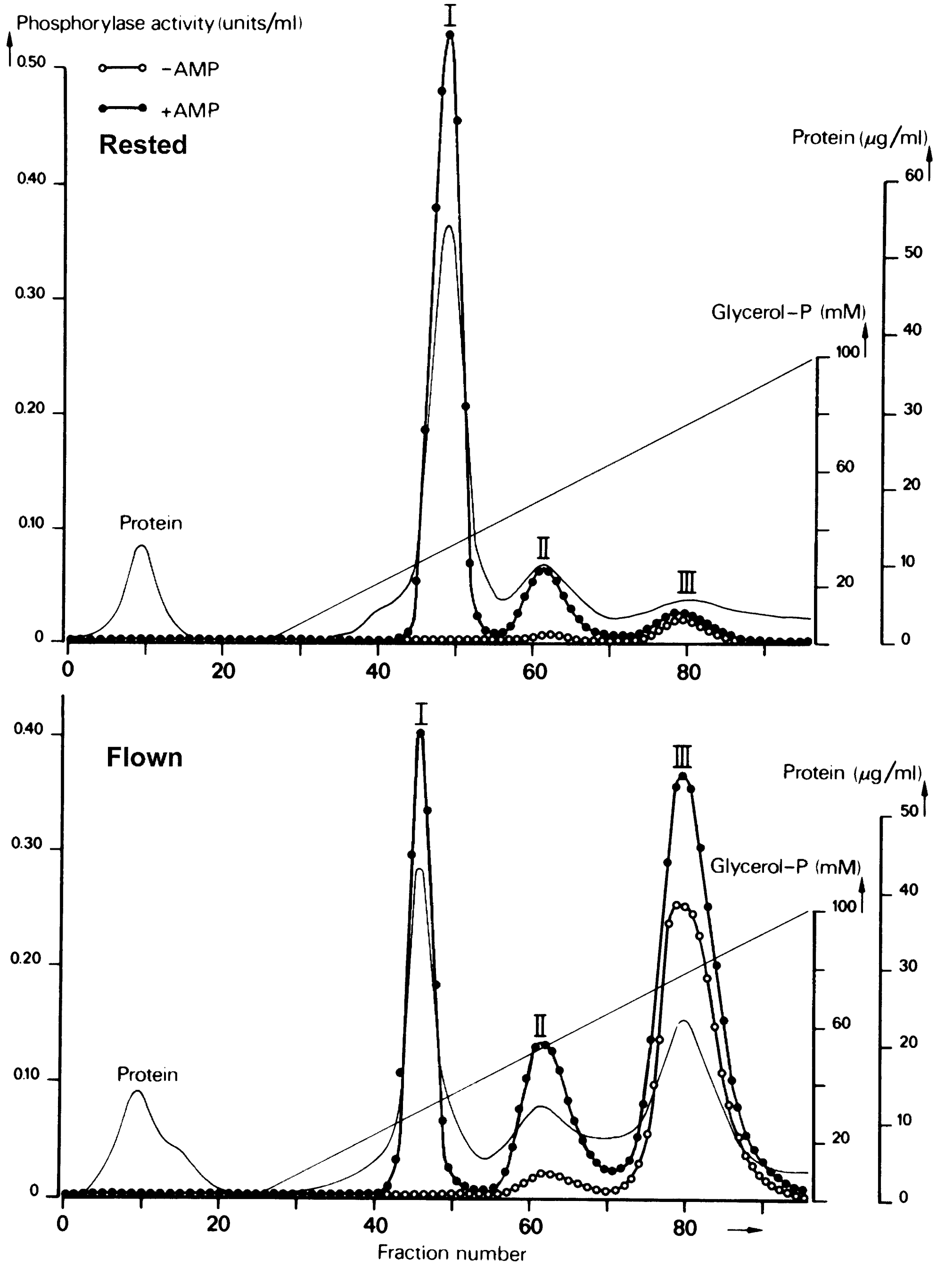

FIGURE 1 Fractionation of locust fat body phosphorylase by DEAE–Sephacel chromatography. Elution profiles were obtained with partially purified enzyme from the pooled fat bodies of 80 locusts at rest (Rested) and of 120 flown locusts (Flown).

phosphorylase *b* and phosphorylated active phosphorylase *a*. Also in locusts, flight activity as well as AKH injection induces the conversion of fat body phosphorylase *b* into the *a* form. This activating conversion indicates phosphorylation of each of the two subunits of the enzyme, which takes place in two steps, thereby giving rise to an intermediate hybrid *ab* form (Fig. 1). Activation of fat body phosphorylase by flight activity or hormone injection, combined with the occurrence of three forms of the enzyme including the partially phosphorylated phosphorylase *ab*, has also been demonstrated in *M. sexta* and *P. americana*. It has been suggested that the presence of this hybrid phosphorylase would provide for a sophisticated way of regulating carbohydrate metabolism in insects.

## III. HORMONAL SIGNAL TRANSDUCTION

Binding of the AKH/RPCH peptides to their plasma membrane receptor(s) in the insect fat body results in the induction of a variety of signal transduction events that ultimately lead to the activation of target enzymes. As discussed above, the target enzyme in the mobilization of carbohydrate reserves is glycogen phosphorylase, which initiates the conversion of glycogen into trehalose. Signal transduction mechanisms have been investigated mainly in locusts and cockroaches, with focus on GTP-binding (G) proteins, cyclic AMP (cAMP), $Ca^{2+}$, inositol phosphates, signaling cross-talk, and hormone receptors. Some major results are discussed in this article.

### A. Cyclic AMP and G-Proteins

In the fat body of *L. migratoria*, an accumulation of the second messenger cAMP, brought about by AKH-I has been demonstrated both *in vivo* and *in vitro*. Moreover, this accumulation of cAMP leads to the activation of glycogen phosphorylase, which is evidence in favor of a role of cAMP in AKH signal transduction. Each of the AKHs stimulates cAMP production in a dose-dependent manner in the fat body within 1 min. At a physiological dose of 4 nM, AKH-III is the most potent and AKH-I the least potent peptide hormone in stimulating cAMP production, and the same order of potency holds for the activation of glycogen phosphorylase by this hormonal dose. The observation that AKH-II is somewhat stronger than AKH-I in activating glycogen phosphorylase is in line with suggestions that the second AKH would be the major trigger for carbohydrate mobilization from the fat body and that the action of cAMP is directed more toward carbohydrate mobilization than lipid mobilization. The involvement of cAMP in the stimulation of glycogen mobilization is not a general feature of insects, since the HTHs of several cockroach species studied do not utilize this second messenger to activate trehalose synthesis in the fat body.

Experiments on locust fat body using cholera toxin (CTX) and pertussis toxin (PTX) suggest that the AKH receptor(s) is coupled to the $G_s$-protein (and not to $G_i$). The demonstration that AKH-I-, AKH-II-, and AKH-III-stimulated phosphorylase activation is ablated by the universal G-protein inhibitor guanosine-5′-*O*-(2-thiodiphosphate) strongly substantiates this suggestion.

### B. Calcium

The relative importance of $Ca^{2+}$ in signal transduction in the fat body is not equal in several closely related insect species. For example, the influx of extracellular $Ca^{2+}$ into the fat body of *L. migratoria* has a much stronger stimulating effect on glycogen phosphorylase activity than the release of calcium ions from intracellular stores, but in *B. discoidalis* the opposite result holds. The presence of extracellular $Ca^{2+}$ ions has been shown to be indispensable for the induction of fat body phosphorylase by AKH in *L. migratoria* and by HTH in *P. americana*. In the absence of $Ca^{2+}$ in the medium, none of the three locust AKHs is capable of enhancing cAMP production and inducing glycogen phosphorylase activation in the fat body, but 1.5 mM $Ca^{2+}$ (which is the $Ca^{2+}$ concentration in hemolymph) is sufficient for complete activation (Fig. 2). Since the induction of

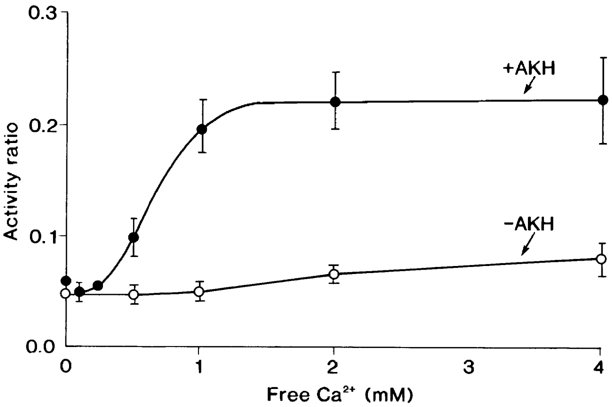

FIGURE 2 Dependence of AKH-induced activation of locust fat body phosphorylase on extracellular $Ca^{2+}$. Fat body was incubated for 15 min in the presence or absence of 40 nM AKH-I. Similar results were obtained with AKH-II and -III.

phosphorylase by cAMP in fat body was shown to be independent of extracellular $Ca^{2+}$, data suggest that at least part of the action of extracellular $Ca^{2+}$ is at a site proximal to cAMP, e.g., the binding of the hormones to their receptor(s) or a $Ca^{2+}$-sensitive adenylyl cyclase.

All three locust AKHs are capable of stimulating the $Ca^{2+}$ inflow into the fat body cells within 30 s with equal potency. This hormone-induced $Ca^{2+}$ influx appeared to be mediated through depletion of intracellular $Ca^{2+}$ stores, suggesting the functioning of a store-operated or capacitative $Ca^{2+}$ entry mechanism. Simultaneously, the AKHs also enhance the efflux of $Ca^{2+}$ from the fat body. At a physiological dose, AKH-III caused the strongest efflux and AKH-I the weakest; at a massive dose, their efficacy was equal. As the influx of $Ca^{2+}$ exceeded the efflux, it is feasible that the intracellular $Ca^{2+}$ concentration rises as a result of incubation of fat body with AKH.

## C. Inositol Phosphates

For a maximal effect of AKH on glycogen phosphorylase activity in locust fat body, the release of $Ca^{2+}$ from intracellular stores is required in addition to the availability of extracellular calcium. The same holds for the activation of glycogen phosphorylase and the stimulation of trehalose synthesis by HTH in *B. discoidalis* fat body. In the regulation of $Ca^{2+}$ mobilization from intracellular stores, inositol phosphates ($InsP_n$) play a pivotal role, and the formation of these putative second messengers is induced by the locust AKHs. Each of the *Locusta* AKHs stimulates the synthesis of total $InsP_n$ within 1 min with different potencies: AKH-II barely induces any $InsP_n$ and AKH-III is more potent than AKH-I. The observation that the activation of glycogen phosphorylase by each of the AKHs is dampened by the phospholipase C (PLC) inhibitor U73122 suggests the involvement of $InsP_n$ (and/or diacylglycerol) in AKH signaling in the locust fat body.

All individual forms of $InsP_n$ are elevated by the AKHs, with $InsP_3$ and $InsP_4$ being the most interesting because of their presumed $Ca^{2+}$ mobilizing actions (Fig. 3). With respect to $InsP_3$, AKH-III is again more potent than AKH-I, and the AKH-II-enhanced $InsP_3$ formation is quite small. The most prolonged effect on $InsP_3$ is caused by AKH-III. The high potency and prolonged effects of AKH-III with respect to the induction of various second-messenger systems apparently compensate (in part) for its low abundance relative to that of the other AKHs, and therefore, the effects of this hormone may be stronger than estimated from its relative amount in the circulation.

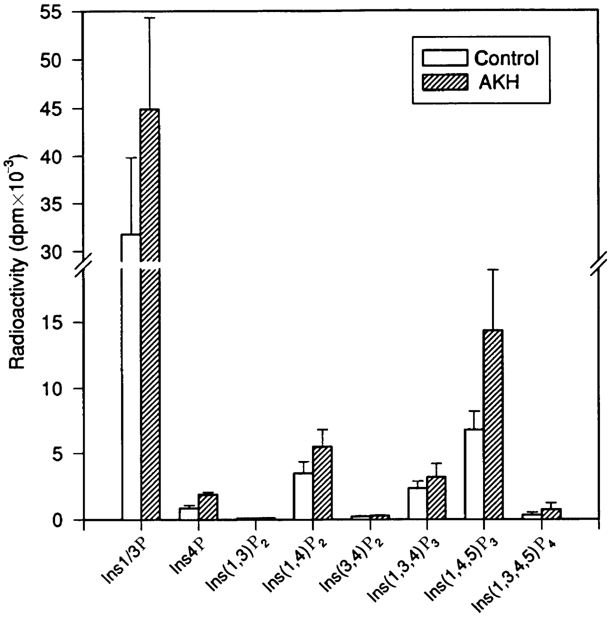

FIGURE 3 Effect of AKH on the formation of inositol phosphate isomers from locust fat body. Fat body tissue prelabeled with *myo*-[2-$^3$H]inositol was incubated for 1 min in the presence or absence (controls) of 40 nM AKH-I; then $InsP_n$ were isolated and separated by high-performance liquid chromatography and their radioactivity was measured. Results are expressed as disintegrations per minute per milligram of protein.

Ins(1,4,5)$P_3$ levels are greatly increased by HTH in the fat body of *B. discoidalis* in a time- and dose-dependent manner, which, along with the strong evidence for $Ca^{2+}$ as component in the HTH second-messenger cascade, argues strongly for $InsP_3$ as a primary second messenger in response to HTH followed by the mobilization of intracellular $Ca^{2+}$.

## D. Signaling Crosstalk

Crosstalk between signal transduction cascades provides the cell with a complex intracellular system for fine-tuning of hormone-induced signals. In locust fat body an elevation of cAMP levels does not influence the intracellular $InsP_n$ content, indicating that the basal PLC activity is not regulated by this cyclic nucleotide. Moreover, none of the signal transducing elements between the AKH receptor and PLC is affected by cAMP, since preincubation of fat body tissue with forskolin or dibutyryl-cAMP does not have an impact on AKH-induced $InsP_n$ production. Proof of a direct linkage between the AKH

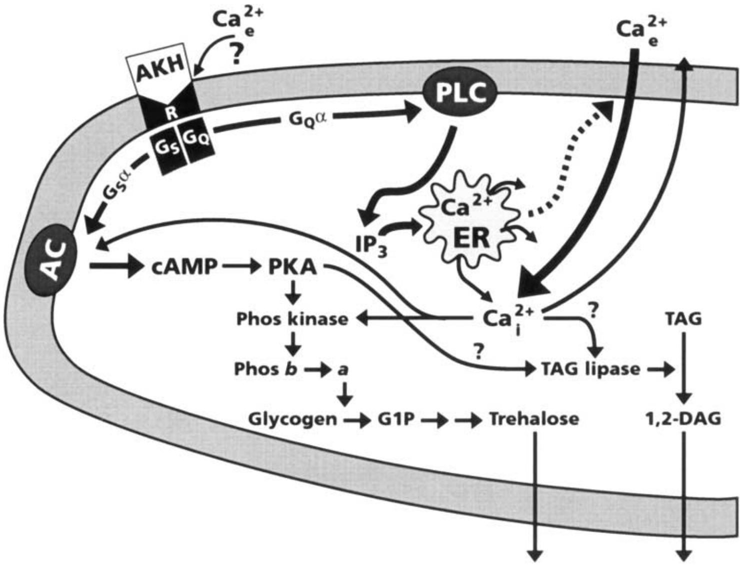

FIGURE 4 Tentative model for the coupling of AKH signaling pathways mediating the mobilization of energy substrates in the locust fat body cell. R, receptor; ER, endoplasmic reticulum; AC, adenylyl cyclase; PLC, phospholipase C; Phos, phosphorylase; G1P, glucose 1-phosphate; TAG, triacylglycerol; DAG, diacylglycerol.

receptor(s) and PLC (instead of a route via cAMP) came from the substantiation that the G-protein activator aluminum fluoride increases $InsP_n$ levels. Experiments using CTX, PTX, and GP antagonist-2A, a specific inhibitor of $G_q$, preclude the involvement of $G_i$ and a $G_s$-sensitive isoform of PLC and evidence the involvement of $G_q$ in the transduction of AKH signals toward fat body PLC (Fig. 4).

## Glossary

**adipokinetic hormone** Peptide hormone in insects involved in the mobilization of substrates (predominantly lipids and carbohydrates) for energy generation needed by contracting flight muscles.

**corpus cardiacum** Neuroendocrine organ situated caudal to the brain to which it is connected by paired nerves. The organ combines the functions of neurohemal storage and adipokinetic/hypertrehalosemic hormone production.

**fat body** Organ whose functional role in insects is analogous to the combined functions of liver and adipose tissue in mammals. Fat body is the chief site of intermediary metabolism, detoxification, storage of nutrient reserves, and, in some species, deposition of nitrogenous waste products.

**glycogenolysis** Breakdown of glycogen; in insect fat body, the resulting glucose monomers are used for the biosynthesis of the disaccharide trehalose, the carbohydrate transport form in insects.

**glycogen phosphorylase** Enzyme that catalyzes the sequential removal of glycosyl residues from the nonreducing end (i.e., with a free 4′-OH group) of the glycogen molecule, using orthophosphate as a co-substrate and releasing glucose-1-phosphate.

**hemolymph** Blood of insects, which circulates in the body cavity between the various organs, bathing them directly. It consists of a fluid plasma in which the blood cells or hemocytes are suspended.

**hypertrehalosemia** Condition characterized by an increase in the level of trehalose in the hemolymph (insect blood) to values that significantly exceed the normal resting level.

**hypertrehalosemic hormone** Peptide hormone from the corpus cardiacum involved in the mobilization of fat body glycogen to fuel flight activity. For transport to the flight muscles, glycogen is converted into the disaccharide trehalose, the carbohydrate transport form in insects, whose level in hemolymph is raised during flight.

**red pigment concentrating hormone** Peptide hormone from the eyestalk of crustaceans; member of the adipokinetic hormone/red pigment-concentrating hormone family that comprises structurally related but functionally diverse peptides.

**signaling cross-talk** Occurrence of communication/interaction between different signaling pathways, resulting in an integrated response to a hormone-induced signal.

**trehalase** Enzyme that catalyzes the hydrolytic reaction by which trehalose is brought into the glycolytic mainstream: Trehalose + $H_2O \rightarrow 2\text{D}$-glucose.

## See Also the Following Articles

**Adipokinetic Hormones and Lipid Mobilization**
**• Adipokinetic Hormones: Structure and Biosynthesis**
**• Insect Endocrine System**

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# Adipokinetic Hormones and Lipid Mobilization

Dick J. Van der Horst and Rob C. H. M. Oudejans
*Utrecht University, The Netherlands*

I. INTRODUCTION
II. ADIPOKINETIC HORMONE SIGNAL TRANSDUCTION IN INSECT FAT BODY
III. EFFECT OF ADIPOKINETIC HORMONE ON LIPID MOBILIZATION
IV. ADIPOKINETIC HORMONE-INDUCED LIPOPHORIN CONVERSIONS

**In the migratory locust, *Locusta migratoria*, the flight activity-induced release of adipokinetic hormones (AKHs; AKH-I, -II, and -III) from the neurosecretory cells of the corpus cardiacum, a neuroendocrine gland located caudal to the insect brain, has been studied extensively. The direct actions of these hormones on their fat body target cells trigger signal transduction processes that lead to the mobilization of lipid (diacylglycerol). This substrate fulfills a major role in energy metabolism of the contracting flight muscles during sustained flight activity. The molecular mechanism of diacylglycerol transport in insect blood involves a reversible conversion of the insect lipoprotein lipophorin, which has revealed a novel concept for lipid transport in the circulatory system.**

## I. INTRODUCTION

The flight activity of insects provides an exceptionally suitable yet relatively simple model system for studying the regulatory phenomena involved in energy expenditure during extreme physical activity. Similar to the processes used by vertebrates to generate energy for sustained locomotion, long-distance flying insects mobilize endogenous lipid (triacylglycerol) reserves to fuel oxidative metabolism during migratory flight. The fat body, which combines many of the properties and functions of vertebrate liver and adipose tissue, plays a fundamental role in lipid storage, as well as in the process of lipolysis controlled by adipokinetic hormones (AKHs). This article discusses the action of AKHs on lipid mobilization in fat body cells and the eventual AKH-induced transformation of the transport system of lipids in the insect blood, which will be exemplified using mainly the migratory locust (*Locusta migratoria*) as an insect model.

## II. ADIPOKINETIC HORMONE SIGNAL TRANSDUCTION IN INSECT FAT BODY

Binding of the peptide adipokinetic hormones to their plasma membrane receptor(s) at the fat body cells results in the induction of a variety of signal transduction events that ultimately lead to the activation of target key enzymes. The first insect AKH receptors have recently been identified at the molecular level. The signal transduction mechanism of the three locust AKHs has been studied extensively and involves stimulation of cAMP production, which is dependent on the presence of extracellular $Ca^{2+}$. Additionally,

## Further Reading

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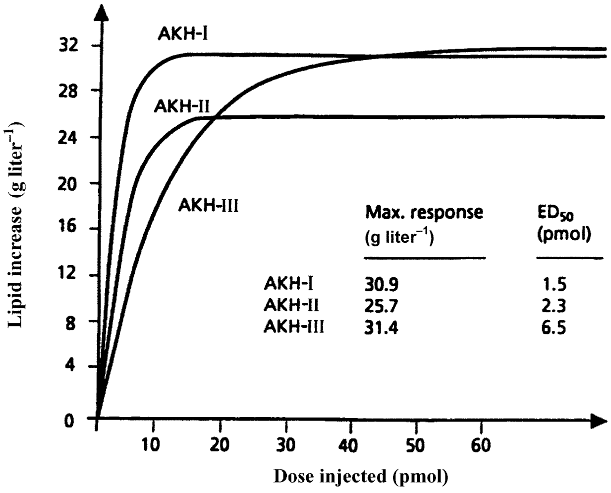
Binding of the peptide adipokinetic hormones to their plasma membrane receptor(s) at the fat body cells results in the induction of a variety of signal transduction events that ultimately lead to the activation of target key enzymes. The first insect AKH receptors have recently been identified at the molecular level. The signal transduction mechanism of the three locust AKHs has been studied extensively and involves stimulation of cAMP production, which is dependent on the presence of extracellular $Ca^{2+}$. Additionally, the AKHs enhance the production of inositol phosphates including inositol 1,4,5-trisphosphate, which may mediate the mobilization of $Ca^{2+}$ from intracellular stores. This depletion of $Ca^{2+}$ from intracellular stores stimulates the influx of extracellular $Ca^{2+}$, indicative of the operation of a capacitative (store-operated) calcium entry mechanism. The interactions between the AKH signaling pathways ultimately result in the mobilization of stored reserves as fuels for flight. Although the carbohydrate (mainly trehalose) in the insect blood (hemolymph) provides the energy for the initial period of flight, additional trehalose is mobilized from fat body glycogen reserves by the AKH-induced activation of glycogen phosphorylase. At the same time, the concentration of lipid (diacylglycerol, DAG) in the hemolymph is increased progressively at the expense of stored triacylglycerol (TAG) reserves in the fat body and gradually constitutes the major substrate during prolonged flight.



| | Max. response (g liter$^{-1}$) | $ED_{50}$ (pmol) |
|---|---|---|
| AKH-I | 30.9 | 1.5 |
| AKH-II | 25.7 | 2.3 |
| AKH-III | 31.4 | 6.5 |

FIGURE 1 Dose–response curves for the lipid-mobilizing effects of AKH-I, -II, and -III in *L. migratoria*. Adult male locusts were injected with different doses of AKH or, in the controls, with saline, and after 120 min, the lipid content in hemolymph was determined. Responses represent increases in hemolymph lipid in AKH-injected locusts, expressed as grams per liter. For clarity, data points and standard error bars were omitted. $ED_{50}$, effective dose eliciting 50% of the maximum response.

## III. EFFECT OF ADIPOKINETIC HORMONE ON LIPID MOBILIZATION

The namesake action of the AKHs on insect fat body cells results in the mobilization of lipid (TAG) stores. The increased production of DAG reflected by the increased concentration of DAG in the hemolymph indicates hormonal activation of the key enzyme, fat body TAG lipase. In a bioassay, all three *L. migratoria* AKHs (-I, -II, and -III) are able to stimulate lipid mobilization, although their relative potencies are different, as inferred from the dose–response curves shown in Fig. 1. This recalls the concept of a hormonally redundant system involving multiple regulatory molecules with overlapping actions. Results obtained with combinations of two or three AKHs, which are likely to occur together in locust hemolymph under physiological conditions *in vivo*, revealed that the maximal responses for the lipid-mobilizing effects were much lower than the theoretically calculated responses based on the dose–response curves for the individual hormones. In the lower (probably physiological) range, however, combinations of the AKHs were more effective than the theoretical values calculated from the responses elicited by the individual hormones.

The mechanism by which TAG lipase catalyzes AKH-controlled production of the DAG on which long-distance flight depends is only poorly understood, mainly due to technical problems in isolating or activating the lipase. In vertebrates, hormone-sensitive lipase (HSL) controls mobilization of TAG stores in adipose tissue, and although, in contrast to the situation in insects, free fatty acids (FFA) are released into the blood for uptake and oxidation in muscle, there is a clear functional similarity between vertebrate adipose tissue HSL and insect fat body TAG lipase. In vertebrate HSL, specific regulatory sites are phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA), resulting in activation of HSL. The mechanism behind this activation of HSL upon phosphorylation by PKA is not well understood, but seems to involve translocation of HSL from the cytosol to the lipid droplet as well as conformational changes in the HSL molecule. Different phosphorylation sites in HSL may play different roles in the process of translocation and the increase in specific activity of the enzyme. Additionally, perilipins, a family of unique proteins intimately associated with the limiting surface of neutral lipid storage droplets, are acutely polyphosphorylated by PKA on stimulation of lipolysis, hinting at a role in this lipolytic process. Conformational changes of phosphorylated perilipins may expose the neutral lipid cores of the lipid droplets, facilitating the ensuing hydrolysis.

Even though a translocation of the insect fat body TAG lipase has not been established, the involvement of AKHs in the process of lipolysis is beyond dispute. The effect of AKHs was demonstrated not only *in vivo* from enhanced levels of DAG in hemolymph of insects injected with the hormones as shown above, but also *in vitro* by the accumulation of DAG in isolated *L. migratoria* fat body tissue that was incubated in the presence of AKH. In later *in vitro* experiments, both cAMP and $Ca^{2+}$ were shown to play an important role in the effect of AKH on lipolysis. The involvement of cAMP suggests a role for PKA in the phosphorylation and activation of fat body TAG lipase. In addition, an increase of fat body TAG lipase activity was measured in gregarious locusts *in vivo* when injected with AKH-I, whereas in solitary locusts, which do not store sufficient amounts of TAG in the fat body, AKH-I administration had no significant effect on lipase activity. The reason that the measured factorial increases of lipase activity after stimulation with lipolytic agents are relatively modest in these and other similar studies may reside in the applied experimental procedure, in which the enzyme is assayed in extracts of fat body or adipose tissue in the presence of optimally accessible TAG substrate. In this way, only the effects of (de)phosphorylation or other conformational changes of the enzyme on its lipolytic activity will be measured, and other potential effects such as enzyme translocation and the involvement of lipid droplet-associated proteins, factors that may be highly important for lipase activity as discussed above for vertebrate HSL, remain confounded. This may also explain why phosphorylation by PKA did not change the activity of TAG lipase that had been purified from fat body of the adult hawkmoth, *Manduca sexta*.

In two insect species that rely on lipid mobilization during sustained flight activity, *L. migratoria* and *M. sexta*, it has been shown that DAG, which is released from the fat body by the action of AKH, is stereospecific and has the *sn*-1,2-configuration. Although conclusive evidence on the pathway for the stereospecific synthesis of this *sn*-1,2-DAG is still lacking, the most probable route involves stereospecific hydrolysis of TAG into *sn*-1,2-DAG by a stereospecific lipase acting at the *sn*-3 position of the TAG.

## IV. ADIPOKINETIC HORMONE-INDUCED LIPOPHORIN CONVERSIONS

For insect species that recruit fat body TAG depots to power their flight muscles during the vast distances covered nonstop by migratory flight, an efficient mechanism for lipid transfer is a premier issue. The action of AKH on lipid release has revealed a novel concept for lipid transport in the circulation of animal organisms. Insect hemolymph generally contains abundant amounts of a single multifunctional lipoprotein particle, high-density lipophorin (HDLp), that performs the tasks of transporting dietary and endogenously produced lipids to peripheral tissues during all developmental stages. A characteristic feature of HDLp is its ability to function as a reusable vehicle for a variety of lipids by the selective loading and unloading of lipid components at target tissues. In the flying insect, however, the increased transport of the released DAG in the hemolymph appears to require an AKH-stimulated transformation of the lipophorin particle, which is capable of alternating between the relatively lipid-poor HDLp form and a lipid-enriched (low-density lipophorin, LDLp) form. In this reversible conversion the exchangeable apolipoprotein apoLp-III, which exists in a lipid-free form and a lipid-bound form, plays an essential role. A schematic overview of the process is depicted in Fig. 2. Recent advances on the structural properties of HDLp and apoLp-III demonstrate a remarkable similarity to their counterparts in the mammalian system (the two nonexchangeable apolipoproteins of HDLp are similar to apoB; apoLp-III is similar to apoE). However, the functioning of the insect lipoprotein as a shuttle mechanism operating in energy transport during flight activity is intriguingly different, since in mammals lipoproteins do not play a role as carriers of mobilized lipids during exercise.

Both HDLp and apoLp-III are abundantly present in the hemolymph of resting insects. The loading of DAG onto HDLp particles at the fat body cell plasma membrane induces the association of multiple copies of apoLp-III with the expanding lipoprotein surface. The resulting LDLp, which is both larger and lipid-enriched, travels in the circulation to the flight muscles, where a lipophorin lipase specifically hydrolyzes the LDLp-carried DAG. The liberated FFA are imported into the muscle cells and oxidized to yield energy. Once the lipid content of the LDLp has diminished, apoLp-III dissociates from the particle, and finally, its HDLp constituent is recovered. Both HDLp and apoLp-III are recycled to the fat body and reutilized for DAG uptake (see Fig. 2). This concept of a reusable lipoprotein shuttle for lipid transport during flight activity was initially reported for the locust, but thereafter found to be present in many other long-distance flying insects.

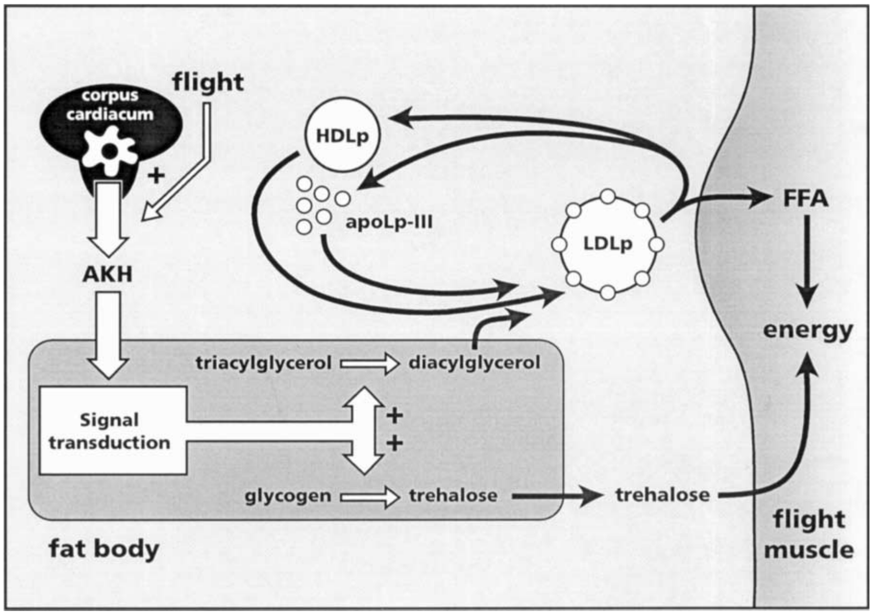


FIGURE 2 Schematic overview of AKH-controlled substrate mobilization from insect fat body during flight activity. AKH, adipokinetic hormones; HDLp, high-density lipophorin; LDLp, low-density lipophorin; apoLp-III, apolipophorin III; FFA, free fatty acids.

This shuttle mechanism is specific to the adult stage of the insect. During all stages of locust development, high circulating levels of HDLp are present to transport lipid components between tissues. However, the apoLp-III gene is expressed only after the first week of adult life and is apparently related to the adult-specific capacity of the insect to fly. The molecular basis of the interaction of apoLp-III with the lipoprotein surface is of great interest and value since the locust apoLp-III represents the only full-length apolipoprotein for which a three-dimensional structure has been disclosed. In the absence of lipid, apoLp-III reveals a globular bundle of five amphipathic α-helices. These helices are oriented such that the hydrophobic amino acid side chains are buried in the bundle interior and the hydrophilic residues face outward. A plausible model postulates that lipid binding induces a significant reorganization of the helical segments, allowing interaction of the hydrophobic face of the helices with the lipoprotein lipid surface. A critical event in lipid surface recognition seems to be the disturbance of the phospholipid monolayer by the appearance of patches of DAG molecules in the lipoprotein surface. Mechanistic details underlying the opening of the amphipathic helix bundle and the lipid-binding activity of apoLp-III, however, remain to be defined.

## Glossary

**adipokinetic hormone** Peptide hormone in insects involved in the mobilization of substrates (predominantly lipids and carbohydrates) for energy generation needed by contracting flight muscles.

**apolipophorin III (apoLp-III)** Exchangeable apolipoprotein (mol wt 17–20 kDa) in the hemolymph of insects engaging in long-distance flights for which lipids are used as an energy source. In the resting blood, apolipophorin III forms a stable particle consisting of a bundle of five amphipathic α-helices; during lipid loading of lipophorin the conformation of apoLp-III changes and allows the peptide to unfold and reversibly bind to the expanding particle.

**fat body** Organ whose functional role in insects is analogous to the combined functions of liver and adipose tissue in mammals. Fat body is the chief site of intermediary metabolism, detoxification, storage of nutrient reserves, and, in some species, deposition of nitrogenous waste products.

**HDLp** High-density lipophorin (density approximately 1.12 g/ml), the abundant and generally single lipoprotein particle in insect hemolymph, transporting several classes of lipids between organs and tissues in the resting situation.

**LDLp** Low-density lipophorin (density approximately 1.04 g/ml), the adipokinetic hormone-induced form of lipoprotein in the hemolymph of insects engaging in long-distance flights for which lipids are used as an energy source. The particle consists of lipid (diacylglycerol)-loaded and apoLp-III-associated HDLp.

**lipophorin** The abundant and generally single lipoprotein particle in insect hemolymph. The density of lipophorin (lipid-bearing protein) is similar to that of human high-density lipoprotein (HDL); for the insect lipophorin, HDLp is used to distinguish it from HDL.

## See Also the Following Articles

**Adipokinetic Hormones and Carbohydrate Metabolism**
- **Adipokinetic Hormones: Structure and Biosynthesis**
- **Insect Endocrine System**

## Further Reading

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# Adipokinetic Hormones: Structure and Biosynthesis

Rob C. H. M. Oudejans and
Dick J. Van der Horst
*Utrecht University, The Netherlands*

I. INTRODUCTION
II. PRIMARY STRUCTURE OF ADIPOKINETIC HORMONES
III. BIOSYNTHESIS OF ADIPOKINETIC HORMONES
IV. STORAGE, RELEASE, AND INACTIVATION OF ADIPOKINETIC HORMONES

**The structure and biosynthesis of the adipokinetic hormones (AKHs) are reviewed and their storage, release, and inactivation in insect blood (hemolymph) are discussed. Since most of our knowledge on the biosynthesis of the AKHs has been obtained from locust species, these insects will serve as a general model system.**

## I. INTRODUCTION

Insect long-distance flight activity involves extremely high metabolic rates, and insect flight muscles are among the most energy-demanding tissues known. The energy substrates needed to fuel the flight muscles are stored in the fat body and their mobilization is under the control of adipokinetic hormones (AKHs). Additional functions of the AKHs are revealed during a variety of other metabolic "stress" situations (energy-consuming processes), such as starvation, diapause, and molting. The AKHs are peptide hormones that (with a few exceptions) are synthesized and stored in the so-called adipokinetic cells of the glandular lobes of the corpus cardiacum, a neuroendocrine organ situated in the insect head and connected to the central nervous system.

## II. PRIMARY STRUCTURE OF ADIPOKINETIC HORMONES

Since the elucidation of the structure of the first AKH in 1976 from 3000 locust corpora cardiaca, analytical techniques have been revolutionized and today it is possible to determine the structure of a new AKH from just a few picomoles of the hormone. To date, the structures of some 37 different AKHs are known from representatives of most insect orders (Table I).

energy source. The particle consists of lipid (diacylglycerol)-loaded and apoLp-III-associated HDLp.

**lipophorin** The abundant and generally single lipoprotein particle in insect hemolymph. The density of lipophorin (lipid-bearing protein) is similar to that of human high-density lipoprotein (HDL); for the insect lipophorin, HDLp is used to distinguish it from HDL.

## See Also the Following Articles

**Adipokinetic Hormones and Carbohydrate Metabolism**
- **Adipokinetic Hormones: Structure and Biosynthesis**
- **Insect Endocrine System**

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## II. PRIMARY STRUCTURE OF ADIPOKINETIC HORMONES

Since the elucidation of the structure of the first AKH in 1976 from 3000 locust corpora cardiaca, analytical techniques have been revolutionized and today it is possible to determine the structure of a new AKH from just a few picomoles of the hormone. To date, the structures of some 37 different AKHs are known from representatives of most insect orders (Table I).

TABLE I Amino Acid Sequences of Peptide Hormones of the AKH/RPCH Family

| Hormone (by acronym) | Amino acid sequence |
|---|---|
| Miv-CC | pGlu-Ile-Asn-Phe-Thr-Pro-Asn-Trp-$NH_2$ |
| Phm-AKH-III | pGlu-Ile-Asn-Phe-Thr-Pro-Trp-Trp-$NH_2$ |
| Poa-HrTH | pGlu-Ile-Thr-Phe-Thr-Pro-Asn-Trp-$NH_2$ |
| Lom-AKH-II | pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-$NH_2$ |
| Tem-HrTH | pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-$NH_2$ |
| Pab-RPCH | pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-$NH_2$ |
| Scg-AKH-II | pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-$NH_2$ |
| Pya-AKH | pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-$NH_2$ |
| Ers-AKH | pGlu-Leu-Asn-Phe-Thr-Pro-Ser-Trp-$NH_2$ |
| Lom-AKH-III | pGlu-Leu-Asn-Phe-Thr-Pro-Trp-Trp-$NH_2$ |
| Mem-CC | pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-Trp-$NH_2$ |
| Pht-HrTH | pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-$NH_2$ |
| Pea-CAH-II | pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-$NH_2$ |
| Taa-AKH | PGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-$NH_2$ |
| Ona-CC-II | PGlu-Phe-Asn-Tyr-Ser-Phe-Asp-Trp-$NH_2$ |
| Ona-CC-I | PGlu-Tyr-Asn-Phe-Ser-Thr-Gly-Trp-$NH_2$ |
| Pea-CAH-I | pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-$NH_2$ |
| Ani-AKH | pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-$NH_2$ |
| Grb-AKH | pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-$NH_2$ |
| Emp-AKH | pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-$NH_2$ |
| Psi-AKH | pGlu-Val-Asn-Phe-Thr-Pro-Gly-Trp-$NH_2$ |
| Lia-AKH | pGlu-Val-Asn-Phe-Thr-Pro-Ser-Trp-$NH_2$ |
| Mas-AKH | pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-$NH_2$ |
| Del-CC | pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-Asn-$NH_2$ |
| Tea-HrTH | pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-Gly-Gly-$NH_2$ |
| Phm-AKH-I | pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Ser-$NH_2$ |
| Lom-AKH-I | pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ |
| Hez-HrTH | pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn-$NH_2$ |
| Phl-CC | pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Ser-$NH_2$ |
| Cam-HrTH-II | pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ |
| Cam-HrTH-I | pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$<br>\|<br>Hexose |
| Taa-HoTH | pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr-$NH_2$ |
| Bld-HrTH | pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr-$NH_2$ |
| Plc-HrTH-II | pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-Asn-$NH_2$ |
| Rom-CC-I | pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ |
| Lom-HrTH | pGlu-Val-Thr-Phe-Ser-Arg-Asp-Trp-Ser-Pro-$NH_2$ |
| Vac-AKH | pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly-Lys |

| | Variation per residue position | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Position: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | pGlu | Ile | Asn | Phe | Ser | Ala | Asn | Trp | Gly | Asn | Lys |
| | | Leu | Thr | Tyr | Thr | Arg | Asp | | Ser | Gly | |
| | | Phe | | | | Phe | Gly | | | Pro | |
| | | Tyr | | | | Pro | Ser | | | Ser | |
| | | Val | | | | Ser | Trp | | | Thr | |
| | | | | | | Thr | | | | Tyr | |

They are short peptides consisting of 8–11 amino acid residues and constitute the so-called AKH/RPCH (red pigment-concentrating hormone) family. The nomenclature of the members of this family (by acronym) is based on the use of the first two characters of the genus name and the first character of the species name of the animal from which the hormone was first isolated, supplemented by an abbreviation of its function.

The name is followed by a roman numeral in case there is more than one hormone per function. So Lom-AKH-I, -II, and -III are the three AKHs isolated from *Locusta migratoria*, and Lom-AKH-I and Scg-AKH-II are the two AKHs from *Schistocerca gregaria*.

In addition to the AKHs, the family contains hypertrehalosemic (HrTH) and hypotrehalosemic hormones (HoTH), corpus cardiacum factors (CC), etc. All members are found in insects, except for RPCH, which is restricted to the X-organ-sinus gland of crustaceans. Curiously, the variation in the structure of AKHs of insects is extremely large, whereas in all crustaceans investigated to date, just one RPCH structure has been found. All members (except Vac-AKH) are fully blocked peptides: They are N-terminally blocked by a pyroglutamate (pGlu) residue and C-terminally blocked by an amide group. Although the variation in structure has increased by an increasing number of new hormones, characteristic features are still present. At the bottom of Table I the variation per amino acid residue position is indicated. At position 1 a pGlu is always found, at position 8 a Trp is always found, and at position 4 an aromatic amino acid residue is always found. At position 2 most members contain a branched-chain amino acid residue, and at position 5 either a Ser or a Thr is present. Except for Lom-HrTH, all longer members possess a Gly at position 9. Most variation occurs at positions 6, 7, and 10. In only one member (Cam-HrTH-I), a sugar moiety with an as yet unknown structure has been found as a posttranslational modification. Except for a few members (Lom-HrTH, Mem-CC, Ona-CC-II, and Pht-HrTH), all peptides are uncharged.

Adipokinetic activity of a substance can be shown in the locust (*L. migratoria*) by using a simple bioassay of injecting the substance into resting animals and measuring the increase in lipid content in the hemolymph. Potential hypertrehalosemic activity can be measured using a bioassay in the cockroach (*Periplaneta americana*). Interestingly, all members of the AKH/RPCH family (including RPCH itself) exert an adipokinetic effect in the locust assay, suggesting that the AKH receptor(s) of *L. migratoria* is (are) less specialized or that all AKHs share structural similarities. In contrast to locusts and cockroaches, some insect species have a low response to their own AKH and a higher response to the hormone from other species. There are even members of the family with an as yet unknown function (for instance, Lom-HrTH, which stimulates in a heterologous assay the mobilization of carbohydrates only in the cockroach). In many insect species a particular physiological function seems to be supported by two, or in some locust and grasshopper species even three, hormones, suggesting sophisticated hormonal control. Whether there are quantitative or qualitative differences that result in the fine-tuning of apparently identical or just pleiotropic functions is still under investigation.

## III. BIOSYNTHESIS OF ADIPOKINETIC HORMONES

All AKHs studied thus far are translated from separate mRNAs. They code for preprohormones with a simple structure: a signal sequence, a monocopy AKH sequence (starting with a Gln to form pGlu), a Gly residue (to form the terminal AKH amide group), a dibasic processing site, and an AKH-associated peptide sequence. In particular, in *L. migratoria* the signal sequences of the three preprohormones are cotranslationally cleaved, and the resulting prohormones of AKH-I and -II dimerize at random by oxidation of their Cys residues and give rise to two homodimeric precursor molecules (AKH-I/I and AKH-II/II) and one heterodimeric precursor molecule (AKH-I/II). This dimerization is a rather unique phenomenon, first established for another locust species (*S. gregaria*). Further proteolytic processing of the dimeric products in the secretory granules of the AKH cells of the corpus cardiacum results in the bioactive hormones and one heterodimeric and two homodimeric AKH-precursor-related peptides (APRPs) with as yet unknown functions (see Fig. 1). The biosynthesis of AKH-III is not yet fully understood. Recently, dimerization of the AKH-III prohormone with itself (it contains two Cys residues itself) has been established. Dimerization with the prohormones of AKH-I and -II has not been observed, and whether a cyclic prohormone of AKH-III can arise by formation of an internal disulfide bond is unknown.

*In vitro* studies on the biosynthesis of AKHs in locusts have revealed that the total time required for their biosynthesis from the starting point when radiolabeled amino acids are made available to the moment that radiolabeled bioactive hormones appear is 30–60 min. In *L. migratoria* the time for biosynthesis of AKH-III is shorter than that for AKH-I and -II, suggesting that there are two different pathways or different processing procedures for AKH-I/II, on the one hand, and for AKH-III, on the other.

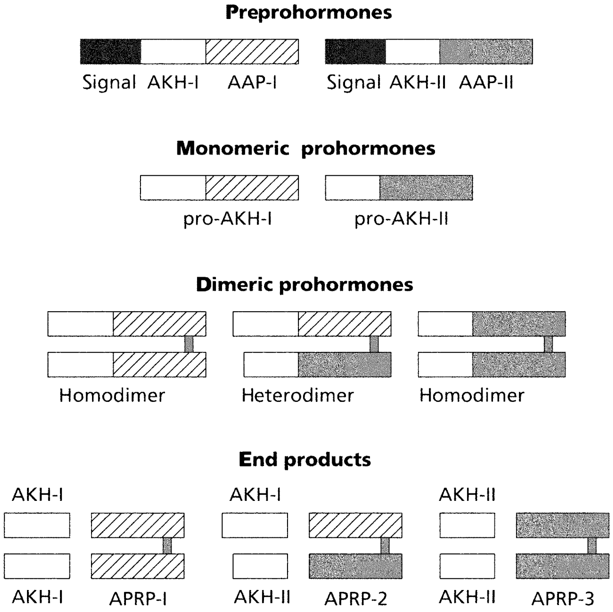

FIGURE 1 The biosynthesis of the locust adipokinetic hormones AKH-I and -II and the adipokinetic precursor-related peptides APRP-1, -2, and -3. After cleavage of the signal peptide from the preprohormones, the resulting monomeric prohormones, which consist of the AKH sequence and the AKH-associated peptide (AAP), form three dimeric prohormones, which are then processed to the AKHs and the APRPs.

## IV. STORAGE, RELEASE, AND INACTIVATION OF ADIPOKINETIC HORMONES

AKHs are stored in the secretory granules of the adipokinetic cells of the corpus cardiacum. In *L. migratoria* the total content of the AKHs increases continuously during the larval stages and throughout adult life. In aging locusts an increasing number of intracisternal granules that contain stores of AKH prohormones/precursors are also found. The three AKHs, colocalized and stored in the same secretory granules, are released during flight. Since the membrane of the pertinent secretory granule fuses with the plasma membrane, the total contents of the granule are released into the hemolymph: the bioactive AKHs, the APRPs, and possibly other end products.

The release of the AKHs in *L. migratoria* is subjected to many regulatory substances, which are of either a stimulatory or an inhibitory nature and can be of both neural and humoral origin. A detailed description falls beyond the scope of this article. The only natural stimulus of release of the AKHs is flight and the relative contributions of all known substances effective in the release process remain to be established *in vivo*.

The situation in *L. migratoria* is even more complex, since secretory granules of only a particular age can be released. Newly formed granules containing the AKHs must mature before they can release their contents (or before they can fuse with the plasma membrane). Granules more than 8 h old are believed to enter a nonreleasable pool. Determination of the total hormone content of a neuroendocrine structure has therefore a limited physiological value, because only the releasable amount of hormone is relevant.

Finally, the balance between released hormones (a mixture of many compounds present in the secretory granule) and the rate of their inactivation prior to reaching the target tissue will be of eminent importance for their ultimate effect. The three AKHs of *L. migratoria* appear to be inactivated differentially after their release, with half-lives during flight of 35, 37, and 3 min obtained for AKH-I, -II, and -III, respectively.

## Glossary

**adipokinetic hormone** Peptide hormone in insects involved in the mobilization of substrates (predominantly lipids and carbohydrates) for energy generation needed by contracting flight muscles.

**corpus cardiacum** Neuroendocrine organ situated caudal to the brain to which it is connected by paired nerves. The organ combines the functions of neurohemal storage and adipokinetic/hypertrehalosemic hormone production.

**fat body** Organ whose functional role in insects is analogous to the combined functions of liver and adipose tissue in mammals. Fat body is the chief site of intermediary metabolism, detoxification, storage of nutrient reserves, and, in some species, deposition of nitrogenous waste products.

**hemolymph** Blood of insects, which circulates in the body cavity between the various organs, bathing them directly. It consists of a fluid plasma in which the blood cells or hemocytes are suspended.

**hypertrehalosemic hormone** Peptide hormone from the corpus cardiacum involved in the mobilization of fat body glycogen to fuel flight activity. For transport to the flight muscles, glycogen is converted into the disaccharide trehalose, the carbohydrate transport form in insects, whose level in hemolymph is raised during flight.

**red pigment concentrating hormone** Peptide hormone from the eyestalk of crustaceans, a member of the adipokinetic hormone/red pigment-concentrating hormone family that comprises structurally related but functionally diverse peptides.

## See Also the Following Articles

**Adipokinetic Hormones and Carbohydrate Metabolism • Adipokinetic Hormones and Lipid Mobilization • Insect Endocrine System**

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# Adrenal Cortex Role in Medullary Synthesis of PNMT

I. C. McMillen and M. B. Adams
*University of Adelaide, Australia*

I. INTRODUCTION
II. THE ADRENAL MEDULLA AND ADRENAL CORTEX—ANATOMY AND DEVELOPMENT
III. SYNTHESIS OF ADRENOMEDULLARY CATECHOLAMINES
IV. GLUCOCORTICOIDS AND PNMT SYNTHESIS
V. GLUCOCORTICOIDS AND PNMT SYNTHESIS DURING DEVELOPMENT
VI. SUMMARY

**During development, two anatomically and functionally distinct structures merge to form the adrenal gland. These structures comprise the central adrenal medulla, which synthesizes and secretes the catecholamines noradrenaline and adrenaline, and the peripheral adrenal cortex, which synthesizes and secretes steroid hormones, most notably the mineralocorticoids (e.g., aldosterone) and the glucocorticoids (e.g., corticosterone or cortisol).**

## I. INTRODUCTION

Although the adrenal medulla and cortex have different embryological origins and are regulated predominantly by separate reflex neurogenic and endocrine mechanisms, respectively, there is substantial functional cross talk between these two separate components of the adrenal gland, which is important in the maintenance of an adequate physiological stress response to a range of metabolic and other physiological stressors. One important aspect of the functional interaction between the adrenal cortex and the medulla is the role of the adrenal cortex in the control of the synthesis of the adrenaline-synthesizing hormone, phenylethanolamine *N*-methyltransferase (PNMT). This article reviews the separate developmental origins of the adrenal medulla and adrenal cortex, the anatomical relationship between these two structures, and the role of the glucocorticoid hormones in the induction and maintenance of PNMT synthesis during different physiological conditions.

## See Also the Following Articles

**Adipokinetic Hormones and Carbohydrate Metabolism • Adipokinetic Hormones and Lipid Mobilization • Insect Endocrine System**

## Further Reading

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# Adrenal Cortex Role in Medullary Synthesis of PNMT

I. C. McMillen and M. B. Adams
*University of Adelaide, Australia*

I. INTRODUCTION
II. THE ADRENAL MEDULLA AND ADRENAL CORTEX—ANATOMY AND DEVELOPMENT
III. SYNTHESIS OF ADRENOMEDULLARY CATECHOLAMINES
IV. GLUCOCORTICOIDS AND PNMT SYNTHESIS
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VI. SUMMARY

**During development, two anatomically and functionally distinct structures merge to form the adrenal gland. These structures comprise the central adrenal medulla, which synthesizes and secretes the catecholamines noradrenaline and adrenaline, and the peripheral adrenal cortex, which synthesizes and secretes steroid hormones, most notably the mineralocorticoids (e.g., aldosterone) and the glucocorticoids (e.g., corticosterone or cortisol).**

## I. INTRODUCTION

Although the adrenal medulla and cortex have different embryological origins and are regulated predominantly by separate reflex neurogenic and endocrine mechanisms, respectively, there is substantial functional cross talk between these two separate components of the adrenal gland, which is important in the maintenance of an adequate physiological stress response to a range of metabolic and other physiological stressors. One important aspect of the functional interaction between the adrenal cortex and the medulla is the role of the adrenal cortex in the control of the synthesis of the adrenaline-synthesizing hormone, phenylethanolamine *N*-methyltransferase (PNMT). This article reviews the separate developmental origins of the adrenal medulla and adrenal cortex, the anatomical relationship between these two structures, and the role of the glucocorticoid hormones in the induction and maintenance of PNMT synthesis during different physiological conditions.

## II. THE ADRENAL MEDULLA AND ADRENAL CORTEX—ANATOMY AND DEVELOPMENT

In early development, ectodermal neural crest cells migrate ventrally from the apex of the neural tube to the dorsal aorta, where they aggregate and differentiate to form sympathetic neurons, or to the adrenal gland primordia, where they differentiate to form chromaffin cells. The migratory primitive chromaffin cells invade the medial side of the developing adrenal cortical anlage and pass between the cortical cells and as the medulla occupy the center of the gland. In the developing mammalian adrenal medulla, three types of cells can be observed depending on the stage of development. The first type includes the primitive sympathetic migratory neurons, which are known as sympathogonia and are totipotent, being capable of differentiating into either sympathetic neurons or chromaffin cells depending on migratory route and the levels of environmental factors, such as the presence of glucocorticoids, nerve growth factor (NGF), and fibroblast growth factors. These cells are characterized by a small rounded nucleus with only a very thin peripheral rim of cytoplasm. The second type of cell is the phaeochromoblast, which has large elongated nuclei and a cytoplasm that is devoid of catecholamine secretory granules. The third and final cell type is the mature "chromaffin" cell or phaeochromocyte, which possesses a smaller ovoid nucleus and contains catecholamine-storing granules, with the cytoplasm giving a positive staining reaction to chromic acid due to the oxidation of the catecholamines to melanin. The mammalian adrenal cortex has its embryological origins in the mesoderm arising from mesenchymal tissue adjacent to the coelomic epithelium lying close to the urogenital ridge. Mesothelial cells between the root of the mesentery and the developing gonad undergo proliferation and subsequently penetrate the underlying mesenchyme. These cells then differentiate to form the adrenocortical masses, which are invaded during development by migrating sympathochromaffin cells. An important feature of the anatomical juxtaposition of the adrenal medulla and cortex is the fact that the continuous networks of blood vessels that supply the adrenal cortex converge and empty into the larger sinusoids present within the corticomedullary and medullary regions, so that the medulla is directly exposed to blood that has passed through the adrenal cortex. The medullary sinusoids then drain into medullary veins.

Aside from the cortical effluent, which drains into the medullary sinusoids, the medulla also receives blood directly from arteries known as arteriae medullae. The presence of arteriae medullae within the adrenal gland is species specific, with relatively few being observed in the rat adrenal gland, whereas substantially greater numbers are found in cat and bovine adrenals. The arteriae medullae derive from branches of the large capsular arteries that pass directly through the adrenal cortex before entering the medulla and branching into arterioles and capillaries. These vessels then empty into the medullary veins, which in turn empty into the central adrenal vein.

From a range of studies in different species, it appears that the adrenal medullary cells are exposed to high concentrations of glucocorticoids delivered either through the vasculature or through paracrine interactions within the gland. In the human adrenal, it has been shown, for instance, that there are variously protrusions, clusters, islets, and single cortical cells that occur diffusely within the adrenal medulla, providing the opportunity for paracrine interactions. Similarly, specific immunostaining for the neuroendocrine protein chromogranin-A has identified the occurrence of chromaffin cells within all three zones of the human adrenal cortex, again supporting a close functional relationship between these cell types. In species such as the sheep or cow, adrenomedullary cells directly adjacent to the zona reticularis and zona fasiculata of the adrenal cortex characteristically synthesize large amounts of adrenaline and the area of the adrenaline-synthesizing zone varies directly with the activity of the adrenal steroidogenic cells. Before discussing the range of mechanisms by which adrenal glucocorticoids may regulate the synthesis of adrenaline, it is necessary to review the synthetic pathway for both of the major adrenal catecholamines, noradrenaline and adrenaline.

Cholinesterase staining and nerve degeneration studies confirm that the majority of the nerve fibers that project to the chromaffin cells of the mammalian adrenal medulla are preganglionic cholinergic sympathetic fibers arising from the splanchnic nerve. Retrograde tracer studies reveal that the preganglionic sympathetic fibers that innervate the adrenal gland arise ipsilaterally from the intermediolateral horn of the spinal cord between thoracic level 3 (T3) and lumbar level 2, with the majority arising from T8–T11. There is a body of evidence to suggest that splanchnic nerve stimulation can enhance ACTH-induced glucocorticoid output from the adrenal cortex. Postganglionic sympathetic fibers containing catecholamines have been found using fluorescence histochemistry to run in close association with vascular supply of the gland. The adrenal gland also appears to have an intrinsic innervation with ganglion cells having been identified within the gland with their numbers being species specific. In the rat, two populations of ganglion cells have been identified. One population of cells, termed the type I ganglion cells, are relatively large and exhibit properties consistent with a postganglionic, noradrenergic phenotype. These cells are probably derived from neural crest cells that invaded the cortical anlage but unlike the chromaffin cells were exposed to a different set of environmental factors that led to their differentiation into neurons. Type II ganglion cells are smaller, have a nonclassical peptidergic transmitter phenotype, and express nitric oxide synthase. Intra-adrenal ganglion cells have fibers that project to cortical, medullary, and capsular regions of the gland, with a number appearing in close proximity to blood vessels and chromaffin cells. Little is known about the physiological function of these intrinsic ganglion cells but they may act to regulate adrenal blood flow and also secretory output from the adrenal medullar and adrenal cortex.

## III. SYNTHESIS OF ADRENOMEDULLARY CATECHOLAMINES

Catecholamines are synthesized in the chromaffin cells of the adrenal gland from the dietary amino acid tyrosine, as illustrated in Fig. 1. The initial step in the synthesis of adrenomedullary catecholamines is the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase [TH; tyrosine 3-monooxygenase; tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2]. TH is a mixed-function oxidase, which requires molecular oxygen and utilizes a tetrahydrobiopterine as a co-substrate. The tetrahydropteridine co-substrate is oxidized to dihydrobiopteridine in the conversion of tyrosine to DOPA; dihydropteredine reductase (EC 1.6.99.7) and nicotinamide adenine dinucleotide phosphate are required to regenerate the pool of the reduced form of the biopterine. Tyrosine hydroxylase is the rate-limiting enzyme in the catecholamine synthetic pathway, as TH has a substantially lower specific activity than any of the other catecholamine synthetic enzymes and the pool of tyrosine is greater than that of any of the other catecholamine synthetic enzyme substrates. Tyrosine hydroxylase is located in the soluble fractions of adrenal medullary homogenates, indicating its presence in the cytoplasm of the chromaffin cells. DOPA is subsequently decarboxylated by DOPA decarboxylase, also known as aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28), to form dopamine. AADC is not specific for catecholamine synthesis as it is involved in the decarboxylation of a number of aromatic L-amino acids and appears to have a wide distribution in a number of tissues. Like TH, AADC activity in the chromaffin cell is found in the water-soluble fraction of adrenal homogenates, indicating that it is located in the cytosol of chromaffin cells. Dopamine is actively transported into the chromaffin granules and converted to noradrenaline by dopamine β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1]. DBH is the only enzyme in the catecholamine biosynthetic pathway located within the chromaffin granules; all of the other enzymes are situated in the cytoplasm of the chromaffin cells. DBH is present in the chromaffin granules in one of two similar forms, either bound to

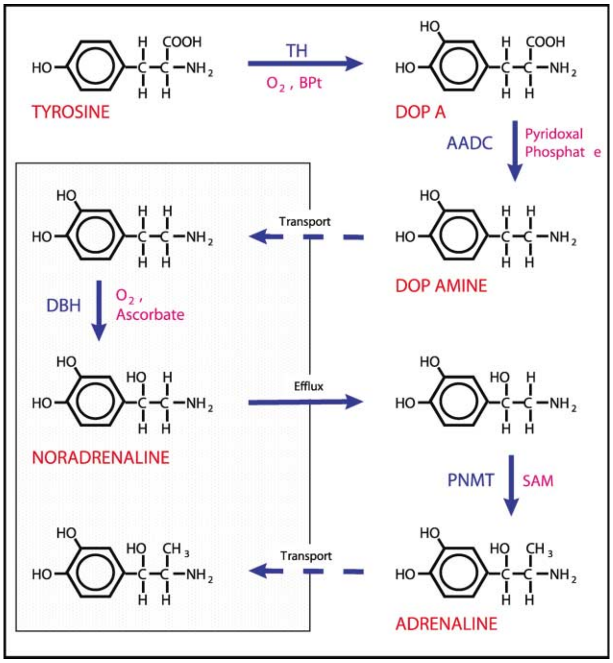

**FIGURE 1** Biosynthetic pathway of adrenomedullary catecholamines. Schematic diagram of the synthesis of catecholamines within the chromaffin cells of the adrenal medulla, with substrates and products, enzymes, and co-factors indicated. Shaded area represents chromaffin granule; the unshaded area depicts the cytosol of the chromaffin cell. DOPA, dihydroxyphenylalanine; TH, tyrosine hydroxylase; AADC, aromatic amino acid decarboxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase; BPt, tetrahydrobiopterine; SAM, *S*-adenosylmethionine.

the internal membrane or free in the internal matrix of the chromaffin granule. The relative proportions of the enzyme that are free versus membrane-bound vary between species, with the free form probably being derived from a membrane-bound precursor that undergoes proteolysis. Like TH, DBH is a mixed-function oxidase and it catalyzes the oxidation of dopamine to noradrenaline, requires molecular oxygen, and utilizes ascorbate as a co-factor. The final step in the catecholamine biosynthetic pathway is the N-methylation of noradrenaline, which passively permeates into the cytosol from chromaffin granules, to adrenaline. This is achieved by transfer of the *S*-methyl group from *S*-adenosylmethionine to the primary nitrogen group of noradrenaline by the enzyme PNMT.

## IV. GLUCOCORTICOIDS AND PNMT SYNTHESIS

Early endocrine ablation and replacement experiments clearly demonstrated that glucocorticoids play a role in the maintenance of the activity of TH and PNMT. Hypophysectomy of the adult rat, which abolishes pituitary ACTH secretion and reduces adrenal corticosterone output, dramatically decreases both adrenaline content and PNMT activity within the adrenal gland. Administration of corticosterone or the potent synthetic glucocorticoid dexamethasone reverses the fall in PNMT activity after hypophysectomy. Glucocorticoids are also found to inhibit the enhanced degradation of the PNMT enzyme that occurs as a consequence of hypophysectomy, by stabilization of the enzyme co-substrate, *S*-adenosylmethionine. A number of studies have found that glucocorticoid administration to hypophysectomized rats also increases PNMT mRNA expression due to increased transcription of the PNMT gene. Analysis of the PNMT gene has revealed the presence of consensus sequences for the glucocorticoid-response element in the $5'$ upstream regulatory/promoter sequence. In primary cultures of isolated bovine chromaffin cells that have been removed from any cortical influence, dexamethasone is a powerful inducer of PNMT activity with an $EC_{50}$ for PNMT mRNA and activity induction of 1–10 nM. Bovine chromaffin cells have been found to possess glucocorticoid-binding receptors with a $K_d$ of approximately 1 nM, and binding of glucocorticoids to the type II glucocorticoid receptors (GRs) present in chromaffin cells results in dimerization and transport into the nucleus, where the complex interacts with glucocorticoid-response elements on the PNMT gene to stimulate transcription. Furthermore, substantially higher levels of GR immunoreactivity have been identified in the adrenaline-containing cells than in the noradrenaline-containing cells of the adrenal medulla of the rat. There is evidence from a range of studies that glucocorticoids appear to play a crucial role in the induction of adrenal PNMT activity and mRNA expression that occurs in response to a range of stressors, thus demonstrating the importance of the anatomical juxtaposition of the adrenal cortex and adrenal medulla in the generation of the adaptive stress response.

## V. GLUCOCORTICOIDS AND PNMT SYNTHESIS DURING DEVELOPMENT

Previous studies have shown that when dissociated chromaffin cells from immature adrenals are cultured in the absence of glucocorticoids they exhibit poor long-term survival in culture. The addition of NGF to these cultures is able to rescue many of the chromaffin cells; however, they subsequently undergo a phenotypical change exhibiting extensive neurite outgrowth. Glucocorticoid administration is able to block or delay NGF-induced neurite outgrowth. Long-term culture of rat adrenal chromaffin cells in the presence of NGF and the absence of glucocorticoids results in a complete transition to a sympathetic neuron phenotype as assessed by a number of morphological and biochemical criteria. Hence, glucocorticoids appear to act as a survival factor for chromaffin cells and repress the neuronal transdifferentiation of these cells. The levels of corticosterone present in the fetal rat adrenal gland at the time of invasion by the SA progenitor cells would be more than sufficient to suppress neural transdifferentiation by these cells. It has therefore been considered that the sympathetic progenitor cells that migrate from the sympathetic ganglion primordium through the developing adrenal anlage develop into chromaffin cells in the adrenal medulla under the influence of the glucocorticoid-rich environment. Recent studies, however, have challenged this view as an analysis of mice carrying targeted mutations of the GR gene found that the mice lacking a GR gene product had normal numbers of adrenal chromaffin cells. The GR mutant mice did, however, lack adrenomedullary PNMT. It therefore appears that the role of glucocorticoids in development is to modulate directly or indirectly the expression of PNMT, but that the expression of PNMT itself does not determine the chromaffin phenotype.

There are also important interactions between the adrenal cortex and the adrenal medulla in those species, such as sheep and human, that have a prolonged gestation period and in which there are intact neuroendocrine responses to intrauterine stressors present before birth. Classical studies in the sheep fetus have shown that adrenaline content in the adrenal gland increased markedly during the 10–15 days preceding parturition coincident with the prepartum surge in adrenal cortisol output. Intrafetal administration of adrenaline to the sheep halts lung liquid secretion and stimulates lung liquid absorption by a β-adrenergic-dependent mechanism. Stimulation of $\beta_2$-adrenoreceptors in the fetal sheep and fetal rabbit lung with adrenaline and specific $\beta_2$-adrenoreceptor agonists also elicits an increase in pulmonary phospholipid synthesis and also the levels of phospholipids in lung lavage fluid. Catecholamines, in particular adrenaline, via $\beta_2$-adrenoreceptor stimulation are able to stimulate an increase in pulmonary surfactant secretion and synthesis. The well-established actions of glucocorticoids on surfactant synthesis in the lungs are therefore enhanced by the concomitant actions of the catecholamines. Newborns that are delivered by caesarean section do not experience the substantial catecholamine surge that occurs in babies that are vaginally delivered and have a lower lung compliance after delivery.

## VI. SUMMARY

The functional interactions between the neighboring cells of the adrenal medulla and adrenal cortex are initiated early in development and are maintained by the paracrine and endocrine actions of the adrenocortical glucocorticoids on PNMT and hence adrenaline synthesis in the adrenomedullary chromaffin cells. The array of strategies that exist across a range of species to ensure that adrenomedullary cells that synthesize adrenaline are in close contact with the adrenocortical cells highlight the importance of a coordinated adrenocortical–medullary response to a range of acute and chronic stressors from before birth and into adult life. The chromaffin cells are therefore well placed to integrate both neurogenic and hormonal stimuli to ensure that adrenaline and the glucocorticoids act in a tightly regulated partnership to enhance the metabolic, cardiovascular, and respiratory responses to physiological challenges that threaten homeostasis.

## Glossary

**chromaffin cell** The functional cell of the adrenal medulla; it contains catecholamine-storing granules, with the cytoplasm giving a positive staining reaction with chromic acid due to the oxidation of the catecholamines.

**dopamine β-hydroxylase** The only enzyme in the catecholamine biosynthetic pathway located within the catecholamine-containing granules of the chromaffin cell, where it catalyzes the conversion of dopamine to noradrenaline.

**glucocorticoid receptors** Proteins that are present in chromaffin cells and which, after binding with the glucocorticoids, dimerize and are transported to the nucleus of the cell, where the complex interacts with the glucocorticoid-response elements on the phenylethanolamine *N*-methyltransferase gene to stimulate transcription.

**phenylethanolamine N-methyltransferase** An enzyme that catalyzes the final step in the catecholamine biosynthetic pathway, i.e., the N-methylation of noradrenaline to result in the synthesis of adrenaline.

**tyrosine hydroxylase** The enzyme that catalyzes the oxidation of tyrosine to dihydroxyphenylalanine; it is the rate-limiting enzyme in the catecholamine synthetic pathway in the adrenal medulla.

## See Also the Following Articles

**Adrenocorticosteroids and Cancer • Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • Glucocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Biosynthesis • Mineralocorticoid Effects on Physiology and Gene Expression**

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# Adrenocorticosteroids and Cancer

THOMAS J. DILLING AND W. GILLIES MCKENNA
*University of Pennsylvania*

I. ADRENOCORTICAL TUMORS
II. PARANEOPLASTIC SYNDROMES
III. MANAGEMENT OF TUMOR SEQUELAE
IV. TREATMENT OF CANCER
V. MANAGEMENT OF SEQUELAE FROM CANCER TREATMENT

**Adrenocorticosteroids play several distinct roles for the clinical oncologist. Rarely, patients may have a primary tumor of the adrenal gland itself, leading to a clinical excess (or dearth) of adrenocortical hormones. Sometimes, too, tumors outside the adrenal axis can produce these hormones, causing well-described clinical syndromes. Expansile tumors in the brain or spinal column are an oncologic emergency, managed in part with adrenocorticosteroids. Sometimes steroids are also used as a primary chemotherapeutic agent in the treatment of the cancer itself. Finally, despite the oncologist's best efforts, patients sometimes experience side effects from chemotherapy and/or radiation, particularly in the lung; these side effects can effectively be managed by adrenocorticosteroids in many cases.**

## I. ADRENOCORTICAL TUMORS

Adrenal cortical carcinoma is a very rare tumor, comprising 0.05 to 0.20% of all cancers. It may be either functional or nonfunctional in terms of hormone production. When functional, the carcinomas may secrete excessive amounts of either adrenocorticotropic hormone (ACTH, or corticotropin) or sex hormones. Children less than 6 years of age can present with evidence of virilization, precocious puberty, or Cushing's syndrome. Adults (typically between the ages of 40 and 50) can also present with these tumors, as evidenced by hormonal syndromes (feminization, virilization, hypercortisolism, or hyperaldosteronism). Surgery is curative if total resection is possible, though 70% of patients present with positive lymph nodes or distant metastasis and therefore require chemotherapy.

## II. PARANEOPLASTIC SYNDROMES

It should be noted that tumors outside the adrenal axis sometimes secrete adrenal corticosteroids. The classic tumors with ectopic endocrine production are some small-cell cancers of the lung and pituitary adenomas, though other less frequent tumors appear on this list as well. Pituitary ACTH overproduction (also known as Cushing's disease) is the most common syndrome, occurring in 55 to 82% of patients, and adrenal dysfunction occurs in 5 to 32% of patients, ectopic ACTH production in 11 to 25% of patients, and CRH overproduction in approximately 1% of patients. The resultant paraneoplastic syndromes are numerous. Cushing's initial description of the peripheral effects of hyperfunctioning pituitary adenoma included truncal obesity, purple striae, hypertension, fatigue, moon facies, buffalo hump, weakness, depression, amenorrhea, hirsutism, and edema. Complete treatment of this vast topic is beyond the scope of this article, though the reader is encouraged to consult the citations under Further Reading for additional information.

## III. MANAGEMENT OF TUMOR SEQUELAE

Two dreaded complications of malignancy include spinal cord compression and life-threatening brain edema. In the former condition, a metastatic clone of malignant cells grows and expands within the spinal column, eventually compressing the spinal cord to the point that permanent paralysis can quickly ensue if not treated urgently. Likewise, primary brain tumors (or lesions metastatic to the brain) can expand within the fixed volume of the skull and cause life-threatening edema, leading to gross alteration of personality, seizures, or death.

Adrenocorticosteroids have a potent anti-inflammatory activity that plays an important role in the management of these conditions. Dexamethasone is commonly prescribed in doses ranging from 16 to

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Adrenocorticosteroids have a potent anti-inflammatory activity that plays an important role in the management of these conditions. Dexamethasone is commonly prescribed in doses ranging from 16 to

100 mg/day, tapering over time. Studies on spinal cord compression have failed to demonstrate a distinct advantage for the higher doses, though patients do appear to experience diminished pain after 24 h on the higher doses. Lower doses are usually used to treat brain edema. In any case, radiation therapy or surgical decompression is then performed urgently to debulk the tumor.

## IV. TREATMENT OF CANCER

In 1943, a corticosteroid (the so-called Compound E) was reported to cause atrophy of lymphatic tissue in mice. Cortisone acetate and adrenocorticotropic hormone became clinically available in 1948. In November 1949, a group of researchers published a report in which they noted cancer regression in a pair of patients with different types of lymphoma. They stated that enlargement of lymph nodes occurred approximately 6 weeks after ACTH was discontinued in the patient with Hodgkin's disease, but found that there was no recurrence in the patient with lymphosarcoma 10 weeks after discontinuation of therapy. A number of studies followed in the 1950s and 1960s, confirming the earlier results regarding the importance of prednisone in treating lymphoma.

Although individual compounds were shown to be helpful, they did not always completely cure patients of these lymphomas or produce lasting remission. In 1968, Vincent DeVita and co-workers reported their landmark study, first published in 1970, in which they combined four different chemotherapeutics that had previously demonstrated individual activity against Hodgkin's disease. They combined cyclophosphamide, vincristine, procarbazine, and prednisone to create the MOPP chemotherapy regime. When tested on 43 patients, it induced complete remission in 35, 17 of whom had lasting remission—a vast improvement over any previously published results. In the years since, prednisone has also been shown to have activity against chronic leukemia and non-Hodgkin's lymphoma. In addition, different combinations (most notably CHOP, which also contains prednisone) have been tested and have become standards in the treatment of some lymphatic/leukemic cancers.

## V. MANAGEMENT OF SEQUELAE FROM CANCER TREATMENT

In the years since chemotherapy and radiation therapy were introduced, physicians and scientists have improved the efficacy of these treatments while simultaneously diminishing their side effects on the patient. Occasionally, however, despite physicians' great determination, patients do experience problems related to the cancer therapy itself. While the specifics of all these potential problems are far beyond the scope of this article, one requires mention here. The lung is a relatively sensitive organ, and the tumors that occupy it can be quite resistant to treatment, requiring high doses of radiation or chemotherapy in an attempt to achieve patient cure. Clinicians, therefore, must balance these opposing problems by examining the therapeutic ratio—the ability to sterilize the tumor while sparing normal tissues.

Chemotherapy and radiation sometimes cause a hypersensitivity-type reaction called radiation pneumonitis. Chemotherapy has been shown to be a radiation sensitizer in the lung, which is important to remember in this era of combination chemotherapy and radiation. Approximately 43% of patients demonstrate radiologic changes within the lung from chemoradiotherapy treatment. Interestingly, symptomatic radiation pneumonitis occurs in only approximately 7% of patients. Patient variability seems to play a role. The volume of lung actually being irradiated, which can vary greatly depending upon the exact disease process, also determines whether a patient will experience this problem. It has also been shown that prior irradiation or chemotherapy can predispose a patient to radiation pneumonitis. Preexisting lung disease has also been implicated. Likewise, it has been shown that subclinical radiation pneumonitis can become symptomatic after steroid withdrawal.

Clinically, the syndrome generally manifests itself within about 2 to 3 months after the completion of therapy. Occasionally it presents as quickly as 1 month or as late as 6 months after the completion of treatment. Patients present with shortness of breath, which can vary from mild to severe. Cough is also typically prominent in about half of these patients. Fever is also sometimes seen. The symptoms can be relatively minor if the area of lung treated is small. On the other hand, the clinical course can be fulminant, leading to respiratory insufficiency and cyanosis, progressing to cor pulmonale (right heart failure) in a matter of days.

The histopathology and cellular biology of this disease process are fairly well elucidated. At 0 to 2 months after radiation, injury to small vessels and capillaries can be seen. This leads to vascular congestion and increased capillary permeability. As a result, proteinaceous material is deposited within the alveoli of the lungs. In the range of 2 to 9 months postradiation, one can note microscopic evidence of obstruction of pulmonary capillaries. In addition, one sees hyperplasia of the vascular endothelium, and the walls of the alveoli become infiltrated with fibroblasts. If the radiation injury is mild, these changes may subside. However, if the injury is severe enough, it can progress to a chronic stage.

Corticosteroid administration dramatically improves the physiologic changes in mice and decreases their mortality. When given prophylactically, the steroids failed to prevent radiation pneumonitis, but when given at the first sign of clinical symptoms, they produced a clinical response. Other reports, however, failed to show amelioration of severe radiation pneumonitis with steroid administration. No controlled clinical trials have been conducted, however, to prove their efficacy in humans. Despite this fact, they are commonly given when the diagnosis is reasonably ascertained, at a dose of 1 mg/kg. This dose is maintained for several weeks and then slowly weaned.

## Glossary

**paraneoplastic syndromes** Clinical syndromes experienced by some cancer patients due to the production of hormones or other substances by a tumor. The symptoms of the syndrome depend upon the exact substance(s) produced.

**radiation pneumonitis** Pathologically demonstrable series of changes seen in the lung tissue of some patients after irradiation. The syndrome can range from subclinical to highly toxic in severity and may be either self-limited or chronic.

**spinal cord compression** Oncologic emergency in which a clone of tumor cells expands within the spinal column, leading to permanent paralysis if not treated urgently.

## See Also the Following Articles

**Adrenal Cortex Role in Medulla Synthesis of PNMT • Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • Androgen Receptors and Prostate Cancer • Apoptosis, Glucocorticoid-Induced • Cancer Cells and Progrowth/Prosurvival Signaling • Estrogen and Progesterone Receptors in Breast Cancer • Glucocorticoid Effects on Physiology and Gene Expression**

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# Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides

Richard G. Allen

*Oregon Health & Science University, Portland*

I. ACTH AND POMC PEPTIDES
II. PHYSIOLOGY OF ACTH AND POMC-DERIVED PEPTIDES
III. REGULATION OF ACTH AND POMC-DERIVED PEPTIDES
IV. SUMMARY

**Adrenocorticotropin (ACTH) is a 39-amino-acid peptide derived from the prohormone proopiomelanocortin (POMC). ACTH stimulates adrenocortical steroid synthesis in response to stress and circadian fluctuations. ACTH is synthesized in corticotropes in the anterior lobe of the pituitary gland and, to a much lesser extent, in melantropes of the intermediate pituitary of many species with the exception of humans. It was first thought that ACTH was exclusively an endocrine hormone; however,**

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Richard G. Allen
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**ACTH and its POMC relatives are expressed abundantly in the nervous system. POMC is a complex prohormone cleaved by specific prohormone convertases. In addition to ACTH, POMC contains the amino acid sequences of β-lipotropic hormone (β-LPH), β-endorphin, the melanocortins, corticotropin intermediate lobe peptide, and joining peptide. POMC is also highly expressed in neurons of the arcuate nucleus of the hypothalamus and the nuclear trachis solitacius. Even though the structure of POMC is the same wherever it is expressed, posttranslational cleavage of POMC can produce a different variety of biologically active peptides in each cell.**

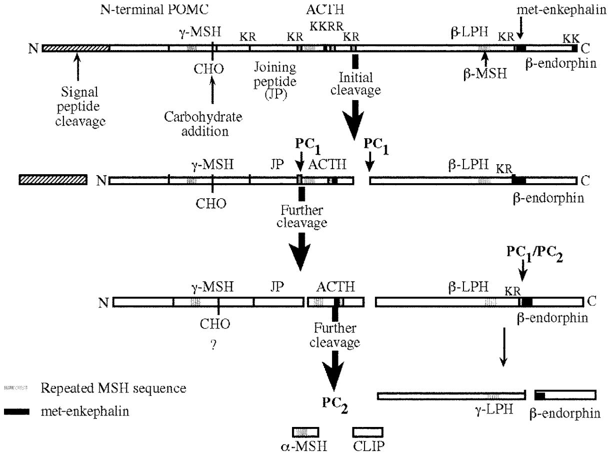

**FIGURE 1** Processing of human proopiomelanocortin (POMC). The processing proceeds in stages, yielding a variety of forms of secreted peptides; N-terminal peptide, adrenocorticotropic hormone (ACTH), and β-lipotropin are the principal circulating forms produced by the anterior pituitary corticotroph. In the rodent, approximately 40% of ACTH has a posttranslational addition of a phosphate moiety. γ-MSH, γ-melanocyte-stimulating hormone; α-MSH, α-melanocyte-stimulating hormone; β-LPH, β-lipotropin, CHO, carbohydrate. In the CNS, ACTH is converted to α-MSH and CLIP.

## I. ACTH AND POMC PEPTIDES

The biologically active peptides derived from POMC are the result of proteolytic processing by the prohormone convertases PC1/3 and PC2. Several such enzymes have been characterized and include the PCs, as well as furan and PACE 4. These enzymes are members of the family of kexin/subtilisin-like serine proteases. PCs cleave at single or multiple basic amino acid residues; however, as is the case with PC2, an accessory protein may be required for activity. The basic amino acid sequence lysine, arginine (KR) is the most often observed cleavage motif, but KK, RR, and RK will subserve this function as well. The PCs have different affinities for these basic cleavage motifs and thus generate different bioactivities from a common prohormone in a given cell type.

Peptide processing studies established that the principal peptide derivatives of POMC in the anterior lobe cells of humans are the N-terminal peptide, ACTH, and β-lipotropin (Fig. 1). The intermediate lobe POMC cells, called melanotropes, are present in other mammals and in the human fetus, but largely are vestigial in the human adult. The melanotropes produce additional products such as α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) from ACTH, and β-endorphin from γ-lipotropin. The smaller POMC peptides are also produced in the human nervous system, with ACTH serving as a biosynthetic intermediate for the production of α-MSH. In rodents, the biological activity of α-MSH is enhanced by α-N-acetylation; however, the acetylation state of human α-MSHs is unclear at present. Human β-MSH probably is not secreted as a distinct hormone but is a by-product of postsecretory proteolysis. It is also interesting to note that the nonhuman primate exhibits the same lobe-specific POMC processing found in rodents and not humans.

In humans, each of the three principal products of POMC contains a common tetrapeptide core (His-Phe-Arg-Trp), which in turn is contained in the three melanocortin peptides, α-MSH, β-MSH, and γ-MSH. In addition, ACTH itself contains the tetrapeptide core and has melanotropic (pigment-producing) activity. This tetrapeptide core is the recognition site for the ACTH and melanocortin receptors. Previously, it was suggested that the hyperpigmentation found in disorders of excess ACTH may be related to ACTH itself or to the other melanotropic derivatives of the POMC molecule. The melanotropic activities of these peptides, particularly γ-MSH, have not been fully elucidated, and the circulating concentrations of these melanotropin peptides have not yet been determined simultaneously. Nonetheless, the absence of significant amounts of γ-MSH, and of β-MSH in humans, support ACTH as the likely candidate.

The cloning of five different melanocortin receptors (MC1–5) began a new era in studying POMC peptides. All of the receptors for POMC peptides that have been cloned belong to the seven-transmembrane G-protein-coupled receptor superfamily. ACTH binds to the adrenal ACTH receptor (MC2) with nanomolar affinity. It then activates adenylyl cyclase via Gαs, increasing intracellular cAMP, activating protein kinase A, and thus resulting in the phosphorylation of proteins. Activation of this pathway induces cortisol synthesis and secretion.

The melanocortins (the MSHs) bind to MC1 and MC3–5 with nanomolar affinity to mediate a variety of physiological actions described below. All of the MC receptors stimulate cAMP production when activated by their respective ligands. In contrast, β-endorphin binds the μ class of opioid receptors and inhibits cAMP production through the Gαi/o class of G-protein-coupled receptors. The receptor for β-LPH has not been cloned.

## II. PHYSIOLOGY OF ACTH AND POMC-DERIVED PEPTIDES

ACTH, released from the anterior pituitary corticotroph, is the major physiologic regulator of the synthesis and secretion of glucocorticoids by the adrenal glands. Glucocorticoids act on many processes mainly by altering gene transcription and protein synthesis in the target cells. In, addition, glucocorticoids permit metabolic adaptation during fasting to prevent low blood sugar. They also play an important role in the body response to physical and emotional stress. Other actions include their inhibitory effect on inflammation and regulation of vascular responsiveness to norepinephrine.

ACTH rapidly stimulates steroidogenesis, which can result in a great rise in blood glucocorticoids within seconds or minutes. To support this function, ACTH stimulates adrenal blood flow and the rate-limiting step of glucocorticoid synthesis: the conversion of cholesterol to pregnenolone. It also exerts several long-term tropic effects on adrenal cells that maintain the cellular machinery necessary to carry out steroidogenesis.

In addition to ACTH, β-lipotropin is cleaved from POMC in anterior pituitary corticotrophs. β-Lipotropin has effects on lipid metabolism, but its physiologic function in humans has not yet been established. When stimulated, corticotrophs secrete ACTH and β-lipotropin in a 1:1 ratio into the bloodstream.

The circulating levels of ACTH and glucocorticoids are greatly influenced by stress. When an individual is exposed to a stressful situation, levels of ACTH and glucocorticoids rise rapidly in the blood. Stress stimulates the hypothalamic–pituitary–adrenal axis regardless of the existing concentrations of circulation glucocorticoids. This occurs because stress increases neural activity in the CNS, stimulating the hypothalamic parvicellular neurons in the paraventricular nucleus to secrete corticotropin-releasing hormone (CRH) at a greater rate. If stress persists, the hypothalamic–pituitary–adrenal axis will function at a higher setpoint, maintaining higher concentrations of ACTH and glucocorticoids, which may eventually have detrimental effects on the individual. The role of the intermediate lobe melanotrope in stress response is not clear. For instance, in all nonhuman species studied, the majority of the endorphins produced by the intermediate lobe are α-N-acetylated and thus do not bind the opioid receptors. In lower organisms, circulating melanocortins may be involved in pigmentation responses to stress.

Whereas the first two decades of POMC research were heavily focused on endorphins, their expression in the pituitary, and the expression of these peptides in the CNS, recently there has been intense interest in POMC peptides and feeding behavior. The cloning of the melanocortin and ACTH receptors contributed greatly to this interest as α-MSH had been implicated in food intake regulation for many years. The melanocortins also have wide variety of physiological effects including alterations in motor activity and sexual behavior, analgesia, memory, nerve regeneration, antipyretic actions, pigmentation, and grooming behavior.

Though many studies have been performed in rodent models examining POMC peptides and obesity, only in the past 5 years has firm evidence for POMC's involvement in obesity emerged. Much of this information comes from genetic studies. A current hypothesis is that mutations in regulatory elements of the POMC gene decrease the levels of POMC expression in the brain, thus causing alterations in energy metabolism.

There have been a small number of children with null mutations in the POMC gene resulting in undetectable levels of ACTH. The common phenotype presented was red hair, adrenal insufficiency, and severe, early onset obesity. Furthermore, recessive and dominant mutations in the MC4 receptor (MC4-R) gene have been seen in humans and these mutations are proposed to cause 5% of childhood obesity. These and other data suggest that obesity may be a genetic disease of the hypothalamus and that POMC may be a major player.

The POMC and neuropeptide Y systems are the most extensively studied regarding energy intake. The POMC system receives input detailing the nutritional status and energy storage via insulin and leptin signaling mediated by receptors on POMC neurons in the arcuate nucleus of the hypothalamus. There is a complex interaction of food intake inhibition by α-MSH and agouti-related protein at the level MC4-R. CRH is the mediator of food intake and environmental conditions. It inhibits food intake and weight gain, activates the hypothalamic–pituitary axis, and modulates energy storage in adipose tissue. The link between the CRH system and POMC neurons in the hypothalamus and other brain areas remains to be completely defined.

## III. REGULATION OF ACTH AND POMC-DERIVED PEPTIDES

Pituitary ACTH secretion is under two primary regulatory influences. There is a positive peptidergic influence from the hypothalamus, mediated by CRH, which varies diurnally and is subject to extreme perturbation by stress. There also is a negative feedback influence of glucocorticoids, acting both at the pituitary level to inhibit the corticotrope and at the hypothalamic level to inhibit CRH neurons. Cortisol is the major physiologic glucocorticoid inhibitor of ACTH secretion; there is no other important endogenous ACTH inhibitor in humans. Figure 2 shows a scheme that depicts a variety of neurotransmitter inputs to the hypothalamus that have been postulated to regulate CRH release and ultimately, the secretion of ACTH. Further, vasopressin (AVP) and melatonin have been implicated in the regulation of ACTH secretion. In particular, AVP and melatonin released from the suprachiasmatic nucleus appear to be intimately involved in the circadian rhythm of glucocorticoids. In addition, AVP released from the neural lobe of the pituitary has long been thought to act synergistically with CRH to enhance ACTH secretion from the anterior lobe.

In humans, ACTH has a diurnal rhythm, exhibiting the highest plasma levels at approximately 6–8 AM and a nadir at approximately midnight. In rats, this pattern is reversed, and both lighting and activity may alter this cycle. In humans, there is also a free-running cycle of plasma cortisol that varies between 25 and 33 h. All of the other peptides derived from pituitary POMC demonstrate these cycles; however, due to differences in the half-lives of the peptides, the plasma concentrations are not superimposable.

The negative feedback effect of glucocorticoids on ACTH secretion results from actions on both the hypothalamus and the corticotroph. Glucocorticoids act directly on the corticotrophs to decrease POMC synthesis and secretion. Glucocorticoids inhibit the release of CRH from the hypothalamus, which also decreases ACTH release. In addition, glucocorticoids inhibit ACTH secretion by acting directly on the corticotroph to inhibit the action of CRH. As a result, CRH becomes less effective at stimulating ACTH release. In species other than humans, the synthesis and secretion of POMC peptides are tonically inhibited by hypothalamic neurons releasing dopamine. Intermediate lobe melanotropes do not express the glucocorticoid receptor and therefore are not regulated by glucocorticoids.

Cortisol and its synthetic analogues (e.g., prednisone and dexamethasone) suppress ACTH secretion. After the administration of a dose of a glucocorticoid, ACTH secretion diminishes within minutes ("fast feedback") and, in a second phase ("delayed feedback"), is suppressed for hours or days, depending on the glucocorticoid used. It is unclear whether the fast and delayed feedback actions are associated with different anatomic sites. It is likely, however, that delayed feedback is an intracellular event operating through changes in nuclear mechanisms, whereas fast feedback may be dependent on

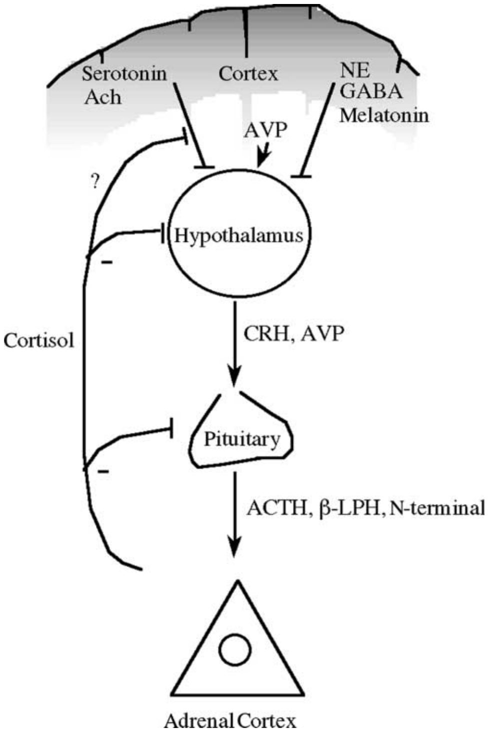

FIGURE 2 Scheme for control of ACTH secretion. Ach, acetylcholine; NE, norepinephrine; GABA, γ-aminobutyric acid; CRH, corticotropin-releasing hormone; AVP, arginine vasopressin.

effects on the cell membrane mediated through other classes of receptors.

Although pituitary POMC regulation by circulating glucocorticoids is fairly well understood, much less is known about POMC peptide regulation in the brain. In the rodent, a large cell group of POMC neurons is bilaterally distributed in the arcuate nucleus of the hypothalamus and projects extensively to other regions of the brain. These projections pass through limbic structures, the diencephalon, and amygdala, as well as the thalamus and periaqueductal gray, areas associated with nociception and sensory integration. In addition, there are projections to the brainstem, which are thought to be involved in the regulation of cardiovascular and respiratory systems. In the nucleus tractus solitarius, POMC neurons send projections to the spinal cord and probably interact with local POMC circuits in the spinal cord itself. It appears that human central nervous system POMC expression resembles that found in the rodent, thus serving as a good model for future POMC peptide research.

## IV. SUMMARY

POMC is a prohormone that gives rise to several biologically active peptides that are expressed primarily in the pituitary and brain. ACTH, the melanotropins, and endorphins are liberated by specific proteolytic enzymes called PCs. By expressing the PCs in a cell-type-specific fashion, a variety of POMC-derived products can be differentially expressed. When combined with the tissue-specific expression of POMC peptides and their cognate receptors, an incredibly diverse array of physiological responses can be produced. Since all of the peptides derived from POMC are co-released from large secretory granules, a myriad of biological activities are modulated simultaneously. In the case of POMC, these are glucocorticoid production, food intake, stress responses, and modulation of pain perception. There is much to learn about how the modulation of these specific POMC-derived bioactivities evolved to be regulated in a coordinated manner.

## Glossary

**adrenocorticotropic hormone** A peptide that elicits glucocorticoid secretion from the adrenal cortex.

**β-endorphin** A peptide possessing analgesic properties mediated through the opioid receptors.

**β-LPH** A peptide derived from proopioimelanocortin containing γ-MSH and β-endorphin.

**α-MSH, β-MSH, γ-MSH** α-, β-, and γ-Melanocyte-stimulating hormones, collectively called the melanocortins. These peptides are thought to stimulate pigmentation and may be involved in appetite and feeding behavior.

**prohormone convertase** A class of proteolytic enzymes responsible for cleaving proopioimelanocortin into biologically active peptides.

**prohormone** A protein that is converted to biologically active molecules via posttranslational processing by specific proteolytic enzymes or other modifications.

**proopioimelanocortin** A prohormone expressed in the neuroendocrine system.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Corticotropin-Releasing Hormone, Stress, and the Immune System • Endocrine Rhythms: Generation, Regulation, and Integration • Glucocorticoid Effects on Physiology and Gene Expression • Melatonin in Humans • Neuropeptides and Control of Anterior Pituitary • Stress • Vasopressin (AVP)**

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# Amino Acid and Nitric Oxide Control of the Anterior Pituitary

Michael Selmanoff
*University of Maryland School of Medicine*

I. HYPOTHALAMIC CONTROL OF PITUITARY GLAND FUNCTION
II. HYPOTHALAMIC GABA NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION
III. HYPOTHALAMIC GLUTAMATE NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION
IV. HYPOTHALAMIC NITRIC OXIDE NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION
V. THE ROLE OF GABA, GLUTAMATE, AND NITRIC OXIDE NEURONS IN THE NEUROENDOCRINE REGULATION OF OTHER ANTERIOR PITUITARY TROPIC HORMONES

**Hypothalamic neurosecretory neurons regulate the secretion of the six anterior pituitary tropic hormones. In turn, these first-order, final common pathway neurons of the neuroendocrine system are immediately controlled by second-order afferent neurons. Most of these hypothalamic afferent neurons utilize either the inhibitory neurotransmitter γ-aminobutyric acid (GABA) or the excitatory neurotransmitter glutamate (Glu). Second-order neurons also regulate the neurosecretory neurons by elaborating the neuroactive gas nitric oxide (NO). Second-order GABA neurons uniformly decrease the secretion of the anterior pituitary tropic hormones, whereas Glu neurons increase their secretion. Second-order NO neurons mostly increase, but in one case decrease, anterior pituitary tropic hormone secretion.**

## I. HYPOTHALAMIC CONTROL OF PITUITARY GLAND FUNCTION

### A. Hypothalamic Releasing and Inhibiting Hormones

Neurons situated in the hypothalamus control the synthesis and secretion of the hormones of the anterior and posterior pituitary gland (Fig. 1). The posterior pituitary hormones, oxytocin and vasopressin, are synthesized in the cell bodies of neurons located in the hypothalamic supraoptic and paraventricular nuclei. Axons of these neurons project to terminals in the posterior lobe (PL), from which oxytocin and vasopressin are released on depolarization. In contrast, the anterior pituitary hormones are controlled by hypothalamic neurons, whose cell bodies are located in arcuate nuclei (AN) and other

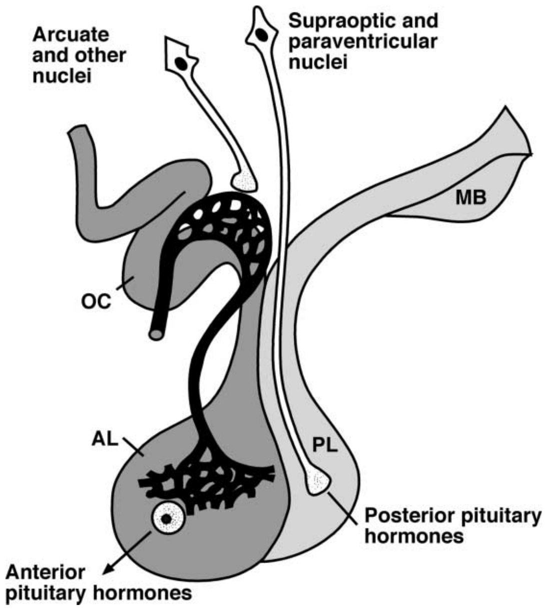
Arcuate and other nuclei
Supraoptic and paraventricular nuclei
MB
OC
AL
PL
Posterior pituitary hormones
Anterior pituitary hormones

FIGURE 1 Secretion of hypothalamic hormones. The hormones of the posterior lobe (PL) are released into the general circulation from the endings of supraoptic and paraventricular neurons, while hypophysiotropic hormones are secreted into the hypophysial portal circulation from the endings of arcuate and other hypothalamic neurons. The hormones circulate in the portal vessels to the anterior lobe (AL), where they control the synthesis and secretion of the anterior pituitary tropic hormones. MB, Mamillary bodies; OC, optic chiasm. Modified from Ganong (2001), with permission.

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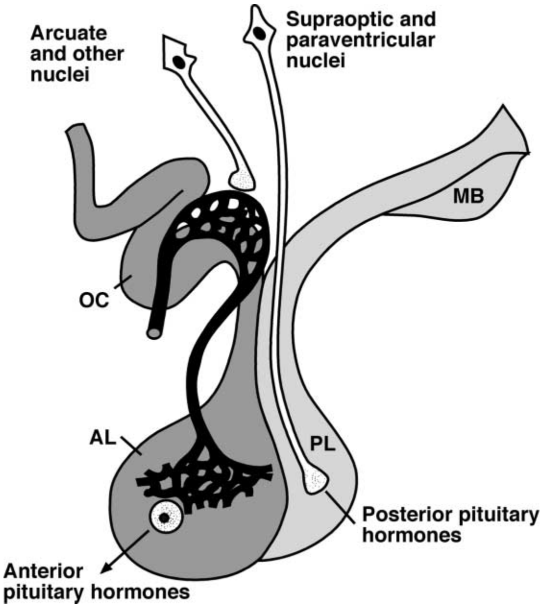
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hypothalamic nuclei, and whose axons project to terminals situated in the median eminence (ME). On depolarization, these neurons release their hypothalamic releasing and inhibiting hormones into the hypophysial portal circulation, which carries them to the anterior lobe (AL). There the hormones bind to specific high-affinity, low-capacity receptors on the AL cells, which synthesize and secrete the anterior pituitary tropic hormones. Although there is some evidence that GABA, Glu, and NO affect anterior pituitary tropic hormone secretion by direct actions at the level of the pituitary, at the present time most of this evidence is not compelling; hence, further discussion here is restricted to the hypothalamic actions of these neurotransmitters.

## B. Tropic Hormones of the Anterior Pituitary Gland

Six anterior pituitary tropic hormones (Fig. 2) control the functioning of the adrenal glands [adrenocorticotropic hormone (ACTH)], the thyroid gland [thyroid-stimulating hormone (TSH)], the mammary glands [prolactin (PRL)], and the gonads [luteinizing hormone and follicle-stimulating hormone (LH and FSH)] and exert widespread effects on body growth [growth hormone (GH)]. These AL tropic hormones are regulated by the hypothalamic releasing and inhibiting hormones corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), prolactin-inhibiting hormone (PIH or dopamine), prolactin-releasing factor (PRF), gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), and somatostatin (SS or growth hormone-inhibiting hormone). The cell bodies of these neurons reside in several hypothalamic structures (Fig. 3). The regulation of these hypophysiotropic hormone-secreting neurons by second-order, afferent neurons, which synapse on them and increase or decrease their firing rate in response to physiological stimuli, is an intensely active area of neuroendocrine research. It is now clear that some of these afferents are neurons utilizing inhibitory (GABA, glycine, taurine, β-alanine) and excitatory (Glu, aspartate, cysteic acid, homocysteic acid) amino acid neurotransmitters, and neurons that elaborate neuroactive gases (NO, carbon monoxide). Most workers in this area to date have studied the principal inhibitory and excitatory amino acid and neuroactive gas neurotransmitters: GABA, Glu, and NO. Electrophysiological and whole-cell patch-clamp work by van den Pol and co-workers indicates that almost all fast synaptic activity in the hypothalamus is generated by the release of the inhibitory transmitter GABA or the excitatory transmitter Glu. Hence, this article focuses on three systems of neuronal afferents, GABA, Glu, and NO neurons, which likely affect the activity of all of the hypophysiotropic hormone-secreting neurons. To date, the majority of the research in this area has focused on the regulation of the GnRH neurons by afferent GABA, Glu, and NO neurons. Evidence for the regulation of GnRH is presented here in some detail; the effect of these afferent neurons on the neuroendocrine control of the other anterior pituitary hormones is limited to a summary table.

## C. The Hypothalamic GnRH Pulse Generator

GnRH is released in episodic bursts that produce the pulses of LH observed in male and female mammals, and in other vertebrate species as well. In humans, the LH pulses in men occur at a frequency of one every 90 min; the LH pulse frequency in women ranges

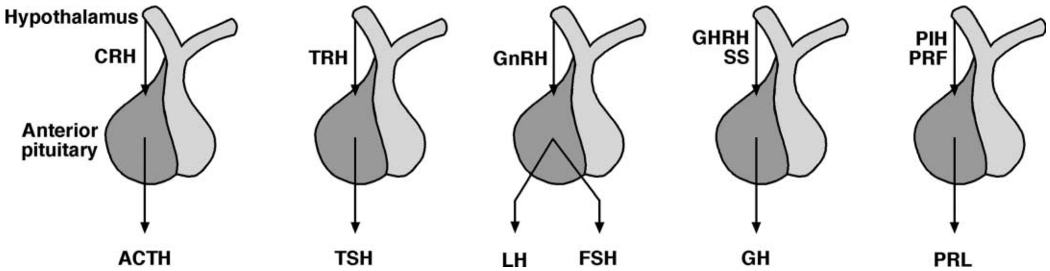

FIGURE 2 The hypothalamic hypophysiotropic hormones that control the secretion of the anterior pituitary tropic hormones. CRH, Corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GHRH, growth hormone-releasing hormone; SS, somatostatin (growth hormone-inhibiting hormone); GH, growth hormone; PIH, prolactin-inhibiting hormone (dopamine); PRF, prolactin-releasing factor (unidentified); PRL, prolactin. Modified from Ganong (2001), with permission.

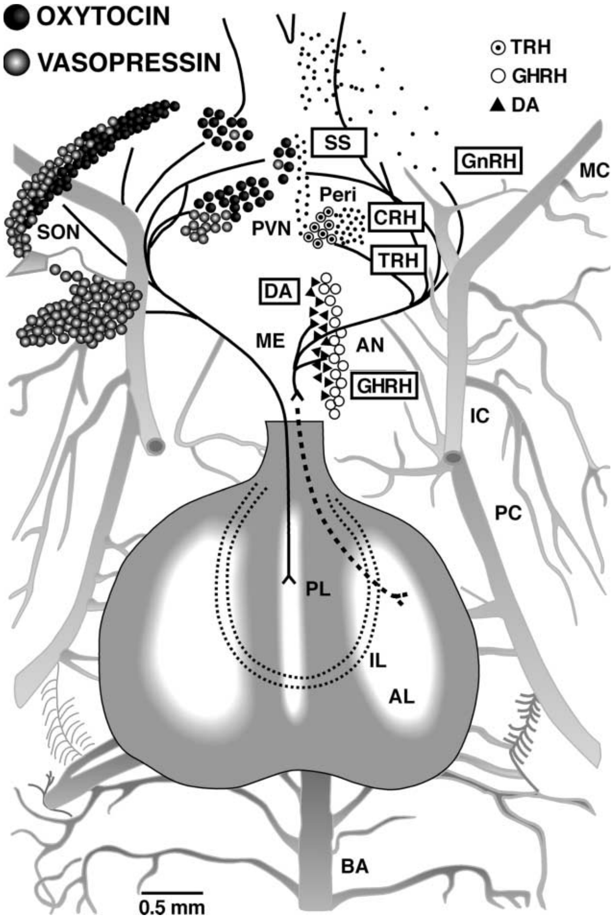

FIGURE 3 Location of the cell bodies of the hypophysiotropic hormone-secreting neurons projected on a ventral view of the hypothalamus and pituitary of the rat. The names of the hormones are enclosed in the boxes. The magnocellular oxytocin and vasopressin neurosecretory neurons that project to the posterior lobe (PL) are indicated on the left side of the figure. The parvicellular neurosecretory neurons that control anterior lobe (AL) tropic hormone secretion are indicated on the right side of the figure. The dashed line from the median eminence (ME) to the AL represents the hypophysial portal circulation. AN, Arcuate nucleus; BA, basilar artery; CRH, corticotropin-releasing hormone; DA, dopamine; GnRH, gonadotropin-releasing hormone; GHRH, growth hormone-releasing hormone; IC, internal carotid artery; IL, intermediate lobe; MC, middle cerebral artery; PC, posterior cerebral artery; Peri, periventricular nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; SS, somatostatin; TRH, thyrotropin-releasing hormone. Modified from Ganong (2001), with permission. Courtesy of L.W. Swanson and E.T. Cunningham, Jr.

from one every 90 min during the follicular phase of the menstrual cycle to one every 3 h during the luteal phase. The frequency and amplitude of the GnRH secretory episodes are essential for maintaining the sensitivity of anterior pituitary gonadotropes to GnRH stimulation and for generating the other hormonal events responsible for reproductive cycles in females and normal testicular function in males. The hypothalamic GnRH pulse generator may be composed solely of the GnRH neurons, or may also consist of additional GnRH afferent circuitry, which, in conjunction with the GnRH neurons, generate the oscillatory bursting patterns of the GnRH neurons. Hence, the GABA, Glu, and NO neurons may indeed be components of the GnRH pulse generator, or may instead be significant modulators of GnRH pulse generator activity by virtue of their being GnRH neuronal afferents. Even relatively small changes in GnRH pulse generator function are associated with menstrual disorders in females and with infertility in both sexes. Dramatic changes in GnRH pulse generator function also are responsible for controlling the onset of puberty, the waning of reproductive function associated with menopause in women, and, to a lesser extent, andropause in men.

The activity of the GnRH pulse generator is modulated by numerous physiological stimuli, including circulating hormones. For example, the sex steroid hormones—estradiol and progesterone in females and testosterone in males—exert negative and positive feedback effects on GnRH pulse generator function as part of homeostatic, physiological regulatory mechanisms coordinating the integrated functions of the hypothalamic–pituitary–gonadal (i.e., the reproductive) axis. Another example is the adipose cell hormone leptin, which exerts a positive effect on the GnRH pulse generator in its role in communicating the nutritional status of the individual to the neurons controlling reproductive function. Hence, the presence of sex steroid or leptin receptors on hypothalamic GABA, Glu, or NO neurons thought to be GnRH afferents, or documented effects of these hormones on these GnRH afferents, may both be considered lines of evidence indicating that specific populations of GABA, Glu, and NO neurons are physiologically significant GnRH afferents.

## II. HYPOTHALAMIC GABA NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION

### A. Hypothalamic GABA Neurons

GABA, a four-carbon amino acid, was discovered in brain tissue in 1950 by Eugene Roberts and coworkers. GABA, produced by the enzymatic decarboxylation of glutamic acid by the rate-limiting enzyme, glutamic acid decarboxylase (GAD), is catabolized primarily to succinic semialdehyde by GABA-transaminase (GABA-T) (Fig. 4). Whereas monoaminergic (dopamine, norepinephrine, epinephrine, and serotonin) and cholinergic neurons constitute relatively small populations of neurons, GABA is thought to be the most ubiquitous transmitter in brain. GABA neurons comprise 30–50% of all nerve cell bodies, and perhaps 20% of the estimated $10^{15}$ central nervous system (CNS) synapses utilize GABA as their transmitter. In the hypothalamus, morphometric analysis at the electron microscopic (EM) level indicates that about 50% of all synapses are GABAergic. Most nuclei of the hypothalamus have high concentrations of GAD and GABA and this brain region has heavy concentrations of GABA cell bodies. Medial (compared with lateral) hypothalamic structures in particular are among the most densely GABA innervated regions in brain. It is in these medial structures that most hypothalamic releasing and inhibiting hormone cell bodies and their dendrites are located (Fig. 3). GABA cells make up 50–90% of all cells in the rostral hypothalamus [the medial preoptic area (MPOA)], where the GnRH cell bodies are located, and 15–50% of all cells in the arcuate nucleus. A tuberoinfundibular system of GABA (TIGA) neurons exists with cell bodies in the AN and axons that project to GABA terminals in the external layer of the ME. In the ME the terminals of the TIGA neurons are observed closely apposed to the GnRH terminals. Double-label immunohistochemistry at the EM level indicates that rostral MPOA GABA neurons form axodendritic and axosomatic synapses with the GnRH neurons. Hence, GABA neurons may provide inhibitory input to the GnRH neurons in either the MPOA or the ME, or both. Indeed, recent quantitative EM work in the primate AN indicates that inhibitory (not excitatory) synapses comprise the dominant input to the GnRH neurons. van den Pol and co-workers have provided compelling electrophysiological evidence that almost all fast inhibition in the hypothalamus is caused by GABA release. Despite GABA being a dominant neurotransmitter in the hypothalamus, this very large population of hypothalamic neurons has been understudied by neuroendocrine investigators.

GABA was first convincingly shown to be an inhibitory transmitter in 1967, and subsequent iontophoretic studies indicate that GABA inhibits virtually every neuron in the adult CNS. GABA is thought to be released by a depolarization-induced, $Ca^{2+}$-dependent exocytotic process, although physiologically significant GABA release also may occur by reversal of the $Na^{+}$-dependent presynaptic GABA transporter (GAT) (Fig. 4). At the level of the membrane, activation of the postsynaptic $GABA_A$ receptor and its integrated $Cl^{-}$ channel produces

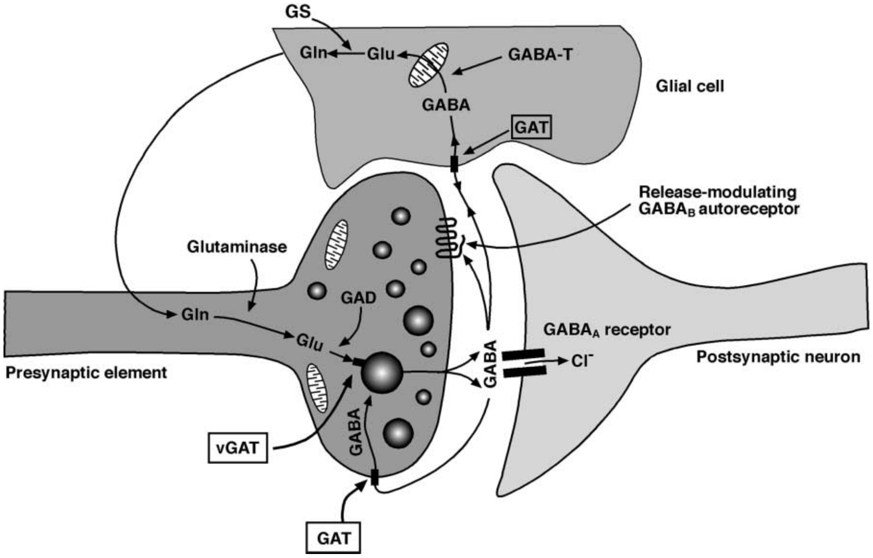

**FIGURE 4** Schematic representation of a GABA synapse. The α-ketoglutarate formed in the Krebs cycle is transaminated to glutamate (Glu) by mitochondrial GABA-transaminase (GABA-T). The transmitter GABA is formed from Glu by glutamic acid decarboxylase (GAD). Released GABA may bind to postsynaptic $GABA_A$ receptors or to presynaptic $GABA_B$ autoreceptors, or may be taken up by high-affinity GABA transporters (GAT) present on neurons and glia. GS, Glutamine synthetase; vGAT, vesicular GABA transporter. Modified from Zigmond *et al.* (1999), with permission.

membrane hyperpolarization, whereas presynaptic $GABA_B$ receptors control synaptic transmission by inhibiting $Ca^{2+}$ influx and/or by affecting $K^+$ channels, thus inhibiting transmitter release at the nerve terminal (Fig. 4). Less is known about the recently cloned G-protein-coupled $GABA_B$ receptor, which is structurally similar to the Glu metabotropic receptors, and the putative $GABA_C$ receptor. The $GABA_A$ receptor, a heteropentameric protein, is more correctly referred to as a receptor complex because it has separate binding sites for GABA agonists and antagonists, benzodiazepines, barbiturates, alcohol, steroids, and certain convulsants. This variety of binding sites on the $GABA_A$ receptor makes it highly susceptible to modulation by physiological and pharmacological parameters. It is composed of combinations of at least 20 different subunits and their functional splice variants: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_1$ and $_3$, $\gamma_{2L}$ and $_{2S}$, δ, ε, θ, π, and $\rho_{1-3}$.

## B. GABA Regulation of GnRH Pulse Generator Function

Several lines of evidence are consistent with the hypothesis that hypothalamic GABA neurons inhibit the GnRH pulse generator, and may indeed comprise pulse generator circuitry. First, GnRH neurons *in situ* express the $GABA_A$ $\alpha_1$, $\alpha_5$, $\beta_1$, $\beta_3$, and $\gamma_2$ receptor subunit mRNAs, demonstrating that GnRH neurons are targets for the neurotransmitter GABA. Second, evidence indicates that MPOA and AN GABA neurons express estrogen receptor (ER) and progesterone receptor (PR) mRNA and protein. In the MPOA, about 40% of all estrogen-receptive cells immunostain for GAD, indicating they are GABA neurons. Third, in several tissues, including brain, one of the physiological actions of estrogen is to induce the progesterone receptor. The TIGA neurons are reported to express such estrogen-inducible PR, as well as the leptin receptor. Indeed, it is reported that all ER immunoreactive cells in the hypothalamus, including the MPOA and AN, also express leptin receptor protein. GnRH neurons studied in hypothalamic slices *in vitro* are directly inhibited by applied GABA, and this effect is $GABA_A$ receptor mediated. This additional evidence demonstrates that the $GABA_A$ receptors expressed in the GnRH neurons are, indeed, physiologically functional. Similarly, GnRH neurons studied in hypothalamic slices are generally silent, suggesting that they are tonically inhibited, but also exhibit episodes of burst firing, which may underlie pulsatile GnRH release. Recent electrophysiological evidence from hypothalamic slices indicates that MPOA GABA neurons may mediate estrogen negative feedback by reducing the autoinhibition of GABA neurons brought about by presynaptic, $GABA_B$ autoreceptor stimulation (Fig. 4). A better understood oscillator in the thalamus and the hypothalamic rhythm generator in the suprachiasmatic nucleus both rely on GABA neurons for their function. A network oscillator model for hypothalamic pulse generators consisting of Glu and GABA neurons would function more reliably than would a single-cell oscillator (i.e., composed solely of the GnRH neurons). Finally, reflecting on the overall function and importance of GABA neurons in brain, Eugene Roberts concludes that "disinhibition, coupled to variability generation... is the major organizing principle in nervous system function."

More physiologically oriented work in intact animals indicates that GABA has an inhibitory action on LH secretion. Administration of GABA or the $GABA_A$ receptor agonist muscimol (MUS) into the MPOA or intracerebroventricularly (icv) inhibits pulsatile LH release, and the $GABA_A$ receptor antagonist bicuculline (BIC) blocks this effect. $GABA_A$ agonists and antagonists inhibit and stimulate, respectively, GnRH pulse generator function as assessed by multiunit activity recordings from the AN–ME region. Systemic administration of the GABA-T (Fig. 4) inhibitor aminooxyacetic acid (AOAA) blocks GABA degradation, resulting in increased brain GABA levels. This GABA increase is associated with inhibition of pulsatile LH secretion. MUS and AOAA administration also block LH surge release and BIC prevents the $GABA_A$ agonist effect. Sex steroid removal down-regulates, and sex steroid administration up-regulates, several components of GABA transmission in MPOA and TIGA neurons: GAD mRNA levels and enzymatic activity, GAT-1 mRNA levels and transport activity, $GABA_A$ receptor binding and the levels of receptor subunit mRNAs, and the rate of GABA turnover. In females, MPOA GAD mRNA levels, the rate of GABA turnover, and the extracellular GABA concentrations in microdialysates all decrease before and during the LH surge. Most recently, a group reported that grafting genetically engineered astrocytes that release GABA in the ME near GnRH terminals disrupts estrous cyclicity in rats; this finding demonstrates that GABA released in the vicinity of the ME can inhibit the GnRH pulse generator, raising the possibility that similar local alterations in GABA neurotransmission may contribute to the pathology of hypothalamic amenorrhea/oligomenorrhea in humans.

## III. HYPOTHALAMIC GLUTAMATE NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION

### A. Hypothalamic Glu Neurons

Iontophoresis of Glu onto many different mammalian neurons rapidly depolarizes them. Perhaps 75% of excitatory transmission in the brain is attributable to Glu synapses, and van den Pol and co-workers have published evidence confirming that Glu is the principal excitatory neurotransmitter in the hypothalamus. Utilizing Glu-specific antibodies, immunohistochemical work reveals relatively dense Glu nerve terminal immunostaining throughout most of the hypothalamus. In neurons, Glu is thought to be synthesized principally from glutamine by the mitochondrial enzyme phosphate-activated glutaminase and also from aspartate by the cytoplasmic enzyme aspartate aminotransferase (Fig. 5). Presynaptic Glu is sequestered in secretory vesicles at concentrations exceeding 20 m*M*. When Glu nerve terminals are depolarized, Glu is released in a $Ca^{2+}$-dependent manner.

Two kinds of Glu receptors are recognized: ionotropic and metabotropic. The ionotropic receptors are ligand-gated cation channels and are of three types: kainate (KA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and *N*-methyl-D-aspartate (NMDA). KA and AMPA receptors permit $Na^+$ influx and $K^+$ efflux; NMDA receptors pass relatively large amounts of $Ca^{2+}$. The ionotropic receptors are either pentamers or tetramers, and to date six NMDA (NR$_1$, NR$_{2A-D}$, and NR$_{3A}$), five KA (GluR$_{5-7}$ and KA$_{1 \text{ and } 2}$), and four AMPA (GluR$_{1-4}$) subunits have been identified and their genes have been cloned. The Glu concentration required for half-maximal (EC$_{50}$) stimulation of AMPA receptors is 200 μ*M*, whereas the EC$_{50}$ for NMDA receptors is only 10–15 μ*M*. Hence, Glu is a much more potent activator of NMDA receptors, compared to AMPA receptors.

Eight metabotropic receptor subunits have been described (mGluR$_{1-8}$). These receptors are G-protein-coupled serpentine receptors that increase intracellular inositol trisphosphate and diacylglycerol levels or decrease intracellular cAMP levels. In addition to binding to these pre- and postsynaptic receptors, released Glu may be taken up by neurons or surrounding glia via the Glu transporters: excitatory amino acid carrier-1 (EAAC1), glutamate transporter-1 (GLT-1), or glutamate–aspartate transporter (GLAST) (Fig. 5).

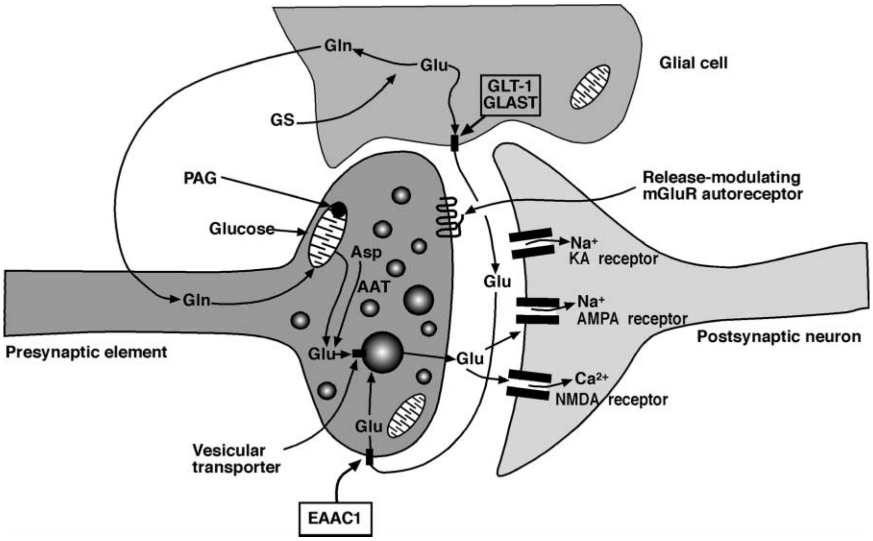

**FIGURE 5** Schematic representation of a glutamate synapse. Glutamate (Glu) is synthesized from glutamine (Gln) by the action of phosphate-activated glutaminase (PAG), and from aspartate (Asp) by the action of aspartate aminotransferase (AAT). Glu is concentrated in secretory granules by a vesicular transporter. Released Glu can bind to postsynaptic *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), or kainate (KA) receptors or to presynaptic metabotropic autoreceptors (mGluR), or can be taken up by the high-affinity Glu transporters, excitatory amino acid carrier-1 (EAAC1), glutamate transporter-1 (GLT-1), or glutamate-aspartate transporter (GLAST), which are present on neurons and glia as indicated. Modified from Zigmond *et al.* (1999), with permission.

Double-label immunohistochemistry at the EM level indicates that Glu-immunoreactive neurons synapse with GnRH dendrites and that ME Glu terminals are closely apposed to GnRH terminals, suggesting that asynaptic neurotransmission may occur in this structure. Autoradiographic mapping demonstrates a relatively dense distribution of Glu receptors in the hypothalamus, particularly in the AN. More specifically, NMDA ($NMDAR_1$ and $NMDAR_{2A-D}$ subunits), AMPA ($GluR_{1-4}$ subunits), KA ($GluR_{5 \text{ and } 6}$ and $KA_2$ subunits), and mGlu ($mGluR_{1,2,3 \text{ and } 5}$ subunits) receptor mRNAs and/or proteins are found widely expressed in hypothalamic neurons. In most hypothalamic regions the NMDA receptor predominates. Finally, mRNA and protein for the Glu transporters EAAC1, GLT-1, and GLAST are detected in the hypothalamus.

### B. Glu Regulation of GnRH Pulse Generator Function

Several lines of evidence are consistent with the hypothesis that hypothalamic Glu neurons stimulate the GnRH pulse generator and may indeed comprise pulse generator circuitry. First, *in situ*, about 50% of GnRH neurons express the kainic acid 2 ($KA_2$) receptor subunit mRNA, whereas about 80% express the $NMDAR_1$ receptor subunit mRNA, demonstrating that GnRH neurons are targets for the neurotransmitter Glu. Second, double-label immunofluorescence work in several hypothalamic structures indicates that an average of 80% of Glu neurons express ER and as many as of 93% of Glu neurons express PR. Third, Glu evokes currents in GnRH neurons studied in hypothalamic slices; subsequent patch-clamp work indicates these currents are mediated by NMDA and AMPA channels, indicating that the Glu receptors expressed by GnRH neurons are physiologically functional. In addition, antisense oligodeoxynucleotides against the $NMDAR_{2A}$ receptor subunit mRNA decrease the protein levels of this receptor subunit and suppress pulsatile GnRH secretion from MPOA–ME hypothalamic fragments studied *in vitro*. Another finding is that NMDA, AMPA, and KA agonists all stimulate GnRH release from hypothalamic fragments incubated *in vitro*. Finally, mathematical modeling of hypothalamic pulse generators indicates that neuronal oscillators consisting of Glu and GABA neurons exhibit greater stability than would a single-cell oscillator composed solely of GnRH neurons.

*In vivo* work in intact animals indicates that Glu stimulates GnRH and LH secretion. Systemic or icv administration of Glu and the Glu receptor agonists NMDA, KA, and AMPA induce a rapid release in LH secretion, and Grattan and co-workers demonstrated that intravenous NMDA directly stimulates GnRH release into anterior pituitary perfusates collected by push–pull cannulae. NMDA microinfusion specifically into the MPOA stimulates LH release. Pulsatile LH release is suppressed by NMDA and AMPA/KA receptor blockers. Microdialysis studies suggest that Glu release increases at the time of the LH surge in the MPOA but not in the AN–ME region. Administration of NMDA or AMPA receptor blockers inhibits LH surge release. Also, sex steroid administration to gonadectomized animals up-regulates components of hypothalamic Glu transmission, including Glu release, the activity of Glu transporters, and the expression of Glu receptors. Specifically, Glu transport into hypothalamic Glu nerve terminals varies across the rat estrous cycle, and hypothalamic $GluR_1$, $GluR_{2/3}$, and AMPA receptor subunit proteins increase with sex steroid administration.

## IV. HYPOTHALAMIC NITRIC OXIDE NEURONS AND THE NEUROENDOCRINE REGULATION OF LH SECRETION

### A. Hypothalamic NO Neurons

In the 1980s it was determined that the potent vasodilatory factor released by endothelial cells was the gas nitric oxide; it was proposed that in brain, Glu acting at the NMDA receptor caused rapid NO synthesis and subsequent NO-induced cGMP level increases in nearby cells, and that an isoform of the enzyme producing NO, neuronal nitric oxide synthase (nNOS), was present in neurons (Fig. 6). These data suggested that NO might be a neurotransmitter in brain. If this were so, however, it was soon appreciated that its neurotransmitter characteristics would be quite unconventional, because NO is not stored in synaptic vesicles, nor indeed in any other cellular compartment, is not released by $Ca^{2+}$-dependent exocytosis, does not bind to membrane receptors on target cells, sometimes functions as a retrograde signaling molecule, and is not inactivated by reuptake into neurons or glia or by enzymatic degradation. Instead, NO is synthesized in a one-step reaction in which nNOS converts L-arginine to NO and citrulline (Fig. 6). nNOS is present in NO neurons but requires calmodulin-bound $Ca^{2+}$ for activation. Once nNOS is activated, NO is immediately synthesized; NO increases cGMP levels by binding to and stimulating the enzyme guanylyl

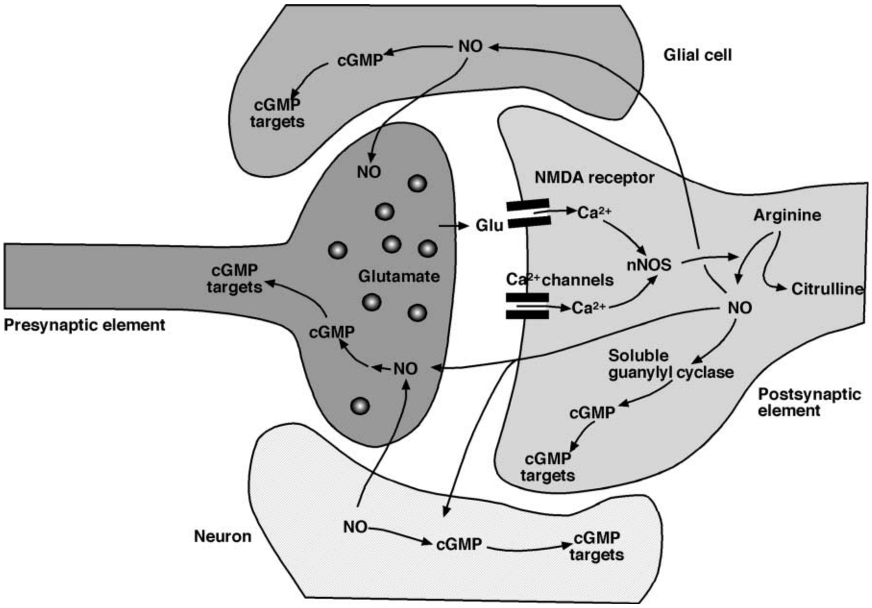

**FIGURE 6** Schematic representation of a nitric oxide (NO)-releasing neuron. NO is formed from arginine by the action of neuronal nitric oxide synthase (nNOS). NO freely diffuses across cell membranes and can thereby influence both presynaptic neurons, such as the glutamatergic presynaptic neuron in the figure, and other cells that are not directly apposed to the nNOS-containing neuron, such as the other neuron and glial cell depicted. In each case NO stimulates guanylyl cyclase, increasing cGMP and activating cGMP-sensitive intracellular targets. As indicated in the figure, Glu can trigger NO formation by activating the NMDA receptor, which results in $Ca^{2+}$ influx and $Ca^{2+}$ activation of nNOS. The half-life of NO is less than 30 s; it decays spontaneously to nitrite. Modified from Zigmond *et al.* (1999), with permission.

cyclase. In this way cGMP levels increase and cGMP-sensitive targets are activated in the NO neuron or in the surrounding cells, including NO afferent neurons. Message termination rapidly occurs by spontaneous degradation to nitrite.

Although nNOS is present in only about 1% of brain neurons, the processes of these cells ramify so extensively that it is estimated that every neuron in brain is likely exposed to NO. Because vesicular release of NO is not regulated in neurons, as is the case for other transmitters, it seems reasonable that the biosynthetic enzyme be highly regulated. Indeed, no other enzyme is known to be regulated by so many factors. nNOS regulators include five oxidative–reductive cofactors, four phosphorylating enzymes, and three binding proteins, and its mRNA is regulated by hormones and other factors. Although stored NO is not released in an impulse-dependent manner, neuronal stimulation indeed causes NO release, by the Glu and NMDA receptor mechanism (Fig. 6) and/or by other as yet unknown mechanisms.

In the hypothalamus, many nNOS immunoreactive neuronal cell bodies, processes, and terminals are observed. Although some macrophage-inducible NOS (miNOS) is observed in microglia, and some endothelial NOS (eNOS) is expressed by CNS vascular endothelial cells, nNOS is found to be the major NOS isoform present in the hypothalamus.

## B. NO Regulation of GnRH Pulse Generator Function

Several lines of evidence are consistent with the hypothesis that hypothalamic NO neurons stimulate the GnRH pulse generator, may comprise pulse generator circuitry, and may mediate NMDA-induced GnRH secretion. nNOS immunoreactive neurons are visualized in several hypothalamic structures, including the MPOA, and some immunostaining is also present in terminals in the ME. Hence, NO neurons may affect the GnRH neurons in either the MPOA cell body and dendrite region or the ME preterminal axon and terminal location. A few GnRH neurons appear nNOS-positive and many nNOS immunoreactive neurons surround GnRH cell bodies in the MPOA. Some hypothalamic NO neurons express ER and PR, and estradiol increases hypothalamic nNOS mRNA and protein levels. Similarly, MPOA nNOS mRNA, protein, and enzyme activity vary across the rat estrous cycle. NO stimulates MPOA and AN–ME cGMP in a dose-related manner and this stimulation is blocked by hemoglobin, an NO scavenger.

*In vivo* work in intact animals indicates that NO stimulates GnRH and LH secretion in rats. First, icv administration of an nNOS inhibitor suppresses pulsatile LH secretion. Second, nNOS inhibitors block LH surge release and icv administration of the NO precursor L-arginine potentiates the LH surge. Third, antisense oligodeoxynucleotides against nNOS mRNA, delivered icv, decrease hypothalamic nNOS protein and diminish LH surge release. Fourth, microinfusion of the NO precursor L-arginine into the AN–ME region increases the amount of cGMP and GnRH recovered in AN–ME push–pull perfusates. Fifth, NO and GnRH are secreted in a copulsatile fashion from ME fragments incubated *in vitro*, and the amplitude of these secretions varies across the estrous cycle.

The precise neuronal mechanism whereby NO stimulates GnRH release is uncertain. NO may directly stimulate guanylyl cyclase in GnRH neurons, resulting somehow in GnRH release. However, there is more evidence for other indirect actions. Evidence exists suggesting that NO induction is required for Glu-induced GnRH secretion, that NO releases prostaglandin $E_2$ (which activates adenylate cyclase and protein kinase A, and then GnRH secretion), and that NO-stimulated GnRH secretion is mediated by neuropeptide Y neurons. Clearly, a great deal of additional work is required before it will be possible to distinguish between these and other neuroendocrine regulatory mechanisms.

## V. THE ROLE OF GABA, GLUTAMATE, AND NITRIC OXIDE NEURONS IN THE NEUROENDOCRINE REGULATION OF OTHER ANTERIOR PITUITARY TROPIC HORMONES

The evidence indicating significant physiological roles for GABA, Glu, and NO neurons in the regulation of GnRH and LH secretion is well documented. Related evidence suggests that these neurons are significant afferents to the other hypothalamic neurons that secrete releasing and inhibiting hormones [CRH, TRH, GHRH, SS (somatostatin), PIH, and PRF], which in turn regulate the secretion of the anterior pituitary hormones (ACTH, TSH, GH, and PRL). However, the data supporting the latter role are fewer and in some cases substantially less well developed, compared to the evidence for GnRH regulation. Some of this evidence is presented in reviews cited in the bibliography of this article. Evidence substantial enough to form a reasoned judgment is included in Table 1, which summarizes the neuroendocrine effect of GABA, Glu, and NO on the secretion of ACTH, TSH, GH, and PRL. So that the table is complete, the effects on LH and FSH secretion are included. Although a great deal is known about hypothalamic GABA, Glu, and NO neurons and how they function as physiologically significant afferents to the hypophysiotropic hormone-secreting neurons, considerably more than that remains to be learned about these neuroendocrine regulatory mechanisms.

TABLE 1 Net *In vivo* Effect of GABA, Glu, and NO on the Secretion of the Anterior Pituitary Tropic Hormones[a]

| Effector | LH/FSH | ACTH | TSH | GH | PRL |
|---|---|---|---|---|---|
| GABA | ↓ | ↓ | ↓ | ↓ | ↓ |
| Glu | ↑ | ↑ | ↑ | ↑ | ↑ |
| NO | ↑ | ↓ | ND | ↑ | ↑ |

[a] LH, Luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; GH, growth hormone; PRL, prolactin; ↑, increases; ↓, decreases; ND, not determined.

## Glossary

| | |
|---|---|
| **afferent neuron** | Neuron that conveys incoming nerve impulses to another neuron or nerve center with which it synapses. |
| **γ-aminobutyric acid** | Principal inhibitory neurotransmitter in the mammalian central nervous system (CNS), inhibiting virtually every neuron in the adult CNS at physiological concentrations. |
| **glutamate** | Principal excitatory neurotransmitter in the mammalian central nervous system, stimulating virtually every neuron in the adult CNS at physiological concentrations. |
| **gonadotropin-releasing hormone** | Hypothalamic releasing hormone that stimulates luteinizing hormone and follicle-stimulating hormone secretion from the anterior pituitary gland. |
| **hypophysiotropic hormone-secreting neurons** | Hypothalamic neurons that secrete into the hypophysial portal circulation various releasing or inhibiting hormones, which in turn control secretion of the six anterior pituitary tropic hormones. |
| **luteinizing hormone and follicle-stimulating hormone** | Anterior pituitary tropic hormones controlling the reproductive functions of the testes and ovaries. |
| **median eminence** | That portion of the medial basal hypothalamus from which the hypophysial portal vessels arise. |

**nitric oxide** Neuroactive gas, released from neurons, which functions as a neurotransmitter, probably affecting every neuron in brain.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Cytokines and Anterior Pituitary Function • Follicle-Stimulating Hormone (FSH) • Gonadotropin-Releasing Hormone (GnRH) • Luteinizing Hormone (LH)**

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# Amphiregulin

EUNKYUNG CHUNG[*], PAUL W. COOK[†], AND ROBERT J. COFFEY[*]

[*]*Vanderbilt University and VA Medical Center* • [†]*Cascade Biologics, Inc., Oregon*

I. INTRODUCTION
II. STRUCTURE AND PROCESSING OF AR
III. SELECTED ASPECTS OF AR SIGNALING
IV. REGULATION OF AR EXPRESSION
V. BIOLOGICAL ROLES FOR AR
VI. SUMMARY

**The epidermal growth factor (EGF) family of polypeptide growth factors includes EGF, transforming growth factor-α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin, epigen, heregulin, neuregulin, and cripto.**

## I. INTRODUCTION

The epidermal growth factor (EGF) receptor ligands can interact with four known EGF receptor (EGFR) subtypes, and, in some cases, distinct intracellular signaling pathways can be activated in a ligand/receptor-dependent fashion. Like the other EGFR ligands, amphiregulin (AR) is synthesized in a proform and is inserted into the plasma membrane, where it is cleaved in its ectodomain to release soluble growth factor. AR was originally identified as an EGFR ligand from a phorbol ester-treated human breast adenocarcinoma cell line, MCF-7. Although the growth inhibitory properties of AR have not been subsequently validated, Shoyab and co-workers named it "amphi" because of its growth inhibitory effects on A431 and several other cancer cell lines and growth stimulation of many other cell lines, normal and transformed. This article will attempt to provide a biological perspective on AR by discussing its

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Eunkyung Chung[*], Paul W. Cook[†], and Robert J. Coffey[*]

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**The epidermal growth factor (EGF) family of polypeptide growth factors includes EGF, transforming growth factor-α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin, epigen, heregulin, neuregulin, and cripto.**

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## II. STRUCTURE AND PROCESSING OF AR

At the genomic level, human AR maps to chromosome 4q13–q21 and is partitioned into six exons that span 10.2 kb of genomic DNA. As deduced from the cDNA, the transcript is 1.4 kb and the translation product is 252 amino acids with a predicted mass of 25.9 kDa without glycosylation. Fig. 1 depicts the chromosomal location of AR in human and mouse. AR is contiguous with epiregulin in human and with betacellulin in mouse, respectively. The tight linkage between these EGFR ligands in both human and mouse suggests that they may have arisen through a tandem gene duplication event. In addition, AR is in close proximity to the chemotactic cytokines for neutrophils, B cells, and melanocytes, such as Gro1, Gro2, Gro3, and interleukin-8 (IL-8) in human and Gro1 in mouse. Of interest, AR has been shown to be pro-inflammatory in the skin of keratin 14 promoter (K14)-AR transgenic mice, in which there is targeted overexpression of human AR in the basal layer of the epidermis.

As illustrated in Fig. 2, mature AR is initially synthesized as a 252-amino-acid transmembrane precursor that is proteolytically cleaved to its mature 78- to 84-amino-acid form. Following the signal peptide, the pro-region consists of potential N- or O-linked glycosylation sites and three potential tyrosine sulfation sites. Subsequent work from the authors' laboratory has revealed that N-linked glycosylation of the pro-region predominates over O-linked glycosylation and that tyrosine sulfation occurs in this region. Mature AR consists of a heparin-binding domain (HBD) made up of positively charged amino acids and followed by the EGF domain, which is composed of the conserved spacing of six cysteine residues ($CX_7CX_4CX_1CX_8C$) shared among EGFR ligands. Plowman and co-workers have shown that the HBD of AR prevents the secretion of mature AR when the pro-region is deleted. In this

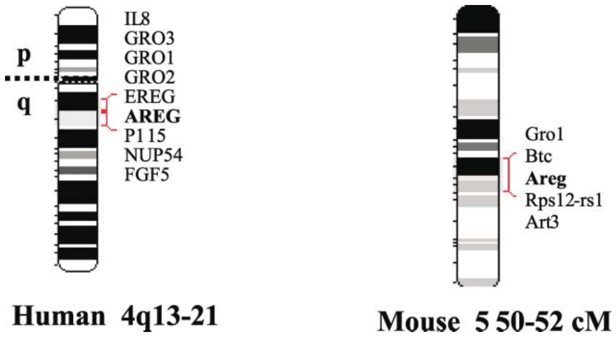

FIGURE 1 Chromosomal location of AR and adjacent genes in human and mouse genomes. Human AR is in close proximity to epiregulin on chromosome 4q13–q21, and mouse AR is adjacent to betacellulin on chromosome 5 at 50–52 cM. (In human) IL-8, interleukin-8; GRO1, 2, 3 oncogene, melanoma growth stimulating activity α; EREG, epiregulin; AREG, amphiregulin; p115, vesicle-docking protein p115; NUP54, nucleoporin p54; FGF5, fibroblast growth factor 5. (In mouse) Gro1, the mouse homologue of human GRO1; Btc, betacellulin; Areg, amphiregulin; Rps12-rs1, ribosomal protein S12; Art3, ADP-ribosyl transferase 3.

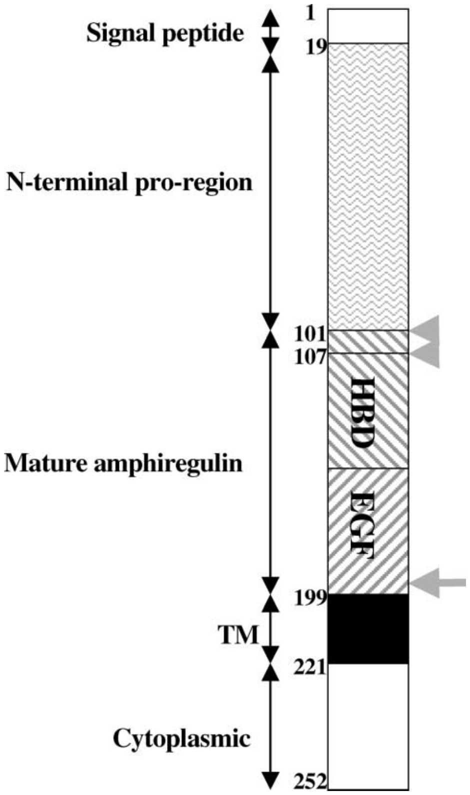

FIGURE 2 Structure of AR precursor. AR is synthesized as a 252-amino-acid, single-pass transmembrane glycoprotein. After the signal peptide (1–19), there is an N-terminal pro-region (amino acids 20–100) containing N- and O-linked glycosylation sites and potential tyrosine sulfation sites, followed by mature AR. Mature AR consists of two distinct parts, a heparin-binding domain (HBD) and an EGF repeat. TM represents the transmembrane domain (amino acids 199–221) that is followed by the cytoplasmic domain (amino acids 222–252). Arrow at a distal site indicates a TACE-mediated cleavage site, and two arrowheads show alternative proteolytic processing sites that give rise to different isoforms of mature AR.

study, deletion of the pro-region resulted in the prevention of secretion of AR into the medium. Moreover, fusion of the HBD of AR to the EGF repeats of transforming growth factor-α (TGF-α) and EGF, along with TGF-β's signal peptide, prevented secretion of these constructs. From these studies, an electrostatic model has been proposed in which the negatively charged pro-region of AR interacts with its positively charged HBD to facilitate proper folding and secretion. However, replacement of AR's pro-region with the pro-region of heparin-binding EGF-like growth factor (HB-EGF) (which is positively charged) allowed secretion of mature AR, whereas TGF-α's pro-region (which is negatively charged) did not. Therefore, it may be that only specific negatively charged amino acid sequences can confer proper folding and secretion of AR.

In the C-terminus of AR's EGF repeat, a leucine that is conserved in all other EGFR ligands is converted to methionine at residue 186, a change that reduces binding to EGFR. The transmembrane domain and cytoplasmic domain of AR are not required for secretion of mature AR, although proteolytic removal of the N-terminal pro-region is less efficient in the absence of the membrane anchor. However, the loss of basolateral sorting was observed by deletion of these regions in polarized Madin-Darby canine kidney (MDCK) II epithelial cells (see below). Fig. 3 depicts various isoforms of AR in the cell lysates and medium following its posttranslational modification. Although some of these isoforms have been shown to possess biological activity, it is not known whether all of these soluble AR forms are biologically active and, if so, whether they may differ in their biological activities.

## III. SELECTED ASPECTS OF AR SIGNALING

The sorting and processing of AR in the context of polarized epithelial cells have been examined. This approach assumes biological relevance when it is recalled that the EGFR is localized preferentially to the basolateral surface of all polarized epithelial cells. Studies have examined the trafficking of wild-type and mutant forms of AR in polarized MDCK II cells that had been stably transfected with these cDNAs, as well as trafficking of endogenous AR in polarized HCA-7 cells, a human colon cancer cell line that was generated from an individual with a well-differentiated rectal cancer. These HCA-7 cells, like MDCK II cells, form a uniform polarizing monolayer when cultured on polycarbonate-coated Transwell filters. Both of these cells contain 20,000–40,000 basolateral EGFRs. In both lines, AR is delivered preferentially to the basolateral surface, where it is cleaved by tumor necrosis factor α-converting

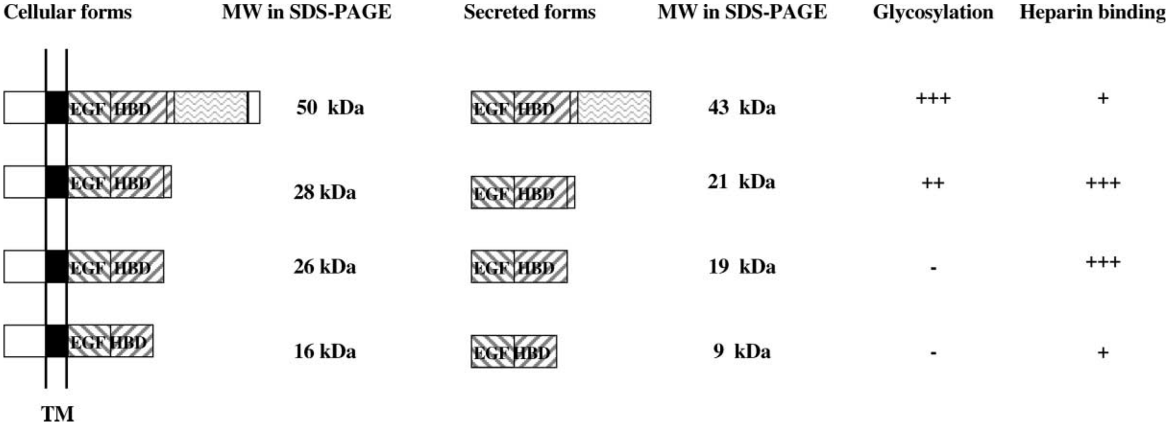

FIGURE 3 Various forms of cellular and secreted AR. Different forms of cell-associated and secreted AR are depicted and the extent of glycosylation and the ability to bind heparin are described quantitatively. Cell surface, membrane-anchored forms of AR are proteolytically processed to multiple soluble isoforms. TACE-mediated cleavage of the ectodomain of 50 kDa pro-AR at a distal site releases 43 kDa soluble AR into conditioned medium. Alternatively, in the absence of primary proteolytic cleavage at the distal site, secondary cleavage at proximal sites removes the N-terminal domain, resulting in 28 and 26 kDa cell surface pro-AR forms, which appear as a doublet. These forms are then released by TACE-mediated cleavage at the distal site. This soluble doublet probably represents the originally described mature form of AR. In the absence of cleavage at the distal site, further proteolytic processing at a proximal site results in a 16 kDa cell surface form of AR, which has a reduced HBD and is not glycosylated. The 16 kDa cell surface form can be cleaved by TACE at a distal site to release a 9 kDa soluble AR.

enzyme/a disintegrin and metalloprotease 17 (TACE/ADAM17) (see Fig. 4). The basolateral sorting information resides in the cytoplasmic tail as AR cDNA constructs that lack the tail are missorted in polarized MDCK II cells. However, cell surface processing of AR appears to be unaffected, suggesting that information in the tail is not required for cell surface proteolytic cleavage. AR exhibits punctate immunoreactivity at both the basal and the lateral surfaces of these polarized epithelial cells, likely due to its ability to bind to heparan sulfate proteoglycans (HSPGs) at the cell surface and in the extracellular matrix. Levels of AR in the basolateral medium are not significantly altered after administration of an antibody to the EGFR that blocks ligand binding (MAb 528 or 225). This contrasts with TGF-α levels that increase promptly in the basolateral medium after EGFR MAb blockade, a finding that has been interpreted as evidence that TGF-α is a locally acting growth factor. These differences in post-cleavage disposition of TGF-α and AR underscore the possibility of distinct biological activities.

A speculative view of possible participants in endogenous AR signaling is presented in Fig. 5. Signaling pathways initiated by endogenous AR are thought to be complex due to a number of factors. These include glycosylation, multiply processed forms, ability to bind HSPGs, and variable-affinity binding to EGFR. Although all seven EGFR ligands bind to the EGFR and thereby activate conserved downstream effectors, such as Ras-mitogen-activated protein kinase and phosphatidylinositol 3-kinase–Akt/protein kinase B, the complexity of the production and processing of endogenous AR allows for activation of alternative signaling pathways. It should be emphasized, however, that activation of such alternative pathways has not been proven. In the following discussion, possible effects due to the ability of AR to bind heparin are considered in light of what has been established for the other heparin-binding EGFR ligand, HB-EGF.

Immunohistochemical studies have identified several binding proteins that may be involved in AR signaling. AR has been shown to co-localize with CD44, a hyaluronan receptor, which is modified by HSPGs in its v3 region. This interaction with CD44 may facilitate the juxtacrine activity of AR as has been shown for HB-EGF. It should be noted, however, that CD44 also has been reported to co-localize with EGFR, erbB-4, and matrilysin/matrix metalloprotease-7. CD9, a tetra-spanning transmembrane protein, has also been found to co-localize with AR by immunofluorescence studies. It has been reported that CD9 co-localizes with α3β1 integrins, which, in turn, increases the juxtacrine activity of HB-EGF and possibly TGF-α, respectively; however, to date, this interaction has not been shown to increase the juxtacrine activity of pro-AR. In addition, cell surface HSPGs, members of the syndecan and glypican family, have been postulated as possible binding partners of AR. In polarized epithelial cells, glycosylphosphatidylinositol (GPI)-linked glypican, which is generally targeted to the apical domain, would be less likely to participate in AR-induced signaling than syndecan-1, which is sorted to the basolateral compartment of polarized epithelial cells like AR and EGFR. It is possible that HSPGs on the cell surface and extracellular matrix (perlecan, agrin) act

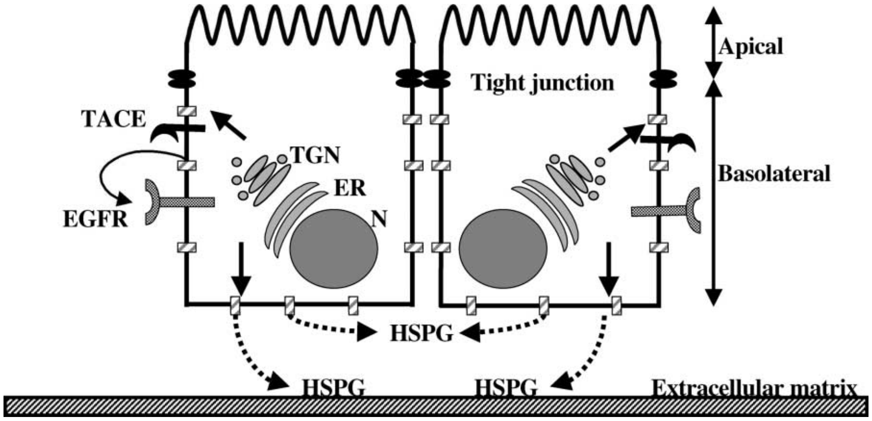

FIGURE 4 Sorting and processing of AR in polarized epithelial cells. This simplified schematic depicts cell surface cleavage of one AR molecule and its binding to EGFR. It should be noted that TACE, AR, and EGFR are all found within the basolateral compartment. N, ER, and TGN represent the nucleus, endoplasmic reticulum, and *trans*-Golgi network, respectively.

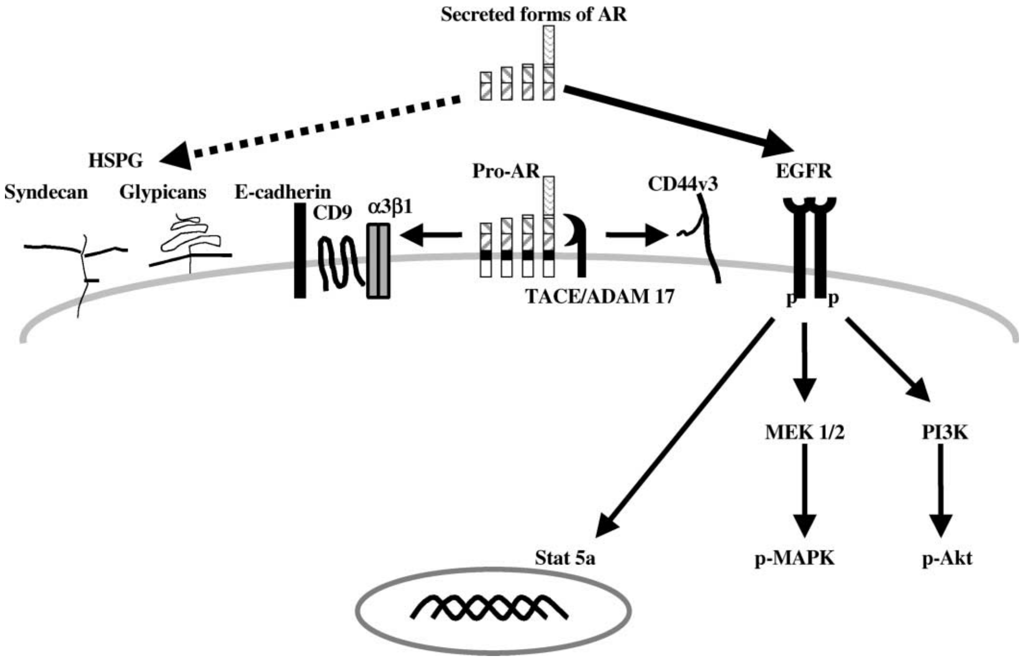

FIGURE 5 Participants in AR signaling. A model of protein interactions that appear to regulate AR signaling is presented (see text for details). Both CD44v3 and CD9 directly interact with AR. Whether TACE interacts directly with AR is not known.

as depots for AR in its pro-AR form or after it is cleaved and released from the cell surface. These depot forms of AR may increase local concentrations of AR or regulate its access to the EGFR. In summary, these accessory molecules presumably influence the nature of the interaction between AR and EGFR, as there is no evidence at the present time for EGFR-independent signaling by AR.

What is the evidence that endogenous AR has effects distinct from those of the other EGFR ligands? *In vitro*, AR differed from EGF in its recruitment of downstream molecules when these ligands were administered to cells expressing an EGFR with a C-terminal truncation (C′1000). It is also possible that distinct actions of AR may ensue from differences that arise after ligand binding to the EGFR. For example, Derynck's group previously reported that TGF-α dissociated from the EGFR earlier in the endocytic pathway than EGF based on differences in their isoelectric points (the p*I* for TGF-α is 5.9 and that for EGF is 4.6). Thus, TGF-α is less likely to be degraded and more likely to be recycled. AR has a p*I* of 9.84 and therefore would be predicted to dissociate from EGFR even earlier than TGF-α during endocytosis. AR also possesses biological properties that in some cases are distinct from those of TGF-α or EGF. These properties include affinity for heparin and heparin-like molecules, heparin-dependent inhibition of mitogenic activity, and cell density-dependent responsiveness in Balb MK-2 cells.

More convincing evidence for distinct biological actions of AR comes from mouse studies in which there was a targeted overexpression or disruption of AR. In the study of triple knockouts of EGFR ligands (EGF, TGF-α, and AR), a specific role for AR was identified in mammary gland development with signal transducers and activators of transcription (Stat) 5a involved in this AR-specific effect. Two separate groups used the K14 promoter to overexpress AR and TGF-α to the basal layer of the epidermis. Both transgenic lines exhibited hyperplasia of the epidermis; however, only AR mice exhibited marked inflammation of the skin and synovium with features strikingly similar to psoriasis and psoriatic arthritis. These features included epidermal T-lymphocytic and neutrophilic infiltration and tortuous dilated blood vessels within the dermis, indicating that AR may exert unexpected and unique pro-inflammatory and angiogenic activities. This study also demonstrated that epidermally targeted AR expression in transgenic mice stimulated psoriatic pathological processes in the skin that were different from those of other transgenically targeted cytokines and growth factors such as vascular endothelial growth factor, keratinocyte growth factor, IL-1α, tumor necrosis factor α, interferon-γ (IFN-γ), and IL-6. Additionally, because

AR is significantly induced by barrier disruption as well as placing human skin into explant culture, it has been proposed that cutaneous injury (wounding) induces AR expression and initiates the isomorphic, Koebner response (wound-dependent lesion formation) in psoriatic individuals. Collectively, these K14–AR transgenic studies suggest that AR may act as a cutaneous injury signaling factor that initiates epidermal proliferation, angiogenesis, and innate immune responses in the skin.

## IV. REGULATION OF AR EXPRESSION

Table 1 lists factors that affect AR expression. Multiple lines of evidence support increased EGF-related peptide signaling and activation of the EGFR as important components of oncogenic Ras transformation of rat intestinal epithelial cells (RIE-1 cells). For example, oncogenic Ras-transformed RIE-1 cells exhibit increased production of EGFR ligands AR, TGF-α, and HB-EGF. EGFR blockade partially reverts the transformed morphology of oncogenic Ras RIE-1 cells and prevents the morphological transformation of parental RIE-1 cells caused by constitutively activated Ras-conditioned medium. Moreover, the growth inhibitory effects of the farnesyltransferase inhibitor L744, 832 for oncogenic Ras-transformed RIE-1 cells is preceded by decreased expression of AR, HB-EGF, and TGF-α. Also, in mouse models of skin carcinogenesis and melanoma, EGFR signaling (with AR likely being an active participant) plays a pivotal role in mediating the Ras-transformed phenotype. Thus, the oncogenic Ras-transformed phenotype is mediated, at least in part, through increased production of EGFR ligands (including AR) that presumably bind to and activate EGFR. It is thought that EGFR signaling will further augment Ras signaling as well as activate Ras-independent signals that contribute to the Ras-transformed phenotype.

**TABLE 1** ***In Vitro* and *In Vivo* Conditions with Altered AR Expression**

| Condition | Target cells | mRNA | Protein |
|---|---|---|---|
| ***In vitro* conditions with altered AR expression** | | | |
| **Up-regulation** | | | |
| Mutated Ras | RIE-1, IEC-6 | + | + |
| EGFR ligands | RIE-1, HK, LIM1215 | + | + |
| Insulin/IGF-I | HK | + | + |
| IL-1α and TNFα | Cervical epithelial cells | + | + |
| IFN-γ | NHBECs | + | + |
| Gastric injury | Rat gastric epithelial cells | + | + |
| *Helicobacter pylori* | MKN 28 | + | + |
| $1,25(OH)_2D_3$ | SCC25, MCF-7, MDA-231 | + | ND |
| Androgen | LNCaP | + | + |
| E2 | MCF-7 | + | + |
| Phorbol esters | MCF-7, T-47D, MDA-231 | + | + |
| Gastrin | Rat stomach cells | + | ND |
| TARP | PC3 | + | ND |
| WT1 | U20S | + | ND |
| **Down-regulation** | | | |
| TGF-β | A549 | − | − |
| PPAR-γ agonists | Ha-Ras-transformed IEC | − | − |
| ***In vivo* conditions with increased AR expression** | | | |
| Psoriasis | Skin | + | + |
| Neoplasia | Pancreatic cancer, | + | + |
| | GI cancer (gastric, CRC), | + | + |
| | mammary gland cancer | + | + |

*Note*: +, induction; −, down-regulation; ND, not determined. RIE-1, IEC-6, rat intestinal epithelial cells; HK, human keratinocytes; NHBECs, normal human bronchial epithelial cells; LIM1215, colon adenocarcinoma cell line; MKN 28, gastric adenocarcinoma cell line; $1,25(OH)_2D_3$, 1,25-dihydroxy vitamin $D_3$; SCC25, head and neck squamous cell carcinoma cell line; MCF-7, estrogen receptor (ER)-positive breast carcinoma cell line; T-47D, ER-positive breast carcinoma cell line; MDA-231 (MDA-MB-231), ER-negative breast carcinoma cell line; LNCaP, androgen-sensitive prostate cancer cell line; E2, 17β-estradiol; phorbol esters, TPA (12-O-tetradecanoylphorbol-13-acetate); TARP, T-cell receptor γ-chain alternate reading frame protein; PC3, androgen-independent prostate cancer cell line; U20S, osteosarcoma cell line; A549, lung adenocarcinoma cell line; PPAR-γ agonists, peroxisome proliferator-activated receptor-γ agonists (troglitazone, resiglitazone); GI, gastrointestinal tract; CRC, colorectal cancer.

In addition, there is extensive autoinduction and cross-induction of EGFR ligands in RIE-1 cells and other EGF growth-responsive epithelial cells. Under EGFR ligand stimulation, there is often a prompt and robust induction of AR. Various pro-inflammatory cytokines, such as IFN-γ and IL-1α, and growth hormones, such as androgen, estrogen, and gastrin, have been reported to induce AR expression in various cell types. TGF-β and peroxisome proliferator-activated receptor-γ agonists have been found to down-regulate AR expression. Table 1 also lists *in vivo* conditions in which AR expression has been reported to be increased.

## V. BIOLOGICAL ROLES FOR AR

### A. AR as an Autocrine Growth Factor

AR has been shown to act as an autocrine growth factor for normal human keratinocytes, mammary epithelial cells, and bronchial epithelial cells. Moreover, evidence has been presented for AR acting as an autocrine growth factor in certain human cancer cell lines. These include ovarian (OVCAR3, OVCAR8), prostatic (DU145 and PC3), colonic (HCA-7 and Caco-2), bladder, cervical, and gastric cell lines.

### B. AR as a Morphogen

Previous studies have shown that AR influences organ development in the lung, mammary gland, and kidney. In lung development, AR contributes to the growth of the mesenchyme and epithelium, thus participating in the process of branching morphogenesis. These effects are mediated through interactions with HSPGs, as glycosaminoglycan-degrading enzymes abolished AR-induced mitogenesis and branching morphogenesis. The role of AR in mammary gland development has been demonstrated by studies of mice with targeted disruption of AR, TGF-α, and EGF. Mice lacking AR singly and in combination with TGF-α showed defects in ductal outgrowth in glands. In triple-null glands, alveoli were poorly organized and poorly differentiated, and milk protein gene expression was decreased. A role for AR in kidney differentiation was found initially in studies using an embryonic epithelial cell line derived from c-met mice. In addition, AR was identified in a search for genes with altered expression following induction of the Wilms tumor suppressor (WT1), a zinc-finger transcription factor implicated in kidney differentiation and tumorigenesis. It appears that WT1 acts as a transcriptional regulator during kidney differentiation through direct binding to the AR promoter. Furthermore, recombinant AR stimulates epithelial branching in organ cultures of embryonic mouse kidney. Taken together, these studies support a role for AR as an important modulator of branching morphogenesis in developing lung, mammary gland, and kidney. AR appears to act on both epithelium and mesenchyme with the participation of HSPGs.

## VI. SUMMARY

Human AR is a heparin-binding, heparin-inhibited, polypeptide growth factor of the EGF family, which was initially isolated from the conditioned medium of human MCF-7 breast tumor cells that had been treated with phorbol ester. The human AR gene is located on chromosome 4q13–q21 and is expressed as a 252-amino-acid precursor that is processed into several different cell surface and soluble isoforms. In polarized epithelial cells, AR is preferentially delivered to and processed at the basolateral surface. AR is thought to associate with extracellular and cell-associated HSPGs (e.g., syndecans, CD44) as well as other accessory molecules (e.g., CD9). Interaction with these molecules may facilitate a depot form of AR and promote productive interactions between AR and cell surface EGFRs.

AR displays some biological properties that are distinct from those of either TGF-α or EGF. These properties include affinity for heparin, heparin-mediated inhibition of mitogenic activity, and cell density-dependent responsiveness. Distinct biological actions of AR were identified from a comparison of mice with targeted overexpression of AR and TGF-α in the skin, where AR mice exhibit an inflammatory psoriasis-like phenotype. Furthermore, experiments with AR null mice have revealed a role for AR in mammary gland development through activation of Stat5a. AR also participates in branching morphogenesis in the lung and kidney. Additional studies will be needed to elucidate mechanisms underlying these biological properties of AR. It is anticipated that AR will be an important target of the protease-mediated cleavage of EGFR ligands that underlie G-protein-coupled receptor transactivation of the EGFR.

AR has been shown to be regulated by a variety of agents and conditions that include up-regulation by EGFR ligands and mutant Ras. AR has also been shown to be up-regulated in pathological states such as psoriasis and a number of neoplasms, where it may act in an autocrine manner to stimulate growth and/or prevent apoptosis.

## Glossary

**epidermal growth factor receptor (EGFR)** Type I transmembrane protein that has tyrosine kinase activity in its cytoplasmic domain. There are four mammalian members of this receptor family. Signaling from the EGFR is initiated by binding of its ligands, which then induces receptor dimerization and autophosphorylation to propagate downstream signaling cascades.

**epidermal growth factor receptor (EGFR) ligands** There are currently seven members of the mammalian epidermal growth factor family that exhibit conserved spacing of six cysteine residues in the mature domain of the growth factor. The ligands are synthesized in a proform, delivered to the cell surface as a transmembrane protein, and then cleaved to release mature soluble growth factor.

**psoriasis** Hyperproliferative skin disorder with infiltrating lymphocytes and neutrophils in the epidermis and dermis.

**tumor necrosis factor $\alpha$-converting enzyme/a disintegrin and metalloprotease 17 (TACE/ADAM17)** Enzyme that is involved in the cleavage of pro-transforming growth factor-$\alpha$ and pro-amphiregulin at the cell surface.

## See Also the Following Articles

**Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor**

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# Androgen Effects in Mammals

SHALENDER BHASIN

*Charles R. Drew University of Medicine and Science, Los Angeles*

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II. TESTOSTERONE AS A PROHORMONE
III. MECHANISM OF ANDROGEN'S ACTION
IV. TESTOSTERONE'S EFFECTS ON REPRODUCTIVE ORGANS

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VI. TESTOSTERONE'S EFFECTS ON BEHAVIOR
VII. TESTOSTERONE REPLACEMENT IN ANDROGEN-DEFICIENT MEN
VIII. ANABOLIC EFFECTS OF ANDROGENS IN PATIENTS WITH CHRONIC ILLNESSES AND AGE-RELATED FRAILTY

**Testosterone, a 19-carbon steroid secreted by the testis, is the predominant androgen in most mammalian species. Androgens directly or indirectly affect almost all body systems during fetal and pubertal development and in adult life. The sexual differentiation of the mammalian fetus is a complex, multifactorial process that requires not only the genetic information carried on the sex chromosomes, but also the hormonal secretions of the fetal testis, namely, testosterone and Müllerian-inhibiting hormone. Testosterone masculinizes the Wolffian structures and causes the external genitalia to form a scrotum and penis. In addition, increasing testosterone levels during puberty promote somatic growth and virilization of boys. Testosterone plays a critical role in mammalian reproduction; it is essential for maintaining sexual function, germ cell development, and accessory sex organs. In the adult animal, testosterone has additional effects on the muscle, bone, hematopoeisis, coagulation, plasma lipids, protein and carbohydrate metabolism, and psychosexual and cognitive function.**

## I. TESTOSTERONE SECRETION, TRANSPORT, AND METABOLISM

### A. Testosterone Secretion

In males of most mammalian species, 95% of circulating testosterone is derived from testicular secretion. Only a small amount of dihydrotestosterone (DHT; approximately 70 μg daily) is secreted directly by the human testis; most circulating DHT is derived from the peripheral conversion of testosterone. Testosterone is produced in the testis by a heterogeneous group of cells that includes the adult Leydig cells, Leydig cell precursors, and immature Leydig cells. Studies in hypogonadotropic (*hpg*) mice suggest that fetal development of both Sertoli and Leydig cells is independent of gonadotropins; however, normal differentiation and proliferation of the adult Leydig cell population require the presence of gonadotropins. The number of Sertoli cells after birth is regulated by gonadotropins. A number of 46,XY male humans with inactivating mutations of the luteinizing hormone (LH) receptor have been studied; these patients have varying degrees of genital ambiguity and Leydig cell agenesis, pointing to the important role of LH in the regulation of Leydig cell development in humans.

In man, 3–10 mg of testosterone is secreted daily by the testis; direct secretion of testosterone by the adrenal and the peripheral conversion of androstenedione, which is secreted by the adrenal and converted to testosterone, collectively account for another 500 μg of testosterone daily. Leydig cells arise from poorly characterized mesenchymal precursor cells under the influence of luteinizing hormone, insulin-like growth factor-I, transforming growth factor-β, transforming growth factor-α, interleukin 1, and basic fibroblast growth factor. Leydig cells exist in two distinct generations in higher mammals, fetal and adult, that are separated in higher mammals by a prepubertal period during which the testis is devoid of Leydig cells.

Testosterone secretion by Leydig cells is under the control of LH, a pituitary glycoprotein hormone. LH binds to specific G-protein-coupled receptors on the Leydig cells and activates the cyclic AMP pathway. Although LH also activates the phopholipase C pathway, it is unclear whether this pathway is essential for LH-mediated stimulation of testosterone production. The rate-limiting step in testosterone biosynthesis is the delivery of cholesterol to the inner mitochondrial membrane, which is the site of the cholesterol side chain cleavage complex that converts cholesterol to pregnenolone. A steroidogenesis acute regulatory protein makes cholesterol available to the cholesterol side chain complex and regulates the rate of testosterone biosynthesis. Peripheral benzodiazepine receptor, a mitochondrial cholesterol-binding protein known to be involved in mediating cholesterol transport, is present in high concentrations in the outer mitochondrial membrane and has also been proposed as an acute regulator of Leydig cell steroidogenesis. In addition, another group of poorly characterized mitochondrial proteins has been implicated in the control of steroidogenesis in Leydig cells.

Leydig cell production of testosterone is modulated by a number of paracrine factors within the seminiferous tubule and the interstitium of the testis, including insulin-like growth factor-I, insulin-like growth factor-binding proteins, inhibins, activins, transforming growth factor-β, epidermal growth factor, interleukin-1, basic fibroblast growth factor, gonadotropin-releasing hormone, and vasopressin.

### B. Androgen Transport in the Body

Ninety-eight percent of circulating testosterone is bound to plasma proteins: the sex hormone-binding globulin (SHBG) and albumin. The sex hormone-binding globulin binds testosterone with much greater affinity than albumin. Only 1 to 3% of testosterone is unbound; although the dogma has been that only the unbound fraction is biologically active, it has been argued that albumin-bound hormone may dissociate readily in the capillaries and thus become bioavailable. In fact, Pardrige *et al.* have demonstrated that albumin- and SHBG-bound androgens represent the major circulating pool of bioavailable hormone for testis or prostate. Furthermore, these investigators have argued that the SHBG–sex steroid complex may be nearly completely available for influx through the blood–testis barrier or prostate plasma membrane; this view is not universally shared.

SHBG is a glycoprotein, synthesized in the liver, that displays high-affinity binding for testosterone and estradiol. Hepatic production of SHBG is regulated by insulin, thyroid hormones, dietary factors, and the balance between androgens and estrogens. SHBG is involved in the transport of sex steroids in plasma and its concentration is a major factor regulating their distribution between the protein-bound and free states, although its functional role in testosterone delivery to target organs is not clear. Plasma SHBG concentrations are decreased by androgen administration, obesity, insulin, and nephrotic syndrome. Conversely, estrogen administration, hyperthyroidism, many types of chronic inflammatory illnesses, and aging are associated with high SHBG concentrations. A locus that is associated with SHBG concentrations in African Americans and Caucasians has been mapped to 1q44. In addition, several other loci in African Americans exhibit linkage with SHBG concentrations, suggesting that many genes likely regulate SHBG levels. The binding of testosterone to SHBG or albumin is not essential for androgen action or steroid homeostasis; rats that are deficient in both sex hormone-binding globulin and albumin are fertile and have normal mating behavior.

### C. Testosterone Metabolism

Testosterone is metabolized predominantly in the liver (50–70%) although some degradation also occurs in peripheral tissues, particularly the prostate and the skin. Liver takes up testosterone from the blood and through a series of chemical reactions that involve 5α- and 5β-reductases, 3α- and 3β-hydroxysteroid dehydrogenases, and 17β-hydroxysteroid dehydrogenase converts it into androsterone and etiocholanolone, which are both inactive metabolites, dihydrotestosterone, and 3-α-androstanediol. These compounds undergo glucoronidation or sulfation before their excretion by the kidneys. Free and conjugated androsterone and etiocholanaolone are the predominant urinary metabolites of testosterone.

## II. TESTOSTERONE AS A PROHORMONE

Testosterone can be converted in many peripheral tissues into its active metabolites, 17β-estradiol and 5α-DHT (Fig. 1). Aromatization of the A ring converts it into 17β-estradiol. In addition, reduction of the δ4 double bond can convert testosterone into 5α-DHT. Testosterone's actions in many tissues are mediated through these metabolites. For instance, testosterone's effects on trabecular bone resorption and the sexual differentiation of the brain require its aromatization to estradiol. Testosterone's effects on plasma lipids, atherosclerosis progression, and some types of behaviors are also mediated through its conversion to estrogen. Considerable gains in insight into the role of estrogen in mammalian physiology have been made through the study of three mouse knockout models: the estrogen receptor-α, estrogen receptor-β, and aromatase knockouts. These models of estrogen deficiency exhibit significant disruption of spermatogenesis and fertility, elevated testosterone and LH levels, decreased bone mass, and increased adiposity, indicating the important role of estrogens in the regulation of bone mass, gonadotropin regulation, body composition, and spermatogenesis. A very small number of humans with inactivating mutations of the CYP19 aromatase gene have been

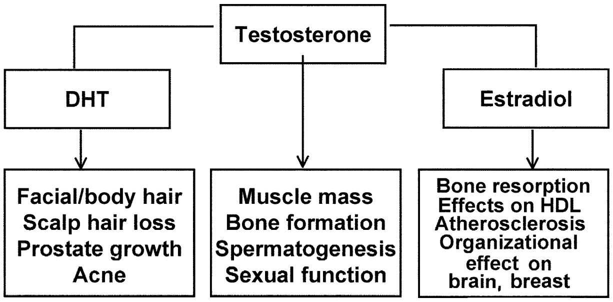

FIGURE 1 Effects of testosterone and its metabolites in men. DHT, dihydrotestosterone; HDL, high-density lipoprotein. Modified from Bhasin and Fisher (2002), with permission.

reported. Females with CYP19 gene mutations are masculinized, fail to undergo female pubertal development, have elevated levels of androgens, LH, and follicle-stimulating hormone, have polycystic ovaries, and are tall in stature. Males with CYP19 aromatase mutations have been characterized by osteoporosis, increased bone turnover, delayed epiphyseal fusion, tall stature, and increased testosterone but markedly decreased estradiol levels.

At least two isoenzymes of steroid 5α-reductase have been cloned and characterized. Type 1 steroid 5α-reductase isoenzyme is expressed in many nongenital tissues, has been mapped to chromosome region 5p15, and has a pH optimum of 8. Type 2 steroid 5α-reductase isoenzyme is expressed in the prostate and other genital tissues, has been mapped to 2p23, and has a pH optimum of 5.0. The biological role of 5α-reductase type 1 has not yet been ascertained. Gene-targeting experiments suggest that type 1 enzyme plays a role in progesterone metabolism at the end of pregnancy. The mice lacking 5α-reductase enzyme type 1 have failure of cervical ripening and fail to deliver.

Testosterone's effects on the prostate and sebaceous glands of the skin require its 5α-reduction to DHT. DHT has been implicated in the pathophysiology of benign prostatic hyperplasia and androgenic alopecia. Type 2 isoenzyme is the predominant form in the prostate and has been implicated in the pathophysiology of benign prostatic hypertrophy, hirsutism, and possibly male-pattern baldness. During embryonic life, testosterone controls the differentiation of the Wolffian ducts into epididymis, vasa deferentia, and seminal vesicles. The development of structures from the urogenital sinus and the genital tubercle such as the scrotum, penis, and penile urethra require the action of DHT. The role of 5α-reduction of testosterone in mediating its effects on muscle and sexual function remains unclear. Although testosterone and DHT can both exercise anabolic effects on the muscle, steroid 5α-reductase activity is very low or absent in the skeletal muscle, and it is not known whether 5α-reduction of testosterone to DHT is obligatory for mediating androgen's effects on the muscle. Similarly, previously published data are unclear on whether androgen's effects on sexual function in adult men are mediated through testosterone or its 5α-reduced metabolite, DHT. Although the enzyme 5α-reductase is expressed in the muscle at low levels, it is generally believed that conversion of testosterone to DHT is not obligatory for mediating its anabolic effects on the muscle.

A large body of information about the role of DHT has emerged from the study of patients with the autosomal recessive genetic disorder of steroid 5α-reductase deficiency. 46,XY males with this syndrome contain normal male internal structures including testes but exhibit ambiguous or female external genitalia at birth. At puberty, these individuals undergo partial virilization, which includes development of a male gender identity in many but not all 46,XY individuals, even if they have been brought up as females. Their development suggests that testosterone itself is able to stimulate psychosexual behavior, libido, development of the embryonic Wolffian duct, muscle development, voice deepening, spermatogenesis, and axillary and pubic hair growth. In contrast, DHT is required for prostate development and growth, the development of the external genitalia, and male patterns of facial and body hair growth or male-pattern baldness. All the 5α-reductase-deficient kindreds that have been studied to date have been shown to have mutations in steroid 5α-reductase type 2, the predominant form in the prostate. Many patients with congenital deficiency of the type 2 isoform of 5α-reductase have low or low normal DHT levels at puberty, either due to the activity of type 1 isoenzyme or due to the residual low activity of type 2 isoenzyme. Therefore, it is not clear whether the normal muscle development and male psychosexual behavior observed in many of these patients after puberty are due to the low normal DHT levels or testosterone itself.

## III. MECHANISM OF ANDROGEN'S ACTION

Most of androgen's actions are mediated through its binding to an intracellular androgen receptor that acts as a ligand-dependent transcription factor. The androgen receptor has homology to other nuclear receptor proteins including the receptors for glucocorticoids, progesterone, and mineralocorticoids. The predominant 919-amino-acid, 110–114 kDa, androgen receptor protein has three conserved functional domains: the steroid-binding domain, the DNA-binding domain, and the transcriptional activation domain; of these, the central, cysteine-rich, DNA-binding domain is the most conserved. The single-copy androgen receptor gene spans a 90 kb region on chromosomal region Xq11–q12. In the absence of its ligand, the androgen receptor protein is distributed both in the nucleus and in the cytoplasm. However, androgen binding to the receptor causes it to translocate within the nucleus; the sequence between amino acids 617 and 633 of the androgen receptor is important for its nuclear migration and transactivation function. There is emerging evidence that some of the effects of androgens may be mediated through nongenomic receptors on the cell membrane.

Binding of androgens by the androgen receptor results in conformational changes in this protein; there is evidence that binding of anti-androgens to the androgen receptor might induce a different set of conformational changes. The androgen receptor can use two transactivation domains, AF1 and AF2. The transactivation domain AF1 (including the so-called 1 and 5 regions) is located in the amino-terminal portion of the receptor, whereas AF2 is located in the carboxy-terminal, ligand-dependent domain. In the intact receptor, both AF1 and AF2 are ligand-dependent and influenced by nuclear receptor co-activators. In contrast, in a truncated androgen receptor that is missing the ligand-binding domain, AF1 becomes constitutively active. Hormone binding to the androgen receptor results in the assembly of tissue-specific co-activators and co-repressors that determine the specificity of hormone action.

In a majority of androgen target tissues, either testosterone or its 5α-reduced metabolite, DHT, binds to the androgen receptor and regulates gene expression. Testosterone binds to the androgen receptor with half the affinity of DHT, although the maximal binding capacity is similar for both androgens. The DHT–androgen receptor complex has greater thermostability and a slower dissociation rate than the testosterone–receptor complex. This may confer greater potency to DHT in mediating androgen's effects in some androgen-sensitive tissues, such as the prostate. However, the reason that 5α-reduction is necessary for mediating androgen's effects in some tissues and not others is unclear. Why this metabolic conversion step evolved for testosterone and not for other steroid hormones is also unknown.

The mutations of the androgen receptor gene have been associated with a wide spectrum of phenotypic abnormalities. Some patients with complete androgen insensitivity present with male pseudohermaphroditism characterized by female external genitalia, a blind vaginal pouch, and well-developed breasts. Other patients with androgen receptor mutations may have a male phenotype and milder abnormalities such as hypospadias, gynecomastia, and infertility.

A number of polymorphisms in the androgen receptor gene have been described; the lengths of the CAG and GCG repeats in exon 1 of the androgen receptor gene have been linked to transcriptional activity of androgen receptor protein. An abnormal length of the polyglutamine tract in exon 1 of the androgen receptor has been associated with spinal and bulbar muscular atrophy, also known as Kennedy's disease. Although some studies have reported an association of androgen receptor polymorphisms in the lengths of polyglutamine and polyglycine tracts with male infertility and risk of prostate cancer, other studies have not confirmed these findings.

## IV. TESTOSTERONE'S EFFECTS ON REPRODUCTIVE ORGANS

### A. Testosterone and the Prostate

Androgens are required for normal prostate development; however, it is not known whether androgens play a role in the initiation of prostate neoplasms. Conversion of testosterone to DHT is obligatory for mediating its effects on the prostate. The prostate increases in size during the peripubertal period in parallel with the rise in serum testosterone levels. Androgen imprinting prior to puberty is an important determinant of the number of stem cells within the prostate and hence adult prostate size in rats and dogs. Castration before puberty, 5α-reductase deficiency, and inactivating mutations of the androgen receptor prevent subsequent development of benign prostatic hypertrophy. Many of the effects of androgen on the prostatic epithelium are mediated through the stromal cells; thus, the interaction between the stromal cells and the epithelial cells is important for normal prostatic development. Androgen's effects on prostatic growth are mediated in part through the regulation of apoptotic genes. Androgen deficiency in adult males is associated with increased expression of many apoptotic genes, resulting in involution of the prostate.

Only the human and the dog develop benign prostatic hypertrophy. Androgens are essential, but not sufficient, for the development of benign prostatic hypertrophy. Abnormal stromal activation and dysregulation of several growth factors play a role in the pathogenesis of this complex disorder. Although androstanediol and DHT can induce prostatic enlargement in the dog, physiologic testosterone administration to hypogonadal men has not been associated with pathologic prostatic enlargement beyond the levels observed in healthy, age-matched controls. Estradiol accentuates prostatic hypertrophy induced by androgen administration in the dog. The critical role of androgens in prostatic growth is supported by clinical findings that lowering serum testosterone levels by surgical orchidectomy or by pharmacologic inhibitors of testosterone production decreases prostate volume in men with benign prostatic hyperplasia. Similarly, clinical trials have established that finasteride, an inhibitor of 5α-reductase enzyme, produces a clinically significant reduction in prostate size in men with benign prostatic hypertrophy.

### B. Testosterone and Spermatogenesis

High intratesticular testosterone concentrations are required for the initiation and maintenance of spermatogenesis. Gonadotropin deficiency resulting from hypophysectomy or administration of a gonadotropin-releasing hormone (GnRH) antagonist is associated with a marked depletion of intratesticular testosterone concentrations and azoospermia in the rat, monkey, and human male. High doses of testosterone can reinitiate and maintain spermatogenesis in rats made gonadotropin-deficient by hypophysectomy or GnRH antagonist treatment. In men with acquired gonadotropin deficiency, luteinizing hormone can reinitiate and maintain spermatogenesis by stimulating testosterone production within the testis. The mechanism by which testosterone maintains germ cell development is not well understood. Testosterone receptors are present on the Sertoli and peritubular cells, some Leydig cells, and endothelial cells of the small arterioles. However, it has not been established whether androgen receptors are also present on germ cells. It is generally believed that androgen's effects on spermatogenesis are mediated indirectly through Sertoli cells, although it is possible that testosterone might also directly affect germ cell development. Testosterone affects protein secretion by both round spermatids and Sertoli cells. The expression of androgen receptors is maximal at stages VI–VII of the seminiferous epithelium; the effects of testosterone might be exerted on the germ cells as they pass through these stages. More recent evidence suggests that testosterone regulates germ cell apoptosis in a stage-specific manner.

### C. Testosterone's Effects on the Epididymis

Androgens are important regulators of epididymal structure and function. A variety of epididymal biochemical processes, such as the transport of small molecules across the epididymal epithelium, DNA and RNA synthesis, protein synthesis, activity of a number of epididymal enzymes, and maturation and storage of spermatozoa, are under androgen control. The regulation of epididymal function by androgens is highly region-specific. Some genes, such as proenkephalin, are down-regulated after castration but are not restored by androgen replacement, whereas expression of other genes, such as E-cadherin, is decreased by castration in a region-specific manner and is restored to varying degrees by androgen administration.

## V. ANDROGEN'S EFFECTS ON NONREPRODUCTIVE BODY SYSTEMS

### A. Androgen's Effects on Bone

Androgens are important regulators of bone maturation and maintenance of bone mass. Androgens promote linear bone growth and endochondral ossification in rats. In humans, the peri-pubertal bone accretion is androgen dependent; consequently, men with androgen deficiency during the peri-pubertal period have diminished peak bone mass. Adult men with testosterone deficiency have lower bone density than age-matched, healthy controls; testosterone replacement therapy of hypogonadal men increases but does not normalize bone density. Suppression of serum testosterone levels by administration of GnRH agonist analogues or surgical orchidectomy leads to a progressive decrease in bone density. Testosterone's effects on bone are mediated in part through its conversion to estradiol. The critical role of aromatization in mediating androgen's effects on bone is supported by observations that 46,XY men with mutations of the genes that code for the estrogen receptor or the aromatase enzyme have delayed epiphyseal fusion and decreased bone density. Also, in epidemiological studies, bioavailable estradiol levels correlate better with bone mineral density than total testosterone levels. Testosterone has additional direct effects in stimulating cortical bone formation. Androgen receptors have been demonstrated on the osteoblasts. Testosterone might also indirectly affect bone mineral density through stimulation of insulin-like growth factor-I and by increasing muscle mass and muscle strength.

### B. Androgen and Body Composition—Effects on Muscle and Fat Metabolism

Testosterone is a major determinant of body composition in male mammals. The sexual dimorphism of several androgen-responsive muscles such as the masseter and levator ani in several mammalian species has been attributed to differences in androgen levels in males and females. Testosterone administration increases nitrogen retention in castrated males of several mammalian species and in hypogonadal men, women, and boys before puberty. Recent studies have established that testosterone replacement in hypogonadal men increases fat-free mass, muscle size, and maximal voluntary strength. Supraphysiologic doses of androgens further increase muscle mass and strength in eugonadal men, especially when given in association with resistance exercise. Controlled clinical trials have demonstrated that testosterone supplementation in older men with low testosterone concentrations and in men with human immunodeficiency virus (HIV) infection or chronic obstructive lung disease and low testosterone concentrations is associated with significant gains in fat-free mass and maximal voluntary muscle strength.

Widespread abuse of androgenic steroids by athletes and recreational body builders is based on the premise that androgens promote muscle hypertrophy. Testosterone's effects on muscle mass, strength, and leg power are dependent on the administered dose of testosterone and the circulating testosterone concentrations. The effects of androgen administration on muscle performance and physical function have not been well studied, although the available evidence indicates that testosterone does not improve aerobic performance. There is some evidence that testosterone might reduce reaction time by its effects on neuromuscular transmission.

A testosterone-induced increase in muscle mass is associated with hypertrophy of both type I and type II muscle fibers but no change in muscle fiber number. Testosterone administration in healthy young men is also associated with an increase in the number of myonuclei and satellite cells. Emerging evidence suggests that testosterone stimulates the differentiation of muscle pluripotent cells into the myogenic lineage and inhibits their differentiation into the adipogenic lineage.

Androgen-deficient men have a greater amount of fat than eugonadal men. In middle-aged men, serum testosterone levels correlate inversely with visceral fat and cardiovascular risk. In healthy, young men, administration of graded doses of testosterone leads to a dose-dependent decrease in whole body fat; the decrease in fat mass occurs in both the trunk and the appendices and is evenly distributed in both the superficial subcutaneous and the deep intermuscular and visceral fat compartments. Physiologic testosterone replacement in middle-aged men with midsegment obesity decreases visceral fat, glucose, and insulin levels. Androgen's effects on body composition in men are modulated by pretreatment body composition, genetic and nutritional factors, the growth hormone secretory status, the existence of co-morbid conditions, cytokines, and exercise status.

## C. Testosterone's Effects on Intermediary Metabolism and Cardiovascular Risk

Testosterone is an anabolic hormone that increases muscle mass by stimulating fractional muscle protein synthesis. The mechanism by which testosterone increases protein synthesis is not known. It has been proposed that testosterone increases muscle protein synthesis by increasing the expression of the insulin-like growth factor-I gene within the muscle. It is not known whether testosterone affects muscle protein degradation. Androgen receptors are present on adipocytes and testosterone stimulates lipolysis in some experimental models. The effects of testosterone on lipid uptake and metabolism are region-specific. Cross-sectional studies of middle-aged men demonstrate a direct correlation of serum testosterone levels with insulin sensitivity and an inverse correlation with visceral fat. However, physiologic replacement or slightly supraphysiologic doses of testosterone have not been shown to affect glucose tolerance or insulin sensitivity in lean, young men.

## D. Androgen's Effects on the Skin

The hair follicles, sebaceous glands, and apocrine sweat glands in the skin are androgen-sensitive structures. Increased testosterone levels during pubertal development stimulate the growth of terminal hair in the axillary and pubic areas in both men and women and on the face in men. Excessive androgen production or action has been implicated in the pathophysiology of clinical disorders such as acne, hirsutism, androgenic alopecia, and suppurative hidradenitis. Many investigators believe that local abnormalities in androgen metabolism within the skin in hirsute women make their skin more sensitive to the effects of circulating androgens. Testosterone's effects on the skin are mediated through its conversion to DHT. Type 1 isoenzyme is the major form of steroid 5$\alpha$-reductase in the skin. The highest level of activity of the steroid 5$\alpha$-reductase enzyme has been located in the apocrine glands and the sebaceous glands. Inhibitors of type 1 steroid 5$\alpha$-reductase enzyme in the skin, such as aliphatic unsaturated fatty acids, are being explored as therapeutic agents for the treatment of androgen-responsive skin disorders such as hirsutism and acne. Finasteride, a weak inhibitor of type 2 isoenzyme, has been approved by the Food and Drug Administration for the treatment of androgenic alopecia.

### E. Androgen's Effects on Hematopoiesis, the Coagulation System, and the Vascular System

Testosterone increases red cell mass; this effect is mediated primarily by increasing the production of erythroid precursors from the pluripotent stem cells in the bone marrow. Testosterone has also been reported to increase erythropoietin production in the kidney, although other data show that testosterone can increase red cell production independent of its effects on erythropoietin. There are numerous anecdotal reports of sudden death, vascular thrombosis, myocardial infarctions, and cardiomyopathy in relatively young athletes abusing large quantities of anabolic steroids, leading to speculation that testosterone increases the thromobogenicity of plasma. However, the effects of testosterone on the coagulation system remain controversial. Fibrinogen and plasminogen activator inhibitor concentrations in plasma correlate directly with endogenous testosterone; these changes may be viewed as favoring plasma thrombogenicity. However, androgens also increase tissue plasminogen activator, protein C, and antithrombin III levels; these factors protect the body from vascular thrombosis. Testosterone and DHT increase platelet aggregation at the site of endothelial trauma. Danazol, a weak synthetic androgen, has been reported to increase platelet count. Androgens may increase vascular reactivity to trauma and decrease aortic smooth muscle prostaglandin $I_2$. Therefore, some effects of androgens appear to favor hypercoagulability and others appear to oppose it. The net effect in a person may vary with the type and dose of androgen used, genetic predisposition, simultaneous use of other medications, and the presence of co-morbid conditions.

### F. Androgens and Plasma Lipids and Cardiovascular Risk

The effects of androgens on plasma lipids depend on the dose (physiologic or supraphysiologic), the route of administration (oral or systemic), and the type of androgen used (aromatizable or not aromatizable). Supraphysiologic doses of testosterone and other androgenic steroids decrease plasma high-density lipoprotein (HDL) cholesterol levels in men. Androgenic steroids that can be aromatized produce a smaller decrease in high-density lipoprotein levels than those that cannot be aromatized. Lowering serum testosterone levels experimentally by GnRH antagonist administration increases plasma HDL cholesterol levels; conversely, testosterone replacement of hypogonadal men modestly lowers plasma HDL cholesterol levels. Other studies using physiologic replacement doses in older men or HIV-infected men have reported either no change or only minor changes in plasma lipid profiles. Cross-sectional epidemiological studies demonstrate a direct relationship between serum testosterone levels and HDL cholesterol levels and an inverse relationship between testosterone levels and visceral fat. Therefore, these data suggest that testosterone levels in the mid- to high normal range may be optimum for cardiovascular health in men. Indeed, physiologic testosterone replacement decreases visceral fat, glucose, and insulin levels in middle-aged men with visceral obesity. It is not known what testosterone levels are consistent with optimum cardiovascular risk in men.

The prevalent dogma that physiologic testosterone replacement increases cardiovascular risk and accelerates atherosclerosis progression is not supported by data. Of the 30 cross-sectional studies reviewed by Alexanderson *et al.*, 18 reported lower testosterone concentrations in men with coronary artery disease than in age-matched controls, 11 reported similar levels, and only 1 study reported higher concentrations of dehydroepiandrosterone sulfate in men with coronary artery disease than age-matched controls. In mice with low-density lipoprotein receptor mutations, surgical orchidectomy is associated with accelerated atherogenesis, and testosterone replacement retards atherosclerosis progression. Testosterone infusion has been reported to improve coronary blood flow in humans and dogs. Testosterone's effects on blood vessel walls require its conversion to estradiol because concomitant administration of an aromatase inhibitor can attenuate the beneficial effects of testosterone in retarding atherogenic lesions. The effects of testosterone replacement on atherosclerosis progression in older men with low testosterone levels have not been studied.

## VI. TESTOSTERONE'S EFFECTS ON BEHAVIOR

### A. Androgen's Effects on Sexual Function

Sexual function in men is a complex multicomponent process that includes central mechanisms for the regulation of sexual desire and arousability and local mechanisms for penile tumescence, orgasm, and ejaculation. The primary effects of testosterone are on sexual interest and motivation. Observations that eunuchs and hypogonadal men can have erections are consistent with the proposal that testosterone regulates libido rather than erectile function. Davidson *et al.* demonstrated that although overall sexual activity is reduced in hypogonadal men, these patients have a normal erectile response to visual erotic stimuli. Testosterone replacement of young, androgen-deficient men improves a wide range of sexual behaviors including frequency of sexual activity, sexual daydreams, sexual thoughts, feelings of sexual desire, spontaneous erections, episodes of nocturnal penile tumescence, and the duration of penile erections in response to visual erotic stimuli. Testosterone regulates nitric oxide synthase activity in the cavernosal smooth muscle, and it is possible that achievement of optimal penile rigidity might require physiologic testosterone concentrations. Orgasm and ejaculation are androgen-independent. In male mammals, relatively low normal levels of serum testosterone can maintain sexual function.

There is considerable confusion among clinicians with respect to the relationship between androgen deficiency and erectile dysfunction. In middle-aged and older men, erectile dysfunction and androgen deficiency are two common but independently distributed clinical disorders that sometimes co-exist in the same patient. Eight to 10% of men presenting with erectile dysfunction have low testosterone levels. The prevalence of low testosterone levels is not significantly different between middle-aged and older men with impotence and those without impotence. Testosterone administration does not improve sexual function in impotent men with normal testosterone levels. On the other hand, many, but not all, older men with low testosterone levels experience improvements in their libido and overall sexual activity with androgen replacement therapy. The response to testosterone supplementation in this group of men is variable because of the co-existence of other disorders such as diabetes mellitus, hypertension, cardiovascular disease, and psychogenic factors. A meta-analysis of the usefulness of androgen replacement therapy concluded that testosterone administration is associated with greater improvements in sexual function than placebo treatment in men with sexual dysfunction and low testosterone levels. However, previous studies of testosterone replacement in older men have been flawed because of failure to include a placebo-control group, small sample sizes, selection of men with erectile dysfunction associated with multiple systemic illnesses, which may attenuate the response to testosterone therapy, and inclusion of men with normal testosterone levels. Testosterone might also favorably affect marital interactions and intimacy due to an overall increase in sexual desire and sense of well-being, independent of the change in erectile function; this hypothesis has not been examined.

In rats, mating behavior can be maintained at serum testosterone levels that are at the lower end of the normal male range. Similarly, in men in whom endogenous testosterone secretion has been suppressed by a GnRH agonist, low doses of testosterone that bring serum testosterone levels to the lower end of the normal range can maintain sexual desire and activity and nocturnal erections. However, supraphysiologic doses of testosterone might further increase some aspects of sexual arousability.

The role of 5α-reductase in mediating androgen's effects on sexual function remains unclear. Although DHT can restore sexual behavior in castrated monkeys, patients with 5α-reductase deficiency have normal libido and erectile function. Furthermore, the relatively low frequency of sexual dysfunction in men treated with finasteride, an inhibitor of 5α-reductase enzyme, suggests that testosterone conversion to DHT is not required for androgen's effects on sexual function. Important insights into the role of 5α-reduction in regulating male sexual behavior have emerged from the studies of a synthetic steroid, 7α-methyl-19-nortestosterone (MENT). This androgen binds the androgen receptor and exerts biologic effects at some peripheral tissues with a greater potency than testosterone. *In vivo*, MENT does not undergo 5α-reduction. MENT, when administered to castrated rats, maintains full copulatory behavior similar to that observed in testosterone-treated rats. These studies of MENT suggest that 5α-reduction of testosterone is not necessary for maintaining normal mating behaviors in the male rat. However, penile nitric oxide synthase activity in the rat is stimulated by testosterone, and it is possible that conversion of testosterone to DHT may be required for achieving penile rigidity.

### B. Testosterone's Effects on Nonreproductive Behaviors

Testosterone is aromatized to estradiol in the brain and some effects of testosterone may be mediated through its conversion to estradiol. However, androgen receptors are expressed in specific regions of the brain and likely mediate some of testosterone's organizational effects during brain development and some activational effects postnatally. There are gender differences in the distribution of androgen receptor in the human hypothalamus.

During fetal life, testosterone is essential for sexual differentiation of the brain along male lines.

There are structural sex differences in the volume and the synaptic organization of many brain nuclei or regions. Testosterone promotes aggression among males, particularly at the time of mate selection. The scent marking behavior, a marker for territoriality, is testosterone-dependent in many mammalian males. The effects of testosterone on human aggression remain controversial. There is a significant, though not strong, correlation between serum testosterone levels and some aspects of aggressive behavior in men. A significant proportion of anabolic steroid users report mood disorders, manic and hypomanic syndromes characterized by aggressiveness, and other psychiatric disorders. Administration of high doses of testosterone to men is associated with increased aggressive responses compared to placebo. However, other well-controlled studies have failed to find clear increases in aggressive behaviors in androgen-treated men. Testosterone affects the development of sex-typed nonreproductive behaviors such as maze learning and juvenile play. Androgens are considered to be responsible for the greater visuospatial ability and lower verbal ability in men than in women. Exposure to testosterone through its conversion to estradiol during the neonatal period is responsible for the development of brain structures that subserve spatial functions. Alterations of androgen levels in adult animals do not affect spatial processing but may influence memory storage and affective properties.

The data on the relationship of circulating androgen concentrations with cognitive function are conflicting. Androgen's effects on cognitive function are domain-specific. Observations that men outperform women in a variety of visuospatial skills suggest that androgens enhance visuospatial skills. Clinical trials in older men suggest that testosterone administration might enhance visuospatial skills and verbal memory. Hypogonadal men perform worse on tests of verbal fluency than eugonadal men and show improvement after testosterone replacement. In transsexual males, administration of anti-androgen and estrogen decreases anger and aggression, sexual arousability, and spatial skills and increases verbal fluency. Conversely, testosterone administration to female transsexuals decreases verbal fluency and increases spatial skills.

The reported literature on testosterone and cognition is equivocal but these inconsistencies should not be interpreted to mean that there is no effect. Furthermore, previous studies of androgen supplementation have focused on a very limited number of domains. Prospective, randomized, placebo-controlled trials are needed to comprehensively determine the effects of testosterone supplementation over a wider range of doses on a number of domains of cognitive function.

## VII. TESTOSTERONE REPLACEMENT IN ANDROGEN-DEFICIENT MEN

Testosterone, when administered by mouth, is absorbed readily from the gastrointestinal tract; however, most of it is destroyed during its first pass through the liver. Therefore, an appropriate delivery system is needed to achieve sustained levels of testosterone in the blood. Because of the potential contraceptive and anabolic applications of androgens, there has been considerable interest in developing more physiologic and long-acting testosterone delivery systems (Table 1).

### A. Testosterone Esters

Esterification of the 17β-hydroxyl group makes the compound more hydrophobic. Therefore, when these esters are injected in an oil suspension in the muscle, they are absorbed very slowly from the muscle depot into the bloodstream. It is the slow release rather the slow deesterification that accounts for the extended duration of testosterone esters. The longer the side chain, the more hydrophobic the compound and the longer the duration of action. Testosterone enanthate and cypionate, the two commonly used testosterone esters, have identical kinetics. After injection of 200 mg testosterone enanthate or cypionate, the usual replacement dose in hypogonadal men, serum testosterone levels rise into the supraphysiologic range within 24 to 48 h and then gradually decline into the hypogonadal range 10–17 days later. These fluctuations in serum testosterone levels are associated with changes in the patient's mood and energy level and are a major drawback of this formulation. Variations in serum testosterone levels during treatment with testosterone esters can be minimized by giving 100 mg of testosterone enanthate or cypionate every week. With administration of physiologic replacement doses, serum levels of estradiol and DHT are normal. Given an adequate dose, testosterone esters can produce virilization, restore sexual function, and increase muscle mass, bone mass, and hematocrit in hypogonadal men.

### B. Testosterone Transdermal Systems

Three transdermal testosterone systems are commercially available to treat hypogonadal men: a scrotal

**TABLE 1 Clinical Pharmacology of the Testosterone Formulations**

| Formulation | Regimen | Pharmacokinetic profile | DHT and estradiol | Advantages | Disadvantages |
|---|---|---|---|---|---|
| Testosterone enanthate or cypionate (127–129) | 100 mg im weekly or 200 mg im every 2 weeks | After a single im injection, serum T levels rise into the supraphysiological range and then decline gradually into the hypogonadal range by the end of the dosing interval (127–129) | DHT and E2 levels rise in proportion to the increase in T levels; T:DHT and T:E2 ratios do not change | Corrects symptoms of androgen deficiency; relatively inexpensive, if self-administered; flexibility of dosing | Requires im injection; peaks and valleys in serum T levels |
| Scrotal testosterone patch (130–131) | One scrotal patch designed to nominally deliver 6 mg over 24 h, applied daily | Normalizes serum T levels in many but not all androgen-deficient men | Serum E2 levels are in the physiological male range, but DHT levels rise into the surpraphysiological range; T:DHT ratio is significantly lower than in healthy men (130–132) | Corrects symptoms of androgen deficiency | To promote optimum adherence of the patch, scrotal skin needs to be shaved; high DHT levels |
| Nongenital transdermal system (133–134) | One or two patches, designed to nominally deliver 5–10 mg T over 24 h, applied daily on nonpressure areas | Restores serum T, DHT, and estradiol levels into the physiological male range | T:DHT and T:E2 levels are in the physiological male range | Ease of application; corrects symptoms of androgen deficiency; mimics the normal diurnal rhythm of testosterone secretion; lesser increase in hemoglobin than injectable esters | Serum testosterone levels in some androgen-deficient men may be in the low normal range; these men may need application of two patches daily; skin irritation at the application site may be a problem for some patients |
| Testosterone gel | Testosterone gel containing 50–100 mg testosterone, should be applied daily | Restores serum T and estradiol levels into the physiological male range | Serum DHT levels and T:DHT ratios are lower in hypogonadal men treated with the testosterone gel than in healthy eugonadal men | Corrects symptoms of androgen deficiency; provides flexibility of dosing; ease of application; good skin tolerability | Potential of transfer to a female partner or child by direct skin-to-skin contact; moderately high DHT levels |
| 17α-Methyl testosterone (135) | Orally active, 17α-alkylated compound that should not be used because of potential for liver toxicity | Orally active | | | Clinical responses variable; potential for liver toxicity; should not be used for treatment of androgen deficiency |
| Testerone pellets | Three or four 200 mg pellets inserted under the skin | Maintains serum T and free T in normal range for 3–4 months | Normal DHT and E2 concentrations | Long duration | Requires insertion through a trocar |
| Testosterone undecanoate | 80–160 mg daily | Serum T increases transiently into normal range, but needs 3–4 times a day administration | High DHT but normal E2 concentrations | Oral route | Requires insertion through a trocar; spontaneous extrusion; suboptimal clinical responses; variability in serum T levels |

*Note*. DHT, dihydrotestosterone; E2, 17β-estradiol; im, intramuscular; T, testosterone.

testosterone patch (Testoderm, ALZA Corp., Palo Alto, CA) and two nongenital patches (Androderm, Watson Pharmaceuticals, Pomona, CA; Testoderm TTS, ALZA Pharmaceuticals, Mountainview, CA).

Scrotal patches deliver either 4 or 6 mg/day of testosterone depending on their size. Serum testosterone levels in hypogonadal men peak in the midnormal range 4 to 8 h after application of the patch and then decline through the day in a pattern similar to the circadian pattern in normal young men. Hypogonadal men treated with the scrotal patch have normal serum estradiol levels but much higher DHT levels than untreated eugonadal men. This is likely the result of the high activity level of 5α-reductase in the scrotal skin. It is not known whether long-term exposure to high serum DHT levels will have deleterious effects on the prostate or whether intraprostatic DHT levels are also elevated.

Two nongenital transdermal systems, Androderm and Testoderm, can maintain physiologic testosterone levels in hypogonadal men. Four to 12 h after application of the patch, serum testosterone and estradiol levels are in the midnormal range. The nongenital patches also produce physiologic levels of serum DHT and estradiol and normal DHT:testosterone and estradiol:testosterone ratios. Some patients treated with the patches may experience skin irritation at the site of patch application.

### C. Oral Testosterone Formulations

17α-Alkylation of testosterone makes it less susceptible to hepatic degradation. However, these 17α-alkylated androgens should not be used because of the potential for hepatotoxicity. Testosterone undecanoate, an oral form of testosterone, when administered in oleic acid, is absorbed preferentially through the intestinal lymphatics into the lymphatic duct and general circulation and is therefore spared the first-pass degradation in the liver. Its short half-life necessitates dosing two to three times per day for clinical effects. Circulating levels of testosterone vary among subjects receiving the same dose of this formulation; overnight levels are low and clinical response is not as good as with other forms of testosterone. A sublingual preparation of cyclodextrin-complexed testosterone is rapidly absorbed from the sublingual mucosa into the bloodstream, leading to rapid peaks in serum testosterone levels followed by a decline within 2 h. Frequent dosing several times a day is necessary to maintain normal serum levels.

### D. Novel Androgen Formulations under Development

Interest in developing more physiologic, sustained-release testosterone formulations has increased due to the potential application of testosterone as an anabolic agent and as a male contraceptive. With long-acting injectable testosterone preparations (testosterone buciclate, 600 mg intramuscularly every 3 to 4 months), serum levels peak at 6 weeks and can remain in the normal range for 12 weeks. Testosterone pellets (three 200 mg pellets or six 100 mg pellets) are implanted under the skin and can provide normal testosterone levels as well as physiologic levels of estradiol and DHT for up to 6 months. A single intramuscular injection of a biodegradable testosterone microsphere formulation produces normal levels of testosterone in hypogonadal men for up to 11 weeks; serum estradiol and DHT levels are maintained in the normal range. A number of transdermal, buccal, sublingual, parenteral, and oral testosterone formulations are currently under development.

## VIII. ANABOLIC EFFECTS OF ANDROGENS IN PATIENTS WITH CHRONIC ILLNESSES AND AGE-RELATED FRAILTY

Many chronic illnesses such as cancer, HIV infection, chronic obstructive lung disease, and end-stage renal disease are associated with substantial loss of muscle mass and function. Although these diseases are not yet curable, disease stability can often be achieved. Muscle wasting in these illnesses produces debility and is associated with poor quality of life, adverse disease outcomes, and increased utilization of health care resources. There is a high prevalence of low testosterone levels in these sarcopenic disorders. Furthermore, low testosterone levels correlate with loss of muscle mass and exercise capacity. Because testosterone replacement in hypogonadal men increases lean body mass and muscle strength, it has been speculated that testosterone supplementation in men with chronic illnesses such as HIV infection will have similar anabolic effects. Clinical trials have shown that androgen administration in HIV-infected men is associated with modest increases in fat-free mass, hematocrit, and some aspects of health-related quality of life. However, further studies are needed to determine whether testosterone replacement can produce clinically meaningful changes in muscle function or disease outcomes in patients with chronic illnesses.

There is agreement that total and free testosterone levels and testosterone production rates are lower in older men than in young healthy men. Some studies that exclusively recruited healthy, middle-income older men failed to detect significant differences in serum testosterone levels between old and young men. Recent studies with more representative population samples have reconfirmed the age-related decrease in serum testosterone levels. Interpretation of serum testosterone levels in the older men in these studies has been complicated by several confounding factors: the cross-sectional nature of these studies, the loss of diurnal variation in serum testosterone levels in the elderly so that studies that obtain samples in the afternoon underestimate the age-related changes in testosterone levels, and higher sex hormone-binding globulin levels in older men so that total testosterone levels underestimate the greater decline in free testosterone levels (both dialyzable and bioavailable). A meta-analysis of 44 studies of testosterone levels in older men demonstrated an unequivocal decrease in morning testosterone levels. Recent longitudinal studies of normal men have verified the age-related decline in serum testosterone levels. Lower testosterone levels are the result of changes at multiple levels of the hypothalamic–pituitary–gonadal axis. Testicular response to gonadotropins is diminished in older men, gonadotrope responsiveness to androgen suppression is attenuated, and the pulsatility of the hypothalamic GnRH pulse generator is altered. In addition, there are abnormalities of feed-forward and feedback systems. Co-existing diseases, malnutrition, and concomitant medications also affect serum testosterone levels. A number of clinical problems prevalent in older men may be related to androgen deficiency, including sexual dysfunction, muscle weakness and wasting, changes in body composition, osteopenia, increased prevalence of hip and vertebral fractures, decreased body hair, decreased hematopoiesis, and memory loss. It has been speculated but not proven that androgen replacement may help prevent or reverse these disorders. Four long-term studies of 1 to 3 years in duration have demonstrated that testosterone supplementation in older men with low testosterone levels is associated with modest increases in fat-free mass, grip strength, and hematocrit and a decrease in fat mass. The frequency of adverse events associated with testosterone administration has been low in these studies, although these studies were not powered to detect significant increases in prostate or cardiovascular event rates. Furthermore, the effects of physiologic testosterone replacement on health-related outcomes such as fracture rates, falls, disability, cognitive function, progression to dementia, and psychosexual function have not been rigorously evaluated in adequately powered studies. Thus, the long-term risks and benefits of testosterone supplementation in older men remain largely unknown.

## Glossary

**anabolic steroid** Any substance related to the male sex hormone testosterone that promotes the growth of skeletal muscle (anabolic effects) and the development of male sexual characteristics (androgenic effects).

**andropause** A controversial term used to describe the clinical syndrome associated with the age-related decline in serum testosterone concentrations in older men. There is considerable debate over whether the age-related decline in serum testosterone concentrations is clinically important and whether this should be corrected by testosterone replacement.

**aromatase CYP19** An enzyme associated with cytochrome P450 that catalyzes the final step in the biosynthesis of C-18 estrogens from C-19 steroids and converts the A ring into an aromatic ring.

**dietary steroidal supplements** Compounds such as dehydroepiandrosterone (DHEA) and androstenedione that can be purchased in the United States without a prescription through many health food stores. They are often taken because the user believes they have anabolic effects. Steroidal supplements such as DHEA and androstenedione can be converted into testosterone and other sex steroids in the body.

**steroid 5α-reductase** An enzyme complex that converts testosterone to 5α-dihydrotestosterone. It has two isoforms that have different biochemical properties and tissue distribution.

**testosterone esters** The compounds that result from esterification of testosterone at the 17β-hydroxy position. Two common, clinically used testosterone esters include testosterone enanthate and testosterone cypionate.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptor-Related Pathology • Androgen Receptors and Prostate Cancer • Androgen Receptor Structure and Function • Androgens: Pharmacological Use and Abuse • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Steroid Hormone Receptor Family: Mechanisms of Action**

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# Androgen Receptor Crosstalk with Cell Signaling Pathways

ZORAN CULIG, HELMUT KLOCKER, GEORG BARTSCH, AND ALFRED HOBISCH

*University of Innsbruck, Austria*

I. STEROID RECEPTORS AND NONSTEROIDAL ACTIVATION
II. PEPTIDE GROWTH FACTORS AND ANDROGEN RECEPTOR FUNCTION
III. ANDROGEN RECEPTOR ACTIVATION AND PROTEIN KINASE A SIGNALING PATHWAY
IV. REGULATION OF ANDROGEN RECEPTOR ACTIVITY BY PLEIOTROPIC COMPOUNDS
V. WIDE SPECTRUM OF ANDROGEN RECEPTOR ACTIVATORS

**The androgen receptor (AR) is a nuclear transcription factor that regulates the expression of specific genes in male reproductive and visceral organs. The function of the human AR has been investigated in models of prostate carcinoma and in heterologous cells that transiently express wild-type or mutated steroid receptors. The AR can be activated in a ligand-independent manner by a number of cellular regulators. AR activation by peptide growth factors, agents that stimulate protein kinase A activity, interleukin-6, and the differentiation agent phenylbutyrate has been investigated in detail. Ligand-independent activation of the AR has implications regarding the regulation of both proliferation and differentiation. Cellular regulators potentiate AR activity induced by low concentrations of androgenic hormones. This mechanism is, at least in part, relevant to the failure of endocrine therapy in prostate cancer but it might also occur in other tissues. Although in some cases AR antagonists inhibit AR activation by intracellular kinase pathways, there are also examples of their diminished efficiency in the presence of nonsteroidal reguators of AR function. It is hypothesized that nonsteroidal AR activators modulate receptor phosphorylation, most probably in the N-terminus.**

## I. STEROID RECEPTORS AND NONSTEROIDAL ACTIVATION

In the 1990s, it became clear that steroid receptors are activated not only in the classical way by their respective ligands but also by a variety of nonsteroidal compounds, which in turn stimulate intermediary signal transducers activating protein kinases. Early studies on steroid receptor activation by cellular regulators were focused mainly on the human estrogen receptor-α, which is a classic example of activation by growth factors and analogues of cAMP in the absence of ligand. In contrast, human progesterone and glucocorticoid receptor display ligand-dependent activation in the presence of growth

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# Androgen Receptor Crosstalk with Cell Signaling Pathways

Zoran Culig, Helmut Klocker, Georg Bartsch, and Alfred Hobisch
*University of Innsbruck, Austria*

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**The androgen receptor (AR) is a nuclear transcription factor that regulates the expression of specific genes in male reproductive and visceral organs. The function of the human AR has been investigated in models of prostate carcinoma and in heterologous cells that transiently express wild-type or mutated steroid receptors. The AR can be activated in a ligand-independent manner by a number of cellular regulators. AR activation by peptide growth factors, agents that stimulate protein kinase A activity, interleukin-6, and the differentiation agent phenylbutyrate has been investigated in detail. Ligand-independent activation of the AR has implications regarding the regulation of both proliferation and differentiation. Cellular regulators potentiate AR activity induced by low concentrations of androgenic hormones. This mechanism is, at least in part, relevant to the failure of endocrine therapy in prostate cancer but it might also occur in other tissues. Although in some cases AR antagonists inhibit AR activation by intracellular kinase pathways, there are also examples of their diminished efficiency in the presence of nonsteroidal reguators of AR function. It is hypothesized that nonsteroidal AR activators modulate receptor phosphorylation, most probably in the N-terminus.**

## I. STEROID RECEPTORS AND NONSTEROIDAL ACTIVATION

In the 1990s, it became clear that steroid receptors are activated not only in the classical way by their respective ligands but also by a variety of nonsteroidal compounds, which in turn stimulate intermediary signal transducers activating protein kinases. Early studies on steroid receptor activation by cellular regulators were focused mainly on the human estrogen receptor-α, which is a classic example of activation by growth factors and analogues of cAMP in the absence of ligand. In contrast, human progesterone and glucocorticoid receptor display ligand-dependent activation in the presence of growth factors or peptide hormones. The function of the human androgen receptor (AR) has been studied in prostate cancer models and in heterologous cells that are transiently transfected with AR cDNA. The AR is a nuclear transcription factor that is expressed in benign prostate tissue and nearly all prostate cancers and is involved in the regulation of proliferation and differentiation. This article will summarize findings on the crosstalk between signaling pathways of peptide hormones and cytokines and that of the AR. It is particularly interesting that nonsteroidal activation of the AR has been observed both with compounds that stimulate and with those that retard tumor cell proliferation. Data regarding the action of AR antagonists in the presence of nonsteroidal receptor activators and the involvement of protein kinases will also be presented.

## II. PEPTIDE GROWTH FACTORS AND ANDROGEN RECEPTOR FUNCTION

The growth of the prostate gland is regulated by, in addition to steroid hormones, several growth factors in a paracrine or an autocrine manner. In the late stages of prostate cancer, expression of growth factors or their receptors is frequently disregulated and therefore their potential interactions with other signaling pathways are of special interest. Interactions between these polypeptides and the AR were initially studied in DU-145 prostate cancer cells, which lack endogenous steroid receptors. In transient transfection experiments, AR ligand-independent activation was caused by insulin-like growth factor-I (IGF-I), keratinocyte growth factor (KGF), and epidermal growth factor (EGF). In that experimental system, IGF-I was a more potent AR activator than KGF or EGF and it was effective with three different androgen-responsive reporters. Thus, although there are structural and functional similarities between the AR and human progesterone and glucocorticoid receptors, only the AR could be activated in the absence of ligand. Proliferation of prostate cells is stimulated by IGF-I, and IGF receptors are detectable in the majority of prostate cancers. IGF-II, which binds to IGF receptor type I with lower affinity than IGF-I, was not effective in co-transfection–transactivation experiments. The importance of IGF-I activation of the AR could reflect the fact that elevated serum IGF-I is associated with prostate cancer risk.

The effect of KGF on the AR was seen with a reporter gene that was driven by a promoter consisting of two androgen-responsive elements in front of the TATA box. KGF is a stromal growth factor that is, in co-culture models, induced by androgenic hormones and whose receptor is expressed on the surface of epithelial cells. Experiments performed on explant cultures of developing rat seminal vesicle and prostate tissues provided evidence that the crosstalk between KGF and the AR is important *in vivo*. KGF-stimulated development was blocked by anti-androgens, in concordance with findings previously reported for transfected prostate cancer cells. The growth of prostate primary epithelial cultures was stimulated by KGF and the effect was abolished by the anti-androgen hydroxyflutamide. Taken together, these findings demonstrate up-regulation of AR function by KGF.

In addition to ligand-independent activation of the AR, growth factors potentiate receptor activation via low concentrations of androgen. Synergistic ligand-dependent activation of the AR was also observed with other cellular regulators and is pathophysiologically significant. Low concentrations of androgenic hormones persist in patients who receive endocrine therapy for advanced carcinoma of the prostate. AR activation in conditions in which androgen levels are substantially reduced might be, at least in part, responsible for failure of androgen ablation. In PC-3 prostate cancer cells stably transfected with the AR, dihydrotestosterone and EGF regulated the proliferative response in a synergistic manner. This might be due to both AR activation by EGF and EGF receptor up-regulation by androgen.

HER-2/neu tyrosine kinase is an EGF receptor-related molecule that is expressed at high levels in androgen-independent sublines of the LAPC-4 prostate cancer xenograft. Similar to previously reported effects of EGF, ligand-independent and synergistic activation of the AR by HER-2/neu was documented (Fig. 1). This activation resulted in induction of expression of the endogenous androgen-regulated prostate-specific antigen (PSA) gene. The AR is involved in the regulation of molecules that govern cell cycle progression, such as cyclin-dependent kinases. Enhanced AR activity induced by growth factors and HER-2/neu might therefore facilitate tumor progression. Interestingly, the nonsteroidal anti-androgens hydroxyflutamide and bicalutamide were less efficient at blocking AR activation by HER-2/neu than at blocking AR activation induced by androgenic hormones. A substantial reduction of the ligand-independent effect was achieved with an inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway and MAPK phosphatase. The effect of HER-2/neu on

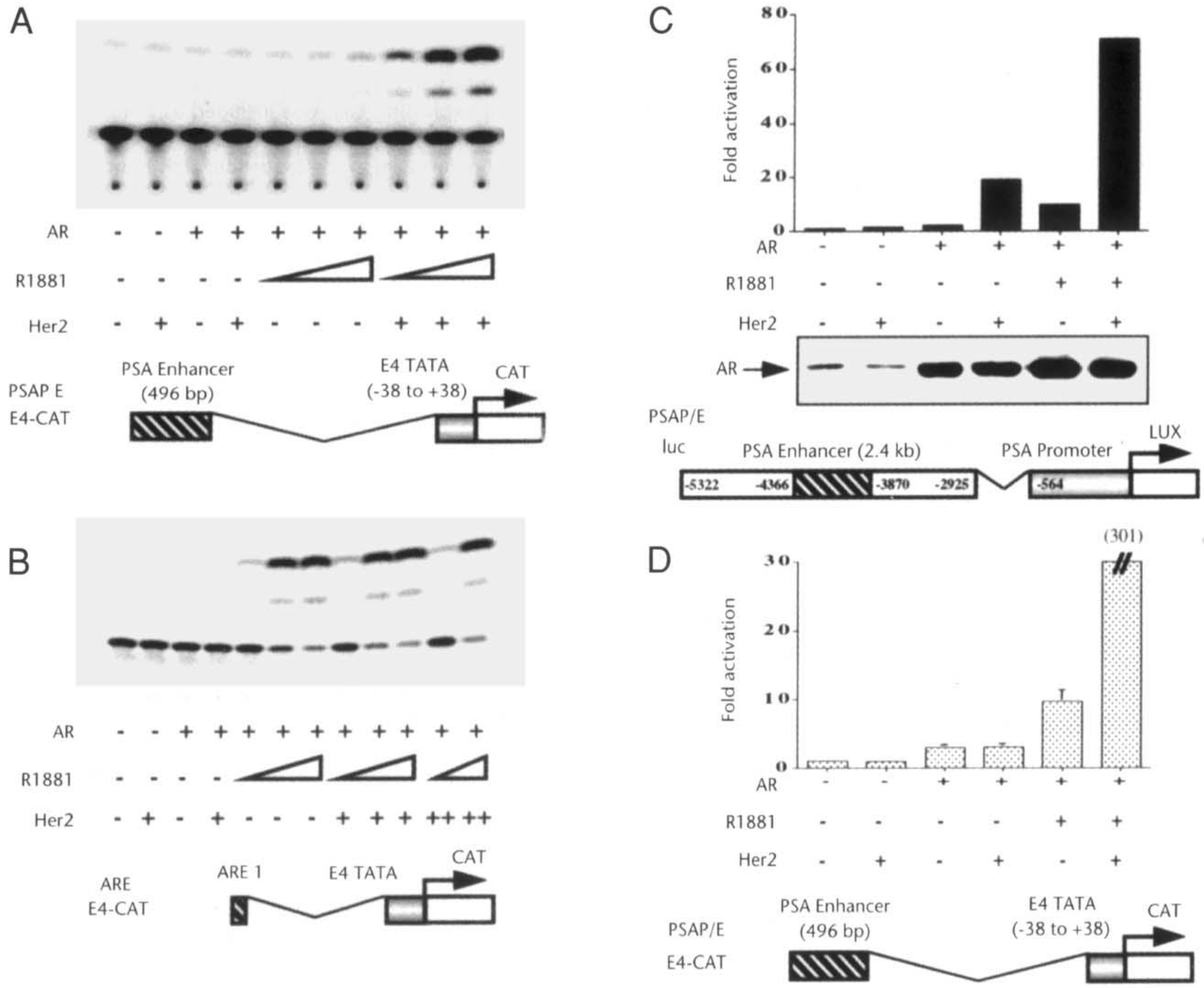

**FIGURE 1** Effects of HER-2/neu on androgen receptor function. (A) TS-13 hamster kidney epithelial cells were transfected with PSA P/E luc in conjunction with plasmids expressing androgen receptor (AR) and/or HER-2 in various combinations and in the presence or absence of 1.0 nM R1881 (+ or −). Expression of AR was measured by immunoblot analysis of whole-cell lysates. Luciferase results are expressed as the fold activation relative to TS13 cells transfected with PSA P/E luc in the absence of AR, HER-2/neu, and R1881, which was designated as onefold. (B) Similarly, TS-13 cells were transfected with the PSA E E4-CAT reporter construct in the presence or absence of AR, HER-2/neu, and/or 1.0 nM R1881. CAT results were analyzed by thin-layer chromatography and quantitated using a phosphorimager. Data are expressed as the fold activation relative to TS13 cells transfected with PSA E E4-CAT in the absence of AR, HER-2/neu, and R1881, which was designated as onefold, and represent the mean of three independent experiments. (C) Dose response of the PSA E E4-CAT reporter to R1881, generated by transfection of TS13 cells with AR [HER-2/neu with R1881 at concentrations of 0.1, 0.3, and 1.0 nM (wedges)]. CAT activity was measured by thin-layer chromatography. At higher doses of R1881, the PSA E E4-CAT construct was maximally activated and no additional effect of HER-2/neu was observed (data not shown). (D) Dose response of a reporter containing a single androgen-response element (ARE E4-CAT) to R1881 with AR [HER-2/neu with R1881 at concentrations of 0.01, 0.1, and 1.0 nM (wedges)]. CAT activity was measured by thin-layer chromatography. The two rightmost lanes contain higher doses of HER-2/neu plasmid (++). Reprinted from Craft *et al.*, 1999, *Nat. Med.* 5, 280–285, with permission.

AR function was also seen in LNCaP cells in which AR protein levels did not change following transfection. The HER-2/neu effect on the AR requires functionality of the phosphatidylinositol (PI) 3-kinase pathway. The AR is phosphorylated at serine residues 213 and 791 by Akt, which is downstream of PI 3-kinase. Consistent with these observations, constitutively active Akt stimulated an androgen-inducible reporter, and a dominant-negative mutant abolished the effect of HER-2/neu. Another example of the inability of AR antagonists to reduce nonsteroidal regulation of AR-mediated gene expression is activation by the protein kinase C pathway inducer, phorbol ester. The outcome of experiments to investigate nonsteroidal activation is cell type-dependent. In CV-1 and HeLa cells, mainly androgen-dependent effects of growth factors on AR transcriptional activity were noted.

## III. ANDROGEN RECEPTOR ACTIVATION AND PROTEIN KINASE A SIGNALING PATHWAY

In addition to being influenced by growth factors, prostate cell growth is affected by peptide hormones and neurotransmitters that increase intracellular levels of cAMP and activate protein kinase A (PKA). AR activation by forskolin (which activates adenylylcyclase to synthesize cAMP and stimulates PKA activity) in monkey kidney and prostate cancer cell lines that transiently expressed the AR was investigated. It was confirmed that the AR could be activated in a ligand-independent manner and that AR antagonists block the activity in these circumstances. Forskolin was maximally efficient at a concentration of 1 μM. In the same series of experiments, forskolin showed a synergistic rather than a ligand-independent effect with the human progesterone receptor, thus pointing to specificity of action. In transfected cells, there were no changes in AR expression following forskolin treatment. AR mutants that lack the DNA-binding domain could not be activated by forskolin, and there is also evidence that residues of the N-terminal region of the AR are needed for AR activation through the PKA pathway. The extent of ligand-independent activation of the AR depends on cell type, promoter, presence of an endogenous or a transfected AR, and the use of a particular inducer of PKA activity. Forskolin was more efficient with reporter constructs that consisted of natural probasin and PSA promoters coupled to the luciferase gene than with the reporter that was driven by a promoter consisting of three androgen-response elements (Fig. 2). It is clear that the PKA pathway has an important role in the regulation of function of the AR since forskolin also up-regulated PSA secretion in prostate explants. From experiments with the PKA inhibitor PKI, it became obvious that the PKA signal transduction cascade is also implicated in receptor activation by ligand. Administration of the inhibitor partially reduced androgen-induced AR activity and similar findings were reported for other steroid receptors.

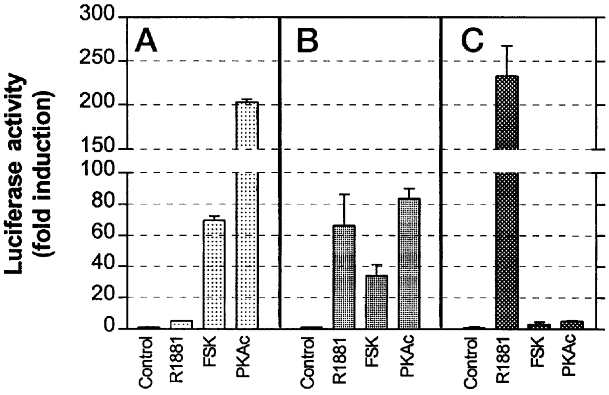

FIGURE 2 Induction of the activities of androgen-responsive promoters by forskolin and PKA. LNCaP cells were transfected with 1.0 μg of PSA (−630 to +12) (A), PB (B), or ARR$_3$-tk-luciferase reporter constructs (C) and either with or without the expression vector for the catalytic subunit of PKA (PKAc, 0.5 μg). Cells were incubated with forskolin (FSK, 50 μM), R1881 (10 nM), or vehicle (Me$_2$SO, 0.5%) for 48 h under serum-free conditions and harvested, and luciferase activities were measured. The normalized luciferase activities were divided by the normalized activity of cells transfected with reporter plasmid and with empty PKAc plasmid to give the fold induction. Reprinted from Sadar, 1999, *J. Biol. Chem.* **274**, 7777–7783, with permission.

## IV. REGULATION OF ANDROGEN RECEPTOR ACTIVITY BY PLEIOTROPIC COMPOUNDS

Luteinizing hormone-releasing hormone (LHRH) analogues are used for prostate cancer therapy because their systemic application leads to long-term suppression of serum androgen levels. Prostate cancer cells express LHRH receptors and their growth is differentially affected by LHRH. Modest AR activation by LHRH analogues was observed in transfected cells, and these compounds reduced the concentration of androgen needed for maximal activation of the AR.

There is an increasing interest in AR activation by interleukin-6 (IL-6). IL-6 is a multifunctional cytokine that is involved in the regulation of immune and inflammatory responses and causes direct effects in target tissues. In prostate cells, IL-6 either induces G1 growth arrest or acts as an anti-apoptotic factor. IL-6 is of special interest in human prostate cancer because its serum levels increase in patients with metastatic disease. IL-6 ligand-independent and synergistic activation of the AR was demonstrated both in cells that transiently express the AR and in LNCaP cells in which expression of the PSA gene was up-regulated in response to IL-6. LNCaP cells were growth-inhibited by IL-6 and it was concluded that IL-6-induced activation of the AR is important for the maintenance of prostatic differentiation function. There is no evidence of changes in AR expression by a nonsteroidal compound in any cell line transiently transfected with AR cDNA. In contrast, IL-6 increases the activity of the endogenous AR gene promoter, AR mRNA, and protein in LNCaP cells. Thus, in that cell line, IL-6-induced AR activity is associated with increased protein expression. In concordance with these observations, nuclear localization of the AR was enhanced by IL-6. The AR nonsteroidal antagonists hydroxyflutamide and bicalutamide nearly completely blocked AR activation. The two major signaling pathways of IL-6 in target cells are those of Janus kinases, which in turn lead to nuclear translocation and activation of signal transducers and activators of transcription (STAT) and MAPK. Inhibitors of these pathways as well as those of PKA and PKC down-regulate the effect of IL-6 on the AR. In contrast, the inhibitor of the PI 3-kinase pathway, wortmannin, was ineffective (Fig. 3).

The presence of IL-6 and its receptor was investigated in prostate cells by immunohistochemistry. It was revealed that, in benign prostate tissue, IL-6 expression is confined to basal cells, whereas tumor cells themselves acquire the ability to produce IL-6. Prostate stromal cells secrete large amounts of IL-6 into their supernatants. The IL-6 receptor, which consists of the ligand-binding subunit gp 80 and the signal-transducing subunit gp 130, is present in benign and malignant prostate tissues. IL-6 binding to the receptor activates intracellular signaling by protein kinase pathways that leads to the activation of the AR. Because of the presence of IL-6 and the AR in prostate tissues, one can conclude that the cross talk between IL-6 and the AR is significant for numerous cellular events. The crosstalk between androgen and the IL-6 signaling cascade is bidirectional; androgenic hormones enhance activation of STAT3 and expression of classic IL-6-target genes. Endogenous protein inhibitors of activated STAT3 down-regulate AR activity induced by IL-6.

## V. WIDE SPECTRUM OF ANDROGEN RECEPTOR ACTIVATORS

One of the experimental therapies for prostate cancer is application of the differentiation agent phenylbutyrate. The butyrate derivative increases PSA gene expression by activation of the AR and induces androgen-inducible promoters to various extents. The binding of the AR to DNA-response elements is enhanced by differentiation-promoting compounds. Butyrate does not change total AR levels but does increase nuclear AR.

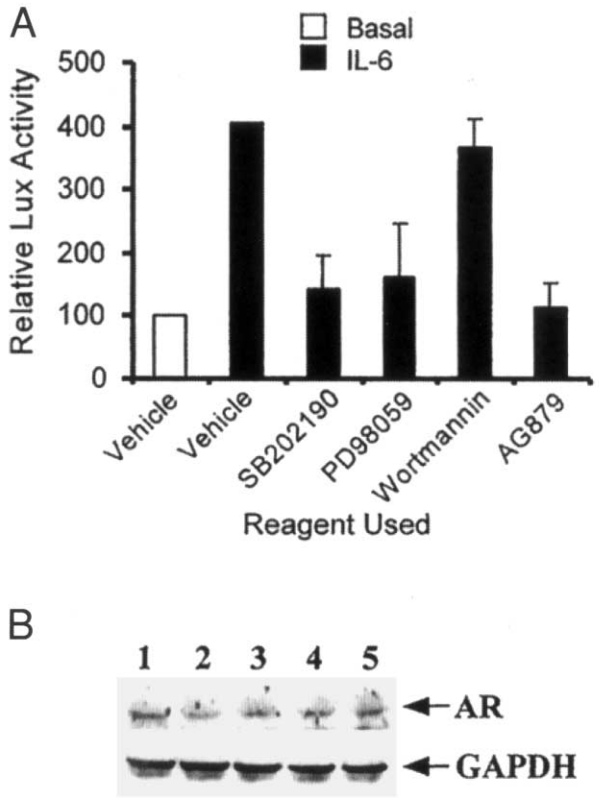

FIGURE 3 PSA promoter induction by IL-6 was blocked by specific inhibitors to signal transducers. (A) LNCaP cells were co-transfected with pRL-CMV (for normalization using the dual lux assay) and p1.5kPSA-Lux. The cells were then incubated with vehicle, SB202190 (5 μM), PD 98059 (15 μM), wortmannin (100 nM), or tyrphostin AG879 (10 μM) for 30 min prior to IL-6 administration (10 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (± SE) lux activity relative to the basal activity, which was set as 100%, of three independent experiments. (B) The immunoblot of AR and GAPDH from 40 μg of cell lysate. LNCaP cells were cultured in medium containing IL-6 (lane 1) or IL-6 plus pretreatment of the inhibitor reagents described above (lanes 2–5, respectively). Reprinted from Lin *et al.*, 2001, *Clin. Cancer Res.* 7, 1773–1781, with permission.

The level of AR ligand-dependent activation is also enhanced by caveolin, which is implicated in the development of therapy-resistant prostate disease. AR co-immunoprecipitates with caveolin through interactions between the ligand-binding domain of the AR and the $NH_2$-terminal domain of caveolin. Examples of nonsteroidal activation of the AR also include cadmium; the thyroid gland hormone T3, which shows a co-stimulatory effect on PSA with androgen; MAPK kinase kinase I, whose overexpression induces apoptosis; and β-catenin.

In conclusion, ligand-independent and/or synergistic activation of the AR by cellular regulators is important in advanced prostate cancer and it offers an explanation of why androgen-responsive genes are up-regulated in late stages of the disease and in respective xenograft models. A better understanding of the mechanisms underlying activation of the AR in the absence of ligand or synergistic regulation of AR activity is crucial for the development of novel endocrine therapies.

## Glossary

**androgen receptor** Nuclear transcription factor that regulates expression of androgen-inducible genes.

**caveolin** The principal protein component of caveolar membranes.

**growth factors** Polypeptide hormones that regulate growth in an autocrine and paracrine manner.

**IGF-I, IGF-II** Growth factors with structures similar to that of insulin.

**interleukin-6** Proinflammatory cytokine that either inhibits proliferation or suppresses apoptosis in target tissues.

**phenylbutyrate** Butyrate derivative used in differentiation therapy.

**protein kinase** Enzymes that catalyze protein phosphorylation.

**xenograft** Tumor maintained in host animals.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor-Related Pathology • Androgen Receptors and Prostate Cancer • Androgen Receptor Structure and Function • Androgens: Pharmacological Use and Abuse • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Insulin-like Growth Factor (Igf) Signaling • Interleukin-6 • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Androgen Receptor Structure and Function

ELIZABETH M. WILSON
*University of North Carolina, Chapel Hill*

I. BIOLOGICALLY ACTIVE ANDROGENS
II. MECHANISMS OF ANDROGEN REGULATION OF GENE EXPRESSION
III. DOMAIN STRUCTURE AND INTERACTIONS OF THE ANDROGEN RECEPTOR
IV. ANDROGEN RECEPTOR MUTATIONS ASSOCIATED WITH DISEASE
V. ANDROGEN RECEPTOR ANTAGONISTS AND AGONISTS IN THE ENVIRONMENT
VI. SUMMARY

**The androgen receptor (AR) is a ligand-activated transcription factor that binds with high affinity to the biologically active androgens, testosterone and dihydrotestosterone. Androgen binding targets the AR to the nucleus, where it binds to specific androgen-response element DNA sequences associated with promoter or intron sequences of androgen-regulated genes. AR-mediated gene activation is required for male sexual development during embryogenesis and sexual maturation at puberty. The domain structure of the AR parallels that of other steroid receptors. An important aspect of AR structure and function is an androgen-specific interaction between the $NH_2$-terminal and carboxyl-terminal regions that contributes to stabilization of the AR. Genetic defects in the AR gene in humans prevent normal male sexual development or cause adult onset muscular atrophy. Androgens are required in prostate development and growth and may contribute to prostate cancer proliferation.**

## I. BIOLOGICALLY ACTIVE ANDROGENS

In the male, testosterone is produced in Leydig cells of the testis in response to placental chorionic gonadotropin during male sexual development of the fetus. The testosterone concentration in circulating blood increases again at the time of puberty under stimulation by pituitary gonadotropin (luteinizing hormone or interstitial cell-stimulating hormone) and remains at relatively high levels until a decline later in life. Testosterone is synthesized by a series of cytochrome P450 enzymes, some of which are also used in the biosynthetic pathways for steroid hormones. Testosterone is the major circulating androgen, the majority of which is bound to serum-binding proteins. Only approximately 1% of the total testosterone is free and thus available to enter cells by diffusion. Most cells are exposed to similar levels of circulating androgen, an exception being the testis, where levels are high due to testosterone production by the Leydig cells. Whether a particular cell responds to testosterone or dihydrotestosterone (DHT) depends on the androgen concentration in the cell and on the presence of the androgen receptor (AR).

In some tissues that are highly responsive to androgen stimulation, such as the prostate and epididymis, testosterone is bioactivated to 5α-DHT by the cytochrome P450 enzyme 5α-reductase type 2. DHT is a more active androgen than testosterone in part because it dissociates more slowly from the AR. That testosterone cannot replace all of the functions of DHT is dramatically revealed in the 5α-reductase syndrome. In this genetic disorder, a mutation in the 5α-reductase gene results in a deficiency in the conversion of testosterone to DHT during embryogenesis. 46,XY genetic males with the 5α-reductase syndrome are born with incomplete development of the male genitalia due to reduced circulating levels of DHT. Remarkably, at puberty, increases in testosterone synthesis by the testes and an increase in the activity of the 5α-reductase type 1 overcome, to a certain extent, the DHT deficiency, resulting in virilization of the male.

A secondary source of androgen in the male is the adrenal gland, but this is not considered a highly significant contribution to overall androgen levels under normal physiological conditions. In the female, by contrast, adrenal androgens contribute a significant portion of circulating androgen levels, which are low compared to those of the male under normal conditions, minimizing the induction of androgen-responsive genes in the female. Adrenal androgens, such as androstenedione, are produced by the same steroidogenic enzyme pathways that function in the testis. Adrenal androgens serve as precursors for testosterone production.

Certain endocrine conditions associated with excess adrenal androgen production are problematic in the female. Congenital adrenal hyperplasia results from a genetic defect in an enzyme in the pathway to hydrocortisone (cortisol) biosynthesis. The most frequently mutated gene that causes congenital adrenal hyperplasia is the 21-hydroxylase gene. This enzyme defect reduces the synthesis of cortisol, which results in the loss of negative feedback to the hypothalamus and anterior pituitary. The consequence is an increase in the secretion of adrenocorticotropic hormone by the anterior pituitary and the subsequent overstimulation of the adrenal glands. This leads to accumulation of 17-hydroxyprogesterone, which results in increased conversion to androstenedione and testosterone in the adrenal gland with increased circulating testosterone. During embryonic development, congenital adrenal hyperplasia causes differing degrees of virilization (development of male external genitalia) in the female fetus and increased virilization in affected males.

## II. MECHANISMS OF ANDROGEN REGULATION OF GENE EXPRESSION

Under normal physiological conditions, the AR is bound to a biologically active androgen, testosterone or DHT, which induces the AR to translocate to the nucleus. In the nucleus, the AR binds as a homodimer to specific DNA sequences known as androgen-response elements. Thus, the AR is a ligand-inducible transcription factor. The androgen-bound AR complex recruits associated co-regulatory proteins that act during the process of chromatin modification necessary for gene activation. Many associated proteins have been reported to have a role in AR-mediated gene activation. These proteins include the family of p160 co-activators, named for their approximate size of 160 kDa and for their capacity to increase the transcriptional response to ligand-activated steroid receptors as measured in transcription assays. The p160 group of transcriptional co-activators include steroid receptor co-activator 1 (SRC1), transcriptional intermediary factor 2 (TIF2, SRC2, GRIP1), and the SRC3/TRAM1/AIB1/pCIP/ACTR/RAC3 group of co-activators. Many of these are associated with histone acetyl transferase activity and can recruit CBP, pCAF, and other co-regulatory proteins required for chromatin modification and gene activation.

The use of two-hybrid screening methods in yeast expression systems has revealed many proteins in addition to the p160 co-activators that associate with the AR and are reported to increase AR-mediated transcriptional activation. The extent of increased gene activation is typically measured using transient transfection DNA transfer methods in cultured cell lines. Plasmid DNA transfected into cells includes expression vectors containing the DNA coding sequence for the AR or other steroid receptors and a reporter plasmid. Reporter plasmids contain a responsive DNA element (DNA sequence that binds the nuclear receptor) linked to a gene such as luciferase or chloramphenicol acetyltransferase, whose activity can be quantitated in *in vitro* assays. Co-transfection of expression plasmids for the interacting proteins is used to determine their effects on ligand-induced receptor regulation of gene transcription.

All but a few tissues in the body express the AR. However, the levels of AR expression differ among tissues. The highest levels are observed in androgen-responsive tissues, such as prostate, seminal vesicle, epididymis, testis, and the genitalia. High AR levels are also reported in the ovary; however, the role of androgen in female reproduction remains to be clearly defined. Genes that are activated by the AR contain an androgen-response element sequence in the promoter or intron region that allows for selective binding of AR to the DNA. In addition, there have been reports of nongenomic effects of the AR; however, it remains to be determined by what mechanisms these effects are mediated.

## III. DOMAIN STRUCTURE AND INTERACTIONS OF THE ANDROGEN RECEPTOR

The AR is encoded on the X chromosome. Thus, 46,XY genetic males have only one allele for the AR gene. 46,XX genetic females have two X chromosomes but also express only one allele due to the random inactivation of the X chromosome. There appears to be a single AR gene and only one form of the AR. This contrasts with other steroid receptors for which there are multiple genes and forms. For example, estrogen receptor α and β are encoded by different but related genes. The A and B forms of the progesterone receptor are synthesized from alternative transcripts and have different cellular effects. Recent evidence supports the existence of only one human AR gene and only the full-length form of the human AR in cells. In some fish, however, there is evidence for more than one form of the AR.

The AR shares with other steroid and nuclear receptors a common overall structural arrangement of its functional domains (Fig. 1). A striking aspect of these domains is a relatively high degree of functional independence. Each of the domains can be expressed and shows its respective activity to a significant degree. However, as described below, important interactions that have an impact on AR function occur among the domains.

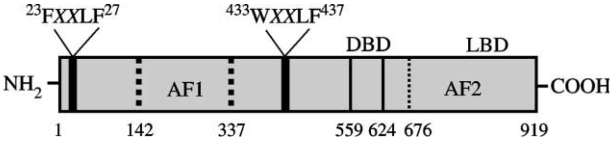

**FIGURE 1** Diagram of the structural domains of the human androgen receptor. Shown are activation functions 1 (AF1) and 2 (AF2), the DNA-binding domain (DBD), and the ligand-binding domain (LBD). Also shown are the N/C interaction motifs *FXX*LF and *WXX*LF. Reprinted from He et al. (2001), with permission.

## A. NH$_2$-Terminal Region

The NH$_2$-terminal region is the largest domain in the AR, making up about 60% of the receptor protein. This contrasts with other nuclear and steroid receptors in which the NH$_2$-terminal region may be short. But like the AR, the glucocorticoid, progesterone, and mineralocorticoid receptors have a similarly large NH$_2$-terminal region. The AR NH$_2$-terminal region is unusual because it contains several repeated sequences, the most striking being the glutamine and glycine repeats. The glutamine repeat is encoded by a CAG trinucleotide repeat whose length is polymorphic in the normal population, ranging from 13 to 35 glutamine residues. An increase beyond 36 glutamine residues is associated with adult onset muscular atrophy or Kennedy's disease (see Section IV.B). The glycine repeat is usually 23 residues in length and is less polymorphic than the glutamine repeat. Significant expansion or contraction of the glycine repeat has not been associated with a disease. A short 8-residue proline repeat, the function of which is not known, also occurs in the NH$_2$-terminal region of the AR.

The NH$_2$-terminal region is the least conserved domain of the ARs of different species and is the least conserved domain among different steroid receptors. Homology comparisons of the amino acid sequence of the NH$_2$-terminal region in primates indicates 92% similarity between human and the prosimian lemur AR amino acid sequence. Lemurs arose during primate evolution from the lower mammals. The glutamine repeat at this position in the NH$_2$-terminal domain expanded throughout primate evolution, increasing from 4 CAG repeats in lemur AR to its present average length of 22 CAG repeats coding for the glutamine repeat region in human AR. In lower mammals, such as rat and dog, a variable-length glutamine repeat also occurs, but remarkably, this glutamine repeat, also encoded by a CAG repeat, is in a different position within the NH$_2$-terminal region. One aspect of the NH$_2$-terminal region that may allow these amino acid repeat expansions to occur without severe disruption of AR function could be a lack of strict structural constraints in the region. Binding of transcription factors to the NH$_2$-terminal region may impose a more structured arrangement. The allowable flexibility in length and position of the glutamine repeat relative to AR function suggests that the NH$_2$-terminal region of AR is tolerant of a significant amount of alteration in protein structure. This contrasts with the very strict structural requirements for the DNA- and ligand-binding domains.

The NH$_2$-terminal region is nevertheless critical for AR function because it contains the major transactivation region referred to as the activation function 1 (AF1) region. Deletion of the NH$_2$-terminal region results in the loss of its major transcriptional activation domain. The NH$_2$-terminal region likely acts to recruit specific transcription factors, some of which have been reported. The AF1 region is located between amino acid residues 142 and 337 in the 1- to 558-residue NH$_2$-terminal region (Fig. 1). Although it is not well defined, the NH$_2$-terminal AF1 region requires androgen binding for transcriptional activity and appears to be critical for AR-mediated gene activation.

## B. DNA-Binding Domain and Hinge Region

The NH$_2$-terminal region is contiguous with the centrally positioned DNA-binding domain that allows for specific binding to androgen-response DNA elements present in androgen-regulated genes (Fig. 1). The AR DNA-binding domain is composed of a two zinc-finger structure where two zinc atoms are held in position by tetrahedral coordination with highly conserved cysteine residues. The zinc-finger structure of the DNA-binding domain is characteristic of all steroid and nuclear receptors. The DNA-binding domain and flanking sequence can be expressed as an isolated domain that binds DNA. A region of genomic DNA associated with androgen-regulated genes that interacts with the AR DNA-binding domain is referred to as an androgen-response element, or hormone-response element. Androgen-response elements vary in sequence, but for many genes, they adhere to the DNA consensus sequence 5′-GGTACAnnnTGTTCT-3′, where n represents an unspecified spacer nucleotide.

A short hinge region at amino acid residues 624–676 (Fig. 1) is positioned between the AR DNA-binding domain and the carboxyl-terminal region. Within the hinge region is the AR nuclear-targeting signal that triggers the subcellular transport of AR from the cytoplasm to the nucleus. Androgen binding to the AR activates this bipartite targeting sequence, which is composed of two closely spaced clusters of the basic amino acids lysine and arginine. Mutation of these basic residues inhibits androgen-induced nuclear translocation of the AR.

## C. Ligand-Binding Domain

The carboxyl-terminal ligand-binding domain at amino acid residues 676–919 (Fig. 1) is approximately one-fourth the size of the AR protein and constitutes the high-affinity hormone-binding site. The ligand-binding region of AR and that of several other nuclear receptors have been crystallized in the presence of bound hormone. The carboxyl-terminal ligand-binding domain forms a series of alpha-helical folds that create a hydrophobic cavity for hormone binding. The size of the binding cavity varies among the steroid receptors, with the smaller binding cavities showing a higher degree of hormone specificity. The ligand-binding domain of the AR can function as an isolated domain with apparent high-affinity androgen binding when the recombinant protein is expressed in the absence of the $NH_2$-terminal region. However, as described below, the $NH_2$-terminal region influences the kinetics of androgen binding.

Steroid hormone binding in the hydrophobic pocket of the ligand-binding domain is relatively specific for the ligand. For example, the AR shows highest binding affinity and specificity for the biologically active androgens testosterone and DHT. However, AR and other steroid receptors also bind additional ligands, but with lower affinity. For example, the mineralocorticoid and glucocorticoid receptors bind each other's preferred hormone, aldosterone and hydrocortisone, with similar affinity. A variety of mechanisms including receptor concentration and selective steroid metabolism contribute to facilitating a specific hormone response. In the case of the mineralocorticoid receptor, there are enzymes in certain tissues, such as the placenta and kidney, that facilitate the rapid metabolism of cortisol to its inactive form, cortisone, thereby allowing the mineralocorticoid receptor to bind aldosterone, the active mineralocorticoid. The AR can bind other hormones and ligands depending on their concentration.

Within the carboxyl-terminal region is an activation region referred to as activation function 2 (AF2; Fig. 1). AF2 forms a hydrophobic binding site for the L*XX*LL motifs of the p160 transcriptional co-activators, where L refers to leucine and *X* is any amino acid. The AF2 region of the AR appears to have a lower binding affinity for the L*XX*LL motifs of the p160 co-activators than do the other nuclear receptors when expressed as a DNA- and ligand-binding domain fragment. It is therefore not clear to what extent the AF2 region in the AR ligand-binding domain contributes to AR transcriptional activity *in vivo*. A mutation at lysine residue 720 in the AR AF2 region of the ligand-binding domain decreased the interaction with p160 co-activators but did not significantly alter AR transactivation when determined in a transient transfection assay, where recombinant AR was expressed in cultured cells in the presence of a reporter vector.

## D. Interaction Between $NH_2$- and Carboxyl-Terminal Regions

As indicated above, two regions of the AR appear to be involved in androgen-induced transactivation of genes, AF1 in the $NH_2$-terminal region and AF2 in the ligand-binding domain. In comparison to other steroid receptors, AF2 in AR is weak, apparently reflecting a reduced ability to recruit p160 co-activators through their L*XX*LL motifs. In addition, inhibition of p160 co-activator binding results from a strong androgen-induced $NH_2$- and carboxyl-terminal (N/C) interaction between an F*XX*LF motif in the $NH_2$-terminus at amino acid residues 23–27 (Fig. 1) and the carboxyl-terminal AF2 hydrophobic binding surface. A second AR $NH_2$-terminal N/C interaction site, W*XX*LF at amino acid residues 433–437, also interacts with AF2 in the ligand-binding domain (Fig. 1).

Androgen dissociation studies provided the first evidence that the $NH_2$-terminal region of the AR interacts with the ligand-binding domain. Deletion of the $NH_2$-terminal domain increases the rate at which bound androgen dissociates, whereas the apparent equilibrium binding affinity is not altered, indicating that the association rate is increased. These studies were followed with a two-hybrid interaction assay that supported a direct interaction between these regions. More recently, F*XX*LF and W*XX*LF motifs in the $NH_2$-terminal region that interact in the presence of testosterone or DHT with the AF2 region in the ligand-binding domain were identified. Recent studies suggest that the N/C interaction stabilizes the AR protein against degradation and interferes with the binding of p160 co-activators to the AF2 region. The F*XX*LF motif appears to have a higher affinity for the AF2 region than do the L*XX*LL motif sequences of the p160 co-activators. A major effect of the androgen-dependent F*XX*LF/W*XX*LF interaction with the ligand-binding domain is to slow the dissociation rate of bound androgen and to stabilize the AR against degradation.

## IV. ANDROGEN RECEPTOR MUTATIONS ASSOCIATED WITH DISEASE

### A. Androgen Insensitivity Syndrome

Steroid receptor-regulated gene transcription is required for cell proliferation, differentiation, and development and involves the formation of active transcription initiation complexes composed of a specific steroid receptor and a host of co-activators. The essential role of the AR in male sex differentiation is well established. Naturally occurring AR gene mutations in 46,XY genetic males result in a phenotypic spectrum known as the androgen insensitivity syndrome, characterized by incomplete male sexual development *in utero*. Because the AR gene is located on the X chromosome (Xq11–q12), there is only one allele in the male. Thus, mutations that abolish AR function can have a dramatic effect on the response to androgen. Androgen insensitivity is an end-organ defect because the individual cannot respond to the high circulating androgen levels due to a genetic defect in the AR protein.

The severity of the defect in male sexual development reflects the importance of specific amino acid residues in AR function. Partial or total loss of AR function can affect any one of a number of functional properties of the AR including high-affinity androgen binding, DNA binding, and AR stability. Depending on the position and type of the substituted amino acid, the degree of phenotypic expression varies. Mutations causing partial androgen insensitivity can alter steroid-binding affinity and specificity. Mutations that severely disrupt androgen- or DNA-binding affinity result in the complete form of the syndrome. This is characterized by a female genital phenotype in a 46,XY genetic male. These individuals lack a uterus and have abdominal testes that produce normal to elevated male levels of testosterone. The majority of AR gene mutations are single-base missense mutations in the DNA-binding or carboxyl-terminal regions (exons 2–8) with more than 200 unique mutations reported. In some cases, there is partial or complete deletion of the AR gene.

Most of the naturally occurring AR gene mutations are in the DNA- or ligand-binding domain, with a surprising absence of missense mutations in the $NH_2$-terminal region. The genetic errors include missense mutations where a new amino acid residue is substituted or nonsense mutations where a premature termination codon is introduced. Many of the premature mutant stop codons occur within the $NH_2$-terminal region. A compilation of the more than 200 different AR mutations that cause partial or complete androgen insensitivity is available on the Internet at http://www.mcgill.ca/androgendb/. The frequency of AR gene mutations in the human population is about 1 in 20,000 individuals. Females can be carriers of an AR gene mutation without significant effects to themselves probably because of the presence of one normal allele for the AR gene and random inactivation of the X chromosome.

Partial forms of the androgen insensitivity syndrome occur in a spectrum of forms, ranging from the mildest form, which causes male infertility in the adult, to a more severe form that leads to ambiguous genitalia in the newborn. The presence of the 46,XY male sexual karyotype with partial disruption or complete loss of the male genital phenotype is the hallmark of the androgen insensitivity syndrome. In some cases, phenotypic expression of the same AR gene mutation varies between individuals from different families and among family members, illustrating that other factors contribute to phenotypic expression of a given AR gene mutation. Mutations that significantly decrease androgen- or DNA-binding affinity result in the complete form of the syndrome.

### B. Kennedy's Disease

Neurodegenerative diseases are sometimes caused by trinucleotide repeat expansions. The first trinucleotide repeat expansion discovered was Kennedy's disease or adult onset spinal/bulbar muscular atrophy. Others include Huntington's disease and spinocerebellar ataxia type 1. The diseases are caused by a CAG repeat expansion that codes for glutamine. Differences occur in repeat length, stability, and location of the expansion. Within the $NH_2$-terminal domain of the AR is a CAG repeat that codes for a glutamine repeat that is polymorphic in the normal population, ranging from 13 to 35 glutamine residues in length. Expansion of the AR CAG repeat in the first exon of the coding region of the $NH_2$-terminal domain to greater than 40 Gln residues results in X-linked adult onset spinal/bulbar muscular atrophy or Kennedy's disease. Clinical features of Kennedy's disease are progressive muscle weakness after the age of 25–30 years, muscle atrophy associated with loss of lower motor and primary sensory neurons, and partial androgen insensitivity, evidenced by elevated serum gonadotropin levels, gynecomastia (breast enlargement in the male), testicular atrophy, impotence, and oligo- or azoospermia (low or absent sperm production). Early onset, severity of muscle weakness, and degree of androgen resistance correlate with longer repeats. Instability of triplet repeats associated with human hereditary disease may result from errors in DNA mismatch repair through the formation of single-stranded hairpin structures and DNA polymerase slippage during replication. Polymorphisms of the AR CAG repeat are stable in the normal population and can be useful as genetic markers in clonality analysis and forensic science. How repeat expansions cause adult-onset muscle wasting is being actively investigated; current evidence suggests that the expanded glutamine repeat contributes to protein aggregation in the nucleus.

### C. Prostate Cancer

There are conflicting reports in the literature concerning the incidence of AR gene mutations associated with prostate cancer. Earlier reports suggested frequencies of AR mutations of up to 50% or more in prostate cancer specimens. However, many of these early reports were confounded by the presence of artifactual polymerase chain reaction DNA incorporation errors. The actual frequency of AR mutations in prostate cancer specimens is probably not more than 5% of cases. Additional studies will help to resolve the conflicting reports but the preponderance of recent information supports the view that AR gene mutations are uncommon, particularly in early stage disease, but may become more prevalent in later forms of the disease when overall genetic instability is increased.

Despite their apparent low incidence, it is nevertheless of interest that the AR gene mutations that have been reported in prostate cancer specimens are sometimes associated with a broadened ligand specificity. In this case, the AR responds by increasing gene transcription in response to not only the biologically active androgens, testosterone and DHT, but also to other circulating steroids. For example, an AR T877A mutation in the ligand-binding domain is present in the LNCaP human prostate cancer cell line. An AR H874Y gene mutation is present in a human prostate cancer xenograft. The xenograft is a transplant of a human prostate cancer into an immune-deficient mouse, where it can be propagated for further study. These mutant ARs show a normal transcriptional response to testosterone and DHT but, in addition, have a greater response than wild-type AR to other ligands, such as estradiol, and to AR antagonists, such as hydroxyflutamide. It is not clear to what extent this broadened ligand specificity contributes to the proliferation of prostate cancer in these cases. Activation of the AR by other circulating hormones increases the possibility for stimulation of tumor growth in the absence of the usual stimulating androgens, testosterone and DHT. This may be particularly critical in late stage prostate cancer where patients have undergone androgen deprivation therapy by castration or chemical treatment. Under these conditions of low androgen levels, other circulating ligands, such as the adrenal androgen dehydroepiandrosterone, might gain the ability to act as a more active androgen and promote the growth of late stage cancer in the androgen-deprived patient.

More recently, it was discovered that certain of the p160 co-activators are highly expressed in the majority of prostate cancer samples analyzed. These highly expressed p160 co-activators include SRC1 and TIF2, whereas AIB1 (amplified in breast cancer 1) was not detected. p160 co-activator expression was low to undetectable in benign prostatic hyperplasia, which is a common form of enlarged prostate in the aging male. It is conceivable that high-level expression of co-activators offers a mechanism for AR activation through other ligands including the adrenal androgens or by ligand-independent mechanisms.

## V. ANDROGEN RECEPTOR ANTAGONISTS AND AGONISTS IN THE ENVIRONMENT

Over the past several years, certain environmental chemicals used as pesticides or herbicides in crop protection have been identified as precursors to chemicals with endocrine disruptor activity involving the AR. A number of environmental chemicals were previously established to have estrogenic activity, but until recently, these activities related only to the estrogen receptor. At least five or more chemicals previously or currently in use in the United States were found to be AR antagonists. An antagonist is a chemical or pharmaceutical ligand that competes for hormone binding and fails to induce receptor-mediated activation of gene transcription. Antagonist activities are detected using *in vitro* assays to demonstrate inhibition of androgen-induced AR transcription activity and inhibition of DNA binding. Thus far, it has been the *in vivo* metabolites of the parent chemicals that interfere with androgen binding to the AR, resulting in antagonist activity. The apparent equilibrium binding affinity of a pesticide or herbicide metabolite is in the micromolar range as opposed to nanomolar binding affinity of androgens for the AR. Like androgens and other antagonists, these chemicals bind to the ligand-binding domain in the carboxyl-terminal region of the AR and block binding of androgen. Binding of these chemicals does not elicit conformational changes in the AR, such as those required for the N/C interaction, and thus these chemicals are ineffective in promoting agonist-related effects on activation of gene transcription.

It is not known whether there is sufficient exposure from pesticide or fungicide residues on fruits and vegetables or in farming practices to elicit AR antagonist activity. The micromolar binding affinity indicates that relatively high levels of pesticide exposure would be required to disrupt androgen-induced effects. Of particular potential concern, however, is the developing male fetus, which depends on the action of androgens produced from the fetal testes during embryonic development for sexual differentiation and development of the external genitalia. Exposure of the pregnant mother to sufficient levels of chemicals with AR antagonist activity during the critical period of male sexual development could possibly inhibit development, resulting in incomplete formation of the external genitalia. In some countries, an increasing incidence of incomplete masculinization has been observed, characterized by the hypospadias deformity, in which the urethra opens on the shaft or base rather than at the tip of the penis. It is not known to what extent this relatively common form of incomplete masculinization is attributable to endocrine disruptor activity. This type of defect can be repaired by surgery, resulting in full male sexual function. However, it is not known whether other anti-androgen effects could occur in the brain to interfere with androgen imprinting or in other organs of the developing male.

Recent evidence suggests, in addition, the presence of environmental androgens. Female Eastern mosquitofish, *Gambusia holbrooki*, present in a Florida river downstream of a paper mill effluent discharge were uniformly masculinized as evidenced by the presence of male-like elongated anal fins. Androstenedione, a known precursor of testosterone, was confirmed by liquid chromatography mass spectrometry to be a component of the river water. Androstenedione was present in the river at a concentration of 0.14 nM. Phytosterols derived from paper pulp, such as β-sitosterol, campesterol, and stigmastanol, can be modified to androgen precursors by the bacteria *Mycobacterium smegmatis* or *Escherichia coli*, which have been shown to produce androstenedione from steroid precursors. Conversion of plant sterols by bacterial metabolism provides a likely source for androstenedione in the contaminated Florida river.

## VI. SUMMARY

The androgen receptor is a ligand-activated transcription factor that binds testosterone or dihydrotestosterone. The androgen-activated AR binds to DNA sequences known as androgen-response elements to regulate gene transcription. The AR has a domain structure characteristic of the nuclear receptor family of transcription factors, with an $NH_2$-terminal transactivation domain, a DNA-binding domain, and a carboxyl-terminal ligand-binding domain. An unusual property of the AR is an androgen-induced interdomain $NH_2$- and carboxyl-terminal interaction that stabilizes the AR against degradation and inhibits p160 co-activator activation of AF2 in the ligand-binding domain. The AR gene is on the X chromosome. In 46,XY genetic males, a single-nucleotide base change causing a missense amino acid mutation can inhibit normal male sexual development, resulting in the androgen insensitivity syndrome. Other diseases related to the AR are Kennedy's disease and prostate cancer. Finally, metabolites of certain chemicals used as pesticides and herbicides have anti-androgen activity that could inhibit sexual development of the male fetus. On the other hand, androgens in the environment have been reported to masculinize entire populations of fish. Thus, the AR is a critical gene regulatory protein. Loss or gain of AR function through genetic mutation or environmental inhibition or activation can have a wide range of adverse effects on sexual development.

## Glossary

**activation function regions** Segments in a steroid receptor that mediate transcriptional activation through their interaction with co-activators or proteins of the basic transcriptional machinery of the cell.

**androgen insensitivity syndrome** 46,XY genetic males with an androgen receptor gene mutation that results in infertility or partial or complete lack of masculinization of the genitalia. The androgen insensitivity syndrome has also been referred to as testicular feminization.

**androgen receptor** A transcription factor that is activated by binding a biologically active androgen, testosterone or dihydrotestosterone; it is required for induction of gene transcription in response to androgen.

**hormone-response element** Segment of DNA sequence that binds to the DNA-binding domain of a steroid receptor and thereby facilitates the activation of a gene. Androgen-response elements are nucleotide sequences that bind the androgen receptor.

**N/C interaction** Interaction between the $NH_2$-terminal and carboxyl-terminal (N/C) regions of the androgen receptor that is selectively induced by the binding of a biologically active androgen, testosterone or dihydrotestosterone. This interaction is mediated by F*XX*LF- and W*XX*LF-binding motifs in the $NH_2$-terminal region and activation function 2 in the ligand-binding domain.

**p160 co-activators** A group of co-regulatory proteins of approximately 160 kDa in size that interact with nuclear receptors to increase the transcriptional response.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptor-Related Pathology • Androgens: Pharmacological Use and Abuse • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Steroid Hormone Receptor Family: Mechanisms of Action**

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# Androgens: Pharmacological Use and Abuse

Maria M. Byrne and Eberhard Nieschlag
*University of Münster*

I. INTRODUCTION
II. PHARMACOLOGY
III. ANDROGEN USE
IV. ANDROGEN ABUSE

**Androgen preparations are widely prescribed for a variety of medical conditions in both men and women. Misuse of androgens, however, has become an extensive problem of increasing proportion. Therefore, it is important for students, scientists, and clinicians to be familiar with the risks and benefits of androgen use.**

## I. INTRODUCTION

The principal medical use of androgens is for the treatment of male hypogonadism and delayed puberty. However, many new indications for androgen treatment are emerging. This article focuses on

# Androgen Receptor-Related Pathology

MICHAEL J. MCPHAUL
*University of Texas Southwestern Medical School*

I. INTRODUCTION
II. PHENOTYPIC SPECTRUM OF ANDROGEN INSENSITIVITY
III. AR MUTATION TYPES
IV. AR MUTATIONS, FUNCTION, AND CLINICAL PHENOTYPE
V. SPINAL AND BULBAR MUSCULAR ATROPHY
VI. SUMMARY

**In mammals, testosterone and 5α-dihydrotestosterone are the principal circulating male hormones (androgens). These hormones serve to modulate a number of important processes, from the development of the male phenotype during embryogenesis to the regulation of spermatogenesis and the secretion of gonadotropins in the adult. Mutations of the human androgen receptor gene have been implicated in pathogenesis of several different diseases.**

## I. INTRODUCTION

Biochemical and genetic data have demonstrated that the male hormones, testosterone and 5α-dihydrotestosterone (DHT), are not equivalent and that they regulate some processes selectively. Although the mechanism of such selective regulation is an area of active investigation, it is clear that the events controlled by each hormone are exerted via the single, X-linked androgen receptor (AR). In mammals, the hormones produced by the gonads during specific periods of embryogenesis govern sexual development. The peptide and steroid hormones that are produced are, in turn, dictated by whether the bipotential gonad differentiates into a testis or ovary, a process that is governed by the fetal sex chromosome composition and distinct genes encoded on autosomes. At approximately 9 weeks of development, the testes begin to secrete testosterone. In combination with its 5α reduced metabolite, 5α-dihydrotestosterone, testosterone acts to induce the virilization of the internal and external genitalia. The Wolffian ducts grow, forming the pelvic portion of the urogenital sinus and giving rise to the seminal vesicles and the epididymis, while the external genitalia respond with the formation and enlargement of the phallus and the fusion of the genital ridges to form the scrotum. In this process, the formation of DHT is particularly important to the virilization of the external genitalia. In addition to events that are mediated by the action of the androgens, a polypeptide hormone produced by the Sertoli cells of the testes, Müllerian inhibiting substance (MIS), induces the regression of structures derived from the Müllerian ducts, particularly the uterus and fallopian tubes.

## II. PHENOTYPIC SPECTRUM OF ANDROGEN INSENSITIVITY

Mutations of the AR associated with androgen resistance have been described in a large number of patients with a wide range of phenotypic abnormalities of male development. At least three different influences are responsible for the relatively large number of clinically apparent defects of AR. The first is the chromosomal location of the AR gene. The AR gene is located on the human X chromosome and is present in only a single copy in 46,XY genotypic males. As such, no second AR allele is present that might serve to mask or compensate the effect of any mutation that impairs the function of the AR gene. In addition, it is apparent that the actions of androgens, although important for normal male sexual development, are not required for life. Even patients with complete defects of AR function mature to adulthood. Finally, many defects of AR function result in abnormalities of sexual development that are evident at birth; such defects have prompted endocrine and genetic studies designed to identify the etiology.

The phenotypic spectrum of androgen insensitivity syndrome (AIS) is most easily conceived as reflecting the degree to which the androgen-regulated events of male sexual development have been disturbed. In individuals with the clinical phenotype of complete androgen insensitivity (complete testicular feminization), the function of the AR is completely defective and there is no development of internal or external male structures that are regulated by androgen. Such individuals show no signs of virilization. The inability to respond to androgens is not sufficient to explain the entire phenotype of complete androgen insensitivity, however. Although circulating androgens cannot act via the AR, testosterone is aromatized to estrogen, which mediates feminization because of the presence of the normal estrogen receptors. As such, affected individuals appear to be normally developed women with normal breast development. Careful evaluation of such subjects will identify testes within the labia majora or the abdominal cavity. In addition, because the testes in such individuals produce Müllerian inhibiting substance, the Müllerian-derived structures (the uterus and fallopian tubes) are absent and the vagina is blind ending.

Other intermediate phenotypes have been described and reflect differing levels of impairment of AR function. These have been referred to using a variety of terms, including incomplete testicular feminization, Reifenstein syndrome, and partial androgen insensitivity. The phenotypes of such individuals are characterized by varying degrees of virilization. Subjects may display a phenotype that is predominantly male in character but with defects of urogenital development, such as perineal hypospadias (the Reifenstein phenotype). Others exhibit phenotypes that are predominantly female with varying degrees of clitoromegaly or labial fusion (incomplete testicular feminization). In recent years, Quigley and colleagues have published a more detailed system with which to categorize patients with partial forms of androgen insensitivity.

Finally, in a small number of patients, specific processes that are regulated by androgen are disturbed, even though male sexual development is normal or near normal. In some, subtle signs of undervirilization, such as gynecomastia, may be present. In other individuals, infertility or oligospermia appear to be the only manifestations of defective AR function.

### A. Characterization of AR Defects: Biochemical Studies

The phenotypic abnormalities that accompany the androgen-resistant states can be viewed as a continuum that relates directly to the extent to which the androgen-mediated processes of male sexual development have been disturbed. In parallel with the description of the clinical syndromes, characterization of the type of AR defect became possible using binding assays to measure the level and character of AR present in patient samples. The application of such techniques has permitted recognition of a number of quantitative and qualitative abnormalities of androgen binding in patients with different forms of androgen insensitivity.

The use of these methods has permitted diagnosis and classification of many types of patients with androgen insensitivity. Despite the utility of these techniques, the information derived from such methods is limited. First, no binding abnormality is evident in a substantial proportion of patients analyzed, even after the application of qualitative and quantitative assessments of AR binding. Second, it is clear that no direct relationship exists between the clinical phenotype and the type of ligand binding abnormality. More detailed studies of the nature of the defects causing androgen insensitivity awaited characterization of the AR at the molecular level.

### B. Structure of the AR

The AR is a member of a large gene family, the nuclear receptor family. In addition to the classic steroid receptors, such as the AR, this family also contains a number of related proteins, including the receptors for thyroid hormone, vitamin D, and retinoic acid. Most members of this family display a similar organization. Each contains a central DNA-binding domain (DBD) and a carboxyl-terminal ligand-binding domain (LBD) domain that mediates the high-affinity binding of ligand. In addition, each protein possesses an amino-terminal segment that is of variable length and is required for full transcriptional activity. In the case of the human AR, the amino terminus is large and comprises nearly half of the molecule (Fig. 1).

## III. AR MUTATION TYPES

Initial efforts categorized the syndromes of androgen insensitivity syndrome on the basis of the associated abnormalities of ligand binding. At present, the number of mutations of the AR that have been identified in patients with different forms of AIS permits additional information to be used in the classification of such patients.

### A. Interruption of the AR Open Reading Frame

A number of distinct mechanisms have been identified that cause an interruption of the primary amino acid sequence of the human AR. Large-scale deletions, small-scale deletions, insertions, and alterations in AR structure caused by changes in mRNA splicing have been reported. Although mechanistically distinct, in each instance the final protein product differs in primary amino acid sequence from that of the normal human AR protein. This difference may be caused by a premature truncation or by the addition or removal of one or more amino acids from the receptor sequence. Because the LBDs and DBDs of the AR are located toward the C-terminus of the receptor, mutations that result in a shift of the open reading frame and premature termination of

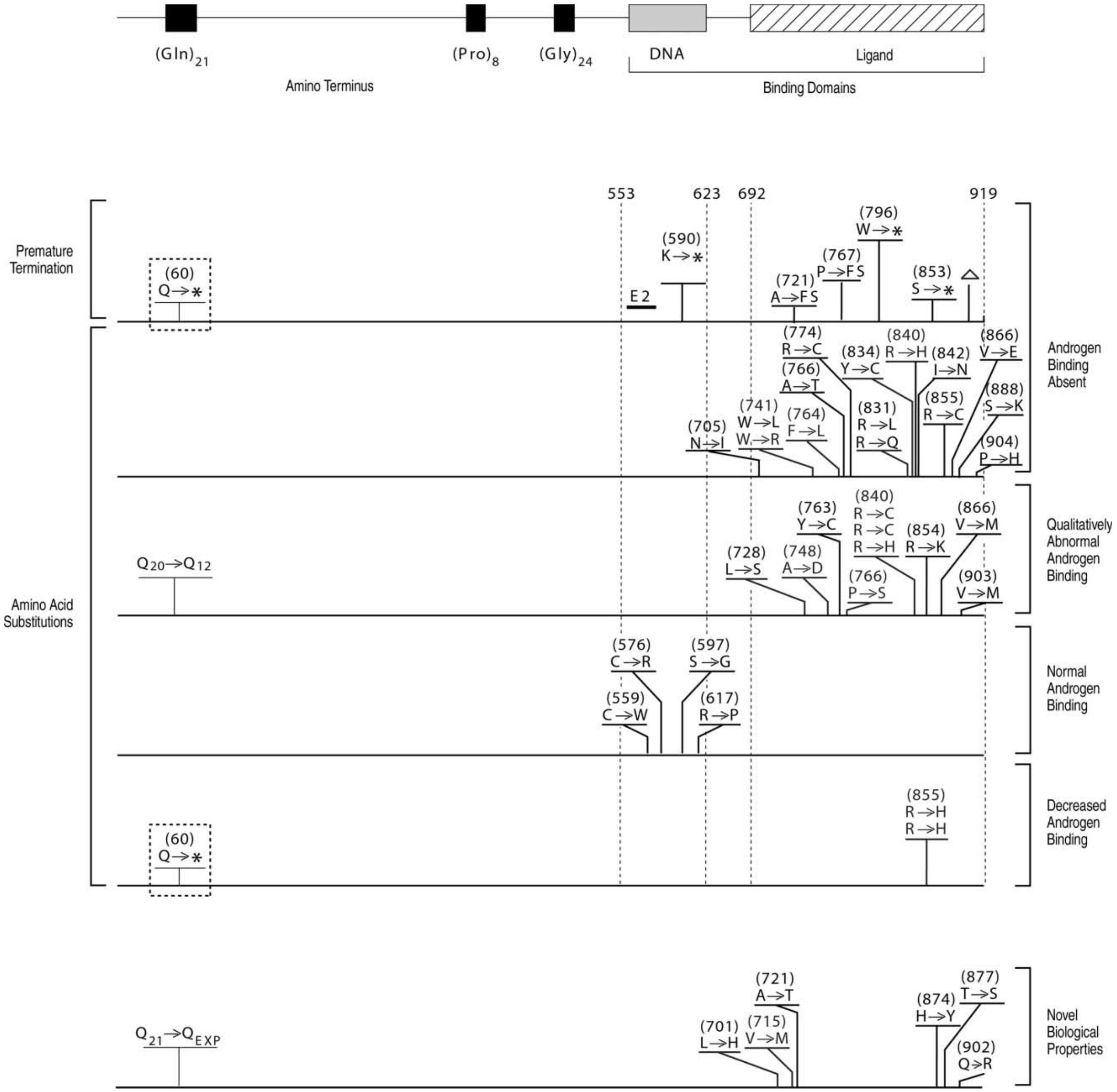
(Gln)21
(Pro)8
(Gly)24
DNA
Ligand
Amino Terminus
Binding Domains
553
623
692
919
Premature Termination
Amino Acid Substitutions
(60) Q->*
E2
(590) K->*
(721) A->FS
(767) P->FS
(796) W->*
(853) S->*
(774) R->C
(766) A->T
(741) W->L W->R
(764) F->L
(705) N->I
(834) Y->C
(840) R->H
(842) I->N
(831) R->L R->Q
(855) R->C
(866) V->E
(888) S->K
(904) P->H
Androgen Binding Absent
Q20->Q12
(728) L->S
(748) A->D
(763) Y->C
(840) R->C R->C R->H
(766) P->S
(854) R->K
(866) V->M
(903) V->M
Qualitatively Abnormal Androgen Binding
(576) C->R
(559) C->W
(597) S->G
(617) R->P
Normal Androgen Binding
(855) R->H R->H
(60) Q->*
Decreased Androgen Binding
Q21->QEXP
(701) L->H
(715) V->M
(721) A->T
(874) H->Y
(877) T->S
(902) Q->R
Novel Biological Properties

**FIGURE 1** Alterations of androgen receptor structure associated with human disease. Top: Schematic of the human AR showing the relative positions of the segments critical to the binding of DNA and ligand by the receptor protein. The predicted amino acid sequence of the AR also encodes a large amino-terminal segment that comprises approximately half of the molecule. This region contains motifs composed of repeated glutamine, glycine, and proline residues. Alterations of the length of the glutamine repeat have been associated with the pathogenesis of spinal and bulbar muscular atrophy, a propensity to develop aggressive forms of prostate cancer, and infertility. Middle: Representative mutations of the AR associated with androgen insensitivity syndrome. Categorization by type of genetic mutation is shown on the left; the observed effects on androgen binding in patients with different forms of androgen insensitivity syndrome are shown on the right. Bottom: A schematic of AR mutations that result in acquisition of novel biological properties. In addition to expansion of the glutamine repeat within the amino terminus of the AR that is associated with spinal and bulbar muscular atrophy ($Q_{21} \rightarrow Q_{EXP}$), also shown are examples of mutations that have been identified in prostate cancer cell lines and tumors that result in novel responsiveness of the AR. (Modified with permission from McPhaul, M. J., Marcelli, M., Zoppi, S., Griffin, J. E., Wilson, J. D. (1993). The spectrum of mutations in the androgen receptor gene that causes androgen resistance. *J. Clin. Endocrinol. Metab.* **76**, 17–23. Copyright The Endocrine Society.)

the receptor have a dramatic effect on receptor function when introduced at virtually any position within the primary amino acid sequence. By contrast, mutations that result in the insertion or removal of single or multiple amino acids from the normal AR sequence behave more like amino acid substitution mutations (see later) and cause syndromes of androgen resistance only when occurring within the DNA- or ligand-binding domains.

## B. Mutations in the DBD of the AR

In the characterization of some patients, endocrine studies or family histories have been suggestive of androgen insensitivity, but abnormalities of ligand binding could not be identified (~20% in one series). This group includes individuals with diverse phenotypes, ranging from the most severely affected individuals to those with much lesser affected phenotypes. At the time, these patients were postulated to harbor either subtle defects of the AR or defects in other genes that are required for the normal AR function.

In pedigrees in which the family history suggests the inheritance of an X-linked trait, mutations within the conserved DBD of the receptor are frequently detected. In one study of four unrelated subjects with complete or near complete forms of androgen resistance, amino acid substitutions were localized to the DNA-binding region of the receptor protein in each instance. When these mutant ARs were analyzed in detail, they were found to bind androgen with normal or near normal kinetics, as predicted on the basis of binding studies performed in fibroblasts established from the individual patients. When assayed in transfection assays using a model androgen-responsive reporter gene, these mutant ARs were found to be markedly impaired. Studies of fusion proteins containing the DBDs of the normal and mutant ARs demonstrated that in each instance the mutant ARs displayed an impaired capacity to bind to target DNA sequences.

AR mutations other than the replacement of individual amino acids can have similar effects on receptor function when they disrupt the structure of the DNA-binding domain while maintaining the AR open reading frame. Such inferences are based on the identification of patients in whom in-frame deletions or insertions have removed or inserted one or more amino acid residues. The characterization of mutant ARs of this type has revealed that regardless of the nature of the causative mutation, changes that alter the structure of only the DNA-binding domain of the receptor cause androgen insensitivity by interfering with a capacity of the receptor to recognize specific target DNA sequences. The published data suggest that the extent to which DNA binding by the mutant receptors is impaired correlates with the degree of receptor function and the phenotype that is observed. The data of Nguyen et al. are interesting in this regard. These scientists identified a mutation in the P-box of the AR DBD in a patient with partial androgen insensitivity. This study demonstrated that the mutant AR (G577R; in the one-letter amino acid code, G is glycine and R is arginine) resulted in the synthesis of a receptor protein that displayed selective impairment of binding of the AR to specific types of androgen response elements. It was further suggested that such differential impairment of DNA binding might contribute to the diversity of androgen-resistant phenotypes.

## C. Mutations in the AR LBD

Mutations that change single amino acid residues in the LBD are the most frequent type of AR mutation. This category accounts for approximately 60% of all AR gene mutations causing clinical androgen resistance. Mutations in this segment of the receptor have been identified in patients with all of the phenotypes associated with androgen insensitivity. These mutations fall into two categories: amino acid substitutions causing absent ligand binding and qualitative abnormalities of ligand binding.

### *1. Amino Acid Substitutions Causing Absent Ligand Binding*

Two broad groups of amino acid substitutions in the AR LBD have been identified in patient fibroblasts that exhibit undetectable levels of ligand binding. Only a few examples of the first type have been characterized and represent the replacement of residues critical to the formation of a LBD that is capable of binding ligand with high affinity. When expressed and studied using eukaryotic or prokaryotic expression systems, such mutant ARs are incapable of binding ligand. The mutant AR containing a single amino acid substitution at residue 739 (W739R; W, tryptophan) is one such mutation. Inspection of the crystal structures of the AR LBD reveals that this mutation results in the substitution of a hydrophobic residue (tryptophan) at the amino terminus of helix 4 with a charged amino acid (arginine). These structures demonstrate that this residue is localized in an area that makes important contacts with the C ring of testosterone and is located deep within the hydrophobic core of the LBD. In addition to disrupting important receptor–ligand contacts directly, the insertion of a charged residue into this region of the receptor is likely to have dramatic effects on the overall tertiary structure of the LBD.

More commonly, when analyses are performed to analyze such mutant ARs by expression in bacteria or in eukaryotes, they frequently reveal that the AR is capable of interacting with ligand with measurable affinity, even when parallel studies performed in patient fibroblasts indicate that the mutant AR lacks the capacity to bind ligand. The mutant receptor containing an amino acid replacement at residue 774 (R774C; C, cysteine) is an illustrative example. When analyzed in cultured fibroblasts, the levels of ligand binding that are measured are below the detection limits of ligand-binding assays. When this mutant receptor (R774C) is expressed in heterologous cells, however, the mutant receptor can be shown to bind ligand. The marked instability of the ligand binding that is detected in these assays is likely the reason that ligand binding is undetectable in the patient samples. Many additional mutant ARs that exhibit the same type of behavior as that of the R774C mutant have been reported, in which normal or near normal-levels of immunoreactive receptor are expressed. This discordance appears simply to reflect differences in the sensitivity of the assays employed and the higher levels of receptor that can be expressed using eukaryotic and prokaryotic expression systems.

### 2. *Qualitative Abnormalities of Ligand Binding*

In some samples obtained from patients with androgen insensitivity, the number of ARs measured in ligand-binding assays is normal. In many instances, when such samples are carefully assessed, the AR that is present can be shown to exhibit qualitative abnormalities of ligand binding (e.g., affinity, reduced increased thermal instability, and accelerated ligand dissociation).

The first study to identify the genetic basis of androgen insensitivity associated with a qualitative abnormality of the AR (increased $K_d$) was reported by Lubahn and colleagues. These workers identified a single nucleotide substitution mutation that resulted in a single amino acid substitution (V866M; V, valine; M, methionine) in the LBD of the mutant AR that was associated with a phenotype of complete androgen insensitivity. Functional studies of this mutant AR demonstrated a reduced capacity to stimulate model androgen-responsive genes in transfection experiments. Of interest, in these analyses the ability of the mutant AR to induce the activity of androgen-responsive genes increased as ligand concentrations were increased.

A large number of the reported different amino acid substitutions are associated with qualitative abnormalities of ligand binding. In virtually all instances, the causative mutations are localized to the LBD of the receptor. An inspection of the locations of mutations in the AR that cause qualitative abnormalities of ligand binding and absent ligand binding shows a similar distribution. This suggests that the degree to which the structure of the LBD is disrupted dictates the type of binding abnormality that is identified. Alterations that cause more dramatic changes of structure are associated with absent ligand binding and those causing less extensive alterations are associated with qualitative defects of the AR. Studies of mutant ARs in which a single residue has been mutated to different amino acid residues in different pedigrees have supported this concept. For example, Prior *et al.* identified a mutation that resulted in the replacement of an arginine residue by a cysteine residue (R774C) in a patient with complete androgen insensitivity. In fibroblasts, this alteration is associated with an absence of ligand binding. By contrast, in a different pedigree, a mutation that causes a replacement of this same arginine by a histidine residue has been shown to be associated with normal levels of AR that exhibit pronounced thermal instability. Similar studies of additional subjects with androgen insensitivity have characterized other mutant ARs in which different amino acid substitutions at the same residue lead to different effects on ligand binding and receptor function.

The study of mutant ARs that exhibit different types of qualitative abnormalities and contain amino acid substitutions in the LBD has provided additional insights. In most target cells, enzymes (such as members of the 17-hydroxysteroid dehydrogenase family) actively convert testosterone and 5α-dihydrotestosterone to inactive metabolites. Several studies have noted that when mutant ARs containing amino acid substitutions in the LBD are stimulated with such androgens, the dosing, concentration, and type of androgen employed have a dramatic effect on the levels of AR function that are measured in functional assays. In the studies of Marcelli and co-workers, testosterone was the least potent, and dihydrotestosterone and mibolerone exhibited greater activity. In several instances, these investigators were able to show that repeated dosing of physiologic concentrations of androgen could restore the function of the mutant AR. These experiments have demonstrated the importance of the stability of the hormone–AR complex. Conditions that enhance the formation and stability of these complexes exert major effects on the function of the mutant receptors in functional assays. In addition, these results suggest that mutant receptors capable of binding hormone can be manipulated pharmacologically to normalize AR function, inferences that have been supported clinically in a limited number of instances. Finally, such experiments demonstrate that care must be taken when considering the relationship between measurements of AR function and patient phenotype. In this regard, it is quite likely that minor changes in the methods used (e.g., the use of different ligands) could result in considerable differences in the levels of receptor function that are measured.

### D. Mutations That Result in the Synthesis of Decreased Levels of AR

Two different mechanisms have been identified in relationship to mutations that result in reduced quantities of AR, underscoring the diversity that is likely to be encountered in this category of genetic alteration. The first mutation associated with this type of abnormality was reported by Zoppi et al. Using monolayer binding assays, affected individuals were found to express reduced amounts of AR. These results contrasted dramatically with the results established by immunoblot assays measuring AR in extracts of fibroblasts in affected family members. In these assays, no immunoreactive AR protein was detected using antibodies directed at the amino terminus of the AR (residues 1–20). This basis for this paradox was identified as an AR open reading frame mutation that introduced a premature termination codon in place of amino acid residue 60. The low level of AR detected in the initial fibroblast monolayer binding assays was shown to be the result of downstream initiation at Met-189. Functional characterization using transfection into heterologous cells suggested that the phenotype associated with this mutation in the pedigree (complete androgen insensitivity) was caused by a combination of reduced receptor expression and a reduced function of the receptor protein that is synthesized. Additional studies demonstrate that this receptor protein, which is analogous to the A-form of the progesterone receptor, is present in normal cells, although more recent reports have raised the possibility that the shortened AR (AR-A) is the result of protein degradation.

The mechanism implicated in the analysis of the patient characterized by Choong and co-workers differs considerably. In this pedigree, the propositus was an individual with partial AIS in which reduced levels of apparently normal AR were synthesized. Analysis of samples from affected subjects revealed a single nucleotide mutation that causes an amino acid replacement at amino acid residue 2 (a lysine residue in place of the normal aspartate residue at this position). On the basis of cell transfection and in vitro translation studies, the authors postulated a reduced efficiency of translation initiation that led to diminished levels of AR.

### E. Additional Perspectives on the Function of Mutant ARs (Interaction of Termini)

Work in a number of laboratories has identified ligand-dependent interactions occurring between the amino and carboxyl termini of the AR. Initially inferred from the influence of changes in the length of the amino-terminal glutamine repeat on the activity of a mutant AR containing an amino acid substitution in the LBD of the receptor, several groups have defined the basis for this interaction and have implicated it as an important component of normal AR function. Despite considerable attention to this interaction, experiments conducted by Chang and McDonnell have failed to demonstrate that alterations of gene activation can be observed to accompany the disruption of the amino- and carboxyl-terminal interaction using small peptides.

In one instance, these results have been extended to examine the interactions between the amino and carboxyl termini in mutant ARs containing LBD mutations. There is evidence that the disrupted amino- and carboxyl-terminal interactions may represent a marker of the potential molecular defect in patients with AIS, in whom normal or near-normal androgen binding is present. Given the limited number of AR mutations that have been analyzed in this fashion, it is not clear whether such studies provide information, or if they simply represent additional properties that reflect the formation of an inherently unstable hormone–receptor complex.

## IV. AR MUTATIONS, FUNCTION, AND CLINICAL PHENOTYPE

It has become evident that the nature of the AR defect does not have a simple relationship to the phenotype exhibited by affected individuals. Despite this, the large number of AR mutations that have been reported in patients with various forms of androgen resistance permits a number of generalizations to be made. First, truncation or interruption of the AR protein is associated with a phenotype of complete androgen resistance. This association is caused by the location of the critical DNA- and ligand-binding domains in the carboxyl-terminal segments of the AR open reading frame. For this reason, interruptions of the coding sequence remove one or both of these important functional domains and result in the synthesis of ARs that are inactive in assays of receptor function. By contrast, amino acid substitutions in the AR can cause variable effects on AR function and have been associated with the complete range of androgen-resistant phenotypes.

The second generalization pertains to the relationship between the AR gene defect and the observed clinical phenotype. In some instances, no AR is expressed or the genetic mutation completely abolishes AR function. In these instances, the clinical phenotype—complete androgen insensitivity—agrees with assessments of receptor function and is consistent between pedigrees. Such considerations do not apply when the AR that is expressed is not completely defective. In these instances, the quantitation of the degree of deficiency is considerably more difficult, because the results of functional assays may show marked differences, depending on the conditions under which such assays are performed. Although a number of examples of this phenomenon can be found in the literature, the experiments of Marcelli are representative, i.e., changes in the incubation conditions or the type of the ligand used can lead to dramatic differences in the level of receptor function measured.

Although in many instances similar phenotypes are observed in different pedigrees in which the same mutation is identified, a significant proportion of patients with identical mutations will exhibit various phenotypes. In the analysis of Gottlieb *et al.*, as many as 10–15% of cases contained in the AR Mutation Database have been reported to have distinctive phenotypes but have identical mutations. The issues raised by these inferences have been more carefully directly assessed in the Netherlands in a nationwide survey of patients with AIS. In this study, fully one-third of families with partial AIS showed considerable variation in phenotype. At least two distinct mechanisms have been identified that may contribute to such differences.

Boehmer *et al.* reported the characterization of two siblings with partial forms of AIS: one with a predominantly female phenotype (classified as grade 5 AIS) and the second with a predominantly male phenotype (perineal hypospadias, micropenis, and bifid scrotum; classified as grade 3 AIS). Investigations of the structures of the AR genes in these individuals identified identical AR mutations (R855H; H, histidine) within the receptor LBD. Analyses of fibroblasts established from both subjects revealed similar levels of androgen binding in both strains that were within the normal range. In these analyses, the mutant AR exhibited only a minor reduction in binding affinity, compared to normal controls. In contrast to these findings, measurements of 5α-reductase levels revealed substantial differences between the strains. In the subject with the greater degree of virilization, levels of 5α-reductase were similar to the levels present in normal male and female fibroblasts. By contrast, the level of 5α-reductase detected in the subject with the predominantly female phenotype was in a range consistent with that observed in patients with 5α-reductase deficiency. This finding could not be explained on the basis of mutations of 5α-reductase 2 gene and was felt possibly to reflect "secondary" 5α-reductase deficiency, as had been observed in other AIS pedigrees. In any event, the differences in local formation of DHT were proposed to underlie the differences in the degree of virilization observed in these two subjects. The postulated effect in these patients would be expected to mirror the effects of DHT versus testosterone in cell transfection experiments using different ligands to stimulate mutant receptors containing amino acid substitution in the LBD.

In some instances, the mutation identified appears inconsistent with the observed phenotype. (e.g., a mutation causing premature termination of the AR in a subject with substantial levels of virilization) This is the circumstance in the pedigree examined by Holterhus and co-workers, who identified a premature termination codon occurring at amino acid 172 in the open reading frame of the AR. Surprisingly, this mutation was identified in a patient exhibiting subtle signs of virilization (clitoromegaly). Investigation of the level and nature of AR expressed in this patient indicated that the reduced level of ligand binding that was detected reflected the presence of reduced amounts of full-length normal AR. The derivation of this normal AR was shown to be the result of a somatic mosaicism in which a proportion of the cells in this patient expressed normal AR. This reduced level of normal AR was proposed to account for the degree of virilization that was observed in this patient. The investigators have identified additional pedigrees in which both normal and mutant ARs are present. The proportion of mutant AR that is present is believed to influence the clinical phenotype.

## V. SPINAL AND BULBAR MUSCULAR ATROPHY

In 1968, Kennedy, Alter, and Sung reported the description of two families with an adult-onset form of spinal and bulbar muscular atrophy. Although similar to other "muscular atrophies" that had been described previously, affected individuals in these pedigrees were noted to exhibit several unusual features. First, the disease became clinically evident in the fourth and fifth decades of life and was slowly progressive. Affected individuals described low backache and muscle cramps. These initial symptoms were followed by the appearance of overt muscle weakness and fasciculations. The muscle weakness was most notable in the proximal musculature; distal muscular involvement occurred later and was less pronounced. Bulbar involvement was present in affected individuals and resulted in both dysarthria and dysphagia. Nerve conduction velocities were normal and electromyelogram (EMG) analyses identified patterns consistent with degeneration of motor neurons within the anterior horns and cranial motor nuclei. Although sensation and cerebellar function were judged to be normal in clinical assessments, subsequent studies identified abnormal sensory action potentials, leading some to term this disorder "bulbospinal neuronopathy." Pathologic findings have supported that changes in ganglia are likely the basis for these changes. Although such sensory abnormalities are consistently demonstrable in a proportion of affected subjects, symptoms and signs referable to the degeneration of affected spinal and bulbar motor nuclei predominate. Importantly, only males are affected in these families, suggesting inheritance as a sex-linked recessive trait. In addition to these neurological features, 3 of the 11 affected individuals were noted to have findings consistent with a defect of androgen action (gynecomastia).

Given the involvement of the AR in mediating the effects of androgen in virilization and fertility, the AR, known to be localized to the human X chromosome, represents a potential candidate gene for this disorder. Seminal work by La Spada and colleagues has demonstrated that an expansion of a CAG triplet repeat in the amino terminus of the AR is the genetic change responsible for the development of Kennedy's syndrome. The increased size of this repeat segment results in the expansion of a segment of repeated glutamine residues (homopolymeric segment) within the open reading frame of the AR, from its normal size (11–33) to a larger size not observed in normal individuals (>38). This expansion is believed to influence at least two distinct properties of the AR that contribute to the overall phenotype that is observed in individuals affected by spinal and bulbar muscular atrophy (SBMA). First is the contribution of this genetic change to the mild androgen resistance that is observed in a proportion of affected individuals. Males carrying this genetic change develop normally and exhibit normal sexual function early in life. Despite this, a proportion of affected subjects begin to exhibit phenotypic changes characteristic of mild androgen resistance as they enter adulthood. In several series, 25–50% of subjects had gynecomastia and several had been investigated for infertility. The expansion of the size of the glutamine repeat has been suggested to contribute to these endocrine effects in two different ways. First, it appears that the glutamine repeat expansion impairs the activity of the receptor protein in transfection assays of receptor function. When examined carefully, these changes appear to reflect true decreases in the specific activity of the receptor protein in modulating the activity of model reporter genes. In addition to these changes, however, it appears that the expansion of the glutamine repeats may in fact impair the efficiency of translation of the AR mRNA, resulting in lower levels of AR expression. Both of these changes may well contribute to the evolution of the mild androgen resistance that is a component of the SBMA phenotype. Perhaps with the decline of testicular function that occurs with age even in normal subjects, the impairment of function of ARs containing the glutamine repeat expansion becomes evident clinically.

La Spada and colleagues were the first to link the expansion of a glutamine repeat within the open reading frame of a protein to the pathogenesis of a disease. In the case of SBMA, they correlated this disorder to the expansion of a homopolymeric domain within the amino terminus of the AR gene (from ~20 in normal controls to >38 in affected individuals). Subsequent investigations have implicated similar genetic changes in the pathogenesis of several progressive neurological diseases, including Huntington's disease, spinocerebellar ataxia-type 1, Machado–Joseph disease, and dentatorubral pallidoluysian atrophy.

The mechanisms by which such glutamine repeat expansions cause neurological symptoms remain the subject of active investigation. Several lines of evidence have suggested that the proteins containing expanded polyglutamine tracts, such as the AR, acquire a toxic property that is not possessed by the normal protein. Several groups have proposed that the central mechanism may involve accumulation of peptides that are produced as a result of the presence of the glutamine repeat expansion. Supporting such a mechanism are studies that have identified intracellular inclusions containing fragments of ARs that contain the glutamine repeat expansion. In such models, the toxicity of these fragments is believed to result in the degeneration of neurons within selected spinal and bulbar nuclei. The mechanism of this toxicity may derive from the sequestration of critical proteins, such as transcription factors by the glutamine repeat-containing peptides. The toxicity may also involve distinct mechanisms that contribute to the neural pathology. It is likely that several different properties of proteins containing glutamine repeat expansions may contribute to the toxicity that these proteins exhibit. Further investigations will be needed to clarify which of these properties represent critical pathologic properties and which are essentially ancillary findings.

Despite the insights that such experiments have provided, a number of important questions still remain pertaining to the mechanisms by which this toxicity emerges. A particularly intriguing and unexplained aspect of this disease is the timing of its onset. The onset of the disease occurs later in life (i.e., middle age), in a time frame that is difficult to explain based on what is known regarding the expression of the gene product (the AR) that has been implicated. Finally, it is remarkable that the toxicity observed in SBMA is restricted to cells of neural lineage, suggesting that some aspect of the neuronal cell type might make it particularly susceptible to the effects of proteins containing the expanded glutamine repeats.

## VI. SUMMARY

Mutations of the human androgen receptor gene have been implicated in pathogenesis of several different diseases. In androgen insensitivity, AR function is defective to varying extents, causing a range of abnormalities of male sexual development. At one end of this spectrum, individuals with complete androgen insensitivity (complete testicular feminization) exhibit a normal female phenotype, with normal breast development and female external genitalia. On the other end of the spectrum, individuals with AR defects have male external genitalia, but exhibit either subtle undervirilization or infertility. Between these extremes are individuals with substantial defects of AR function that are associated with phenotypes between that of a normal male and a normal female. Although diverse at the molecular level, the genetic alterations causing androgen insensitivity can be explained as reflecting loss-of-function mutations.

The same is not true for spinal and bulbar muscular atrophy (Kennedy's disease). This disease is characterized by the appearance in adulthood of signs and symptoms of mild androgen insensitivity and the progressive deterioration of function of muscles innervated by spinal and bulbar motor nuclei. The pathogenesis of this disorder has been linked to the expansion of a triplet repeat encoding a series of repeated glutamine residues. As with other diseases caused by the expansion of glutamine repeats, the basis of this toxic gain-of-function mutation has been the subject of intense mechanistic studies.

## Acknowledgment

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## Glossary

**17-hydroxysteroid dehydrogenases** Group of enzymes responsible for the reversible conversion of testosterone and 5α-dihydrotestosterone to inactive metabolites (androstenedione and 5α-androstenedione, respectively). In specific tissues, selected members of this enzyme group are responsible for synthesis of active androgens (e.g., 17β-hydroxysteroid dehydrogenase type 3 in the testis).

**P-box** Segment of the DNA-binding domain of nuclear receptors that is responsible for the specificity of DNA binding by the receptor protein.

**5α-reductase** Enzyme responsible for the conversion of testosterone to 5α-dihydrotestosterone.

**target cell** Type of cell in which a specific hormone exerts its actions (e.g., the prostate is a classic androgen target tissue).

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptors and Prostate Cancer • Androgen Receptor Structure and Function • Androgens: Pharmacological Use and Abuse • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology**

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# Androgen Receptors and Prostate Cancer

DOLORES J. LAMB
*Baylor College of Medicine*

I. INTRODUCTION
II. CHANGES IN THE ANDROGEN RECEPTOR GENE
III. CHANGES IN THE RECEPTOR PROTEIN
IV. CHANGES IN INTERACTING FACTORS THAT MODULATE RECEPTOR FUNCTION
V. CHANGES IN STEROID BIOAVAILABILITY
VI. ALTERNATIVE ROUTES TO GROWTH STIMULATION
VII. SUMMARY

**Diseases of the prostate account for an enormous portion of the morbidity, mortality, and economics in health care of males in the United States. Prostate cancer represents a major health problem for adult men and is the second leading cause of cancer deaths in the United States. If men are castrated before puberty, prostate cancer does not develop. Accordingly, androgens are thought to be required for the initiation and initial progression of prostate cancer.**

## I. INTRODUCTION

Androgens are required for the normal development and differentiated function of the prostate. It is not surprising that tumors that form in the prostate respond to androgens during the early stages of disease. For tumors that have not spread outside of the prostate, radical prostatectomy is a favored form

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# Androgen Receptors and Prostate Cancer

Dolores J. Lamb
*Baylor College of Medicine*

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**Diseases of the prostate account for an enormous portion of the morbidity, mortality, and economics in health care of males in the United States. Prostate cancer represents a major health problem for adult men and is the second leading cause of cancer deaths in the United States. If men are castrated before puberty, prostate cancer does not develop. Accordingly, androgens are thought to be required for the initiation and initial progression of prostate cancer.**

## I. INTRODUCTION

Androgens are required for the normal development and differentiated function of the prostate. It is not surprising that tumors that form in the prostate respond to androgens during the early stages of disease. For tumors that have not spread outside of the prostate, radical prostatectomy is a favored form of treatment. If the tumor has spread and metastasized, androgen ablation therapy, first proposed by Charles Huggins (who received the Nobel Prize in 1966 for this hypothesis), is a treatment for this disease. Like metastatic breast cancers, initially the tumors respond to steroid deprivation, with regression resulting from apoptosis of androgen-dependent prostate cancer cells. Although nearly all tumors continue to express the androgen receptor, some tumors may contain a heterogeneous mixture of androgen-dependent and androgen-independent cells. The androgen-independent cells will escape apoptosis induced by androgen ablation. Other androgen-dependent cells may adapt to a state of androgen depletion and then gain an androgen-independent phenotype. Ultimately, prostate cancer progresses to a state of androgen independence with uncontrolled growth. For the patient, this represents the failure of their therapy and a probable poor outcome. The molecular basis for this progression to androgen independence is largely unknown at this time. One possibility is that mutations in the androgen receptor contribute to this progression of the prostate cancer to an androgen-independent state.

The androgen receptor is a member of the steroid receptor superfamily. The gene is located on the X chromosome and there are eight exons that encode a protein of 919 amino acids. Because the gene is located on the X chromosome, mutation of only one allele is required for a phenotypic change. Importantly, androgen action is dependent on not only a functioning receptor with high affinity and steroid-binding specificity, but also on the interactions of growth factor signaling pathways and the complex interaction of co-activators, co-repressors, and other factors to regulate specific gene expression. Changes in any of these factors could influence androgen action.

Mutant receptors with an altered function were first described in breast cancer, leading investigators to propose that point mutations in the androgen receptor may account for progression from androgen-dependent to androgen-independent growth in prostate cancer. Molecular changes in receptor structure or concentration may result in part in hormone-independent tumor growth. The types of androgen receptor mutations/alterations and their functional consequences are described here. In addition to mutations, there are a number of other potential changes in the androgen receptor signaling pathway that can stimulate androgen receptor activity at suboptimal levels of hormone. The potential role of these alterations in the androgen receptor in prostate cancer progression is also discussed.

## II. CHANGES IN THE ANDROGEN RECEPTOR GENE

### A. Amplification of the Androgen Receptor Gene

An increased copy number or amplification of the androgen receptor gene has been observed in some prostate cancers. Investigators have proposed that this amplification may lead to overexpression of the androgen receptor and, ultimately, to tumor growth stimulation at low levels of circulating androgens. Some investigators have questioned whether the amplification has physiologic relevance and it is somewhat controversial in the literature. An increase in androgen receptor copy number does not correlate with the response to initial androgen ablation therapy, although it does appear to correspond to a positive outcome of secondary treatment with complete androgen blockade. The increase in androgen receptor copy number was correlated with an increased secretion of prostate-specific antigen that did not correlate with tumor burden, again suggesting a functional consequence of this amplification. Although androgen receptor gene amplification and an increased level of the androgen receptor gene expression occur in a significant percentage of samples obtained from hormone-independent tumors, the frequency of androgen receptor gene amplification is too low to account for all prostate cancer progression to androgen independence.

### B. Point Mutations Producing Superactive Androgen Receptors

Theoretically, an androgen receptor mutation could enhance the transcriptional activation of the receptor, especially at low ligand concentrations. In a transgenic adenocarcinoma of the prostate mouse, mutation was detected at residues (668)QPIF(671) at the boundary of the hinge and ligand-binding domain, resulting in a receptor that exhibits two- to fourfold increased activity compared with wild-type AR. This enhanced activity was noted in response to administration of dihydrotestosterone, estradiol, progesterone, and adrenal androgens, as well as the androgen receptor antagonist hydroxy flutamide without an apparent effect on receptor levels, ligand-binding kinetics, or DNA binding. The receptor could be activated by low circulating levels of androgen. Thus, if such a mutation were present in a human prostate cancer, the growth of the tumor might be stimulated by the low levels of androgen remaining following androgen ablation. Preliminary studies suggest that these types of mutations may occur in some advanced human prostate cancers, but further investigations are required to confirm that this occurs and, if so, to define the frequency. This would be considered to be a "gain of function" mutation.

### C. Point Mutations Generating a Promiscuous Receptor Protein That Is Activated by Ligands Other Than Androgen

Mutations that change steroid-binding specificity are thought to be important in the progression of some prostate cancers. In fact, the first mutation found in the androgen receptor in a prostate cancer was identified in the LNCaP cell line. This cell line is an androgen-responsive cell line derived from a human prostate cancer that was metastatic to bone. The functional consequence of this mutation in exon 8 of the androgen receptor is to change the steroid-binding specificity of the androgen receptor, resulting in estradiol and the anti-androgens hydroxy-flutamide, nilutamide, and cyproterone acetate becoming agonists. Since the identification of this first prostate cancer androgen receptor mutant, mutations have been identified in the androgen receptor in other specimens of prostate cancer and cell lines, and these mutations broaden or change the binding specificity of the androgen receptor for glucocorticoids, some adrenal and weaker androgens, anti-androgens, estrogens, and progesterone. In human prostate cancer, the consequence of this type of mutation is obvious. Treatment of the patient with an anti-androgen or an estrogen would have a detrimental effect. Rather than inhibiting tumor growth, the antagonist has become an agonist for the mutated receptor and might enhance the growth of the tumor. These mutations would all be considered to be in the category of gain of function.

### D. Inactivating Mutations of the Androgen Receptor

A mutation in the androgen receptor may result in either the total loss of receptor protein (such as in a total deletion) or, more commonly, a truncation of the receptor or a "loss of function" in one of the functional domains of the protein. An example of this type of mutation is C619Y, found in a metastatic lymph node of a patient with prostate cancer. This androgen receptor mutation resulted in a receptor that could bind steroid and translocate to the nucleus but was localized in abnormal discrete foci, resulting in cytoplasmic and nuclear aggregates, perhaps due to protein misfolding. The mutated androgen receptor could not activate androgen-regulated transcription. Since androgens are normally thought to enhance prostate growth, the presence of an "inactivating" mutation in advanced prostate cancer may be surprising. Recently, Tindall and colleagues showed that disruption of the androgen receptor protein in prostate cancer cell lines inhibited their proliferation, suggesting that these loss of function mutations may not provide a growth advantage to the tumor. Accordingly, the mechanism of androgen regulation of prostatic growth is unclear, and loss of androgen receptor function in the tumor may result in a progressive loss of differentiated function and loss of growth control or it may inhibit tumor growth.

### E. Androgen Receptor Polymorphisms That Modulate Transcriptional Activation

The length of a polymorphic polyglutamine tract in exon 1 of the androgen receptor influences the relative transcriptional activity of the androgen receptor *in vitro*. The length of the tract is inversely correlated with the transcriptional activation of the receptor by androgen. The shorter the tract, the more active the receptor; receptor transcriptional activation is diminished as the tract lengthens. There are racial differences in the average length of the tract, with African American males having approximately 18 repeats, Caucasian males having an average of 21 repeats, and Asian males having the highest repeat number within the normal polymorphic range. Expansion of these repeats to 40 or more results in Kennedy disease (spinobulbar muscular atrophy), a progressive degenerative neuromuscular illness. Some, but not all, investigators have reported that the CAG repeat length (within the normal range) might be related to the age of the patient at diagnosis of prostate cancer and their response to endocrine therapy. It has been reported that a shorter polyglutamine tract is associated with the presence of metastatic disease at the time of diagnosis, a younger age of onset, increased incidence, higher mortality, and a more aggressive nature of prostate cancer in the African American population; however, not all authors agree. In general, it is accepted that the shorter polyglutamine repeat correlates with an earlier age of onset and a diminished response of the prostate cancer to endocrine therapy.

## III. CHANGES IN THE RECEPTOR PROTEIN

### A. Intracellular Changes That Influence Androgen Receptor Sensitivity or Protein Stability

Receptor function may be modulated indirectly by changes in the expression of specific proteins, such as caveolin, that enhance androgen receptor action by influencing androgen receptor sensitivity. Similarly, enhanced expression of the receptor, stabilization of the protein, and nuclear localization of the receptor at very low ligand concentrations enhanced androgen-mediated growth in human prostate cell lines tested *in vitro*. These types of changes in receptor characteristics, whether through a direct action on the receptor or an indirect action, serve to enhance androgen receptor action at low concentrations of androgen, and presumably this contributes to enhanced growth of prostate cancer after endocrine ablation treatment.

## IV. CHANGES IN INTERACTING FACTORS THAT MODULATE RECEPTOR FUNCTION

### A. Co-activator or Co-repressor Modulation of Androgen Action in Advanced Prostate Cancer

Steroid receptors require co-activators to mediate their transcriptional activation functions with proteins in the general transcription complex to stimulate the steroid-regulated transcription of target genes. These factors interact with either the amino- or the carboxy-terminal regions of the receptor to enhance transcription. Some co-activators modulate a broad range of transcription factors, others stimulate the activities of nuclear receptors in general, and others show specificity for one or a few nuclear receptors. Many proteins have been identified that enhance androgen receptor transcriptional activity *in vitro*. Loss of co-activator interaction may diminish androgen receptor transcriptional activation or may broaden the ligand specificity of the receptor. Conversely, overexpression of specific co-activators, such as steroid receptor co-activator 1 and transcriptional intermediary factor 2, enhance androgen receptor transactivation at physiological concentrations of adrenal androgen, providing an alternative mechanism for enhanced prostate cancer growth despite androgen ablation therapy. It is clear that these co-activators play a critical role in androgen action and this is an area of ongoing investigation in several laboratories.

### B. Ligand-Independent Activation of Androgen Receptor

The direct or indirect transcriptional activation of the androgen receptor by growth factors, neurotransmitters, or other agents that increase intracellular kinase activity or decrease phosphatase activity has been shown for chicken progesterone receptor and the human estrogen receptor. Similarly, the androgen receptor can be activated in the absence of androgen by several growth factors thought to be acting through the mitogen-activated protein kinase pathway, through the protein kinase A signaling pathway, or through Her-2-neu overexpression signaling androgen receptor phosphorylation by Akt. Support for a role of kinases in androgen receptor action comes from the observation that the tumor suppressor gene PTEN appears to inhibit androgen receptor action. As a result of this complex cross-talk between different signal transduction pathways, the androgen receptor has the potential for activation.

## V. CHANGES IN STEROID BIOAVAILABILITY

### A. Altered Steroid Bioavailability or Circulating Levels

Only a small portion of circulating androgen is unbound in the circulation and it is the “free” testosterone that is available to diffuse into the cells, bind to the receptor, and initiate androgen action. Changes in the relative concentrations of free to bound androgen may influence prostate cancer growth. There may be local increased or decreased intraprostatic bioavailability of dihydrotestosterone to activate the androgen receptor. Similarly, racial differences in the average levels of circulating androgens may lead to conditions more permissive of prostate cancer development and/or progression. Thus, conditions that modulate the relative concentration of androgen available to the cell may influence prostate cancer.

## VI. ALTERNATIVE ROUTES TO GROWTH STIMULATION

### A. Activation of Growth-Stimulating Signaling Pathways with the Ability to Bypass Androgen Receptor-Regulated Growth and Differentiation

Growth factors may act directly to enhance prostate cancer growth. Conceptually, androgens are normally considered to be the major stimulator of prostate cancer growth; however, it is known that there are many alternative pathways (growth factors, cAMP, kinases, apoptosis, etc.) involved in growth regulation. Accordingly, all of these pathways may contribute to the loss of growth regulation by androgens that occurs in advanced prostate cancer. Since there is a limit to enhancement of the rate of cell proliferation by these growth stimulatory agents, maximal stimulation of growth by an alternative pathway may not be impacted by the loss of androgens upon ablation therapy. Furthermore, the cells may adapt to a state of androgen deprivation and seek to maximize their growth rate through these alternative pathways.

## VII. SUMMARY

These studies demonstrate the important, but relatively poorly understood, function of the androgen receptor in the development and maintenance of differentiated function of the normal prostate, as well as the development of prostate cancer and the progression of this disease. It is clear that there are multiple routes to androgen-independent disease. Alterations of the androgen receptor structure and modulation of its function provide an important route for the development of new therapies to treat advanced prostate cancer.

## Glossary

**androgen** A steroid hormone, such as testosterone or dihydrotestosterone, that promotes the development and maintenance of male secondary sex characteristics and structures.

**androgen receptor** A member of the steroid receptor superfamily of genes that mediates the action of androgens on the target cell; a ligand-activated transcription factor.

**apoptosis** Programmed cell death that requires gene transcription and specific protein synthesis (physiologically regulated event). The cells shrink and convolute, the nucleus condenses, the DNA fragments, and the cell membrane swells and blebs. Ultimately, proteases are activated.

**casodex, cyproterone acetate, hydroxyflutamide, and nilutamide** Androgen receptor antagonists that block androgen receptor function.

**co-activators and co-repressors** Factors that positively or negatively influence androgen receptor transcriptional activity.

**Kennedy disease (spinal bulbar muscular atrophy)** A progressive degenerative neuromuscular disease of genetic origin resulting from expansion of a polyglutamine repeat in the androgen receptor gene.

**prostate-specific antigen** A protein that serves as a tumor marker for prostate cancer; elevated concentrations in the bloodstream may indicate the presence of prostate cancer.

**transgenic adenocarcinoma of the prostate (TRAMP) mouse** A transgenic mouse model of prostate cancer.

## See Also the Following Articles

**Adrenocorticosteroids and Cancer • Androgen Effects in Mammals • Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptor-Related Pathology • Androgen Receptor Structure and Function • Androgens: Pharmacological Use and Abuse • Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Estrogen and Progesterone Receptors in Breast Cancer**

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**hormone-response element** Segment of DNA sequence that binds to the DNA-binding domain of a steroid receptor and thereby facilitates the activation of a gene. Androgen-response elements are nucleotide sequences that bind the androgen receptor.

**N/C interaction** Interaction between the $NH_2$-terminal and carboxyl-terminal (N/C) regions of the androgen receptor that is selectively induced by the binding of a biologically active androgen, testosterone or dihydrotestosterone. This interaction is mediated by F*XX*LF- and W*XX*LF-binding motifs in the $NH_2$-terminal region and activation function 2 in the ligand-binding domain.

**p160 co-activators** A group of co-regulatory proteins of approximately 160 kDa in size that interact with nuclear receptors to increase the transcriptional response.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptor-Related Pathology • Androgens: Pharmacological Use and Abuse • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Steroid Hormone Receptor Family: Mechanisms of Action**

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# Androgens: Pharmacological Use and Abuse

MARIA M. BYRNE AND EBERHARD NIESCHLAG
*University of Münster*

I. INTRODUCTION
II. PHARMACOLOGY
III. ANDROGEN USE
IV. ANDROGEN ABUSE

**Androgen preparations are widely prescribed for a variety of medical conditions in both men and women. Misuse of androgens, however, has become an extensive problem of increasing proportion. Therefore, it is important for students, scientists, and clinicians to be familiar with the risks and benefits of androgen use.**

## I. INTRODUCTION

The principal medical use of androgens is for the treatment of male hypogonadism and delayed puberty. However, many new indications for androgen treatment are emerging. This article focuses on the pharmacology of the various androgen preparations currently available and includes a discussion of the variety of clinical indications (accepted and experimental) for androgen use. In addition, the escalating problem of androgen abuse is addressed.

## II. PHARMACOLOGY

In human males, gonadotropin-releasing hormone (GnRH) released from the hypothalamus stimulates the pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates the Leydig cells of the testes to secrete approximately 7 mg of testosterone per day. This amount is necessary to induce and maintain secondary sexual characteristics, sexual behavior, muscle development, bone density, and male phenotype. The biological action of testosterone is due to its direct binding to the androgen receptor (AR), to its conversion to dihydrotestosterone (DHT) by the 5α-reductase enzyme, or to its aromatization to estrogen. DHT has a higher affinity, compared to testosterone, for the AR and is therefore a more potent androgen. The principal biological effects of testosterone include (1) stimulation of prenatal differentiation and pubertal development of the testes, penis, epididymis, seminal vesicles, and prostate, (2) increased retention of nitrogen and increase in lean body mass and body weight, (3) larynx growth, sebum production, beard growth, and pubic hair growth via DHT, (4) increased bone mineral density (BMD) via estrogen, and (5) reduction in high-density lipoprotein (HDL) and increase in low-density lipoprotein (LDL) cholesterol levels, with increases in triglycerides. The 5α-reductase enzymes are most abundant in the prostate, skin, and reproductive tissues. DHT is required for fetal development of external genitalia, prostate, and seminal vesicles, and adult secondary hair growth. Aromatization of testosterone to estradiol is necessary for sexual differentiation of the brain, for bone mass accretion, and for fusion of the epiphyses at the end of puberty. Testosterone circulates bound to sex hormone-binding globulin (SHBG), with only 1–2% remaining unbound and hence biologically active. Whereas androgen status is typically defined solely on the basis of serum testosterone levels, the AR is clearly another important determinant of androgen action. The majority of studies demonstrate no correlation between endogenous testosterone levels and the subsequent development of prostate cancer. However, an inverse correlation exists between the length of the AR polyglutamate tract and both an increased risk and an earlier onset of prostate cancer. CAG repeats are considered to play a modifying role in prostate cancer risk. Therefore, polymorphisms in the AR may be as important as absolute testosterone levels in mediating androgen effects.

Because of the short duration of action of testosterone, pharmacological strategies have been developed to produce more sustained blood levels of testosterone, resulting in prolongation of its androgenic action. These strategies include (1) new modes of testosterone administration and (2) chemical modification of the testosterone molecule, including esterification of the 17β-hydroxyl group or alkylation of the 17α position of the D ring of the molecule, with or without other modifications of the ring structure (Fig. 1).

### A. Oral Testosterone

Orally administered unmodified testosterone is rapidly absorbed from the gastrointestinal tract into the portal blood and then degraded by the liver (first-pass effect), resulting in a minimal amount of testosterone reaching the systemic circulation. Large doses (200–400 mg/day), taken several times a day, are necessary to achieve physiological levels. Oral testosterone induces production of the liver enzymes that are responsible for testosterone metabolism. Although long-term hepatotoxicity of large doses is unknown, unmodified oral testosterone is not recommended to achieve physiological testosterone levels in blood.

#### 1. *Testosterone Undecanoate*

Testosterone is esterified in the 17β position with a long aliphatic side chain to produce an oral hydrophobic 17β-hydroxyl testosterone ester. It is administered in doses of 80–240 mg/day for androgen replacement therapy, and 40 mg/day for the treatment of constitutionally delayed puberty. Due to the nonpolar nature of the ester, it is preferentially absorbed through the lymphatic system, thereby avoiding first-pass metabolism in the liver. Although testosterone has been widely used for the treatment of male hypogonadism in Australia, Canada, and Europe since the 1970s, its clinical use is limited by a short half-life, necessitating multiple daily dosing. Maximum serum levels were obtained 4 to 5 h after ingestion, with huge interindividual variability. The DHT/testosterone ratio is increased secondary to intestinal 5α-reductase activity, and uncommon gastrointestinal side effects include flatus, oily stool, and diarrhea.

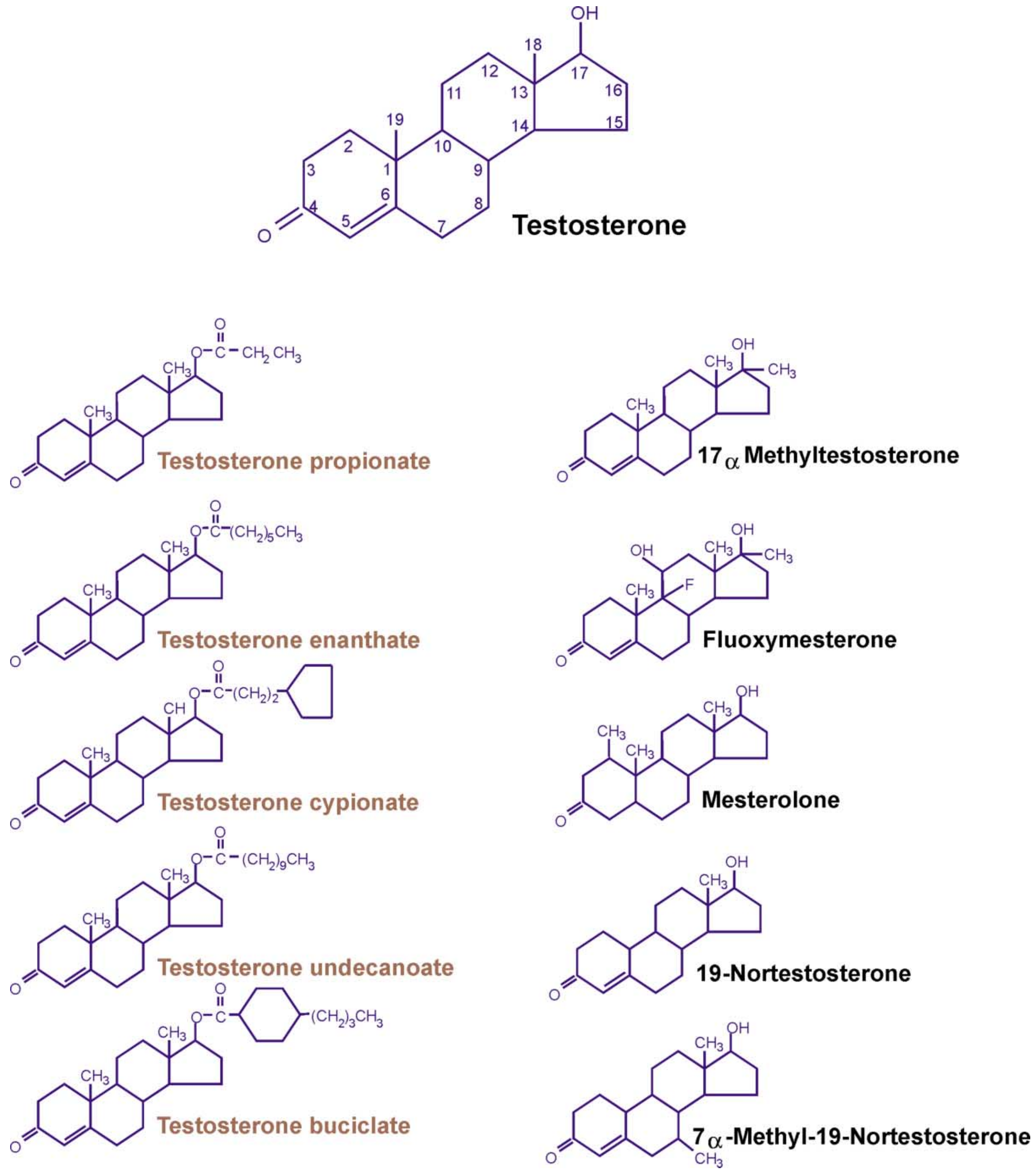
Testosterone
Testosterone propionate
Testosterone enanthate
Testosterone cypionate
Testosterone undecanoate
Testosterone buciclate
17α Methyltestosterone
Fluoxymesterone
Mesterolone
19-Nortestosterone
7α-Methyl-19-Nortestosterone

FIGURE 1 Molecular structures of testosterone and some of its modifications.

### 2. *17α-Methyltestosterone*

Alkylation at the 17α position reduces hepatic metabolism, resulting in improved systemic concentrations of testosterone. 17α-Methyltestosterone is rapidly absorbed, with maximal testosterone levels achieved 90–120 min after ingestion; plasma half-life is 150 min. 17α-Alkylated derivatives of testosterone and fluoxymestrone (addition of a fluorine and a hydroxyl group into the steroid skeleton of testosterone) are rarely used due to lack of potency and potential serious hepatotoxicity (hepatitis, cholestasis, peliosis, and benign and malignant hepatic tumors). Use of these derivatives has been discontinued in Europe. Other 17α-alkylated derivatives include oxandrolone, oxymetholone, stanozolol, and danazol, which are less androgenic and more anabolic.

Mesterolone, a 1α-methyl derivative of 5α-DHT, contains only modifications in the A ring of testosterone, without modification of position 17. Mesterolone and DHT cannot be metabolized to estrogen and therefore will exert questionable clinical effects on bone and brain. Mesterolone is considered to be a relatively weak androgen and therefore not suitable for the treatment of male hypogonadism.

## B. Transdermal Testosterone

The development of transdermal testosterone preparations in the late 1980s was an important advance in androgen replacement therapy. Transdermal substitution of estradiol is a well-established method of treating ovarian insufficiency. However, only micrograms of estradiol are required for replacement therapy. In contrast, up to 7 mg/day of testosterone is required for treating male hypogonadism. Due to the superficial vascularity of the scrotal skin, there is a 5- to 40-fold increase in steroid absorption when compared to other skin sites. The scrotal transdermal testosterone system (TTS) is a 40 or 60 $cm^2$ large polymeric membrane loaded with 10 or 15 mg of testosterone. The patches are applied daily in the morning to the shaved scrotum and result in physiological levels of testosterone (mimicking the normal circadian rhythm), rising to a maximum 2–4 h after application and remaining within the normal range until the patch is removed. DHT levels rise above normal within a few hours of application and remain constantly elevated during the treatment period. This is the result of the high levels of 5α-reductase activity in the scrotal skin. The clinical significance of supraphysiological levels of DHT remains currently unknown, but use over several years has not resulted in untoward side effects. Careful monitoring is still recommended.

In recent years the availability of nongenital patches has led to a decline in the use of scrotal preparations. Permeation-enhanced transdermal delivery systems with a reservoir containing testosterone (12.2 mg) in an alcohol base (Androderm) have been shown to maintain serum testosterone levels more consistently within the normal range, compared to an equivalent intramuscular (im) dose. The recommended dosage for adult hypogonadism is two 2.5-mg patches or one 5-mg patch applied at night to nonscrotal skin (e.g., abdomen, thighs, upper arm, or back). This regimen delivers 5 mg/day of testosterone, with 60% being absorbed within the first 12 h, maintaining serum testosterone levels in the physiological range and mimicking the normal circadian rhythm. In contrast to scrotal TTS, DHT levels also remain within the physiological range. The main drawback of this patch is local skin irritation, which occurs in ~30% of patients compared to ~5% of patients using scrotal TTS. The patch can also cause an allergic contact dermatitis (~12% of patients). Simultaneous treatment with 0.1% triamcinolone acetonide under the patch will improve local tolerability without altering the pharmacokinetics. Skin irritation limits the acceptability of this treatment, resulting in 10–15% of patients having to discontinue treatment.

The recently approved nonscrotal patch Testoderm TTS, which has a larger surface area but does not contain a reservoir, causes less skin irritation (itching and erythema, in ~12% and ~3% of patients, respectively). However, adherence of the patch to the skin poses a problem in some patients, especially those engaged in strenuous exercise. When applied nightly it provides a steady-state delivery of testosterone to the circulation that mimics the normal diurnal rhythm. The long-term use of these transdermal patches has been efficacious in maintaining sexual function, secondary sexual characteristics, and bone and muscle mass in hypogonadal young and elderly men.

The latest development in androgen replacement therapy is an open testosterone delivery system using a hydroalcoholic gel (Androgel 1%), which is now licensed in the United States and several other countries for the treatment of hypogonadal men. When applied to the skin, the gel dries rapidly and the steroid is absorbed into the stratum corneum, which serves as a reservoir. Pharmacokinetic studies of this gel (50 or 100 mg) applied to hypogonadal men indicate that testosterone levels increase into the normal range within 30 min, with steady-state levels achieved by 24 h. Hypogonadal men treated for 6 months with this gel had improved sexual function, increased lean body mass and muscle strength, and decreased fat mass. There was a significant dose-related increase in hematocrit levels reported. Serum DHT levels were also increased. Transdermal gel is user friendly, providing flexibility in dosing with little skin irritation and thereby good compliance. One concern may be the contact transfer of androgens and the induction of androgenic side effects in females or children.

DHT is available as a 2% hydroalcoholic gel that is applied to a large area of skin and washed off

10 min after application. This results in stable DHT plasma levels for 24 h, an elevated DHT/testosterone ratio, lack of aromatization to estrogen, and suppression of endogenous estrogen and testosterone. There is also a risk of contact transfer to female partners. DHT has been shown to maintain sexual function in hypogonadal men but is less efficient than testosterone in preventing bone loss. Prostate volume and prostate-specific antigen (PSA) remain unchanged with long-term DHT gel treatment in older men. A 0.7% hydroalcoholic dermal gel is being developed for the treatment of aging men. Daily application for 3 months to elderly partially androgen-deficient men results in stable pharmacokinetic features with consistent negative feedback on the pituitary–testicular axis and reduced body fat, but minimal effects on muscle mass, strength, or function. Prostate volume and PSA levels remain unchanged.

## C. Intramuscular Testosterone

The most widely used testosterone replacement therapy is in the form of im injection of testosterone esters. Esterification at the 17β-hydroxyl group increases the solubility of testosterone in oil and thereby slows its release into the circulation, prolonging its duration of action. However, only the unesterified testosterone is biologically active. Testosterone propionate must be injected every 2–3 days, but testosterone enanthate (doses of 200–250 mg) and testosterone cypionate have longer durations of action and can be injected every 2–3 weeks, for replacement therapy of hypogonadism.

The disadvantage of all these esters is that they produce initially supraphysiological testosterone levels, which then decline slowly to the hypogonadal range prior to the next injection. Some patients correlate these fluctuations in testosterone levels with changes in well being, mood, and sexual activity. For these reasons, preparations containing a combination of a short- and a long-acting 17β-hydroxyl testosterone ester have been developed and are available in Europe (e.g., Testoviron Depot 50 and 100, containing a combination of testosterone propionate and enanthate, and Sustanon 100 and 250, containing combinations of testosterone propionate, phenylpropionate, isocaproate, and decanoate). These preparations result in even higher initial testosterone peaks with no prolongation in the duration of action and are therefore not recommended ahead of testosterone enanthate or cypionate for the treatment of male hypogonadism.

Longer acting preparations, including testosterone buciclate and testosterone undecanoate, are being developed. Single injections of testosterone buciclate (600 mg) have been shown to maintain serum testosterone levels in the low normal range for 12 weeks in hypogonadal men. This is the longest duration of action of any injectable ester tested so far and this ester appears to have great potential for clinical use. In China, injection of testosterone undecanoate dissolved in teaseed oil (125 mg/ml of teaseed oil) in doses of 500–1000 mg results in serum testosterone levels within the normal range for 6–8 weeks. However, this is limited by an injection volume of 8 ml, which renders its use impractical. Treatment of hypogonadal men with 1000 mg of testosterone undecanoate dissolved in 4 ml of castor oil (250 mg/ml), every 6 weeks for 6 months, produced serum testosterone levels above the lower limit of normal, with good tolerability. The slowly increasing testosterone levels at the end of the injection interval suggested that the interval could be extended. A recent study has shown that injections every 12 weeks for 2 years resulted in stable testosterone levels within the normal range. Maximal serum testosterone levels were lower than with the Chinese preparation and did not exceed 25 nmol/liter. Slight increases in body weight, hemoglobin, hematocrit, prostate volume, and PSA were observed.

## D. Testosterone Subcutaneous Implants

A single implantation procedure delivering three to six 200-mg pellets of unmodified testosterone provides stable, effective, and well-tolerated testosterone replacement for 4 to 6 months. Disadvantages include the need for a minor surgical procedure and the cumbersome delivery system, requiring the use of a large trocar, leading to a small risk of bleeding and infection. The major side effect is the extrusion of pellets, which occurs after ~8.5% of implant procedures. 7α-Methyl-19-nortestosterone (MENT), a long-acting sc implant, is currently under investigation. This is a modified androgen that can be aromatized to estrogen but not 5α-reduced. The relative sparing effects of MENT on the prostate, compared with testosterone, suggest that it may be useful for androgen therapy and for male contraception.

## E. Sublingual Testosterone

Testosterone cyclodextrin (2.5–5.0 mg given three times daily) is also being developed. Testosterone has been complexed with hydroxypropyl-β-cyclodextrin to enhance its solubility and sublingual absorption. Testosterone rises into the supraphysiological range in ~20 min and falls to baseline within 4–6 h. This may be useful for the treatment of delayed puberty, of postmenopausal women with reduced libido, and of elderly men with hypogonadism.

## III. ANDROGEN USE

The principal uses of androgens are for the treatment of adult male hypogonadism and for the treatment of delayed puberty.

### A. Treatment of Adult Male Hypogonadism

Before treating hypogonadal men, it is important to distinguish between primary hypogonadism (low testosterone with elevated LH and FSH concentrations) and secondary hypogonadism (low testosterone with serum LH and FSH concentrations that are low, or low normal), as shown in Table 1. In males with primary hypogonadism, androgen-dependent processes can be stimulated and maintained by exogenous hormone replacement; however, fertility cannot be induced by hormonal therapy. For secondary hypogonadism in males who are potentially fertile, there are two treatment options, exogenous androgens or exogenous gonadotropins; these hormones can stimulate spermatogenesis and endogenous androgen secretion until paternity is achieved.

The principle of testosterone replacement in the treatment of male hypogonadism is to induce and maintain secondary sexual characteristics, sexual behavior, muscle development, bone density, and male habitus. Testosterone will inhibit endogenous gonadotropin secretion and thereby suppress spermatogenesis, resulting in infertility. The preparations commonly used in the treatment of hypogonadism in adult males are shown in Table 2.

Traditionally, long-acting esters such as testosterone enanthate and testosterone cypionate have been classically used im every 2–3 weeks (in doses of 200–250 mg) for the treatment of adult hypogonadism. Their use has been safe and free of significant side effects, and they are currently the cheapest form of testosterone replacement. Patients can usually be taught to self-administer im injections, or a family member or friend may be trained to give them. The disadvantage of these esters is that they produce supraphysiological testosterone levels for 2–3 days after injection. The levels then decline slowly into the hypogonadal range prior to the next injection. In some men, these large fluctuations in testosterone levels result in undesired changes in mood, libido, and energy levels. This form of replacement therapy is not optimal because it does not mimic stable physiological levels and is associated with only minor diurnal variations. If patients have symptoms of androgen deficiency with documented low serum testosterone levels a few days before the next injection, the dosage interval can be reduced. In elderly hypogonadal men with symptoms of bladder outlet obstruction or enlarged prostate glands, prostate carcinoma must be excluded, and then it is recommended to commence therapy with 50–100 mg im every 2–4

**TABLE 1 Causes of Hypogonadism**

| Primary (hypergonadotropic) | Secondary (hypogonadotropic) | Combined (primary and secondary) |
|---|---|---|
| Klinefelter's syndrome | Panhypopituitarism | Aging |
| XX males | Hyperprolactinemia | Hepatic cirrhosis |
| XY/XO mixed gonadal dysgenesis | Isolated gonadotropin deficiency | Sickle cell disease |
| XYY syndrome | Kallman's syndrome and variants | |
| Ullrich–Noonan syndrome | Idiopathic hypothalamic hypogonadism | |
| Myotonic dystrophy | Isolated LH or FSH deficiency | |
| Sertoli-cell-only syndrome | Genetic disorders | |
| Enzymatic defects in testosterone biosynthesis | Prader–Willi syndrome | |
| Male pseudohermaphroditism (with AR defects or LH receptor defects) | Laurence–Moon–Biedl syndrome | |
| Testicular feminization | Severe systemic illness | |
| Viral orchitis (mumps most common) | Massive obesity | |
| Cryptorchidism | Nutritional deficiency or starvation | |
| Polyglandular autoimmune disease | Constitutional delay of puberty | |
| Testicular trauma | | |
| After chemotherapy or testicular irradiation | | |
| Congenital or acquired anorchia | | |

TABLE 2 **Preparations Commonly Used in the Treatment of Adult Male Hypogonadism**

| Preparation | Route | Dosage |
|---|---|---|
| **Currently approved** | | |
| Testosterone undecanoate (Restandol, Andriol) | Oral | 80–240 mg/day |
| Testosterone enanthate | Intramuscular | 200–250 mg every 2–3 weeks |
| Testosterone cypionate | Intramuscular | 200 mg every 2 weeks |
| Transdermal testosterone patch | | |
| (Testoderm) | Scrotal skin | 1 patch/day |
| (Androderm, Testoderm TTS) | Nonscrotal | 1–2 patches/day |
| Testosterone gel (Androgel 1%) | Skin | 50–100 mg/day |
| Dihydrotestosterone (Andractim) | Skin | 125–150 mg/day |
| Testosterone pellets | Subcutaneous implants | 3–6 doses of 200 mg every 6 months |
| **Investigational** | | |
| Testosterone undecanoate | Intramuscular | 1000 mg every 8–12 weeks |
| Testosterone buciclate | Intramuscular | 600 mg every 12 weeks |
| Testosterone cyclodextrin | Sublingual | 2.5–5.0 mg twice daily |
| Dihydrotestosterone 0.7% gel | Skin | 16–64 mg/day |
| **Obsolete** | | |
| 17 α-Methyltestosterone | Oral | 10–40 mg/day |
| Fluoxymesterone | Oral | 10–20 mg/day |

weeks. Prostate examination and a PSA level assay are recommended after 3, 6, and 12 months and then at yearly intervals for men <45 years old and at 6-month intervals for men >45 years old during testosterone replacement therapy. All patients should be informed about the expected somatic and behavioral changes that occur during treatment.

Transdermal testosterone applied to scrotal (Testoderm) and nonscrotal skin (Testoderm, Androderm) is now widely used for the treatment of male hypogonadism. However, some men find the scrotal application cumbersome, and in men with hypogonadism of prepubertal onset, the surface area of the scrotum may not be large enough to accommodate the patch. Androderm is applied once nightly to nonscrotal skin (e.g., abdomen, thighs, upper arm, or back) and the recommended dosage for adult hypogonadism is two 2.5-mg patches or one 5-mg patch. This regimen delivers 5 mg/day of testosterone, with 60% being absorbed within the first 12 h, maintaining serum testosterone levels in the physiological range and mimicking the normal circadian rhythm. The main side effect of Androderm is local skin irritation in ~30% of patients; however, pretreatment of the skin with triamcinolone cream reduces adverse skin reactions and only 10% of subjects have to discontinue therapy. Nonscrotal Testoderm TTS is associated with less skin irritation but does not always adhere well to the skin. Testosterone patches have the disadvantage of relatively limited flexibility in dosing and are 10 times more expensive than testosterone esters. In comparison with testosterone esters, the patches provide similar symptomatic relief of androgen deficiency; increase energy, libido, and sexual function; and improve BMD.

Daily administration of 50, 75, or 100 mg of 1% Androgel to hypogonadal men for 180 days results in constant testosterone levels in the middle to upper physiological range throughout the entire day. Androgel provides flexibility in dosing and little skin irritation. Within days or weeks of starting therapy, men will notice an increase in libido, improved energy levels, and sense of well being. Changes in physical appearance, including breaking of the voice, will evolve over 6 months. Almost all studies of androgen replacement therapy in hypogonadal men have shown a significant increase in hematocrit, due to stimulation of erythropoiesis, which can potentially cause symptoms of hyperviscosity, and a modest increase in prostate volume comparable to that seen in age-matched eugonadal men. Despite an increase in PSA levels within the normal range, there are no data to suggest a link between testosterone administration and the induction of prostate cancer, although longer term follow-up is necessary. However, once a carcinoma has developed, testosterone will promote its growth. The impact of androgens on lipids varies depending on the dose, route of administration, and whether aromatizable or nonaromatizable androgens are used. Most studies describe a decrease in total cholesterol with a decrease in high-density lipoprotein cholesterol. Hepatotoxicity is generally limited to oral androgens. Therefore, patients should be carefully monitored by physicians with expertise in this area for changes in hematocrit, liver function, lipid parameters, and PSA. The prostate should be evaluated by digital examination and by ultrasound to detect changes in volume or development of nodules or carcinoma. Absolute contraindications to testosterone use include prostate carcinoma and elevations in PSA.

## B. Treatment of Aging Males

Although elderly men with hypogonadism are treated like younger men according to the principles just described, it is currently disputed whether aging males in general should receive hormone substitution. Male aging is associated with a gradual, progressive decline in circulating testosterone concentrations. After decades of controversy, epidemiological studies clearly show that, in the general male population, total testosterone concentrations decrease by up to 1% per year. This decline in testosterone is due to a combination of a primary steroidogenic defect in the aging Leydig cells and a decreased pituitary secretory capacity and responsiveness to GnRH. Normal aging is associated with decreased lean body mass, muscle mass, and strength; diminished libido and erectile function; decreased bone mass; increased fracture risk; and diminished sleep quality. Controlled clinical trials of testosterone replacement in healthy older males with testosterone deficiency have demonstrated some equivocal moderate improvement in muscle mass and strength. However, the clinical and functional relevance of these changes and the risks of testosterone replacement therapy in elderly men in general remain to be determined.

## C. Treatment of Delayed Puberty in Boys

Androgen replacement therapy in male adolescents with constitutional delay of growth and adolescence has been shown to be beneficial psychologically as well as physiologically, and should be initiated promptly on diagnosis. Boys with delayed puberty are at risk for not obtaining adequate peak bone mass and for having deficiencies in developing social skills, an impaired body image, and low self-esteem. Younger boys with short stature, delayed bone age (at least 10.5 years), and delayed pubertal development in the absence of other endocrinological abnormalities can be treated with 50–100 mg of testosterone enanthate or cypionate (im, every 4 weeks for 3 months), whereas boys >13 years old may be treated with 250 mg (im, every 4 weeks for 3 months). After a 3-month “wait and see” period, another course of treatment may be offered if pubertal development does not continue. An increase in testes size is the most important indicator of spontaneous pubertal development, (testes volume >3 ml). Overtreatment with testosterone may result in premature closure of the epiphyses of long bones, resulting in reduced adult height. Therefore, treatment of patients who have not yet reached full adult height has to be undertaken carefully. At the beginning of therapy it is often difficult to distinguish between boys with delay of growth and puberty, who require only temporary androgen replacement, and boys with idiopathic hypogonadotropic hypogonadism, who require lifelong androgen therapy to stimulate puberty and to maintain adult sexual function. However, boys with permanent hypogonadotropic hypogonadism will not have testicular growth with androgen therapy. Because pubertal growth is a product of the interaction of growth hormone (GH) and insulin-like growth factor I(IGF-I) and the hypothalamic–pituitary–gonadal axis, boys with concomitant GH deficiency will require the simultaneous administration of GH and androgens for the treatment of delayed puberty.

## D. Treatment of Osteoporosis

Testosterone and its conversion to estradiol are important for the attainment of peak bone mass and for the maintenance of bone density. Delayed puberty is associated with a reduction in peak cortical and trabecular bone mass, and testosterone therapy is associated with an increase in BMD. Bone mass in men >20 years old declines linearly with age, and the decrease in trabecular mass is greater than that in cortical mass. Androgen deficiency is associated with osteopenia and osteoporosis and increased risk of vertebral and hip fractures. Administration of testosterone to hypogonadal men improves levels of bone turnover markers (causing a dampening of bone resorption) and causes increases in particular trabecular bone and also cortical BMD. However, it still remains unknown whether testosterone replacement reduces the fracture risk in osteoporotic hypogonadal men. Administration of androgens to eugonadal men with idiopathic osteoporosis increases lumbar spine BMD, with no change in the femoral neck, trochanter, or total hip. Further long-term studies are necessary to establish if testosterone therapy can be recommended for eugonadal men with idiopathic osteoporosis or glucocorticoid-induced bone loss.

## E. Treatment of Hereditary Angioneurotic Edema

Hereditary angioneurotic edema is characterized by markedly reduced levels of the serum C1 esterase inhibitor and by a syndrome of episodic angioedema of the skin and mucous membranes, commonly affecting the extremities, face, pharynx, and gastrointestinal tract. 17α-Alkylated androgens stimulate the production of C1 esterase inhibitor and can therefore be used prophylactically in the treatment of this condition. To minimize virilizing effects, danazol, a weak androgen, is used in women.

## F. Treatment of Hematological Disorders

Androgens enhance erythropoiesis via indirect stimulation of erythropoietin secretion from renal and extrarenal sources. Androgens also act directly on the bone marrow to increase red blood cell production in response to erythropoietin. They are therefore beneficial for the treatment of anemia due to chronic renal failure, raising hemoglobin levels by approximately 1–2 g/dl in the presence of adequate iron and folate stores. With the availability of recombinant erythropoietin, androgens are now used infrequently for the treatment of anemia associated with chronic renal failure, especially in women, due to virilizing side effects. However, erythropoietin is very expensive and androgens can be used in combination with lower doses of erythropoietin. Androgens can also be beneficial to treat patients with aplastic anemia, Fanconi's anemia, hemolytic anemia, and sickle cell anemia, and some anemia secondary to hematological malignancies. Danazol (600–800 mg/day) has been used to treat patients with autoimmune hemolytic anemia and idiopathic thrombocytopenia purpura by directly lowering autoantibody titers.

## G. Micropenis and Microphallus

Enlargement of a micropenis or microphallus can be achieved by treatment with 25–50 mg of testosterone enanthate or cypionate (im, every 3 to 4 weeks for 3 months) or with 1.25–5% testosterone cream, 5% DHT cream, or 10% testosterone propionate cream (twice daily for 3 months). High-dose androgen therapy may be necessary to achieve some adrogenization in male pseudohermaphroditism due to 5α-reductase deficiency and certain androgen receptor defects.

## H. Experimental Use of Androgens

### *1. Male Contraception*

Hormonal male contraceptives include exogenously administered testosterone alone or in combination with progestogens or GnRH analogues. Gonadotropin secretion is inhibited, resulting in inhibition of spermatogenesis. Androgens are essential to inhibit gonadotropin secretion and to replace endogenously suppressed testosterone. Clinical trials using intramuscular testosterone injections or subcutaneous testosterone implants in combination with oral, intramuscular, or subcutaneously implanted gestagens are under way and result in high rates of azoospermia, the prerequisite for effective contraceptive protection. Self-administered oral or transdermal regimes have been less successful in inducing azoospermia.

### *2. Treatment of Wasting Syndromes*

Recent studies have shown that replacement doses of testosterone in hypogonadal men and supraphysiological doses in eugonadal men increase fat-free mass, muscle size, and strength. It has therefore been proposed that androgens may be useful in conditions associated with muscle wasting (sarcopenia) and protein catabolism. Interventions that prevent or reverse the sarcopenia associated with aging, human immune deficiency virus (HIV) infection, cancer, chronic illness, major surgery, or burns could result in an improved quality of life, improved disease outcomes, and reduced health care costs.

Hypogonadism occurs in approximately 30% of HIV-infected men. Therapy with supraphysiological doses of testosterone, nandrolone decanoate, or oxandrolone can benefit HIV-infected men and women with wasting and/or low levels of circulating androgen. However, it remains unclear whether physiological testosterone replacement can produce clinically meaningful changes in quality of life, independent living, body composition, and muscle function in HIV-infected patients.

### *3. Overtall Stature*

In some European countries supraphysiological doses of testosterone (500 mg of testosterone enanthate, im, every 2 weeks for 1–4 years until epiphyseal closure) have been used to treat constitutional tall stature in boys (predicted adult height of 205 cm). Because tall stature is often desirable, this treatment is very rarely indicated. Follow-up studies have shown that this treatment has no long-term negative effect on testicular development.

### 4. *Androgen Therapy in Women*

Evidence is emerging that testosterone plays a physiological role in female brain development, sexual function, mood, cognitive function, and well being. Premenopausally, 50% of testosterone is secreted directly by the ovaries and an equal amount is produced by the peripheral conversion of androstenedione and dehydroepiandrosterone (DHEA, produced in the ovary and the adrenals). Testosterone levels decline with increasing age in women during the reproductive years such that levels in women aged 40 years are only 50% of those in women aged 21. This decline in androgen levels in females appears to be largely associated with increased age, rather than being linked to menopause. DHEA sulfate (DHEAS) levels fall linearly with age, and probably contribute to the decline of their main metabolite, testosterone. The clinical indications for testosterone replacement therapy in women include adrenal insufficiency and premature ovarian failure, including Turner's syndrome, premenopausal iatrogenic androgen deficiency (surgical menopause, chemotherapy, irradiation), and symptomatic deficiency following natural menopause. Estrogen replacement therapy further reduces free testosterone levels and thereby may enhance the symptoms of androgen deficiency (i.e., reduced libido and sexuality). These symptoms are improved with androgen replacement therapy. Testosterone is usually recommended in combination with estrogen. Examples of preparations used in studies include oral esterified estrogen–methyltestosterone, combined estradiol (E2) and subcutaneous testosterone implants, transdermal matrix delivery of testosterone, and intramuscular nandrolone decanoate, tibolone, and stanazol. For the treatment of adrenal insufficiency, 50 mg/day of DHEA for months increased both androstenedione and testosterone levels, improved the sense of well being, and increased the frequency of sexual thoughts, interest, and satisfaction. With regard to osteoporosis, the combined administration of testosterone and estrogen increases trabecular and cortical BMD in postmenopausal women to a greater extent compared to estrogen alone. However, because there are no prospective data confirming a reduction in fracture risk with this therapy, it cannot currently be recommended for protecting against bone fracture. Side effects include masculinization, hirsutism, acne, temporal balding, deepening of the voice, and hepatotoxicity (particularly if 17α-alkylated steroids are used). The effects on body composition, lipids, risk of coronary heart disease (CHD), and breast cancer remain unclear.

## IV. ANDROGEN ABUSE

The observation that androgens promote nitrogen retention and muscle mass led to their use to improve physical performance as early as the 1940s. Androgenic anabolic steroids (AASs) have been classically used by weight lifters, bodybuilders, and hammer and discus throwers, but today their use is more prevalent, extending to football players, swimmers, and track-and-field participants (both male and female). AAS abuse is also prevalent in peripubertal boys to enhance athletic performance and to improve appearance. In one survey in the United States, 7% of male high school seniors reported having used AAS, and the majority had started at or before the age of 16 years. AASs are most frequently distributed illegally to athletes via a "black market." Androgen sales due to increasing misuse and abuse in the United States are reported to be increasing by 20–30% each year.

It still remains controversial as to whether androgens improve athletic performance. It is known that androgens have muscle-growth-promoting effects in boys, women, and hypogonadal men. The assumption that exogenous androgens have less effect on muscle growth in normogonadal men is based on the fact that the androgen receptor in striated muscle is down-regulated after puberty and is nearly completely saturated, within physiological concentrations of testosterone. Therefore, the biological response may well have reached a plateau within the physiological range. Nandrolone, the 19-nor analogue of testosterone, was the first compound to show enough myotrophic–androgenic dissociation in animal experiments to justify its introduction in clinical therapy as an anabolic steroid. Supraphysiological doses of testosterone enanthate (600 mg/week) for 10 weeks, with or without resistance exercise training, have been shown to increase fat-free mass, muscle size, and muscle strength in normal men. The effects of exercise were shown to be additive. However, the long-term effects on muscle strength and the side effects of such doses remain unknown. Some of the reported enhancement in performance may have been due to the effects of anabolic steroids in increasing aggression and competitiveness, to differences in intensity of training or dietary intake, and to important placebo effects. The problem with studying the effects of androgens in athletes is that the athletes often take multiple anabolic agents simultaneously ("stacking"). Frequently, the regimen starts with lower doses, with doses progressively increasing over a period of several weeks. The most frequently misused androgens in controlled competition sports are testosterone, nandrolone, stanozolol, and metandienone. Doses used may be 100 times the doses used for hormone replacement therapy.

Although no systematic studies have been performed (the difficulty being that many preparations are abused simultaneously), the androgenic adverse effects of anabolic steroids are known to include virilization (e.g., acne, hirsutism, and alopecia, sometimes irreversible) in women and premature epiphyseal closure with stunted final height, gynecomastia, decreased testicular volume, impaired spermatogenesis resulting in infertility (reversible), increased sexual interest, and decreased HDL and cholesterol in men. The toxic effects of anabolic steroids established from single reports include (1) cardiovascular effects, including cardiomyopathy, acute myocardial infarct, cerebral vascular accident, and pulmonary embolism, (2) hepatic effects, including cholestatic jaundice, peliosis hepatitis, and tumors, and (3) psychological effects, including increased aggression, dysphoria, psychosis, addiction, withdrawal-like symptoms, and depression; a fourth negative impact relates to needle sharing, i.e. hepatitis and HIV infection.

Because the misuse of androgens is clearly an escalating problem, there has been a marked increase in urine testing prior to many athletic events. Many athletes previously stopped the administrations of AASs in the last few weeks before a competition and changed to androgens that are difficult to detect in the urine or those that are rapidly metabolized and excreted within a few days (e.g., stanozolol and testosterone). These deceptions were termed “precompetition bridging programs” in former Eastern Block countries. Today, the introduction of out-of-competition testing has improved the detection rate of androgen misuse. Analytically, testosterone administration is much more difficult to detect compared to doping with synthetic steroids. The ratio of testosterone to epitestosterone (an inactive metabolite of testosterone secreted largely from the gonads, with only small amounts produced from the peripheral metabolism of testosterone) is used to detect exogenous androgen misuse. The normal ratio is approximately 1:1 in men and women. A ratio of 6:1 is considered to be indicative of steroid abuse. However, competitors are known to titrate their dose to below the testosterone:epitestosterone ratio of 6, or to coadminister epitestosterone in an attempt to normalize their testosterone:epitestosterone ratio. Direct methods such as gas chromatography/combustion/carbon isotope ratio mass spectrometry are currently available to identify unambiguously doping with exogenous steroids. Administration of human chorionic gonadotropin (hCG) will stimulate testicular steroidogenesis without altering the testosterone:epitestosterone ratio. However, hCG can be detected by immunoassay in the serum and urine. Synthetic androgens are extensively metabolized and doping tests are focused on urinary metabolites. Derivatization methods for steroid analysis improve the detection limits for anabolic steroids. The potential risks of high-dose anabolic steroids clearly outweigh their potential benefits.

## Glossary

**anabolic androgenic steroids** Synthetic derivatives of testosterone having pronounced anabolic properties and relatively weak androgenic properties.

**dehydroepiandrosterone** The most abundant human steroid hormone and its sulfated derivative are produced by the adrenal gland (approximately 30 mg/day), and are universal precursors for androgenic and estrogenic steroids. Conversion and metabolism occur in the peripheral tissues.

**dihydrotestosterone** Testosterone is metabolized via 5α-reductase to the potent androgen dihydrotestosterone, which binds directly to the androgen receptor and cannot be aromatized to estrogen.

**testosterone** The major androgenic steroid hormone produced by the Leydig cells of the testes (~ 7 mg/day in an adult man).

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor Crosstalk with Cellular Signaling Pathways • Androgen Receptor-Related Pathology • Androgen Receptor Structure and Function • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Sex Hormones and the Immune System • Steroid Hormone Receptor Family: Mechanisms of Action**

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# Angiogenesis

Lena Claesson-Welsh
*Uppsala University, Sweden*

I. INTRODUCTION
II. FORMATION OF BLOOD VESSELS
III. BLOOD VESSEL COMPOSITION
IV. ANGIOGENIC GROWTH FACTORS
V. MOLECULAR MECHANISMS IN BLOOD VESSEL FORMATION
VI. CLINICAL THERAPIES TO SUPPRESS OR STIMULATE ANGIOGENESIS
VII. PERSPECTIVES

**Physiological processes such as embryonic development, wound healing, and menstruation are known to require the formation of new blood vessels to meet increased needs for oxygen and nutrients. Pathological processes involving growth of tissues, such as tumors and chronic inflammatory diseases, also depend on the blood vessel supply.**

## I. INTRODUCTION

A number of different growth factors are implicated in blood vessel formation and one of these, vascular endothelial growth factor (VEGF), is a focus of attention due to its critical and specific role both in vasculogenesis (establishment of the vascular tree during embryogenesis) and in angiogenesis (formation of new vessels from preexisting vessels). A number of approaches have been taken to inhibit the function of VEGF in order to inhibit angiogenesis and thereby halt the progress of diseases such as cancer.

## II. FORMATION OF BLOOD VESSELS

During growth and development of the embryo, there is a continuously increasing need for oxygen and nutrients. This metabolic need, together with genetic programming, results in the maturation of endothelial cells from stem cells, the angioblasts. The maturation probably occurs in discrete steps, but much work remains to be done until this process is understood completely. Once formed, the endothelial cells assemble into crude vascular plexa with coarse tubes, which are remodeled into finer vessels. The "*de novo*" establishment of the vascular tree is denoted vasculogenesis. Interruption of vasculogenesis results in

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# Angiogenesis

LENA CLAESSON-WELSH
*Uppsala University, Sweden*

I. INTRODUCTION
II. FORMATION OF BLOOD VESSELS
III. BLOOD VESSEL COMPOSITION
IV. ANGIOGENIC GROWTH FACTORS
V. MOLECULAR MECHANISMS IN BLOOD VESSEL FORMATION
VI. CLINICAL THERAPIES TO SUPPRESS OR STIMULATE ANGIOGENESIS
VII. PERSPECTIVES

**Physiological processes such as embryonic development, wound healing, and menstruation are known to require the formation of new blood vessels to meet increased needs for oxygen and nutrients. Pathological processes involving growth of tissues, such as tumors and chronic inflammatory diseases, also depend on the blood vessel supply.**

## I. INTRODUCTION

A number of different growth factors are implicated in blood vessel formation and one of these, vascular endothelial growth factor (VEGF), is a focus of attention due to its critical and specific role both in vasculogenesis (establishment of the vascular tree during embryogenesis) and in angiogenesis (formation of new vessels from preexisting vessels). A number of approaches have been taken to inhibit the function of VEGF in order to inhibit angiogenesis and thereby halt the progress of diseases such as cancer.

## II. FORMATION OF BLOOD VESSELS

During growth and development of the embryo, there is a continuously increasing need for oxygen and nutrients. This metabolic need, together with genetic programming, results in the maturation of endothelial cells from stem cells, the angioblasts. The maturation probably occurs in discrete steps, but much work remains to be done until this process is understood completely. Once formed, the endothelial cells assemble into crude vascular plexa with coarse tubes, which are remodeled into finer vessels. The "*de novo*" establishment of the vascular tree is denoted vasculogenesis. Interruption of vasculogenesis results in the death of the embryo. In the later stages of development and during adulthood, new blood vessels are formed from already existing blood vessels, by migration of endothelial cells away from the mother vessel, followed by cell division and organization into a new vessel. The formation of blood vessels from existing blood vessels is known as angiogenesis. Recent findings also indicate that the formation of new blood vessels depends on or at least involves circulating, bone marrow-derived precursor cells.

Angiogenesis is important in wound healing and for the normal growth of tissues, such as during the later stages of embryo development. Growth of the endometrium during the menstrual cycle and the process of ovulation are accompanied by angiogenesis. In recent years, there has been an increased awareness of the role of angiogenesis in a number of diseases. These include diseases that are characterized by the presence of too many blood vessels, such as cancer, chronic inflammatory diseases, retinopathy, and psoriasis. In these conditions, the tissues secrete an increased amount of angiogenic growth factors. There are also conditions characterized by a deficiency in the blood vessel supply. Such conditions include coronary and peripheral ischemia.

## III. BLOOD VESSEL COMPOSITION

Blood vessels occur in three principal variants, arteries, veins, and capillaries. All vessels contain a single layer of endothelial cells, which face the lumen of the vessel. Arteries transport the oxygenated blood from the heart throughout the body. The main artery, the aorta, branches out into finer vessels (arteriolae). In arteries, several layers of contractile smooth muscle cells, which are important for maintaining the blood pressure, surround the endothelial cell layer. Arterioles branch into a plexa of thread-thin vessels, called capillaries. The exchange of oxygen and nutrients occurs through the capillary wall. In addition, new blood vessels are formed from capillaries and not from larger vessels. Capillaries are composed of a single layer of endothelial cells, surrounded by a specialized coat containing extracellular matrix proteins (laminin, fibronectin, collagen, perlecan, etc.), called the basement membrane. Pericytes, which are supporting cells, may be embedded in the basement membrane or rest on the membrane. The function of pericytes is not clear, but it has been suggested that both pericytes and the basement membrane are critical for the stability of the capillary.

Different types of capillaries can be distinguished (Fig. 1). Continuous capillaries, which occur in the skin, muscles, lungs, and central nervous system, have a low permeability, due to the presence of tight junctions between the cells. The tight junctions are specialized structures that bridge adjacent cells and keep them in close contact. A specialized variant of these junctions, the blood–brain barrier, occurs in the central nervous system. Fenestrated capillaries are found in endocrine glands, kidney, intestine, and other tissues where large amounts of fluid or metabolites enter or leave capillaries. Discontinuous capillaries or sinusoids are found in the liver, spleen, and bone marrow. These contain gaps between endothelial cells that are wide enough to allow large proteins to cross the capillary wall.

The capillaries fuse into venules and then into larger vessels, veins, which transport the blood back to the heart and lungs for renewed oxygenation. Similar to arteries, veins have a muscular coat, although it is somewhat thinner. The pressure is lower in veins than in arteries, and therefore, the lumen of veins is often collapsed on sectioning and histological analysis of the tissue. The veins of the limbs contain paired valves, which ensure that the blood does not move backward.

It is generally thought, but has not yet been formally proven, that endothelial cells from different

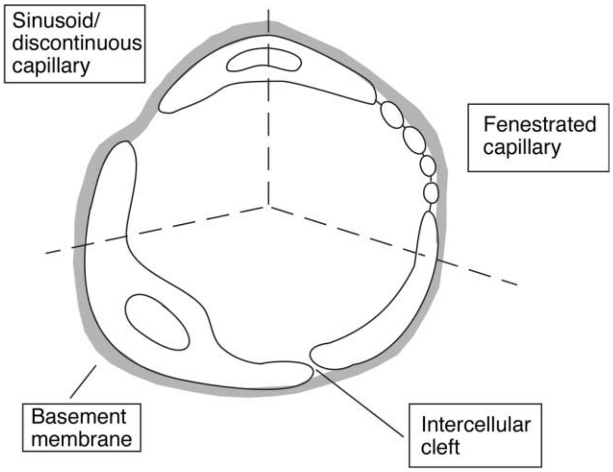

**FIGURE 1** Schematic illustration of a capillary composed of endothelial cells surrounded by the basement membrane (shaded). Different types of capillaries are indicated, such as sinusoid and fenestrated capillaries, which have gaps of different sizes allowing the flow of proteins (sinusoid) and fluid and metabolites (fenestrated). In the continuous capillaries, individual cells meet in the intercellular clefts.

types of vessels, such as arteries, veins, and capillaries, have different properties. These distinct properties cannot be distinguished morphologically when endothelial cells are explanted and cultured *in vitro*. During development, arterial and venous specification can be traced before the onset of circulation by their specific expression of the growth factor ephrin-B2 and its receptor ephB-4 on primitive arteries and veins, respectively.

## IV. ANGIOGENIC GROWTH FACTORS

Many growth factors have been implicated in the stimulation of angiogenesis. Some of these are considered to act directly and some indirectly, depending on whether or not receptors for these growth factors have been detected on endothelial cells. Growth factors bind to receptors that belong to a class of proteins called the tyrosine kinases. Thus, binding of the growth factor to the extracellular domain of the receptor leads to the activation of an enzymatic activity that is harbored in the intracellular domain of the receptor. This enzymatic activity, the tyrosine kinase activity, catalyzes the transfer of a phosphate group to particular tyrosine residues, either within the receptor molecule itself or on cytoplasmic proteins, the so-called signal transduction molecules. In many cases, these signal transduction molecules are enzymes themselves, and phosphorylation on tyrosine residues leads to their activation. In this manner, a signal is propagated in the cell, which eventually results in a cellular response of some kind, such as migration or division.

The directly acting endothelial growth factors include the vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) family and the angiopoietins, for which there are receptors on endothelial cells. However, there may not be receptors for these factors on all kinds of endothelial cells in, e.g., arteries, veins, and capillaries, or on endothelial cells in vessels with specialized functions such as in capillaries forming the blood–brain barrier. Furthermore, receptors may not be expressed on endothelial cells at all different developmental stages.

The VEGF family currently encompasses six members, and for several of these members, there are a number of splice variants that may have distinct functions. The first identified VEGF family member was originally denoted VPF, but is now most commonly referred to as VEGF-A. However, the original nomenclature, VPF, indicates what is perhaps the most important function of this factor, namely, to induce vascular leakage. VEGF-A is produced by many cells in the body in a manner that is dependent on the oxygen tension in the tissue. Thus, the promoter element in the VEGF-A gene contains a binding site for the hypoxia-induced factor. Solid tumors often have a central hypoxic core, which leads to increased VEGF-A production by the tumor cells and therefore increased angiogenesis. Genetically unstable cells, such as tumor cells, may also start to produce VEGF-A or other angiogenic growth factors as a consequence of chromosomal rearrangements that may lead to altered regulation of growth factor production. As a rule, tumors can grow only up to a certain size, a few $mm^3$, unless they start producing angiogenic growth factors, i.e., VEGF-A. This restriction point, the angiogenic switch, must be overcome to allow further tumor growth. This is the rationale for the interest in developing therapies for treatment of cancer based on inhibition of VEGF function.

The additional members of the family are VEGF-B, -C, and -D and placenta growth factor. The sixth family member is VEGF-E, which thus far has been detected only as a virus-encoded protein and not in mammalian cells. None of the VEGF family members, except for VEGF-A, appear to be regulated by hypoxia. The VEGF family members bind in a particular pattern to three receptors, which belong to the receptor tyrosine kinase family. Two VEGF receptors, VEGF receptor-1 and -2, are expressed in vascular endothelial cells, whereas VEGF receptor-3 is expressed in endothelial cells in lymph vessels. The essential role of VEGF-A and the VEGF receptors in vascular development has been demonstrated genetically, in experiments in which the individual genes for VEGF-A or the three receptors have been destroyed. Mice lacking expression of VEGF-A and VEGFR-2 die at approximately embryonic day 8.5 due to the lack of blood vessels. Mice lacking VEGFR-1 die slightly later. In these animals, the endothelial cell pool is enlarged and the cells fill the lumen of the vessels, rendering them nonfunctional. Elimination of VEGFR-3 leads to embryonal death due to abnormal vessel formation.

Tyrosine phosphorylation sites in the VEGF receptors have been identified and to some extent signal transduction molecules interacting with these sites have also been identified. A theme appears to be that the signaling molecules bind with low affinity to the phosphorylated tyrosine residues. Phospholipase C$\gamma$1 has been shown to be an important signaling molecule downstream of VEGFR-2. These types of studies are complicated by the fact that the properties of VEGFR-2-mediated signal transduction appear to be correctly revealed only when primary endothelial cells are studied, rather than the more convenient standard tissue culture models based on transformed cells. In addition, VEGFR-2 signal transduction appears to be channeled through or modified by a number of co-receptors, such as the neuropilins, heparan sulfated proteoglycans, integrins, and cell–cell adhesion molecules.

The Tie family of receptor tyrosine kinases and their ligands, the angiopoietins, are indispensable for normal embryonic blood vessel development, by their critical function in the regulation of angiogenic remodeling and vessel stabilization. All known angiopoietins bind to one of the two known Tie receptors, Tie2. Interestingly, the Tie2 ligands have opposing actions on endothelial cells. Accordingly, angiopoietin-1 and -4 function as activating ligands for Tie2, whereas angiopoietin-2 and -3 behave as competitive antagonists. Activation of Tie2 facilitates the association between endothelial cells and adjacent supporting cells and leads to decreased permeability of the vessel, even in the presence of VEGF-A.

Other growth factors are assumed to act indirectly on endothelial cells, but in most cases, the mechanisms involved are unresolved. The exception may be platelet-derived growth factor-BB (PDGF-BB), which is known to act on supporting smooth muscle cells such as pericytes. The endothelial cells produce PDGF-BB, and the PDGF β-receptor is expressed on the pericytes. There are also growth stimulatory loops in the other direction, as the smooth muscle cells produce angiopoietins, for which there are receptors on the endothelial cells.

How fibroblast growth factor (FGF), hepatocyte growth factor, epidermal growth factor, and other growth factors act on endothelial cells is unclear. It is possible that an important mechanism for the angiogenic effect of these growth factors is the stimulation of VEGF-A production. It is also possible that endothelial cells in certain circumstances, for example, during development or in pathological conditions such as in tumors, express receptors for these growth factors.

## V. MOLECULAR MECHANISMS IN BLOOD VESSEL FORMATION

A large array of molecules regulate vasculogenesis, as can be inferred from the results of gene inactivation of many different classes of proteins, which affect vascular development in different ways. Apart from growth factors and their receptors such as the VEGF/VEGFR family and the angiopoietin/Tie receptor family described above, other important proteins are required, for example, growth modulatory factors such as the transforming growth factor-β family of ligands and receptors, the cell–cell adhesion protein vascular endothelial cadherin, and different second-messenger proteins. Nuclear factors such as the Id1–Id3 complex, which exert their effects by regulating the binding of helix-loop-helix factors to DNA, are essential for proper vascular development.

Angiogenesis involves the stimulation of endothelial cells in capillaries by growth factors produced by the adjacent tissue or released by, e.g., platelets. Another possible growth factor source is a nearby, growing tumor. Morphologically, angiogenic capillaries are composed of a stalk, where the endothelial cells proliferate. The cells in the tip of the stalk do not proliferate, but send out cytoplasmic extensions (sprouts) toward the source of the stimulus. Proliferation of endothelial cells in response to growth factor stimulation is probably regulated in a manner similar to that shown for other cell types and involves progression through the cell cycle, which eventually leads to cell division. The first important step in this process is the binding of the growth factor to its receptor, leading to activation of the receptor tyrosine kinase and recruitment of signaling molecules. To start the cell cycle, specific sets of signaling molecules interact in a chain reaction, involving the small, guanine triphosphate-hydrolyzing (GTPase) molecule Ras, which eventually results in changes in the transcriptional machinery and induction of cell cycling. It has been questioned whether Ras is critical for the proliferation of VEGF-A-stimulated endothelial cells; it is possible that the signaling chain leading to proliferation in this case is dependent on phospholipase Cγ1 rather than Ras. Thus, it is possible that the design of signaling chains is specific to endothelial cells.

The cells in the tip of the angiogenic stalk are in the process of differentiation to form a new lumen-containing vessel, which is dependent on changes in how the cytoskeleton is arranged. There are abundant data indicating that cytoskeletal regulation in cell types other than endothelial cells involves Ras-related GTPases of the Rho family. It is likely that these proteins are also very important in endothelial cells. Much work remains to be done to dissect the roles of other signal transduction molecules activated by angiogenic growth factors that stimulate angiogenesis. A considerable problem in the field is the lack of appropriate models for endothelial cell differentiation. Studies based on microarray techniques in which gene expression in endothelial cells forming vessel-like structures in a three-dimensional extracellular matrix was compared to that in proliferating endothelial cells indicate that thousands of genes are specifically induced during endothelial cell differentiation. These genes encode proteins with diverse functions and include proteases and novel gene products.

## VI. CLINICAL THERAPIES TO SUPPRESS OR STIMULATE ANGIOGENESIS

The steadily increasing number of clinical trials to test the effects of drugs targeted to regulate the vascular bed demonstrates the enormous potential of basic research in this area. As discussed above, pathological conditions involving the vascular bed may be characterized by either too many or too few vessels. Clinical trials aimed at alleviating ischemic conditions, such as coronary ischemia that may precede heart infarction, are based on the delivery of VEGF-A and other growth factors to the ischemic site, to allow ingrowth of new blood vessels. The mode of delivery may be based on different types of vectors, such as novel forms of adenovirus vector, naked DNA, or purified protein.

Drugs aimed at suppressing angiogenesis are targeted to inhibit the function of growth factors or growth factor receptors (Fig. 2) or inhibit endothelial cell function in an unknown manner (see NIH Cancer Website at http://www.cancer.gov/clinical_trials/doc). The targeted growth factor is in most cases VEGF-A, although the importance of inhibiting the function of a broader spectrum of growth factor receptors, such as FGF, PDGF, as well as VEGF-A receptors, has also been stressed.

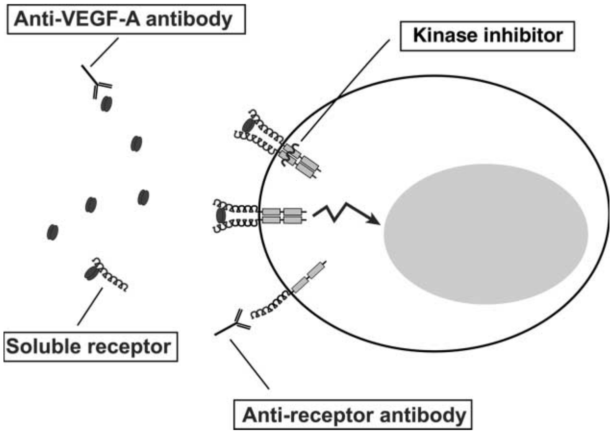

FIGURE 2 Schematic illustration showing different strategies for inhibiting the effect of VEGF-A on an endothelial cell, such as the administration of neutralizing anti-VEGF antibodies, kinase inhibitors that block the activation of VEGF receptors, soluble receptors that bind and neutralize VEGF, and anti-receptor antibodies that block VEGF binding to the receptor.

## VII. PERSPECTIVES

The increasing awareness of the role of blood vessel formation in diseases, together with refined tools to study endothelial cells, has led to an explosion in the number of studies about angiogenic growth factor function in health and disease. The many ongoing clinical trials of drugs aimed at suppressing or enhancing blood vessel formation point to the difficulties in reestablishing vascular balance once it is lost. Many different proteins are involved in regulating this balance, and modulating just one protein, such as VEGF-A, is not sufficient. Another emerging complication is that the growth factors thought to be specific for endothelial cells are also found on other cell types. Thus, it is striking that many proteins are shared between endothelial cells and neuronal cells.

## Glossary

**angiogenesis** Formation of new blood vessels from existing capillaries.

**capillary** The smallest-diameter vessel in the body; origin of new vessels.

**endothelial cells** Cells that line the inner face of a blood vessel.

**vascular endothelial growth factor** A protein acting on endothelial cells to induce vascular leakage; it is of critical importance for vasculogenesis and angiogenesis.

**vascular permeability factor** Another name for vascular endothelial growth factor.

**vasculogenesis** Establishment of the vascular system during embryonic development.

## See Also the Following Articles

**CXC Chemokines • Erythropoietin, Genetics of • Flt3 Ligand • Stem Cell Factor • Vascular Endothelial Growth Factor B (VEGF-B) • Vascular Endothelial Growth Factor D (VEGF-D)**

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# Angiotensins

Nadheige Lochard and
Timothy L. Reudelhuber
*Clinical Research Institute of Montreal*

I. INTRODUCTION
II. ALTERNATE BIOSYNTHETIC PATHWAYS AND ADDITIONAL ANGIOTENSIN PEPTIDES
III. TISSUE SOURCES OF ANGIOTENSIN PEPTIDES
IV. CONCEPT OF A HYBRID CIRCULATING/TISSUE RAS
V. PROPOSED TISSUE-SPECIFIC ROLES OF ANGIOTENSIN PEPTIDES
VI. CONCLUSION

**In 1898, Tigerstedt and Bergman first made a correlation between hypertension and a soluble component in kidney extracts. This pioneering work and that of many others eventually led to the biochemical description of the renin–angiotensin system, an enzymatic cascade that results in the synthesis of a family of vasoactive peptides called angiotensins. The activity of this system plays a key role in the regulation of blood pressure and fluid volume in mammals, and its inhibition has become a primary tool for the treatment of hypertension and, more recently, myocardial infarction in North America.**

## I. INTRODUCTION

All of the components necessary for the production of angiotensin peptides are found in the circulation (Fig. 1). Juxtaglomerular cells in the kidney release the aspartyl protease renin, as well as its enzymatically inactive precursor prorenin, into the circulation. Once there, renin acts on its only known substrate, the 60,000 Da liver-derived glycoprotein angiotensinogen, releasing the decapeptide angiotensin (1–10) (Ang I) from the amino terminus of angiotensinogen. Ang I is in turn converted to the octapeptide angiotensin (1–8) (Ang II) by the removal of two carboxy-terminal amino acids by angiotensin-converting enzyme (ACE). ACE is a metalloprotease bound to the plasma membrane of endothelial cells throughout the circulation. Ang II is the principal effector of the renin–angiotensin system (RAS) through its interaction with angiotensin (AT) receptors type 1 and type 2 ($AT_1$ and $AT_2$), members of the seven-transmembrane G-protein-coupled receptor

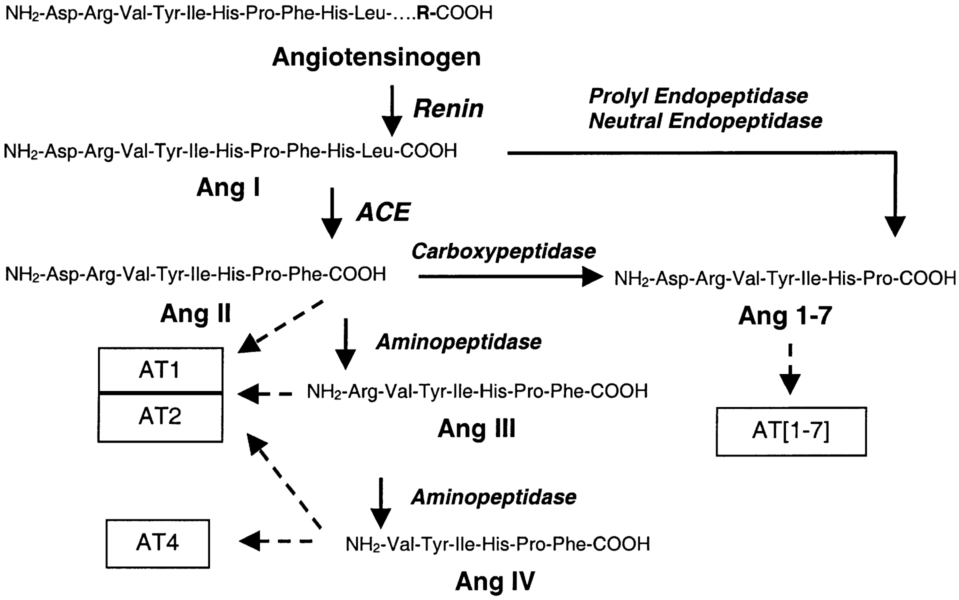
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-....R-COOH
Angiotensinogen
Renin
Prolyl Endopeptidase
Neutral Endopeptidase
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Ang I
ACE
Carboxypeptidase
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH
Ang II
Ang 1-7
Aminopeptidase
AT1
AT2
NH2-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Ang III
AT[1-7]
Aminopeptidase
AT4
NH2-Val-Tyr-Ile-His-Pro-Phe-COOH
Ang IV

FIGURE 1 Generation of angiotensin peptides (Ang) from angiotensinogen and activation of various angiotensin receptors (AT) by angiotensin II and its metabolites.

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# Angiotensin II Receptor Signaling

ALESSANDRO M. CAPPONI
*University Hospital, Geneva*

I. INTRODUCTION
II. ANGIOTENSIN II RECEPTOR SUBTYPES
III. COUPLING MECHANISMS
IV. INTRACELLULAR EFFECTORS COUPLED TO $AT_1$ RECEPTORS
V. SIGNALING THROUGH THE $AT_2$ RECEPTOR SUBTYPE
VI. SUMMARY

**Angiotensin II, an octapeptide hormone, is the product of two successive enzymatic proteolytic cleavages. Angiotensin II is produced in blood and in some tissues by the action of renin and angiotensin-converting enzyme on angiotensinogen (an $\alpha_2$-globulin) and angiotensin I (a decapeptide), respectively. Initially described as a hypertensive factor, angiotensin II has proved to be a remarkably pleiotropic peptide. It exerts pressor effects by acting peripherally (in vascular smooth muscle, adrenal cortex, and kidney tissues) as well as centrally, but is also involved in the regulation of metabolic pathways (in liver and adipose tissues), inflammatory responses (by macrophages), tissue growth, and developmental processes.**

## I. INTRODUCTION

In spite of the spectrum of different responses elicited by angiotensin II in a host of distinct tissues, only two major angiotensin (Ang II) receptor subtypes, $AT_1$ and $AT_2$, have been identified to date. The vast majority of the biological functions of the hormone are mediated through the $AT_1$ receptor subtype, whereas the $AT_2$ subtype is essentially involved in developmental and remodeling processes. Clearly, therefore, the specificity of the response within a given tissue or cell type cannot be conferred by the receptor but must rely on the intracellular signaling mechanisms activated beyond the receptor. In this respect, signal transduction events following binding of the hormone to the $AT_1$ receptor have been extensively studied and are fairly well understood; less is known of the downstream events triggered by activation of the $AT_2$ subtype.

## II. ANGIOTENSIN II RECEPTOR SUBTYPES

Angiotensin II receptor subtypes were first characterized pharmacologically in the early 1990s, when nonpeptidic Ang II antagonists were developed that were able to bind to Ang II receptors and block differentially Ang II binding, depending on the tissue examined. A few years later, the $AT_1$ subtype was first identified, followed by the $AT_2$ subtype, both by expression cloning. Another receptor subtype, $AT_4$, mainly located in the brain and involved in memory retention processes, has been recently cloned and identified to be insulin-regulated aminopeptidase (IRAP). By binding to IRAP, the angiotensin (3–8)-hexapeptide and its analogues may inhibit IRAP and thus prolong the half-life of some neuropeptides. Although the existence of other atypical Ang II receptors has also been reported, their characterization is still incomplete.

Both the $AT_1$ (359 amino acids) and the $AT_2$ (363 amino acids) subtypes are classic seven-transmembrane-domain receptors. They share only 32–34% sequence homology. Although they display the same binding affinity for Ang II, they discriminate among nonpeptidic Ang II antagonists, losartan being the archetypal blocker for the $AT_1$ subtype and the PD123177 compound being, among others, specifically recognized by the $AT_2$ receptor subtype.

The two Ang II receptor subtypes also differ in their tissue distribution. $AT_1$ receptors are found throughout life in all tissues involved in the cardiovascular actions of Ang II, i.e., adrenal cortex, brain, liver, kidney, and vascular smooth muscle. In contrast, $AT_2$ receptors are highly expressed in various tissues in the fetus but their level of expression decreases considerably after birth; in adults they are found only in a few organs, e.g., brain, adrenal gland, or heart. Interestingly, $AT_2$ receptor expression increases in certain tissues (heart, vasculature, and kidney) after injury and wound healing.

## III. COUPLING MECHANISMS

The $AT_1$ receptor subtype belongs to the ever-growing family of G-protein-coupled receptors (GPCRs). The message carried by the hormone that binds to the receptor is transduced to intracellular effectors via heterotrimeric guanosine triphosphate (GTP)-binding proteins (G-proteins) composed of three subunits, $\alpha$, $\beta$, and $\gamma$. For each of these subunits, various isoforms have been described, and it is the cell-, receptor-, and effector-specific assembly of one particular $\alpha$, $\beta$, and $\gamma$ combination that is responsible for conveying the information to a given effector. Interestingly, the $AT_1$ receptor can couple to more than one G-protein, even within the same cell type. For example, in adrenal glomerulosa cells, which make aldosterone in response to Ang II, the $AT_1$ is coupled to phospholipase C-$\beta$ (see later) via a $G_{q11}$-protein and to L-type calcium channels via a $G_i$-protein and/or a $G_o$-protein. Similarly, in vascular smooth muscle, activation of phospholipase C-$\beta$1 is mediated through a G-protein of the $G_{\alpha q11/\beta\gamma}$ and/or $G_{\alpha 12/\beta\gamma}$ isoform. Besides G-protein-mediated activation of intracellular effectors, direct physical interaction of the $AT_1$ receptor with either an effector—mainly various cytosolic tyrosine kinases—or an adapter protein can also occur, essentially via the C-terminal cytoplasmic tail of the receptor. This interaction then triggers kinase cascades within the cell.

The $AT_2$ receptor displays structural features that are related to those of GPCRs and, indeed, it appears to couple to G-proteins of the inhibitory type ($G_i$). In contrast to the $AT_1$ receptor, the major effectors of $AT_2$-mediated angiotensin II action are tyrosine or serine/threonine phosphatases.

## IV. INTRACELLULAR EFFECTORS COUPLED TO $AT_1$ RECEPTORS

Occupancy of the $AT_1$ receptor subtype triggers most known signaling processes, leading to activation of various phospholipases and recruitment of the calcium messenger system and/or to stimulation of numerous kinase cascades (Fig. 1).

### A. Phospholipases

Phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol, is the best known effector enzyme activated by angiotensin II (Ang II). Depending on the cell type (adrenal glomerulosa cell, vascular smooth muscle cell) and the enzyme subtype, this occurs through coupling to a $G_{q/11}$-protein (for PLC-$\beta$2) or through tyrosine phosphorylation mediated by the cytoplasmic tyrosine kinase $pp60^{c\text{-}src}$ (for PLC-$\gamma$1). The activation of PLC is generally rapid and transient. In contrast, in adrenal glomerulosa cells and in vascular smooth muscle cells, Ang II also activates a less rapid generation of diacylglycerol through hydrolysis of phosphatidylcholine by phospholipase D. This response is sustained and displays slower kinetics.

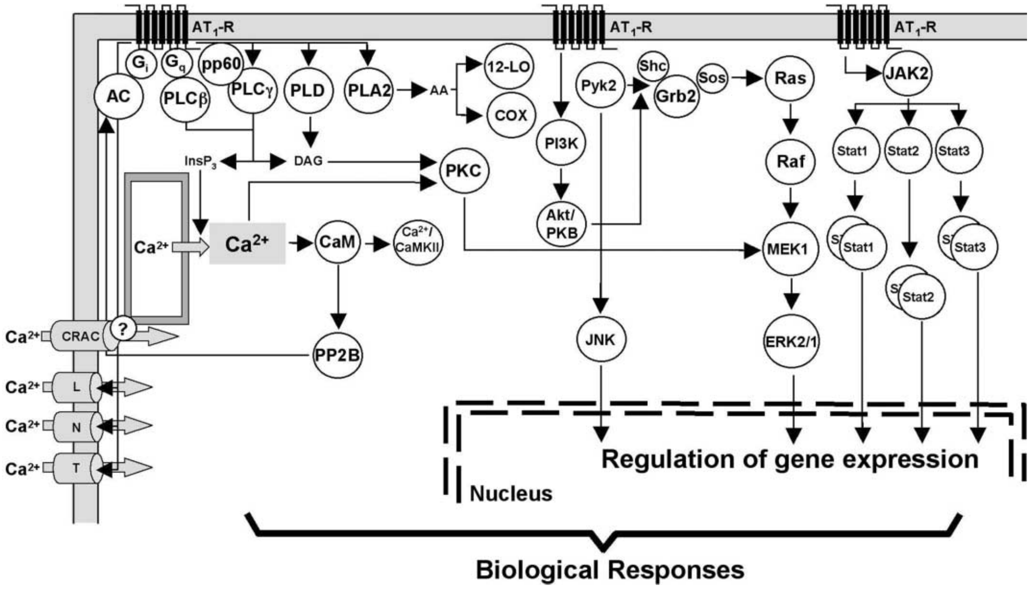

**FIGURE 1** Intracellular signaling mechanisms coupled to the AT$_1$ receptor (AT$_1$-R) subtype. The main signaling pathways described in this article are illustrated, but most cells do not express all of these pathways. AA, arachidonic acid; AC, adenylyl cyclase; CaM, calmodulin; CaMKII, Ca$^{2+}$/calmodulin-dependent kinase II; COX, cyclooxygenase; CRAC, calcium release-activated calcium channel; DAG, *sn*-1,2-diacylglycerol; ERK2/1, extracellular signal-regulated kinases 2 and 1; G$_q$ and G$_i$, GTP-binding protein of the q11 and i family; InsP$_3$, inositol 1,4,5-trisphosphate; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; Shc, Src homology and collagen protein; Grb2, growth factor receptor-binding protein 2; L, N, and T, voltage-operated calcium channel of the L-, N-, and T-types; 12-LO; 12-lipoxygenase; MEK1, mitogen-activated protein kinase kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PP2B, phosphoprotein phosphatase 2B (calcineurin); Pyk2, proline-rich tyrosine kinase 2; Raf, Raf protein kinase; Ras, small G-protein belonging to the Ras family of G-proteins; Sos, son of sevenless; Stat, signal transducer and activator of transcription.

The result of the combined activation of phospholipases C and D is a rapid and transient increase in intracellular inositol 1,4,5-trisphosphate, superimposed over a biphasic, sustained production of diacylglycerol. These two metabolites will in turn activate the calcium messenger system and protein kinase C, respectively (see later). Phospholipase A2, which releases arachidonic acid from membrane phospholipids, is also a major effector of Ang II action in various cell types. Arachidonic acid then enters either the cyclooxygenase pathway to be converted into prostaglandins, as in vascular smooth muscle and mesangial cells, or the 12-lipoxygenase pathway, to give rise to metabolites such as 12-hydroxyeicosatetraenoic acid (12-HETE).

## B. The Calcium Messenger System

Although the signaling mechanisms mediating Ang II action have long remained elusive, once tools became available to record changes in intracellular calcium homeostasis, the hormone soon became an archetype for calcium-mobilizing agents. What is now known of the initial steps of this process is indeed an exemplar of phenomena that occur practically in every cell type.

The inositol 1,4,5-trisphosphate (InsP$_3$) that is produced on activation of PLC binds to tetrameric receptors that are located on some compartments of the endoplasmic reticulum. These receptors function as calcium storage organelles and are actually channels that open on binding of InsP$_3$, allowing calcium efflux into the cytosol. InsP$_3$ binding to its receptor is inhibited by elevated concentrations of cytosolic calcium, $[Ca^{2+}]_c$, so that, once a substantial amount of calcium has been emptied from the stores, InsP$_3$ receptor channels will close again. Through mechanisms that are still poorly understood, the emptying of intracellular calcium stores activates a "capacitative" influx of calcium from the extracellular medium, through so-called calcium release-activated calcium (CRAC) channels, which will replenish the stores. In addition to activating CRAC channels, Ang II also modulates the activity of various voltage-gated membrane calcium channels (of the L-, N-, and T-types), either directly, through cell membrane depolarization, or indirectly, via G-proteins or via protein kinases or phosphatases. This control can be either positive (channel activation) or negative (channel inhibition).

The net result of these numerous impacts of Ang II on regulators of calcium fluxes is a dramatic alteration of intracellular calcium homeostasis. In most cases, this can be observed with fluorescent probes in intact cells, as repetitive spikes of $[Ca^{2+}]_c$, often described as oscillations. At the subcellular level, videomicroscopy imaging reveals that these oscillations actually correspond to localized, confined rises of $[Ca^{2+}]_c$ and are sometimes seen propagating through the cell as "waves" or "tides."

Special mention should be made here of the adrenal glomerulosa cell. Because most enzymes that are required for the biosynthesis of aldosterone are located in the mitochondrial matrix, this organelle appears as a most likely target for the calcium messenger system. Indeed, the changes in $[Ca^{2+}]_c$ elicited by Ang II are relayed and even amplified within the mitochondrial matrix. Concomitantly, there is an increased supply of cholesterol precursor to the mitochondrial steroidogenic machinery, through an increased expression and mitochondrial importation of the steroidogenic acute regulatory (StAR) protein, a crucial factor for the rate-limiting step in the steroidogenic cascade.

## C. Protein Kinase C

The biphasic, sustained elevation in the membrane of *sn*-1,2-diacylglycerol resulting from the activation of phospholipases C and D on Ang II challenge contributes to the recruitment and anchoring to the cell membrane of various isoforms of the calcium-sensitive, phospholipid-dependent protein kinase C (PKC). A rapid and marked redistribution of PKC enzyme and activity from the cytosol to the cell membrane is observed, for example, in adrenal glomerulosa cells and vascular smooth muscle cells after Ang II stimulation. The $AT_1$ receptor bears consensus PKC phosphorylation sites in its sequence and PKC-mediated phosphorylation of these sites results in receptor desensitization. However, the identity and nature of the substrates that contribute to the downstream induction of biological responses to Ang II (steroidogenesis, vasoconstriction, etc.) following PKC phosphorylation remain mostly unknown.

## D. Kinase Cascades

As in most intracellular signaling systems, various kinases are mobilized as a consequence of the initial events following Ang II binding to the $AT_1$ receptor. These kinases can be activated either by intracellular messengers generated through the induction of a first series of effectors (phospholipases, membrane channels, etc.) or by protein-protein interactions.

### 1. $Ca^{2+}$/Calmodulin-Dependent Kinase II

The rises in $[Ca^{2+}]_c$ elicited by Ang II in practically all major target cells of the hormone are sensed by the ubiquitous calcium-binding protein, calmodulin. Once it has bound four atoms of calcium, calmodulin becomes able to interact with, among others, $Ca^{2+}$/-calmodulin-dependent kinase II (CaMKII), a major transducer of extracellular signals to functional cellular responses. In adrenal glomerulosa cells, in which the involvement of CaMKII has been most extensively studied, the enzyme is clearly involved in Ang II-induced modulation of voltage-gated calcium channel activity.

### 2. Extracellular Signal-Regulated Kinases

Activation of serine-threonine extracellular signal-regulated kinases [ERK2/1 or p44/p42 mitogen-activated protein (MAP) kinases] is one of the earliest events that occurs following Ang II challenge. This has been shown in numerous cell types, such as adrenal glomerulosa cells, cardiac myocytes and fibroblasts, vascular smooth muscle cells, and mesangial cells. The response is usually transient, peaking within 5 to 10 min and decreasing thereafter. The p38 MAP kinase, another member of the MAP kinase family, undergoes activation in response to Ang II, following the same pattern. Although pharmacological studies with selective inhibitors have clearly demonstrated that MAP kinase activation is a necessary prerequisite to the induction of a full biological response to Ang II, the protein mediators that are phosphorylated by MAP kinases remain to be identified in most target cells.

MAP kinases are activated by phosphorylation of adjacent threonine and tyrosine residues. In the case of Ang II action in adrenal glomerulosa cells, cardiac myocytes, and vascular smooth muscle cells, this activation has been shown to occur through a proximal signaling module linking the activated receptor to the small GTPase, Ras, via an Src family kinase, which phosphorylates the adapter molecule Shc. The latter then binds to the Grb-Sos complex and activates Ras. The more distal signaling cascade linking Ras to MAP kinase activation involves several kinases that are sequentially activated: activated Ras interacts with and activates the serine-threonine kinase Raf, which in turn phosphorylates and activates the dual-specificity kinase MEK1. Once activated, MEK1 will phosphorylate the adjacent threonine and tyrosine residues in ERK2/1 or p42/p44. Moreover, in vascular smooth muscle cells, an alternative Raf-independent mechanism involving a specific isoform of PKC, PKCζ, can be recruited by Ras to stimulate MEK1 and ERK2/1.

#### 3. *Signal Transducers and Activators of Transcription (JAK–Stat Pathway)*

In vascular smooth muscle cells and in cardiomyocytes—where, in addition to its specific effects, Ang II also plays the role of a growth factor—ligand binding to the $AT_1$ receptor activates members of a family of intracellular tyrosine kinases, the Janus kinases (JAKs). Specifically, the occupied receptor associates with JAK2, which then autophosphorylates, becomes activated, and phosphorylates proteins of a family of signal transducers and activators of transcription (Stat), in particular Stat1, Stat2, and Stat3. Once phosphorylated, Stat proteins form homo- or heterodimers and translocate to the nucleus, where they bind to specific DNA response elements and stimulate the transcription of the c-*fos* gene, for example, an important factor in cell proliferation.

#### 4. *Phosphoinositide 3-Kinase*

In adrenal glomerulosa cells as well as in vascular smooth muscle cells, Ang II has been clearly shown to activate yet another kinase, phosphatidylinositol 3-kinase (PI3-K), and its effector, Akt/protein kinase B, which may represent, besides PKC, one of the upstream elements linking the binding of Ang II to its GPCR receptor to activation of the Ras–Raf–ERK2/1 cascade.

#### 5. *Pyk2 Tyrosine Kinase and C-Jun N-Terminal Kinase*

Activation of tyrosine kinases also appears to play an important role in Ang II signaling. In particular, in vascular smooth muscle cells, the proline-rich tyrosine kinase 2 (Pyk2) has been shown to act as an upstream regulator of the activation by Ang II of both ERK2/1 and yet another kinase, the c-Jun N-terminal kinase (JNK). These kinases may be involved in the growth and vascular remodeling responses elicited by the hormone.

#### 6. *Tyrosine Phosphatases*

Kinase activation is often coupled to the concomitant induction of phosphatases, as a counterregulatory mechanism. This is indeed the case for Ang II, which mobilizes the $Ca^{2+}$/calmodulin-dependent protein phosphatase, calcineurin, and possibly other serine-threonine phosphatases in adrenal glomerulosa cells, as well as phosphotyrosine phosphatase 1D.

### E. Adenylyl Cyclase

Ang II is known to modulate cAMP production, either through a $G_i$-mediated inhibition of adenylyl cyclase or through potentiation of the activity of a $Ca^{2+}$-sensitive adenylyl cyclase involving Ang II-induced capacitative $Ca^{2+}$ influx.

## V. SIGNALING THROUGH THE $AT_2$ RECEPTOR SUBTYPE

The $AT_2$ receptor subtype plays its major roles in developmental processes and in vascular, cardiac, and renal remodeling. It is now fairly established that the $AT_2$ receptor subtype couples to G-proteins, a characteristic shared by most seven-transmembrane-domain receptors, although in an atypical way (Fig. 2).

### A. Phosphoprotein Phosphatases

The binding of Ang II to its $AT_2$ subtype receptors leads to activation of tyrosine or serine-threonine phosphatases, three of which have been identified: SH2-domain-containing phosphatase 1 (SHIP-1), protein phosphatase 2A (PP2A), and mitogen-activated protein kinase phosphatase 1 (MKP-1). With distinct substrate specificities and kinetics, all these phosphatases mediate the inactivation of the ERK pathway that is observed with $AT_2$ stimulation in various cells types and that often leads to growth inhibition or to an apoptotic response.

### B. Nitric Oxide Synthase

Some effects of Ang II mediated by the $AT_2$ receptor subtype involve the activation of nitric oxide (NO) synthase and generation of NO, which will in turn activate a cytosolic guanylyl cyclase and lead to cyclic guanosine monophosphate (cGMP) production. The latter messenger may participate with the signaling mechanisms responsible for the observed natriuretic effect observed with $AT_2$ receptor stimulation.

### C. Phospholipase A2

In epithelial cells of the renal proximal tubule and in cardiac myocytes, $AT_2$ receptor activation triggers

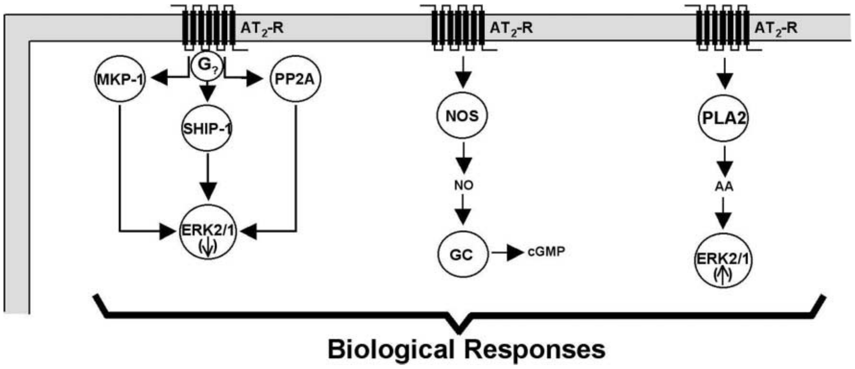

**FIGURE 2** Intracellular signaling mechanisms coupled to the $AT_2$ receptor ($AT_2$-R) subtype. The main signaling pathways described in this article are illustrated, but most cells do not express all of these pathways. AA, arachidonic acid; cGMP, cyclic GMP; ERK2/1, extracellular signal-regulated kinases 2 and 1; GC, soluble guanylyl cyclase; MKP-1, mitogen-activated protein kinase phosphatase 1; NO, nitric oxide; NOS, nitric oxide synthase; PLA2, phospholipase A2; PP2A, protein phosphatase 2A; SHIP-1; SH2-domain-containing phosphatase 1.

the generation of arachidonic acid through stimulation of PLA2. This represents an unusual signaling pathway because arachidonic acid targets and activates the MAP kinase cascade as a downstream effector, in contrast to the mostly inhibitory effects of $AT_2$ on MAP kinases observed in numerous other cellular systems.

## VI. SUMMARY

The intracellular signaling mechanisms triggered by the octapeptide hormone angiotensin II that lead to its pleiotropic effects in various target cells are mediated by two receptor subtypes, $AT_1$ and $AT_2$. $AT_1$ receptor activation is responsible for the vast majority of the cardiovascular effects of Ang II and is associated, via G-proteins, with an intricate network of calcium- and phosphorylation-sensitive effector systems. The $AT_2$ receptor is predominantly involved in developmental processes and is mainly coupled to intracellular phosphatases.

## Glossary

**aldosterone** The main salt-retaining hormone; a steroid synthesized from cholesterol in the zona glomerulosa of the adrenal cortex under the control of three principal physiological activators: angiotensin II, extracellular potassium, and adrenocorticotropic hormone.

**cytosolic calcium concentration $[Ca^{2+}]_c$** The concentration of free calcium ions found in the cytosol; maintained in resting cells at levels 10,000 times lower than those occurring in the extracellular medium (0.1 $\mu M$ vs 1–2 m$M$). Changes of $[Ca^{2+}]_c$ during stimulation serve as intracellular signaling processes.

**CRAC channels** Calcium release-activated calcium channels located in the cell membrane; CRAC channels are activated on release of calcium from intracellular stores, thus contributing to the replenishment of these stores.

**losartan** Nonpeptidic, selective antagonist of the $AT_1$ receptor subtype; widely used to block cardiovascular effects of angiotensin II.

**renin** Proteolytic enzyme that is released by the kidney in response to a drop in blood pressure, sympathetic nerve activity, or decreased sodium excretion; generates angiotensin I in the circulation by cleaving the 10 N-terminal amino acids of a large $a_2$-globulin produced by the liver, angiotensinogen.

## See Also the Following Article

**Angiotensins**

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# Anorexia Nervosa

See *Eating Disorders*

# Anti-Diuretic Hormone (ADH)

See *Vasopressin*

# Anti-Inflammatory Actions of Glucocorticoids

ANN-CHARLOTTE WIKSTRÖM, JAN-ÅKE GUSTAFSSON, AND SAM OKRET

*Karolinska Institutet, Huddinge, Sweden*

I. INTRODUCTION
II. GENE REGULATION BY GLUCOCORTICOID HORMONES
III. NONGENOMIC EFFECTS
IV. ENDOGENOUS GLUCOCORTICOIDS AS ANTI-INFLAMMATORY AGENTS
V. GLUCOCORTICOIDS AND SPECIFIC CYTOKINES
VI. GLUCOCORTICOID REPRESSION OF GENES INVOLVED IN INFLAMMATORY RESPONSES
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IX. GLUCOCORTICOIDS AS THERAPEUTIC AGENTS
X. ADVERSE EFFECTS OF GLUCOCORTICOID TREATMENT
XI. DEVELOPMENT OF GR LIGANDS THAT DISSOCIATE ACTIVITIES

**Glucocorticoid hormones serve as important regulators of inflammatory responses. They act to repress inflammation by inhibiting an array of inflammatory mediators, such as adhesion molecules, prostaglandins, cytokines, and cytokine receptors, as well as by inducing apoptosis of certain inflammatory effector cells. Glucocorticoid effects are mediated mainly by transrepressive genomic mechanisms acting via the intracellular glucocorticoid receptor, and, to a lesser extent, possibly also by nongenomic mechanisms.**

## I. INTRODUCTION

Glucocorticoids (GCs) regulate and suppress nuclear factor κB (NF-κB), a major proinflammatory mediator. Synthetic GCs are often used as drugs for immunosuppression and for treating inflammatory and allergic conditions. However, adverse side effects following therapeutic use can diminish the clinical efficacy of GCs, and thus future development of more selective ligands is desirable.

## II. GENE REGULATION BY GLUCOCORTICOID HORMONES

Glucocorticoids play a key role in regulating diverse physiological processes such as metabolism, salt and water balance, cellular proliferation and differentiation, inflammation, and immune responses. The anti-inflammatory and immunosuppressive actions of GCs are essential; inflammatory reactions with loss of GC production proceed in an uncontrolled way and can lead to death. The GC signal is transduced via the glucocorticoid receptor (GR), which regulates the expression of a subset of genes in both a promoter- and a tissue-specific manner. The general mechanism of GC action involves passive uptake and binding of GC to the GR, which is localized in the cytoplasm. Binding of the hormone to the receptor leads to a conformational change, dissociation of associated chaperone proteins, and translocation into the nucleus, where the GR directly or indirectly (through other transcription factors) binds to DNA in target genes. This results in enhancement or repression of gene transcription.

Stimulation of gene transcription by the GR most often involves binding of a homodimer of the hormone–receptor complex to one or more so-called GC-responsive elements (GREs), which consist of two more or less palindromic hexamers spaced by three nucleotides, usually located in the promoter

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# Angiotensins

NADHEIGE LOCHARD AND
TIMOTHY L. REUDELHUBER
*Clinical Research Institute of Montreal*

I. INTRODUCTION
II. ALTERNATE BIOSYNTHETIC PATHWAYS AND ADDITIONAL ANGIOTENSIN PEPTIDES
III. TISSUE SOURCES OF ANGIOTENSIN PEPTIDES
IV. CONCEPT OF A HYBRID CIRCULATING/TISSUE RAS
V. PROPOSED TISSUE-SPECIFIC ROLES OF ANGIOTENSIN PEPTIDES
VI. CONCLUSION

**In 1898, Tigerstedt and Bergman first made a correlation between hypertension and a soluble component in kidney extracts. This pioneering work and that of many others eventually led to the biochemical description of the renin–angiotensin system, an enzymatic cascade that results in the synthesis of a family of vasoactive peptides called angiotensins. The activity of this system plays a key role in the regulation of blood pressure and fluid volume in mammals, and its inhibition has become a primary tool for the treatment of hypertension and, more recently, myocardial infarction in North America.**

## I. INTRODUCTION

All of the components necessary for the production of angiotensin peptides are found in the circulation (Fig. 1). Juxtaglomerular cells in the kidney release the aspartyl protease renin, as well as its enzymatically inactive precursor prorenin, into the circulation. Once there, renin acts on its only known substrate, the 60,000 Da liver-derived glycoprotein angiotensinogen, releasing the decapeptide angiotensin (1–10) (Ang I) from the amino terminus of angiotensinogen. Ang I is in turn converted to the octapeptide angiotensin (1–8) (Ang II) by the removal of two carboxy-terminal amino acids by angiotensin-converting enzyme (ACE). ACE is a metalloprotease bound to the plasma membrane of endothelial cells throughout the circulation. Ang II is the principal effector of the renin–angiotensin system (RAS) through its interaction with angiotensin (AT) receptors type 1 and type 2 ($AT_1$ and $AT_2$), members of the seven-transmembrane G-protein-coupled receptor

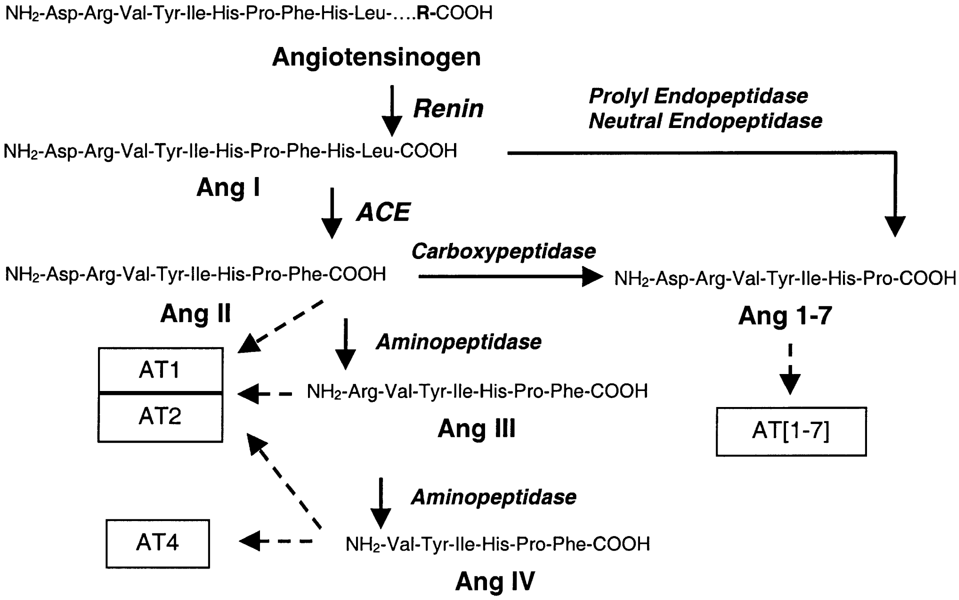
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-....R-COOH
Angiotensinogen
Renin
Prolyl Endopeptidase
Neutral Endopeptidase
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Ang I
ACE
Carboxypeptidase
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH
Ang II
Ang 1-7
Aminopeptidase
AT1
AT2
NH2-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Ang III
AT[1-7]
Aminopeptidase
AT4
NH2-Val-Tyr-Ile-His-Pro-Phe-COOH
Ang IV

FIGURE 1 Generation of angiotensin peptides (Ang) from angiotensinogen and activation of various angiotensin receptors (AT) by angiotensin II and its metabolites.

family. A vast majority of the cardiovascular effects of Ang II are mediated by the $AT_1$ receptors. This activity represents the peripheral (classical) components of the RAS.

## II. ALTERNATE BIOSYNTHETIC PATHWAYS AND ADDITIONAL ANGIOTENSIN PEPTIDES

In the formation of Ang II, there may exist alternate biosynthetic pathways that have important physiological functions: Hussain and colleagues have described an enzyme called chymase, which may partially substitute for ACE in the generation of Ang II in certain tissues, including the heart. In addition, other proteases, such as cathepsin D and tonin, exhibit renin-like activity *in vitro*, leading to the suggestion that Ang II could be synthesized in some tissues by a renin-independent mechanism. Recent studies by Fukamizu and colleagues lend support to this possibility: mice in which the renin gene has been inactivated by homologous recombination are unable to generate angiotensin peptides in the circulation, but still make them in the brain.

Indeed, although Ang II is the major product of the classical pathway of the RAS, this pathway also leads to the formation of other angiotensin peptides that may have biological roles distinct from those of Ang II (Fig. 1). For example, Ang I can be converted by prolyl endopeptidase (EC 3.4.21.26) and various neutral endopeptidases (NEPs) (EC 3.4.24.11, EC 3.4.24.15, and EC 3.4.24.26) to the heptapeptide Ang (1–7). The abundance of Ang (1–7) increases dramatically in animals treated with ACE inhibitors, and it has been proposed to have its own receptor through which it mediates vasodilation. Ang(1–7) has also been reported to bind and inhibit ACE, which could contribute to its role in vasodilation by acting as a natural inhibitor of the RAS. The heptapeptide Ang (2–8), or Ang III, is produced by the action of aminopeptidase A on Ang II. Aminopeptidases can act also on Ang I to generate Ang (2–10), which can in turn be converted into Ang III by ACE. Ang III binds to $AT_1$ and $AT_2$ receptors, although with reduced affinity. Ang II injected directly into the brain has been associated with increases in blood pressure, the stimulation of sympathetic nervous system (SNS) activity, stimulation of vasopressin release, and an increase in water and sodium appetite. These effects of Ang II in brain are mediated by the $AT_1$ receptor. Ang III in the brain can also elicit many of these responses, although higher doses are required. Because Ang III can interact with $AT_1$ receptors and because the brain is particularly rich in aminopeptidases, it is possible that the biologically active angiotensin peptide in brain is Ang III. Interestingly, coinjection of Ang II with aminopeptidase inhibitors dramatically reduces the response to this peptide, raising the additional possibility that Ang III is acting through a signaling pathway distinct from that used by Ang II. Aminopeptidases can further cleave Ang III to generate the hexapeptide Ang (3–8), or Ang IV. Ang IV has also been proposed to bind to a specific receptor ($AT_4$) distinct from $AT_1$ and $AT_2$ with a variety of resulting actions, including increasing cerebral and renal blood flow. Interestingly, very recent data suggest that the $AT_4$ receptor may in fact be an aminopeptidase and that the biological effects of Ang IV may be secondary to inhibition of this enzyme. Ang IV can be further degraded into smaller peptides with currently unknown functions, if any.

## III. TISSUE SOURCES OF ANGIOTENSIN PEPTIDES

For many years the biological actions of the RAS were believed to be solely due to the activity of the circulating RAS components. However, in recent years evidence has accumulated that angiotensin peptides are synthesized within tissues, where they might have local actions that are distinct from the known cardiovascular effects of the RAS. These observations have changed the way that RAS is evaluated and impact the clinical management of RAS activity. The components necessary for the formation and action of Ang II have been detected in a number of tissues:

### A. Brain and Pituitary

Although angiotensins do not readily cross the blood–brain barrier, all of the components of the RAS, including Ang II receptors, have been identified in brain, with the possible exception of renin. Interestingly, the fetal brain is particularly rich in $AT_2$ receptor, which virtually disappears after birth. The pituitary has a high concentration of Ang II per gram of tissue, which has bolstered the suggestion of local angiotensin synthesis. In the pituitary, the mRNA for renin colocalizes with Ang II in the cells (gonadotropes) that produce luteinizing hormone, whereas angiotensinogen is found in a different and as-yet unidentified cell type.

### B. Heart and Blood Vessels

Although the messenger RNAs for angiotensinogen, ACE, and $AT_1$ and $AT_2$ receptors are expressed in the heart, the local synthesis of renin by the heart remains quite controversial. Angiotensin receptors have been described on both cardiac myocytes and fibroblasts. In blood vessels, ACE can be found in abundance on endothelial cells, and angiotensinogen is particularly abundant in the surrounding adipose tissue. Interestingly, although renin activity is easily measured in the lumen of blood vessels, its mRNA there is difficult to demonstrate.

### C. Kidney

In addition to releasing renin into the circulation, the kidney also contains all the necessary components for the local generation of Ang II. Angiotensinogen is synthesized in the epithelial cells of the proximal tubule. ACE is present throughout the renal vasculature and Ang II receptors are found on both the basolateral and lumenal membranes of renal tubules as well as on renal blood vessels. The finding that the concentration of Ang II in renal interstitial and tubular fluid is hundreds to thousands of times higher than that found in the plasma also supports the model of local angiotensin peptide synthesis in the kidney.

### D. Adrenal Gland

The adrenal gland is the tissue with the highest content of Ang II per gram of wet weight. Angiotensinogen mRNA is present in fibroblast-like cells adjacent to the adrenal capsule and in the adrenal cortex and medulla. ACE is present in the adrenal medulla and also at lower levels in the adrenal cortex. The majority of renin is present within the outer adrenal cortex and renin mRNA is present within the steroid-producing adrenocortical cells. Both Ang II $AT_1$ and $AT_2$ receptors are expressed in the outer adrenal cortex. Interestingly, the renin gene is expressed at higher levels in the fetal adrenal gland of the mouse than in any other tissue, raising the possibility that it is somehow important during fetal development.

### E. Reproductive Organs

Ang I and Ang II and $AT_1$ receptors have been localized on Leydig cells and sperm tails. The testis expresses a unique but fully functional form of ACE (a hemienzyme) in the lumenal wall of the epididymis. All of the soluble components of RAS have been found in ovarian follicular fluid. Granulosa and theca cells contain renin and angiotensinogen and the ovary secretes large amounts of prorenin and renin, particularly during pregnancy. Prorenin, found in placental fluid in very large amounts, increases in concentration as pregnancy proceeds to term.

## IV. CONCEPT OF A HYBRID CIRCULATING/TISSUE RAS

The synthesis of angiotensin peptides requires the concerted actions of two enzymes (renin and ACE) on the substrate angiotensinogen. In addition, renin is synthesized as an inactive precursor, and to be activated it must be modified by the precise proteolytic removal of its prosegment by an as-yet unidentified enzyme (or enzymes). These components must also meet in the appropriate compartments to be active. This presents a mechanistic problem, as can be illustrated by considering the cellular distribution of the components of the brain RAS. Although ACE is widely distributed in the brain, angiotensinogen is made in astrocytes and Ang II immunoreactivity has been detected primarily in neurons. Two hypotheses have been proposed to explain these findings. Angiotensinogen might be released by the astrocyte in the extracellular space, where it is converted into Ang II and subsequently internalized in neurons after binding to its specific receptors. The other possibility is that angiotensinogen is taken up by the neurons, metabolized to Ang II inside the cells, and released at the nerve endings to act on receptors in adjacent areas. In fact, there is evidence to suggest that angiotensinogen, renin, Ang I, and Ang II can be taken up by several tissue types to be further metabolized locally for the generation of bioactive peptides (Fig. 2). Specific acceptor proteins have been described for both renin and its precursor prorenin in a number of tissues. These may have the role of concentrating renin on the cell surface for local action or perhaps may be involved in renin internalization for the intracellular generation of peptides. Evidence has also accumulated that angiotensins can be formed within certain cell types for later release. For example, renin, ACE, and Ang II can all be detected in juxtaglomerular cells of the kidney afferent arteriole. Treatment of animals with ACE inhibitors causes the accumulation of Ang I in these cells, suggesting that they may contain a complete intracellular RAS pathway. Thus, it is difficult (and perhaps not so important) to distinguish between a

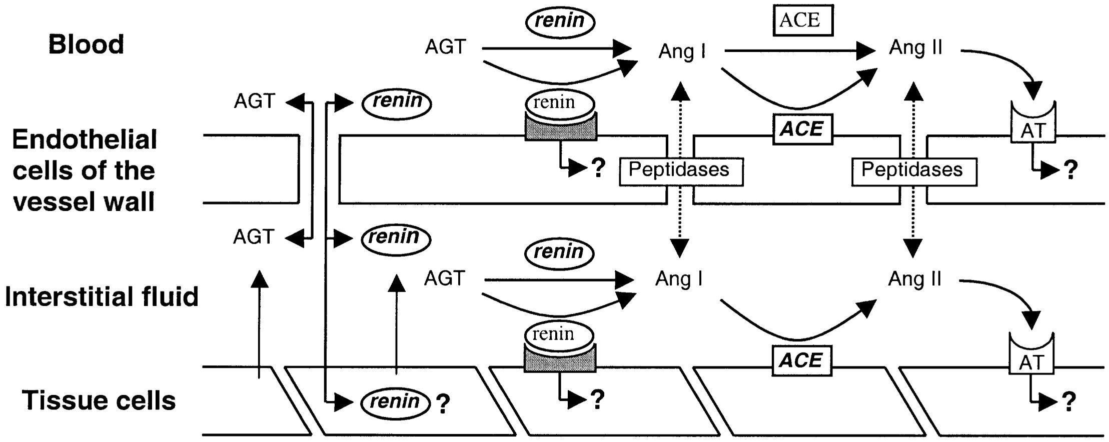

FIGURE 2 Alternative pathways of angiotensin (Ang) I and II production in tissues. Angiotensinogen (AGT), renin, Ang I, and Ang II either traverse the endothelial cell layer to the interstitial space or are produced locally to react with other components of the RAS. Renin may also bind to acceptor proteins on certain cells and either acts on the cell surface or is internalized. Ang I and II may also be metabolized by peptidases while passing through the vascular wall. Tissue Ang I and Ang II generation may occur not only in the interstitial fluid or on the cell surface, but also within cells. Binding of Ang II to the $AT_1$ receptor is followed by intracellular signaling.

circulating and a local RAS, because many tissues are capable of using both locally synthesized and "imported" RAS components to complete angiotensin peptide synthesis locally.

## V. PROPOSED TISSUE-SPECIFIC ROLES OF ANGIOTENSIN PEPTIDES

Ang II acts on numerous tissues to affect salt and water balance and blood pressure. Tissue-specific actions of Ang II include the stimulation of drinking behavior and salt appetite (by actions on the brain), facilitation of sympathetic transmission in the central and peripheral nervous systems, stimulation of aldosterone release from the adrenal gland (which acts on the kidneys to increase sodium retention), direct effects on the kidney to increase sodium retention, stimulation of cardiac output, and vasoconstriction (Fig. 3). The Ang II responsible for these actions can be derived from the circulating renin–angiotensin system in most cases, with the possible exception of the brain, because angiotensin peptides do not readily cross the blood–brain barrier.

There are also several tissue-specific actions of angiotensin peptides that are unrelated to blood pressure regulation. Perhaps the most useful insight into this topic comes from mice, which are deficient for a functional RAS due to inactivation of genes for either angiotensinogen, renin, ACE, or $AT_1$ and $AT_2$ receptors. What is strikingly absent in mice deficient for a functional renin–angiotensin system is any apparent gross effect on reproduction and fetal development. Thus, in spite of the expression of RAS components in reproductive tissues, placenta, and fetal organs, angiotensinogen-deficient mice (thus completely devoid of a RAS) are fertile and deliver pups that are histologically normal at birth. However, these mice do display some deficiencies as they age, some of which are fairly subtle: For example, inactivation of the gene coding for the $AT_2$ receptor in mice leads to reduced exploratory behavior, suggesting that Ang II could affect learning through the $AT_2$ receptor. Ang IV may also play a unique role in the brain: although complete ablation of the RAS (by inactivation of the angiotensinogen gene) leads to a defect in the ability of mice to repair the blood–brain barrier after a cold injury, this defect can be corrected by provision of the mice with Ang IV, even in the presence of antagonists for the $AT_1$ and $AT_2$ receptors.

There are also a number of more dramatic age-related defects in mice that are entirely deficient for a functional RAS. As many as 85% of mice deficient for angiotensinogen die before weaning. This high neonatal mortality may be related to a deficiency in salt retention (perhaps due in part to a lack of Ang II-stimulated aldosterone release), because it can be largely reversed with intraperitoneal injection of saline into neonatal mice. Virtually all RAS-deficient mice also display late-stage renal defects within 3 weeks of birth, including atrophic renal papillae, mesangial expansion, dilated collecting ducts in

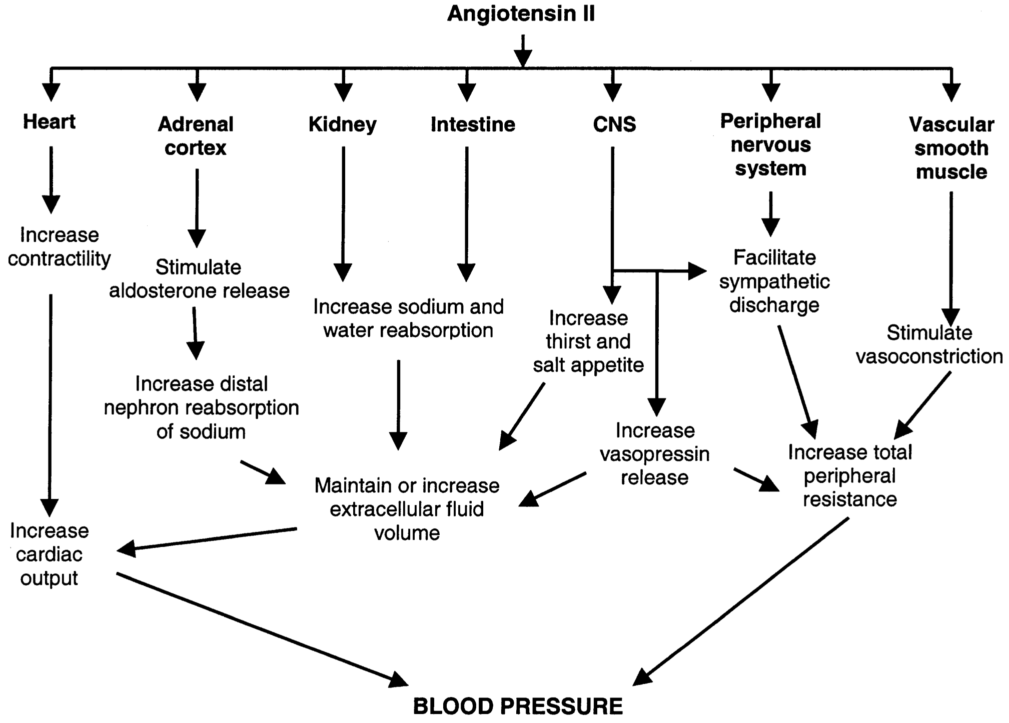
Angiotensin II
Heart
Adrenal cortex
Kidney
Intestine
CNS
Peripheral nervous system
Vascular smooth muscle
Increase contractility
Stimulate aldosterone release
Increase distal nephron reabsorption of sodium
Increase sodium and water reabsorption
Increase thirst and salt appetite
Facilitate sympathetic discharge
Stimulate vasoconstriction
Increase vasopressin release
Maintain or increase extracellular fluid volume
Increase total peripheral resistance
Increase cardiac output
BLOOD PRESSURE

FIGURE 3 Tissue actions of angiotensin II that affect blood pressure and fluid volume.

the outer renal medulla, mild interstitial fibrosis, and hyperplasia of interlobular arteries, all of which are associated with impaired renal function. Ichikawa and co-workers have shown that RAS-deficient mice display impaired urinary peristalsis and propose that Ang II may be a necessary chemotactic or differentiation agent in this correct formation of a smooth muscle cell layer in the renal pelvis. By 7–8 weeks of age, surviving angiotensinogen-deficient mice have lower blood pressure than do their control littermates, confirming the expected role of the RAS in blood pressure modulation. Finally, mice deficient in RAS signaling are anemic, possibly due to a missing and crucial Ang II-signaling step in erythropoiesis, and exhibit a decreased immune response due to an impairment in lymphocyte activation.

## VI. CONCLUSION

The concept of a circulating RAS with a role designed solely to generate the vasoactive peptide Ang II has undergone significant revision in the past two decades. It is now clear that many tissues have the capacity to generate a variety of functional angiotensin peptide hormones and that these peptides have roles beyond the regulation of salt and water balance. Because of the pleiotropic effects of angiotensin peptides, it has been very difficult to sort out their tissue-specific functions in whole animals, and although the use of homologous recombination experiments in mice have helped to rule out some proposed roles for the RAS, the dissection of the role of angiotensin peptides in learning, blood–brain barrier maintenance, erythropoiesis, immunity, and renal development may ultimately make the clinical use of RAS inhibitors safer and more effective.

## Glossary

**angiotensins** A family of peptide hormones derived from the protein angiotensinogen.

**homologous recombination** A natural chromosomal recombination mechanism that has been harnessed to “knock out” genes in mice in order to test for their functional importance.

**renin–angiotensin system** The enzymatic cascade composed of the substrate (angiotensinogen), two processing proteases (renin and angiotensin-converting enzymes), and receptors; mediates the hormonal action of angiotensin peptides.

## See Also the Following Articles

**Angiotensin II Receptor Signaling • Mineralocorticoids and Hypertension • Vasoactive Intestinal Peptide**

## Further Reading

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# Angiotensin II Receptor Signaling

ALESSANDRO M. CAPPONI
*University Hospital, Geneva*

I. INTRODUCTION
II. ANGIOTENSIN II RECEPTOR SUBTYPES
III. COUPLING MECHANISMS
IV. INTRACELLULAR EFFECTORS COUPLED TO $AT_1$ RECEPTORS
V. SIGNALING THROUGH THE $AT_2$ RECEPTOR SUBTYPE
VI. SUMMARY

**Angiotensin II, an octapeptide hormone, is the product of two successive enzymatic proteolytic cleavages. Angiotensin II is produced in blood and in some tissues by the action of renin and angiotensin-converting enzyme on angiotensinogen (an $\alpha_2$-globulin) and angiotensin I (a decapeptide), respectively. Initially described as a hypertensive factor, angiotensin II has proved to be a remarkably pleiotropic peptide. It exerts pressor effects by acting peripherally (in vascular smooth muscle, adrenal cortex, and kidney tissues) as well as centrally, but is also involved in the regulation of metabolic pathways (in liver and adipose tissues), inflammatory responses (by macrophages), tissue growth, and developmental processes.**

## I. INTRODUCTION

In spite of the spectrum of different responses elicited by angiotensin II in a host of distinct tissues, only two major angiotensin (Ang II) receptor subtypes, $AT_1$ and $AT_2$, have been identified to date. The vast majority of the biological functions of the hormone are mediated through the $AT_1$ receptor subtype, whereas the $AT_2$ subtype is essentially involved in developmental and remodeling processes. Clearly, therefore, the specificity of the response within a given tissue or cell type cannot be conferred by the receptor but must rely on the intracellular signaling mechanisms activated beyond the receptor. In this respect, signal transduction events following binding of the hormone to the $AT_1$ receptor have been extensively studied and are fairly well understood; less is known of the downstream events triggered by activation of the $AT_2$ subtype.

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# Anorexia Nervosa

See *Eating Disorders*

# Anti-Diuretic Hormone (ADH)

See *Vasopressin*

# Anti-Inflammatory Actions of Glucocorticoids

Ann-Charlotte Wikström, Jan-Åke Gustafsson, and Sam Okret

*Karolinska Institutet, Huddinge, Sweden*

I. INTRODUCTION
II. GENE REGULATION BY GLUCOCORTICOID HORMONES
III. NONGENOMIC EFFECTS
IV. ENDOGENOUS GLUCOCORTICOIDS AS ANTI-INFLAMMATORY AGENTS
V. GLUCOCORTICOIDS AND SPECIFIC CYTOKINES
VI. GLUCOCORTICOID REPRESSION OF GENES INVOLVED IN INFLAMMATORY RESPONSES
VII. MECHANISMS INVOLVED IN GLUCOCORTICOID REPRESSION OF NF-κB
VIII. GLUCOCORTICOIDS AND T-HELPER CELL SUBSETS
IX. GLUCOCORTICOIDS AS THERAPEUTIC AGENTS
X. ADVERSE EFFECTS OF GLUCOCORTICOID TREATMENT
XI. DEVELOPMENT OF GR LIGANDS THAT DISSOCIATE ACTIVITIES

**Glucocorticoid hormones serve as important regulators of inflammatory responses. They act to repress inflammation by inhibiting an array of inflammatory mediators, such as adhesion molecules, prostaglandins, cytokines, and cytokine receptors, as well as by inducing apoptosis of certain inflammatory effector cells. Glucocorticoid effects are mediated mainly by transrepressive genomic mechanisms acting via the intracellular glucocorticoid receptor, and, to a lesser extent, possibly also by nongenomic mechanisms.**

## I. INTRODUCTION

Glucocorticoids (GCs) regulate and suppress nuclear factor κB (NF-κB), a major proinflammatory mediator. Synthetic GCs are often used as drugs for immunosuppression and for treating inflammatory and allergic conditions. However, adverse side effects following therapeutic use can diminish the clinical efficacy of GCs, and thus future development of more selective ligands is desirable.

## II. GENE REGULATION BY GLUCOCORTICOID HORMONES

Glucocorticoids play a key role in regulating diverse physiological processes such as metabolism, salt and water balance, cellular proliferation and differentiation, inflammation, and immune responses. The anti-inflammatory and immunosuppressive actions of GCs are essential; inflammatory reactions with loss of GC production proceed in an uncontrolled way and can lead to death. The GC signal is transduced via the glucocorticoid receptor (GR), which regulates the expression of a subset of genes in both a promoter- and a tissue-specific manner. The general mechanism of GC action involves passive uptake and binding of GC to the GR, which is localized in the cytoplasm. Binding of the hormone to the receptor leads to a conformational change, dissociation of associated chaperone proteins, and translocation into the nucleus, where the GR directly or indirectly (through other transcription factors) binds to DNA in target genes. This results in enhancement or repression of gene transcription.

Stimulation of gene transcription by the GR most often involves binding of a homodimer of the hormone–receptor complex to one or more so-called GC-responsive elements (GREs), which consist of two more or less palindromic hexamers spaced by three nucleotides, usually located in the promoter region of GC-responsive genes. GR interaction with co-activators results in the establishment of contact with and regulation of the basal transcription machinery, with increased transcriptional initiation as the outcome. In addition, in most natural genes regulated by GCs, the GR establishes, and may even require, an interaction with other transcription factors binding in the proximity of the GRE, in order to allow proper GC regulation. Furthermore, in some cases the GR may even enhance gene expression without having to bind to the DNA. Instead, in these cases, enhancement occurs through a direct protein–protein interaction between the GR and other transcription factors binding to their cognate sites within the regulatory regions of the gene (Fig. 1).

As in the case of activation of gene transcription, repression of gene transcription by the GR occurs via GR binding in a DNA-dependent or -independent mechanism (Fig. 1). For a few genes, GR interaction with a so-called negative GRE seems to be required for repression to occur. This is the case for the human osteocalcin gene, the human interleukin-1β gene, and the bovine prolactin gene. It is generally thought that repression by the GR occurs by displacement of transcription factors binding to an element overlapping a GRE. However, most cases of GR-mediated repression seem to involve GR binding to other transcription factors, such as NF-κB or activator protein-1 (AP-1), which are important for regulating the transcriptional activity of genes. This involves protein–protein interactions without GR binding to DNA. This mechanism has been referred to as "tethering." In fact, repression of target genes by the GR seems to be more important than activation. This was initially suggested by G. Schütz and colleagues, who replaced the wild-type GR in a mouse with a GR mutant ($GR^{dim/dim}$), which maintains its ability to transrepress but has lost its ability to transactivate. These mice develop normally and are generally healthy, revealing that GR-mediated transactivation is not necessary for development or survival. Importantly, these mice also retain their ability to repress inflammatory reactions, suggesting that protein–protein interactions with other transcription factors, rather than GR–GRE interactions, form the basis of the anti-inflammatory activity of the GR (see also the following discussion).

## III. NONGENOMIC EFFECTS

The response to glucocorticoid treatment is generally the result of genomic effects, i.e., specific direct or indirect regulation of target genes via the GR (cf. above). However, when using very high doses of

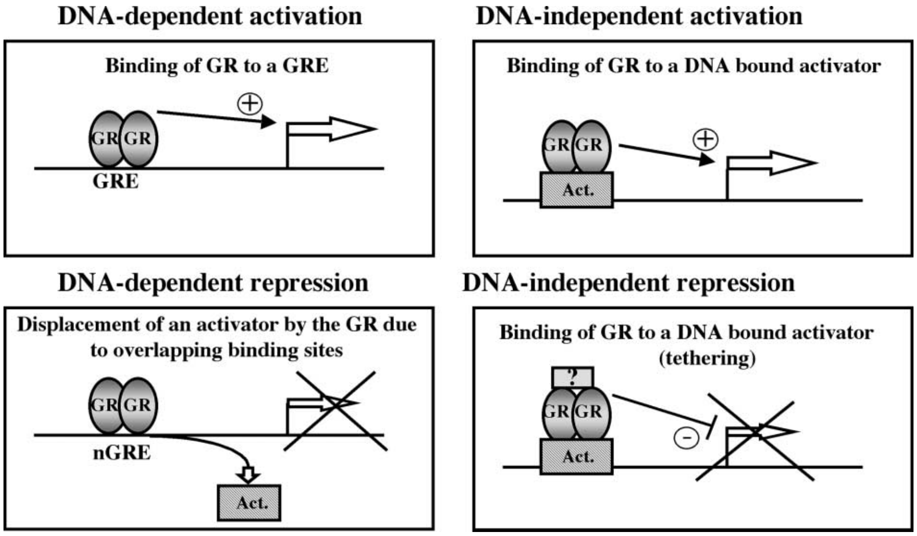
DNA-dependent activation
Binding of GR to a GRE
GR GR
GRE
⊕
DNA-independent activation
Binding of GR to a DNA bound activator
GR GR
Act.
⊕
DNA-dependent repression
Displacement of an activator by the GR due to overlapping binding sites
GR GR
nGRE
Act.
DNA-independent repression
Binding of GR to a DNA bound activator (tethering)
?
GR GR
Act.
⊖

FIGURE 1 Transcriptional activation and repression of target genes by the glucocorticoid receptor (GR). Activation or repression of GR target genes can be DNA dependent or DNA independent. The classical activation of GR target genes involves binding of a GR homodimer to a glucocorticoid response element (GRE). However, the GR may also further stimulate gene expression by binding to another transcriptional activator (Act) on DNA, without binding to the DNA. Repression of gene expression can also be DNA dependent or DNA independent. In the first case, it is thought that the GR may displace binding of another transactivator due to overlapping DNA binding sites. In the latter case, the GR interacts through a protein–protein contact with another transactivator without binding to DNA (tethering) and represses gene expression. This may involve recruitment of additional unknown proteins to the complex.

GCs in acute clinical situations—for example, in severe exacerbation of multiple sclerosis or after spinal cord injury—it has been suggested that nongenomic effects are also of importance. Certain GC effects are obtained within minutes, a time span that does not allow genomic effects to take place. The rapid effects are obtained via mechanisms involving phosphorylation events, $Ca^{2+}$ signaling, and other second-messenger systems. The existence of steroid-binding membrane receptors has been proposed, but no convincing data are yet available to support this notion. Also, a nonspecific, nongenomic effect of steroids has been proposed, whereby the GC molecule would interact directly with and thus stabilize lipophilic biological membranes.

## IV. ENDOGENOUS GLUCOCORTICOIDS AS ANTI-INFLAMMATORY AGENTS

Purified GC has been available since 1936, when Kendall's compound E was synthesized in a crystalline form; the correct structure of this compound was described in 1938 by Steiger and Reichstein. Since 1946, large-scale synthesis of compound E, today called cortisone, has been possible. Long before that, in 1915, Corbett had noted that "suprarenal exhaustion" was an important parameter in the bad prognosis for patients with severe shock. In 1932, Whitehead and Smith administered adrenal extract to five patients with severe infectious disease, all of whom initially improved and four of whom survived!

Compound F (corresponding to cortisol) and compound B [corresponding to corticosterone and deoxycorticosterone (DOC)] were also used in early studies of endogenous corticoids. The endogenous steroid of primary importance in humans is cortisol, also called hydrocortisone. Cortisol and cortisone differ only in their 11-carbon substitution. Cortisol has an 11β-OH group, whereas cortisone has an 11-keto group. This difference is biologically important: cortisone is relatively inactive biologically, whereas cortisol is a biologically active molecule. These forms of GCs are interconvertible by the action of both type 1 and type 2 of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 11β-HSD2), which have been extensively studied during recent years. The conversion of cortisone to cortisol by 11β-HSD1 acting as an 11β-reductase exploits the substantial circulating levels of 11-keto steroids and explains the therapeutic effects of cortisone, which, when used as a drug, requires this activating metabolic step. This conversion takes place mainly in the liver, brain, and adipose tissue, which are organs expressing high levels of 11β-HSD1. Kidney 11β-HSD2, on the other hand, converts cortisol to inactive cortisone, allowing kidney-specific effects of aldosterone to be exerted.

There exists a species-dependent difference regarding which particular GC is the main active form. In rodents, the predominant active form is corticosterone, which differs from cortisol by lacking a hydroxyl group in the 17α position due to a lack of 17α-hydroxylase in rodent adrenals. This active form of GC can be rapidly converted to an inactive form by an 11β-hydroxy → 11-keto conversion catalyzed by 11β-HSD2.

Approximately 8–30 mg/day of GCs is produced in the zona fasciculata in the adrenal cortex. The serum level of cortisol varies in a diurnal pattern, depending on the sleep/wake cycle, and is related to food intake. Peak values of 280–700 nmol/liter are obtained in the morning around 8 AM, whereas after midnight and during the following 4 h serum levels are very low, sometimes not measurable even by sensitive radioimmunoassay techniques. The late-night values are usually in the range of 0–280 nmol/liter, thus there is a 5- to 10-fold variation in serum levels during 24 h. Healthy individuals tend to adhere to an individual diurnal variation and repeatedly maintain a pattern of low-, intermediate-, or high-level cortisol secretion from childhood through puberty and into adult life. Diseases such as depression, posttraumatic stress syndrome, metabolic syndrome, and even aging may lead to perturbations of the diurnal rhythms and secretion levels of GCs.

There have been attempts to exploit the endogenous diurnal rhythm of GC secretion, and studies have been performed to test if the therapeutic effect of GC administration to patients with asthma, for example, varies during the day. Timing the inhalation of a single dose of GCs to the evening, instead of the morning, leads to a better therapeutic effect. However, for oral or parenteral GC therapy this may be a dangerous approach, because administration of GCs in the morning, preferably only every second day, has been shown to be optimal in preventing development of adverse effects, especially the iatrogenic hypothalamic–pituitary–adrenal (HPA) axis depression that occurs with continued high-dose GC therapy.

Animal studies have shown that adrenalectomy is associated with an exacerbation of inflammatory responses. Metyrapone, a cortisol synthesis inhibitor, has been used to study late-phase reactions to antigen in animals. These studies show that blocking cortisol synthesis leads to an enhanced late-phase response.

The interplay of inflammation and cortisol response is complex; allergic or other types of inflammation may lead to increased levels of cortisol via HPA axis up-regulation, whereas inflammatory mediators such as histamine, interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNFα), and others lead to release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), which, in turn, lead to increased levels of cortisol. Cortisol in turn can differentially down-regulate these cytokines, especially IL-1 and TNFα, whereas IL-6 seems to be more resistant to GC regulation. Some studies also indicate that certain cytokines can modify the affinity of the GR to its ligand as well as induce functional resistance to GC effects (see the following discussion).

Both excess and inadequate levels of glucocorticoids can lead to disease. Excess GC levels increase the risk for infections, whereas suboptimal levels increase the risk for inflammatory, allergic, and autoimmune diseases. The data from studies of patients with asthma, rheumatoid arthritis, systemic lupus erythematosus, and other inflammatory/autoimmune disorders and from animal model systems, suggest that the HPA axis as such may influence disease pattern and severity. Humans undergoing long-term stress have an increased susceptibility to viral infection, impaired wound healing, and a poorer response to vaccination. It has also been demonstrated in a rat experimental model by Ester Sternberg and others that endogenous GC levels and the HPA axis have an impact on disease. These studies have compared the Lewis (LEW/N) rat, which has a blunted HPA axis response and a high susceptibility to autoimmune diseases, to the histocompatible Fischer (F344/N) rat, which has a hyperactive HPA axis and a low susceptibility to the same diseases. Interference with the HPA axis, leading to low levels of circulating GCs, renders the previously resistant F344/N strain more prone to inflammatory disease, and exposure to bacterial lipopolysaccharide (LPS) results in a high mortality rate. The susceptible strain LEW/N, when transplanted with fetal hypothalamic tissue from F344/N rats, decreases its inflammatory response and can withstand LPS exposure.

In summary, there is interplay between the immune system and the neuroendocrine GC system. An HPA axis resulting in low levels of circulating GCs may predispose to inflammatory disorders. On the other hand, secretion of specific inflammatory cytokines such as IL-1, IL-6, and TNFα may affect the HPA axis and up-regulate GC secretion, which then can serve to control and down-regulate these and other cytokines and thereby maintain homeostasis and regulate the magnitude of the inflammatory response.

## V. GLUCOCORTICOIDS AND SPECIFIC CYTOKINES

GCs can down-regulate expression of specific cytokines via negative regulation of promoters of cytokine genes. Several cytokines have also been reported to be down-regulated by GCs through a reduction of the half-life and the translatability of the cytokine mRNAs. Among cytokines affected by GCs are IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-13, IFN-γ, granulocyte/macrophage colony-stimulating factor (GM-CSF), and TNFα. In the case of IL-4, for instance, up-regulation by GCs has also been reported. GCs can also inhibit the synthesis of cytokine receptors such as the IL-2 receptor. GC down-regulation of the production of cytokines has secondary effects on cells that are dependent on certain cytokines for survival, i.e., eosinophils and subsets of T lymphocytes; eosinophils, for instance, when deprived of GM-CSF and IL-5, die by apoptosis.

The interplay of cytokines and GCs is complex, as exemplified by the reported down-regulation by GCs of constitutive and IL-1β-induced IL-6 in certain cell types. IL-6 in turn can up-regulate the number of GR sites per cell, as measured by a ligand-binding assay in osteoblastic cell lines, most notably in Saos-2 cells. An up-regulation of the receptor number in this case does not necessarily reflect an increased ability to respond to GCs, but may be due to an inability of the receptor to translocate to the nucleus. The cytokine IL-4, in the presence of IL-2, has been reported to decrease GR ligand affinity in T cells concomitantly with an increase in the number of GR binding sites. These changes seem to affect GR function, because the ability of a GC to inhibit the proliferation of T cells in the presence of these two cytokines is hampered. Also, IL-13 has been reported to have a similar effect, decreasing GR ligand binding affinity.

Other interleukins, e.g., IL-10, have been reported to increase the concentration of GR binding sites in human monocytes without affecting the binding affinity. These cells, when treated with IL-10, have an increased sensitivity to GCs; this effect has been suggested to be mediated via an up-regulation of the GR-stabilizing chaperone, heat-shock protein 90 (Hsp 90). A similar effect on human monocytic cells has been reported to take place when the cells are exposed to transforming growth factor-β (TGF-β), an effect involving activation of the activator protein-1 and Smad, leading to an increased number of GR sites and an enhanced ability to transactivate GRE-dependent promoters.

## VI. GLUCOCORTICOID REPRESSION OF GENES INVOLVED IN INFLAMMATORY RESPONSES

As already indicated, cortisol (corticosterone in rodents) is a key molecule involved in feedback regulation of inflammatory and immune responses. The efficiency of GCs in this respect has widely encouraged their use as drugs in the treatment of inflammatory diseases or when immunosuppression is required. The cellular responses to inflammatory reactions are several, and include migration of cells to the site of inflammation (T cells and dendritic cells), expression and/or release of numerous inflammatory mediators, e.g., adhesion molecules, cytokines, and chemokines (endothelial cells, macrophages, and T cells), and proliferation (T and B cells). GC hormones repress most of these inflammatory responses. In addition, GCs may induce apoptosis of peripheral T cells. The effects are mainly mediated by the ability of GCs to repress transcription of the genes involved in the inflammatory process, e.g., those for intercellular, vascular cellular, and endothelial leukocyte adhesion molecules (ICAM-1, VCAM-1, and ELAM) and proinflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-8, as well as for other molecules involved in inflammatory reactions, such as inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), GM-CSF, cytokine-induced neutrophil chemoattractant/growth-regulated oncogene (CINC/gro), and macrophage inflammatory protein-1 (MIP-1, also known as RANTES, for receptor-activated neutrophil T-cell expression and secretion).

Promoter and ablation analyses (knockouts) have identified some key transcription factors that are responsible for activation of a large part of the genes involved in inflammatory and immune reactions. These include the transcription factors NF-κB, AP-1, nuclear factor of activated T cells (NF-AT), $Ca^{2+}$/cAMP response element binding protein (CREB), and CCAAT/enhancer binding protein β (C/EBPβ). Indeed, mutational studies of inflammatory genes have demonstrated that the ability of GCs to repress transcription involves the binding sites for the above-mentioned transcription factors. However, these sites lack the ability to bind the GR. Instead, as previously discussed, repression occurs through a tethering mechanism by which GR interferes with the transcription factor through a direct or indirect protein–protein interaction, without actually contacting DNA. This interaction between two signaling pathways is often referred to as "crosstalk." Usually this crosstalk is mutual, meaning that the transcription factors also repress the activity of a GRE-bound GR, thus resulting in impaired transcriptional activation by the GR. It is thought that an abortive nonproductive complex is formed and is unable to maintain transcription. The best *in vivo* evidence that tethering is the main operating mechanism in the anti-inflammatory action of GC is the generation of $GR^{dim/dim}$ mice, in which the wild-type GR gene has been replaced by the gene for a GR mutant that is unable to dimerize and bind to GRE. These mice are unable to transactivate GC-responsive target genes but maintain the ability to repress target genes through the tethering mechanism.

Interestingly, these mice have a normal anti inflammatory response, measured as the ability of GCs to repress phorbol ester-induced skin or ear edema, serving as models of acute inflammation. Furthermore, TNFα release after systemic injection of lipopolysaccharide, a model of bacterial infection and subsequent systemic inflammatory response, was also repressed to a similar degree in $GR^{dim/dim}$ mice and in wild-type mice. Analysis of GC repression of cytokine gene expression in primary cells derived from the $GR^{dim/dim}$ mice [TNFα, IL-1β, IL-6, COX-2 from macrophages, or IL-2 and interferon γ (IFNγ) from T cells] confirmed the ability of the transactivation-deficient $GR^{dim/dim}$ mice to repress genes activated during the inflammatory response. These results were also obtained in the presence of a protein synthesis inhibitor, demonstrating that synthesis of a secondary protein is not required. These findings confirm a number of earlier studies based on cell transfection experiments showing that GR mutants unable to bind GRE and to transactivate may retain their ability to repress both NF-κB, AP-1, NF-AT, CREB, or C/EBPβ activity. Although DNA binding per se is not required, regions within the DNA-binding domain (DBD) of the GR seem important for the interference.

## VII. MECHANISMS INVOLVED IN GLUCOCORTICOID REPRESSION OF NF-κB

The expression of most proinflammatory genes depends on the activation of NF-κB. Thus, NF-κB most likely represents the major target for the GR in the inhibition of inflammatory responses. Because of the central role of NF-κB in signal transduction in inflammatory and immunological responses, the focus here is on GC repression of this activity.

NF-κB is activated by numerous signals, including proinflammatory cytokines such as TNFα and IL-1β, viruses, viral proteins, double-stranded RNA, and phorbol esters, as well as physical and chemical stress. In nonstimulated cells, NF-κB is kept inactive in the cytoplasm in a complex with its inhibitor protein, IκBα (Fig. 2). The signal transduction pathway involved in activation of NF-κB includes stimulation of the IκB kinase (IKK) that phosphorylates Ser-32 and Ser-36 on IκBα. These phosphorylations lead to degradation of IκBα through the 26S proteasome machinery, resulting in the release of NF-κB, the translocation of NF-κB into the nucleus, and the binding to NF-κB binding sites in target genes. Through recruitment of co-activators and stimulation of the basal transcription machinery, transcriptional initiation occurs (Fig. 2). The members of the mammalian NF-κB/Rel family consist of RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). The NF-κB/Rel transcription factors are characterized by a conserved stretch of 300 amino acids, the Rel homology domain (RHD), which is responsible for DNA binding and interaction with IκB. NF-κB exists as a dimer, most often a heterodimer, predominantly consisting of one p65 and one p50 protein. The GR has been shown to interact with RelA (p65) and c-Rel, whereas interaction with p50 is a matter of controversy. The physical interaction between the GR and RelA seems to involve the GR DBD and the RHD of RelA, although carboxy-terminal transactivation domains of RelA also seem to be required for a functional interference. It is not known whether the interaction between the GR and RelA proteins is direct or whether intermediary proteins are involved.

Although some previous reports based on *in vitro* experiments have suggested the formation of an abortive NF-κB/GR complex that is unable to bind DNA, more recent results from *in vivo* experiments, including *in vivo* footprinting and chromatin

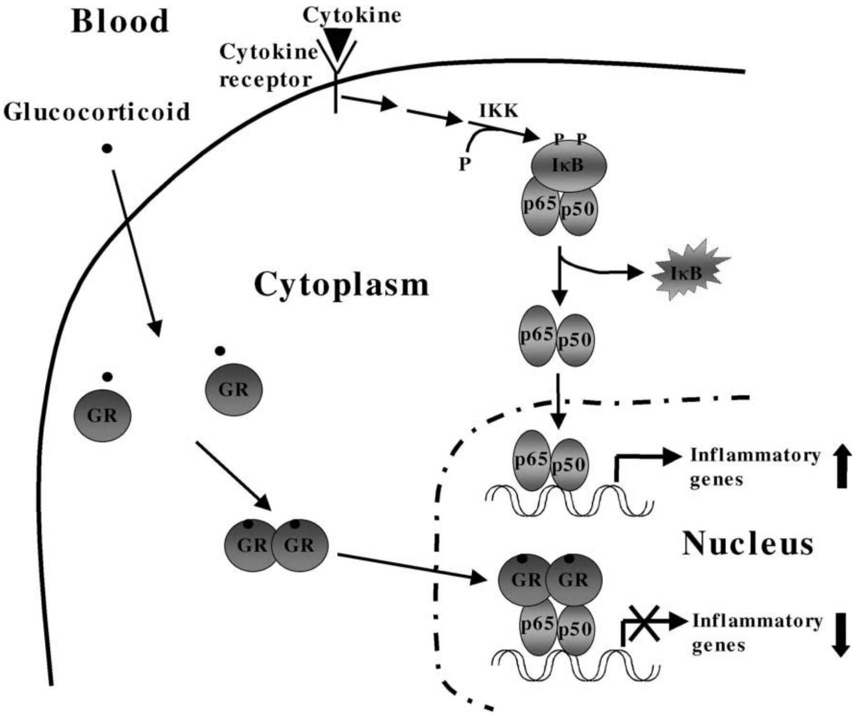

**FIGURE 2** Crosstalk between nuclear factor κB (NF-κB) and glucocorticoid receptor (GR) signaling pathways. Binding of a cytokine to its membrane receptor triggers several steps that lead to activation of the NF-κB signal transduction pathway. This includes phosphorylation of NF-κB inhibitory protein IκBα in the cytosol by an IκB kinase (IKK) at Ser-32 and Ser-36. This triggers degradation of IκBα. This in turn allows the NF-κB complex (most often consisting of a p65/p50 dimer) to translocate to the nucleus, where it binds to NF-κB binding sites, usually located in the promoter region, and stimulates expression of inflammatory genes. Simultaneous administration of glucocorticoid activates the GR, which homodimerizes, translocates to the nucleus, and binds the NF-κB complex on DNA, without binding to the DNA (tethering). This interaction leads to repression of NF-κB activity and thus to reduced expression of inflammatory genes.

immunoprecipitation (ChIP) techniques, show that the GR does not disrupt NF-κB binding to its target genes or alter the assembly of the preinitiation complex formed following TNFα treatment (Fig. 2). Interestingly, GR interaction with NF-κB seems rather to interfere with the phosphorylation of Ser-2 on the carboxy-terminal domain (CTD) of RNA polymerase II, a required step for transcriptional initiation. It is not known whether the NF-κB complex harbors or brings in a kinase activity that is inhibited following GR interaction or whether the GR harbors or brings in a corepressor/phosphatase. It is also possible that the NF-κB interaction with its target sequence is slightly modulated following GR interaction. An additional level of regulation may involve GC-mediated modulation of cofactor histone acetylase activity or DNA methylation. The observation that GR tethering to transcription factors does not result in displacement from DNA is supported by earlier *in vivo* footprinting results from the collagenase I promoter. In this case, the GR tethered to the AP-1 complex did not result in AP-1 displacement from its binding site when GC repression of collagenase I expression took place. Although the GR represses both NF-κB and AP-1 activity, without displacing the factors from the target genes, recent results have indicated that the mechanisms are not completely identical.

The ability of transactivation-deficient GR mutants to repress inflammation also shows that GC induction of the NF-κB inhibitor protein, IκBα, as suggested previously, is not a major mechanism involved in the anti-inflammatory action of GCs. However, this mechanism may, to a lesser extent, contribute to the repressive mechanism in cells in which it is induced following GC administration. This is in line with the observation that IκBα induction by GCs is highly cell and tissue specific, in contrast to GC repression of NF-κB-dependent genes in most cell types and in cells not inducing IκBα in response to GCs. Finally, some GC analogues are able to induce IκBα without repressing NF-κB activity, whereas "classical" GCs affect both proteins in the same cell type. These results show that GC-mediated transrepression of NF-κB, and IκBα up-regulation, respectively, are two separate processes, the latter of which is not necessary for GC-mediated repression of NF-κB activity.

It has also been suggested that a molecular mechanism responsible for repression of NF-κB or AP-1 activity following GR activation by GC administration would involve competition for a limiting amount of a co-activator. This is supported by the mutual nature of the crosstalk between GR and NF-κB or AP-1. In this model, a limiting amount of a general co-activator—e.g., CREB-binding protein (CBP), p300, or steroid receptor co-activator-1 (SRC-1)—that binds to NF-κB or AP-1 and is required for efficient transactivation by these factors would instead bind to the activated GR, thereby reducing transcriptional activity of NF-κB or AP-1. Although some initial reports found experimental support for such a mechanism, more recent results have questioned this model. Overexpression of CBP, p300, or SRC-1 did not impair GC-mediated inhibition of the IL-6 promoter nor of a Gal/DBD/Jun fusion-dependent reporter gene. The model also does not explain the synergistic effect of GR on an AP-1 complex consisting of a c-Jun homodimer or the ability of retinoic acid receptor (RAR) to distinguish between AP-1 and NF-κB in the crosstalk. Binding of co-activators by RAR is similar to that by GR, and RAR represses AP-1 but not NF-κB. In addition, in contrast to the competition model for a limiting co-activator, most recent results have shown that GR repression of the AP-1 complex on the collagenase 3 gene is enhanced rather than repressed by an excess of co-activator, the TIF2/GRIP1. In summary, results show that although the co-activators may be limiting for transactivation, this seems not to be the case for transrepression. Furthermore, because the co-activators interact with many different transcription factors and activities, a competition model would constitute a rather nonspecific way to mediate repression. However, squelching of an unknown cofactor as an explanation for the crosstalk can still not be fully excluded.

Another possible mechanism that may be involved in the anti-inflammatory or immunosuppressive effects of GCs is a rapid GR-mediated inhibition of Jun phosphorylation and of c-Jun N-terminal kinase (JNK) activity, independent of ongoing transcription. The GR has also been reported to interfere with extracellular signal-related kinase (ERK) activity. However, there are conflicting results regarding the requirement for protein synthesis in the inhibition of mitogen-activated protein (MAP) kinase. This may simply be due to use of different investigative model systems. In cases in which ongoing protein synthesis is required for GC-mediated repression, induction of a phosphatase may be a plausible explanation. Another level of GC regulation of NF-κB activity may be exerted by the catalytic subunit of protein kinase A (PKAc). This subunit has been shown to interact with NF-κB and to phosphorylate Ser-276 of RelA. Interestingly, PKAc has also been shown to interact with the GR in a ligand-independent way, and deletion of the conserved Ser-276 in RelA abolishes RelA repression of GR activity. Overexpression of PKAc stops GR/RelA crosstalk.

## VIII. GLUCOCORTICOIDS AND T-HELPER CELL SUBSETS

The profile of an immune response is to a large extent determined by which subset of T-helper cells has been activated and, more specifically, by the balance between $T_H1$ and $T_H2$ cells. The $T_H1$ response is characterized by a cytokine profile that supports inflammation and preferentially activates other T cells and macrophages. A $T_H2$ profile is characterized by the secretion of cytokines that activate B cells and antibody-dependent immune responses. The most important cytokines secreted by $T_H1$ cells are IL-2, IFNγ, and IL-12, whereas $T_H2$ cells produce IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13. In principal, these cytokines promote the development of subsets of the cells that produce them, but they can also inhibit both production of and functional effects of the opposite subset; this effect is called cross-regulation, which explains the inverse relationship between antibody production ($T_H2$ profile) and delayed-type hypersensitivity ($T_H1$ profile). $T_H1$ and $T_H2$ responses are both suppressed by regulatory $T_H$ cells that secrete TGF-β.

Increased levels of GCs after therapeutic administration act in an immunomodulatory or immunosuppressive way, depending on the dose. When employing moderate GC levels, the balance of $T_H1$ and $T_H2$ cell subsets shifts so that $T_H2$ responses become predominant. GCs, norepinephrine, epinephrine, histamine, and adenosine inhibit the production of IL-12 and TNFα, which normally promote $T_H1$ cell development, whereas they do not affect IL-10 production and thus not $T_H2$ development. GCs have also been reported to target macrophages and monocytes as well as IL-1 and IL-1 receptor antagonists. The down-regulation of $T_H1$ cells leads to a subsequent lower production of IFNγ, IL-2, and IL-12. A down-regulation of the $T_H1$ response acts permissively for $T_H2$ responses, and some researchers have reported an up-regulation of the production of IL-4, a $T_H2$-type cytokine. It has also been reported that GCs down-regulate TGF-β secretion and this may serve to down-regulate both $T_H1$ and $T_H2$. Furthermore, it has been shown that memory T cells are less susceptible to inhibition by GCs, implying that GCs may be of special importance in primary responses.

The GC-induced shift to predominant $T_H2$ responses under stressful conditions and during GC treatment may lead to an increased susceptibility to various infections because infections are usually defeated via $T_H1$-dependent mechanisms; the GC-induced shift may also impact susceptibility to autoimmune, allergic, and malignant diseases.

## IX. GLUCOCORTICOIDS AS THERAPEUTIC AGENTS

Due to the potent anti-inflammatory and immunosuppressive effects of GCs, they are widely used in the clinic to treat asthma, rheumatoid arthritis, systemic lupus erythematosus (SLE), dermatitis, polymyositis, glomerulonephritis, and rejection of organ transplants. The use of endogenous-type GCs as drugs is not ideal. These GCs show a high degree of binding to the serum transporter protein, cortisol-binding globulin (CBG). This renders a large fraction of the administered drug inactive. Cortisol also has a relatively rapid turnover. By chemical modifications of the original cortisol structure, it has been possible to synthesize GCs with increased stability and a low binding affinity for CBG. The substitutions have involved insertion of halogen in the C-9α position, as in fluorohydrocortisone, as well as the introduction of a double bond between C-1 and C-2, as in prednisolone and prednisone. The substituted or derived GCs show a whole spectrum of therapeutic potential, ranging from mild steroids, such as the original hydrocortisone, to very potent steroids, such as betametasone and dexametasone.

The routes of glucocorticoid administration determine the requirements for further modifications of steroidal drugs. Originally, most steroids were ingested or injected, and hydrophobic compounds were sufficient. Development of steroid succinates and phosphates enabled various kinds of parenteral and topical treatments. The need for GCs for localized treatment of the colon, in diseases such as inflammatory bowel disease, and of the lungs, as in asthma, led to further pharmaceutical steroid developments. In generating these topical steroids, the aim of the organic chemists has often been to maximize the local effect and to minimize the systemic effect of the new drug. Comparisons of the potencies of GCs that are topical anti-inflammatory (cause cutaneous “vasoconstriction”) and systemic (cause depression of plasma cortisol and changes in differential white blood cell count) formulations have been performed for several of the new compounds. New types of GCs (e.g., 16,17-acetals) have an improved topical/systemic activity ratio as compared to GCs with fluorine substitution in 9α or 6α, 9α positions.

## X. ADVERSE EFFECTS OF GLUCOCORTICOID TREATMENT

When using glucocorticoids as anti-inflammatory drugs, it is important to remember that GCs do not simply regulate the inflammatory response or the immune system. In fact, they affect almost all tissues in the body, influencing energy metabolism, electrolyte and water balance, central nervous system functions, proliferation, and differentiation. Severe side effects thus result following long-term treatment with GC in chronic diseases. Adverse effects of GCs are reminiscent of the disease syndrome that can be found in patients with Cushing's disease and includes a perturbation of the HPA axis. After long-term treatment, even with moderate doses of GCs, the adrenals become atrophic and adrenal production of GCs is depressed. This necessitates specific care when tapering off GC therapy or when the treated individual is exposed to stress. Lack of adequate GC production affects the ability to cope with stress and may even be lethal in cases of severe stress. Another common adverse effect of GC therapy is osteoporosis, which can be both rapid and dramatic. Growth inhibition in children has also been claimed to occur, but recent studies show that this is a rather limited problem. Other side effects of GCs include an increased risk for opportunistic infections, muscle wasting, neuropsychiatric complications with behavioral and cognitive changes, impaired wound healing, skin atrophy, hypertension, bruises, moon face, striae (especially on the trunk), and perturbations in lipid and glucose metabolism. The latter may even lead to GC-induced diabetes. Due to these adverse effects it is recommended to regularly monitor weight, blood pressure, signs of infections, electrolyte concentration (especially $Na^+$ and $K^+$), glucose levels, serum lipid levels, and signs of osteoporosis, osteonecrosis, and cataract.

Preventive measures may be effective to address the side effects of GC therapy, especially for preventing osteoporosis. When more than 4 weeks of GC therapy is planned, dietary supplements of calcium and vitamin D are often recommended. Specific drugs, such as estradiol, bisphosphonates, raloxifen, or parathyroid hormone, may be prescribed with longer term treatment.

## XI. DEVELOPMENT OF GR LIGANDS THAT DISSOCIATE ACTIVITIES

Although several severe side effects occur after long-term treatment with ingested or parenterally administered GCs, the anti-inflammatory effects are in some cases still unsurpassed, and GCs may thus be the only efficient therapy available. Because of this, there is a large degree of interest by pharmaceutical companies in developing GCs that maintain their anti-inflammatory action, but without side effects. Support for such a possibility is based on the fact that GR mutants have proved to be able to dissociate the transrepressive activity of GR, which is responsible for the anti-inflammatory activity, from the transactivating activity of GR. In fact, experimental cell studies have demonstrated the existence of synthetic GC derivatives that can dissociate GR-mediated transrepression from GR-mediated transactivation of genes. Furthermore, claims have been made that a more potent transrepressive effect is exerted by GCs such as RU24858, RU486, and ZK98299, as well as by certain topical steroids such as fluticsone propionate and budesonide, compared to the transactivating effect of these compounds. However, whether all of these compounds are efficient as anti-inflammatory agents *in vivo*, with fewer side effects, remains to be seen. Furthermore, it is not known which mechanism, transrepression or transactivation of GR target genes, is mainly responsible for the side effects of GCs.

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## Glossary

**crosstalk** The ability of various signal transduction pathways to influence the signaling processes of one another. Crosstalk often occurs via phosphorylation/dephosphorylation events or through a physical interaction, e.g., tethering.

**glucocorticoid** A steroid hormone produced in the adrenal cortex. Glucocorticoids bind the glucocorticoid receptor and affect cellular growth and differentiation as well as metabolic and inflammatory processes. They are essential for life and are key regulators in the stress response. The term also encompasses synthetic hormones with a similar structure and function.

**glucocorticoid receptor** The intracellularly located receptor for glucocorticoid hormones belongs to the family of nuclear receptors. Acts as a ligand-activated transcription factor and binds both agonists and antagonists.

**glucocorticoid response element** A conserved palindromic set of nucleotides; each half-site of six conserved nucleotides is separated by three nondefined nucleotides to which the glucocorticoid receptor can bind as a homodimer via its DNA-binding domain.

**nuclear factor-κB** A complex of the family of Rel-related transcription factors and an important signaling molecule in inflammatory responses. NF-κB was first described to be required for B-cell-specific gene activation, but, as with all Rel-related transcription factors, it is a critical factor for the inducible expression of many different genes. NF-κB most often consists of a heterodimer between p65 (RelA) and p50.

**tethering** Physical interaction between two different transcription factors, forming a heteromeric complex independent of DNA binding. Tethering may also occur on the DNA at the point where one of the transcription factors is attached. This interaction often leads to a mutual repression of the transcriptional ability of either of the factors.

**transactivation** The ability of a factor, e.g., the glucocorticoid receptor, to induce gene expression.

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# Anti-Müllerian Hormone

Rodolfo Rey[*] and Jean-Yves Picard[†]

[*]*Hospital de Niños R. Gutiérrez, Buenos Aires* • [†]*Institut National de la Santé de la Recherche Médicale Unité 493, Clamart, France*

I. INTRODUCTION
II. AMH: THE HORMONE
III. AMH RECEPTORS
IV. BIOLOGICAL ACTIONS OF AMH
V. REGULATION OF AMH PRODUCTION
VI. PERSISTENT MÜLLERIAN DUCT SYNDROME
VII. CLINICAL APPLICATIONS OF AMH
VIII. SUMMARY

**Anti-Müllerian hormone (AMH) is involved in the differentiation of the internal genitalia in the fetus; it is produced by fetal testicular Sertoli cells and ovarian granulosa cells. AMH produced by the fetal testis induces the regression of the Müllerian ducts. Granulosa cells produce only small amounts of AMH late in fetal life, when the Müllerian ducts are no longer sensitive to the hormone. In the ovary, AMH down-regulates luteinizing hormone receptor and aromatase activities, leading to a decrease in estrogen production. AMH exerts its action by binding to a specific membrane receptor, AMHR-II, inducing the recruitment of a non-specific type I receptor and leading to the phosphorylation of Smad1, which binds to**

of nuclear receptors. Acts as a ligand-activated transcription factor and binds both agonists and antagonists.

**glucocorticoid response element** A conserved palindromic set of nucleotides; each half-site of six conserved nucleotides is separated by three nondefined nucleotides to which the glucocorticoid receptor can bind as a homodimer via its DNA-binding domain.

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# Anti-Müllerian Hormone

RODOLFO REY* AND JEAN-YVES PICARD†

**Hospital de Niños R. Gutiérrez, Buenos Aires • †Institut National de la Santé de la Recherche Médicale Unité 493, Clamart, France*

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**Anti-Müllerian hormone (AMH) is involved in the differentiation of the internal genitalia in the fetus; it is produced by fetal testicular Sertoli cells and ovarian granulosa cells. AMH produced by the fetal testis induces the regression of the Müllerian ducts. Granulosa cells produce only small amounts of AMH late in fetal life, when the Müllerian ducts are no longer sensitive to the hormone. In the ovary, AMH down-regulates luteinizing hormone receptor and aromatase activities, leading to a decrease in estrogen production. AMH exerts its action by binding to a specific membrane receptor, AMHR-II, inducing the recruitment of a nonspecific type I receptor and leading to the phosphorylation of Smad1, which binds to**

**Smad4, is translocated to the nucleus, and regulates target gene expression. The biological actions of AMH in fetal sex differentiation, the regulation of AMH expression, and the clinical applications of AMH are discussed.**

## I. INTRODUCTION

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS) or Müllerian inhibiting factor (MIF), owes its name to its first described and best-known action during fetal male sex differentiation. The first author to suggest that a second testicular factor—apart from testosterone—should be involved in the differentiation of internal genitalia was Alfred Jost, in the late 1940s. By means of a refined fetal surgery technique, Jost castrated rabbit fetuses before the time of sex differentiation and observed that—whatever the genetic sex was—the absence of gonads resulted in a newborn with female internal and external genitalia, whereas the presence of testes resulted in newborns with male genitalia; he therefore deduced that the male gonad was the "sex differentiator." When a crystal of testosterone was implanted in a normal or castrated female or castrated male fetus, Wolffian ducts gave rise to the epididymis, vas deferens, and seminal vesicle and the external genitalia became virilized, yet the Müllerian ducts formed the Fallopian tubes, uterus, and upper portion of the vagina. Hence, Jost concluded that the fetal testis produces androgens that masculinize the genital tract and another factor, which he called "hormone inhibitrice" or "Müllerian inhibitor," that is responsible for the regression of Müllerian derivatives. More than 50 years later, the picture remains essentially the same, with a more detailed knowledge of the hormones and receptors involved in male sex differentiation (Fig. 1). In this article, we discuss the "AMH pathway" of fetal sex differentiation, as well as other more recently discovered actions of AMH, the regulatory mechanisms controlling AMH expression, and the applications of AMH in clinical practice.

## II. AMH: THE HORMONE

AMH is a 140 kDa glycoprotein homodimer, composed of 550–580 amino acids depending on the species, that belongs to the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors. The maturation process of AMH involves the removal of a 24-amino-acid leader prior to secretion by Sertoli cells; although this full-length secreted dimer has some active potential, to be fully active, it must be cleaved—most probably in target organs—at a monobasic site located 109 amino acids upstream from the C-terminus. The resulting 25 kDa C-terminal dimeric fragment is the bioactive moiety, whereas the 110 kDa N-terminal dimeric fragment is inactive. However, the presence of the N-terminal fragment in the medium significantly enhances the bioactivity of the C-terminal domain.

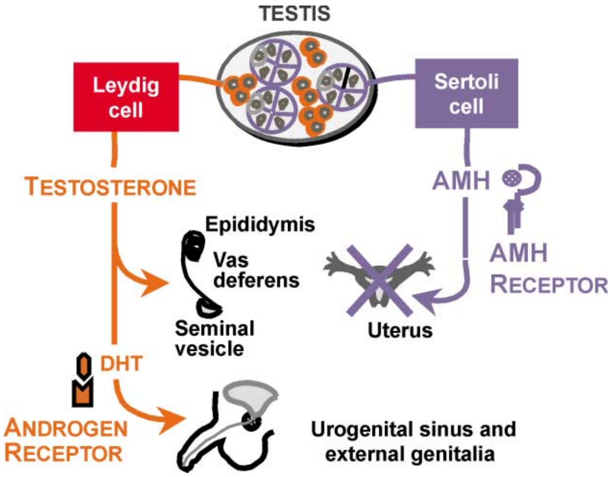

**FIGURE 1** Hormonal control of male sexual differentiation. Reprinted from R. Rey and J. Y. Picard, Embryology and endocrinology of genital development. *Baillière*'s *Clinical Endocrinology and Metabolism* **12**, 17–33. Copyright 1998, by permission of the publisher Baillière Tindall.

The gene for AMH has been cloned in human and several other mammalian and nonmammalian species. It is composed of five exons and four introns spanning approximately 2.8 kb and maps to chromosome 19, band 19p13.3 in the human and to different autosomes in other species. The human AMH promoter lacks a classical TATA-box and exhibits several transcription initiation sites, in contrast to what is observed in the bovine, rat, and mouse genes, which contain a canonical TATA element and a single transcription initiation site. Although more than 3 kb of the 5′-flanking region of the AMH gene have been sequenced, only the proximal promoter region has been functionally studied. Response elements for GATA-4, steroidogenic factor-1 (SF-1), and the SRY-related factor SOX9 are present in a sequentially conserved manner in several mammalian species within the 280 bp proximal promoter of AMH. The importance of the existence of a gene coding for a ubiquitously expressed spliceosome protein, SAP62, 762 bp upstream of the transcriptional start site of the human AMH gene and 328 bp upstream of the mouse gene, remains to be elucidated.

## III. AMH RECEPTORS

Like most glycoprotein hormones, AMH was expected to have a membrane-bound receptor in its target cells. Members of the TGF-β superfamily usually signal through a receptor complex formed by two serine/threonine kinase proteins, named type I and type II receptors according to their molecular weight. The type II receptor, of higher molecular weight, is responsible for ligand binding. Not all of the corresponding type I receptors have been identified yet. Furthermore, most type I receptors can interact with more than one type II receptor and can therefore be involved in signaling different members of the superfamily.

The specific type II receptor for AMH (AMHR-II) is a single-transmembrane domain serine/threonine kinase encoded by a gene of 8.2 kb, arranged in 11 exons and 10 introns, mapping to chromosome 12, band q13, in the human. Of the 573 amino acids that form the receptor, 17 correspond to the signal peptide (5′ sequences of exon 1), 127 to the extracellular domain (3′ sequences of exon 1 and exons 2 and 3), 26 to the transmembrane domain (exon 4), and 403 to the intracellular domain (exons 5 to 11), which bears the serine/threonine kinase activity. AMHR-II has also been cloned in other mammalian species. In the rabbit and the dog, two splice variants exist: the full-length protein and a shorter variant, owing to an alternative splicing resulting in skipping of exon 2. The presence of exon 2 is essential: In humans, a splice mutation leading to the lack of exon 2 results in a nonfunctional receptor.

At least two type I receptors, also involved in intracellular signaling of bone morphogenetic proteins (BMPs) and activin, have been reported to have the capacity to transduce AMH action on AMHR-II. ALK2 (activin receptor-like kinase 2), also known as ActR-IA, and ALK3, also known as BMPR-IA, and ALK6, also known as BMPR-IB, are nonspecific type I serine/threonine kinases with a single transmembrane domain, present in AMH target cells, which are recruited by AMHR-II upon binding of AMH.

## IV. BIOLOGICAL ACTIONS OF AMH

AMH, produced by the fetal testis, provokes the regression of Müllerian ducts in the male fetus. The period of Müllerian duct sensitivity to AMH is limited: In the human, AMH is secreted from the end of the seventh week and acts on its target organ during the following week. By the end of the eighth week, the fate of the Müllerian ducts has already been determined: If no AMH has been produced by then, the uterus and Fallopian tubes will develop, whereas withdrawal of AMH after the eighth week will not stop Müllerian duct regression.

AMH is produced by testicular Sertoli cells and ovarian granulosa cells. Sertoli cells secrete AMH from the time of fetal testicular differentiation, but granulosa cells begin to express AMH only in late fetal life in mammals, when Müllerian ducts are no longer sensitive to the hormone. In birds, AMH is produced by the fetal ovaries at early stages although in lower amounts than in males, which may explain the sexual dimorphism in the evolution of Müllerian derivatives. In all species, the testes secrete significantly higher levels of AMH than the ovaries until puberty. Then, AMH production decreases in Sertoli cells and sex differences are no longer observed in AMH levels in the adult until menopause, when the ovaries cease to produce AMH.

AMH binds to its specific receptor, AMHR-II, present on mesenchymal cells surrounding the Müllerian duct epithelium (Fig. 2A), with a relatively high dissociation constant (approximately 2.5 nM); this explains why AMH must be at a high level to be active, behaving as a local factor. In Jost's experiences and in human pathology, the existence of only one functional testis during fetal life results in the regression of the homolateral Müllerian duct and the development of a Fallopian tube and a hemi-uterus on the side where there is insufficient or no testicular tissue. At variance with this is the observation in bovine and ovine fetuses of "freemartinism," consisting of AMH-like effects in a female twin to a male fetus. A clear explanation of this particular phenomenon is still lacking.

The effects observed in female freemartin fetuses and in transgenic female mice chronically overexpressing AMH confirm that the AMH receptor signaling pathway is present in the target organs of both sexes and that male-specific regression of Müllerian ducts in fetal life is due to the sex differences in AMH gonadal secretion. AMHR-II expression begins in mesenchymal cells of the cranial end of Müllerian ducts in early fetal life and subsequently spreads caudally. In clear correlation with AMHR-II temporal expression is the pattern of regression of Müllerian ducts. When AMH binds to its type II receptor, mesenchymal cells produce an as yet unknown signal that induces apoptosis in the neighboring epithelial cells of Müllerian ducts, followed by basal membrane disruption and epithelial–mesenchymal transformation, which

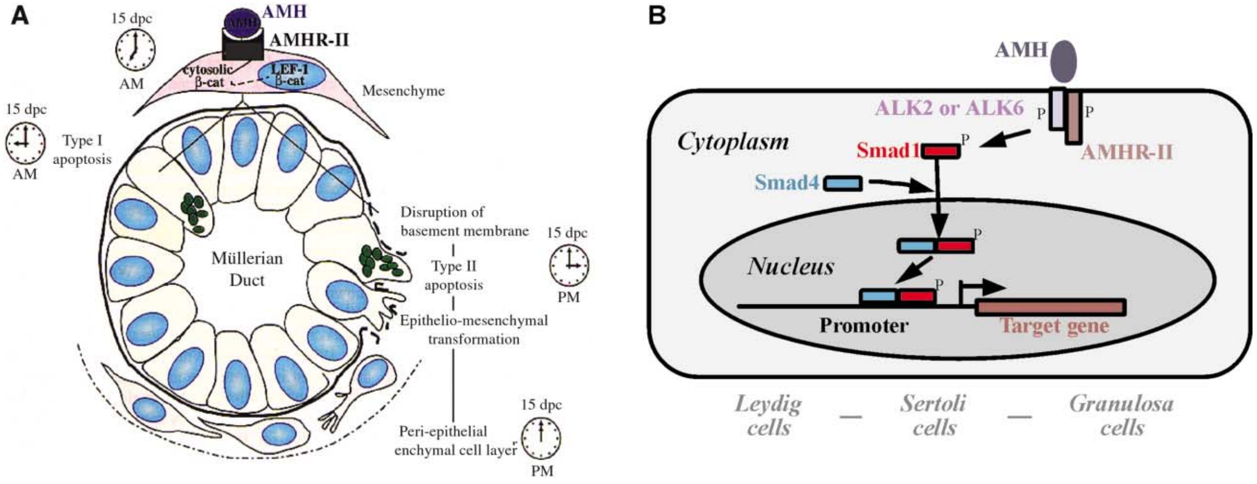

FIGURE 2 (A) Mechanism of action of AMH in Müllerian ducts. Modified from S. Allard, *et al.* Molecular mechanisms of hormone-mediated Müllerian duct regression: Involvement of β-catenin. *Development* **127**, 3349–3360. Copyright 2000, by permission of The Company of Biologists Ltd. (B) Mechanisms of action of AMH in Sertoli, Leydig, and granulosa cells. Modified from J. Massagué. TGFβ signal transduction. *Annual Review of Biochemistry* **67**, 753–791. Copyright © 1998 by permission of the publisher Annual Reviews, www.AnnualReviews.org.

finally results in Müllerian duct regression (Fig. 2A). The expression of AMHR-II is regulated by SF-1 and Wnt-7a.

Naturally occurring mutations in AMH or AMHR-II and transgenic mice in which these genes have been knocked out clearly indicate that the essential role of AMH is the regression of the uterus and Fallopian tube anlagen in the male fetus (see Section VI). However, AMHR-II is also expressed in granulosa cells of the ovary and Sertoli and Leydig cells of the testis. In the female gonad, AMH downregulates LH receptor and aromatase activities, inducing a decrease of estrogen production. An excess of AMH (e.g., in freemartins or in transgenic mice overexpressing AMH) provokes a dramatic reduction of ovarian size owing to a decrease in germ cell number and a depletion of ovarian follicles; cord-like structures resembling Sertoli-cell-only seminiferous tubules may subsequently appear. In the testis, AMH exerts a negative effect on Leydig cell differentiation from mesenchymal precursors and decreases the expression of steroidogenic enzymes, mainly cytochrome P450 17α-hydroxylase/C17–20 lyase (P450c17), resulting in lowered testosterone production. Transgenic male mice chronically overexpressing AMH may have Leydig cell hypoplasia and decreased androgen levels, which can result in hypovirilization. Conversely, transgenic mice with null mutations of AMH or AMHR-II develop a hyperplasia of Leydig cells. As in granulosa cells, aromatase activity is also decreased in Leydig and Sertoli cells exposed to AMH. The mechanism of action of AMH has recently been described (Fig. 2B): In granulosa and Sertoli cells, AMH binding to AMHR-II induces the recruitment of a nonspecific type I receptor, ALK2 or ALK6, resulting in the phosphorylation of Smad1, which—upon binding to Smad4—is translocated to the nucleus and regulates target gene expression.

## V. REGULATION OF AMH PRODUCTION

The need for sex-specific expression of AMH during a short critical period of sensitivity to the hormone in order for normal sex differentiation to occur suggests that AMH expression is tightly regulated. In mammals, but not in birds, SOX9 seems to be one of the most important activators of AMH expression in the fetal testis. SF1 and GATA-4 synergistically increase SOX9-induced AMH expression. All three of these factors have binding elements on the AMH promoter. GATA-4, WT-1 (Wilms' tumor suppressor-1), and DAX-1 (dosage-sensitive sex-reversal, adrenal hypoplasia, critical region on the X chromosome, gene 1), which do not bind to the AMH promoter, can also indirectly influence AMH expression. GATA-4 and WT-1 always function as enhancers, whereas DAX-1 acts as a repressor of AMH transcription, via interactions with SOX9 or SF-1.

While these transcription factors regulate baseline AMH expression, androgens, follicle-stimulating hormone (FSH), and primary spermatocytes influence production in Sertoli cells. When intratesticular testosterone concentration increases in the male gonad, AMH is down-regulated, provided that the androgen receptor is expressed at sufficient levels in Sertoli cell nuclei. This is why testicular AMH production decreases during normal puberty in males. In the fetus and newborn, as in patients with androgen insensitivity, the lack of activity of the androgen receptor prevents testosterone from inhibiting AMH expression in Sertoli cells. FSH is capable of enhancing AMH production, but its effect is observed only when the inhibitory effect of testosterone, which is largely predominant in normal puberty, is absent. Independently of testosterone, the entry of germ cells into meiosis is also followed by AMH down-regulation. Although the molecular events responsible for hormonal and cellular regulation of testicular AMH output need further investigation, serum AMH appears to be a reliable marker of FSH and androgen effects on Sertoli cells as a well as of meiotic entry of male germ cells.

## VI. PERSISTENT MÜLLERIAN DUCT SYNDROME

The persistent Müllerian duct syndrome (PMDS) is a rare form of internal male pseudo-hermaphroditism in which the Fallopian tubes and uterus are present, indicating a failure in the AMH-dependent sex differentiation pathway. In these patients, external genitalia have normal male characteristics, proof that the androgen-dependent pathway is intact. PMDS should be distinguished from other forms of male pseudo-hermaphroditism with alterations in androgen synthesis or action resulting in ambiguous or female external genitalia. In PMDS, the Müllerian derivatives are generally discovered unexpectedly during surgical correction of cryptorchidism and/or inguinal hernia. Two different anatomical forms have been described: In the most common presentation, one testis descends into the scrotum and drags the homolateral Fallopian tube, the uterus, and consequently the contralateral Fallopian tube and testis. Less frequently, both testes remain in ovarian position embedded in the broad ligament.

PMDS is an autosomal recessive condition resulting from a lack of production of AMH or a resistance to AMH in target organs. The former, known as AMH-negative PMDS, is due to mutations in both alleles of the AMH gene: These patients represent approximately 50% of all PMDS cases and have undetectable serum AMH levels. Resistance to AMH, or AMH-positive-PMDS, is due to mutations in AMHR-II, present in nearly 40% of the cases. Serum AMH is normal or high. Except for a 27 bp deletion in exon 10 of AMHR-II, responsible for almost 20% of PMDS cases, there are no hotspots in the AMH or AMHR-II genes, and mutations have been found along the entire length of both genes (Fig. 3). In approximately 10% of the PMDS cases described, no mutation has been found, suggesting that mutations in a type I receptor, in components of the transduction cascade, or in the proteolytic enzyme involved in AMH processing may also be responsible for this syndrome. Mice models for PMDS have been obtained by transgenic engineering: Gene knockout of either AMH or AMHR-II results in the same phenotype. PMDS has also been described as a naturally occurring condition in two strains of dogs.

## VII. CLINICAL APPLICATIONS OF AMH

As a marker of the existence of functional Sertoli cells, determination of serum AMH is extremely useful in boys with nonpalpable gonads (Fig. 4): Before puberty, undetectable serum AMH is highly indicative of anorchia, except for rare cases of AMH-negative PMDS. Serum AMH may also be used to estimate the functional mass of Sertoli cells in prepubertal boys: Low serum AMH is found in patients with small, dysgenetic testes, and elevated AMH has been detected in rare cases of prepubertal Sertoli cell hyperplasia. Because AMH production is normally low in the first 10–15 days following birth, determination of serum levels may not be informative in this period of life. The advantage of AMH over androgens during the rest of childhood is that no human chorionic gonadotropin stimulation test is necessary.

Intersex disorders in patients with an XY lineage may result from gonadal dysgenesis affecting both the tubular and the interstitial compartments of the testes or from a dissociated tubular–interstitial dysfunction in which only one compartment is affected. The existence of ambiguous or female external genitalia indicates that the androgen pathway of sex differentiation is impaired. Low testosterone production may result from either gonadal dysgenesis or mutations affecting Leydig cell differentiation or steroidogenesis. AMH determination is useful in these cases, because serum levels are low in patients with gonadal dysgenesis but normal or high if the condition is due to an isolated defect of the androgen pathway (Fig. 4 and Table 1). In intersex patients with normal or high levels of both testosterone and AMH, androgen

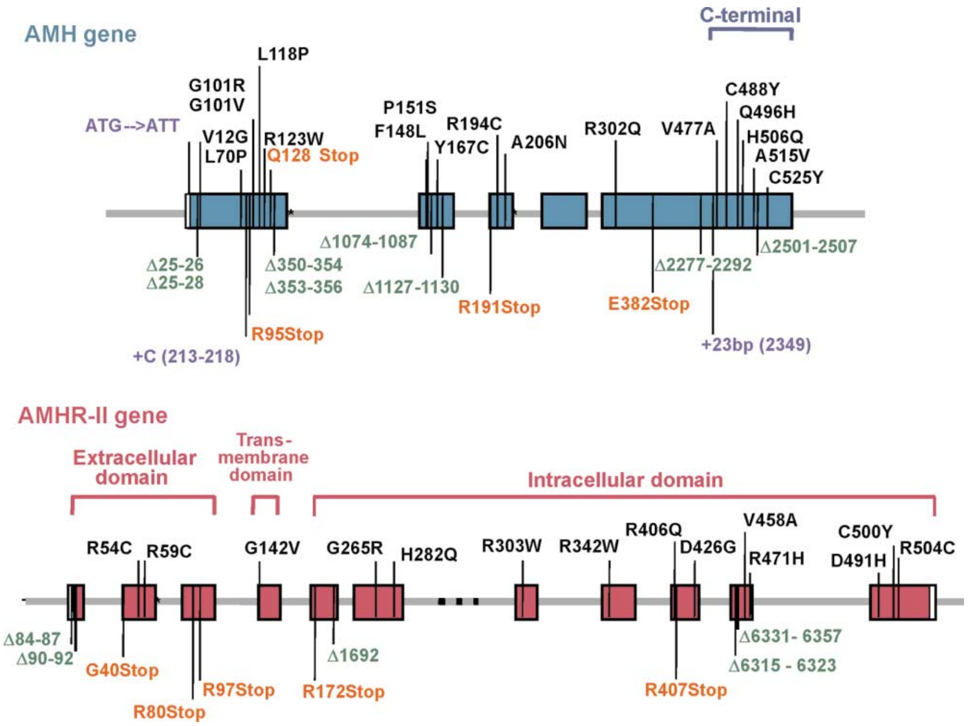
AMH gene
C-terminal
L118P
G101R
G101V
ATG-->ATT
V12G
L70P
R123W
Q128 Stop
P151S
F148L
R194C
Y167C
A206N
R302Q
V477A
C488Y
Q496H
H506Q
A515V
C525Y
Δ25-26
Δ25-28
Δ350-354
Δ353-356
Δ1074-1087
Δ1127-1130
R191Stop
R95Stop
+C (213-218)
Δ2277-2292
Δ2501-2507
E382Stop
+23bp (2349)
AMHR-II gene
Extracellular domain
Trans-membrane domain
Intracellular domain
R54C
R59C
G142V
G265R
H282Q
R303W
R342W
R406Q
D426G
V458A
R471H
C500Y
D491H
R504C
Δ84-87
Δ90-92
G40Stop
R80Stop
R97Stop
Δ1692
R172Stop
R407Stop
Δ6331- 6357
Δ6315 - 6323

**FIGURE 3** Mutations in human genes for AMH and AMHR-II responsible for the persistent Müllerian duct syndrome. From C. Belville, N. Josso, and J. Y. Picard. Persistence of Müllerian derivatives in males. *American Journal of Medical Genetics* **89**, 218–223, 1999. Copyright © 1999. Reprinted by permission of Wiley–Liss Inc., a subsidiary of John Wiley & Sons, Inc.

insensitivity is the most probable etiology (Fig. 4 and Table 1). The ovary produces very low quantities of AMH. The detection of serum AMH levels over 100 pmol/liter is clearly indicative of the existence of testicular tissue in XX patients. Congenital adrenal hyperplasia can be ruled out, and a diagnosis of true hermaphroditism or XX male should be considered.

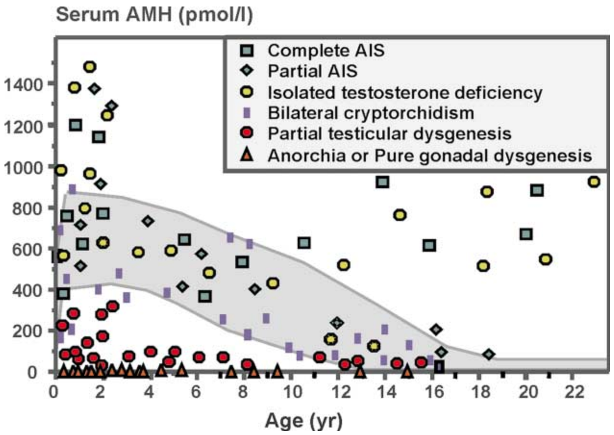

**FIGURE 4** Serum AMH in patients with intersex disorders and/or abnormal testicular function. AIS, androgen insensitivity syndrome. The shaded area represents normal values of serum AMH

Because it is a reliable marker of androgen concentration within the testis, serum AMH is useful in the follow-up of patients treated for precocious puberty or hypogonadotropic hypogonadism. High serum AMH is indicative of low androgen production by Leydig cells; a decrease in serum AMH suggests that the intratesticular concentrations of androgens and Sertoli cell expression of the androgen receptor reach pubertal levels.

Sertoli and granulosa cells may originate sex-cord stromal tumors that conserve AMH expression. Immunohistochemical study of biopsies using anti-AMH antibodies proves useful in the identification of these tumors. Furthermore, the serum level of AMH correlates with tumor progression and is a reliable marker of the success of surgical treatment or chemotherapy (Fig. 5). Since granulosa cell tumors are frequently observed postmenopause or require bilateral ovariectomy—both situations resulting in no detectable AMH in serum—the determination of AMH using an ultrasensitive enzyme-linked immunosorbent assay represents a powerful tool for the early detection of recurrences.

**TABLE 1** Diagnosis of Intersex Disorders Based on Serum AMH and Testosterone Levels in Patients with a 46,XY Karyotype

| | External genitalia | | | |
|---|---|---|---|---|
| | Female | | Ambiguous | |
| | High testosterone | Low or undetectable testosterone | High testosterone | Low testosterone |
| Normal or high AMH | Complete AIS | Leydig cell aplasia; severe steroidogenic enzyme deficiency | Partial AIS | Leydig cell hypoplasia; mild steroidogenic enzyme deficiency |
| Low or undetectable AMH | | Pure gonadal dysgenesis | | Partial gonadal dysgenesis |

*Note*. AMH and testosterone levels are considered high, normal or low, compared to those observed in normal age-matched boys. AIS, androgen insensitivity syndrome.

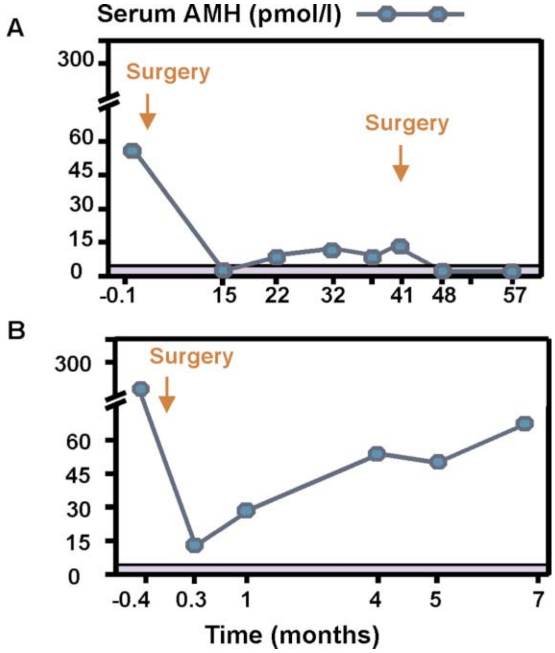

FIGURE 5 Serum AMH in female patients with granulosa cell tumors. In Patient A, surgical removal of the tumor was initially successful, and AMH levels became undetectable; however, the tumor recurred and could be detected by serum AMH determination before clinical manifestations became apparent. In Patient B, a complete surgical removal of the tumor could not be achieved. The shaded area represents expected values in postmenopausal or bilaterally ovariectomized women. Time 0 indicates the moment of surgery. Reprinted from Long, W. Q., Ranchin, V., Pautier, P., Belville, C., Denizot, P., Cailla, H., Lhommé, C., Picard, J. Y., Bidart, J. M., and Rey, R. Detection of minimal levels of serum anti-Müllerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay. *The Journal of Clinical Endocrinology and Metabolism* 85, 540–544, 2000. Copyright 2000, The Endocrine Society.

## VIII. SUMMARY

AMH is produced by testicular Sertoli cells and ovarian granulosa cells. As soon as the testes differentiate in the fetus, Sertoli cells secrete large amounts of AMH, inducing the regression of Müllerian ducts, the anlagen of the uterus and Fallopian tubes, after binding to a specific membrane receptor, AMHR-II. Mutations in the genes coding for AMH or AMHR-II result in males with a uterus. In mammals, granulosa cells secrete only low quantities of AMH from late fetal life, when Müllerian ducts are no longer sensitive to the hormone. AMH also acts as a moderator of the activities of granulosa and Leydig cells, which also express AMHR-II. Owing to its specificity of expression, AMH is a useful serum marker of Sertoli and granulosa cell function under normal and pathological conditions.

## Glossary

**dissociated tubular–interstitial testicular dysfunction** Disorder of gonadal development affecting only one gonadal compartment.

**fetal sex differentiation** In mammals, although defined from the time of fertilization by the existence of a heterogametic (XY) or homogametic (XX) chromosome pair, the sex of the embryo remains indistinguishable during the first stages of development. When the testis differentiates, it begins to secrete AMH, responsible for the regression of Müllerian ducts, and androgens, which virilize the external and internal genitalia. In the absence of the secretion or action of these hormones, the genitalia acquire a female phenotype.

**gonadal dysgenesis** Disorder of gonadal development affecting all gonadal compartments.

**granulosa cells** Somatic cells of the ovary that form the ovarian follicles, in which germ cells are embedded.

**hermaphroditism** The existence of testicular and ovarian tissue in one individual.

**Leydig cells** Somatic cells of the testes secreting testosterone after stimulation by luteinizing hormone or chorionic gonadotropin.

**Müllerian ducts** Paired ducts, formed of epithelial cells surrounded by mesenchymal cells, present in male and female embryos before sexual differentiation. Also known as paramesonephric ducts. In the male fetus, they degenerate under AMH action; in the female fetus, they give rise to the Fallopian tubes, the uterus, and the upper third of the vagina.

**pseudo-hermaphroditism** Disorder characterized by the existence of female or ambiguous genitalia in individuals with male gonads, or vice versa.

**Sertoli cells** Somatic cells of the testes that form the seminiferous tubules where the germ cells giving rise to the gametes are embedded. The tissue surrounding the seminiferous tubules is called interstitial tissue and contains Leydig cells.

**transforming growth factor-$\beta$ (TGF-$\beta$)** The first isolated member of a family of factors controlling the growth, differentiation, and death of most tissues in invertebrates and vertebrates.

## See Also the Following Articles

**Sexual Differentiation, Molecular and Hormone Dependent Events in • Spermatogenesis, Hormonal Control of • Testis Descent, Hormonal Control of**

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# Apoptosis

DEREK LE ROITH* AND MICHAEL KARAS†

*National Institutes of Health, Maryland • †Cambrex Bioscience, Maryland*

I. CELLULAR EVENTS IN APOPTOSIS
II. DETECTION METHODS
III. BIOCHEMICAL ASPECTS

**granulosa cells** Somatic cells of the ovary that form the ovarian follicles, in which germ cells are embedded.

**hermaphroditism** The existence of testicular and ovarian tissue in one individual.

**Leydig cells** Somatic cells of the testes secreting testosterone after stimulation by luteinizing hormone or chorionic gonadotropin.

**Müllerian ducts** Paired ducts, formed of epithelial cells surrounded by mesenchymal cells, present in male and female embryos before sexual differentiation. Also known as paramesonephric ducts. In the male fetus, they degenerate under AMH action; in the female fetus, they give rise to the Fallopian tubes, the uterus, and the upper third of the vagina.

**pseudo-hermaphroditism** Disorder characterized by the existence of female or ambiguous genitalia in individuals with male gonads, or vice versa.

**Sertoli cells** Somatic cells of the testes that form the seminiferous tubules where the germ cells giving rise to the gametes are embedded. The tissue surrounding the seminiferous tubules is called interstitial tissue and contains Leydig cells.

**transforming growth factor-$\beta$ (TGF-$\beta$)** The first isolated member of a family of factors controlling the growth, differentiation, and death of most tissues in invertebrates and vertebrates.

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**Sexual Differentiation, Molecular and Hormone Dependent Events in • Spermatogenesis, Hormonal Control of • Testis Descent, Hormonal Control of**

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# Apoptosis

Derek Le Roith* and Michael Karas†

*National Institutes of Health, Maryland • †Cambrex Bioscience, Maryland

I. CELLULAR EVENTS IN APOPTOSIS
II. DETECTION METHODS
III. BIOCHEMICAL ASPECTS

**Apoptosis is an essential process in the development and homeostasis of multicellular organisms. In embryogenesis, apoptosis ensures the correct development of organisms, counteracting proliferation by removing unnecessary cells. In adults, apoptosis is also crucial in counteracting unrestricted proliferation as well as in the cyclic growth and involution of endocrine-dependent tissues. Apoptotic cell death differs from necrotic cell death in that specific, characteristic morphological changes occur, including the following: There are changes within the nucleus, in particular, chromatin condensed as DNA is degraded into small nucleosomal fragments. Changes occur in specific organelles, in particular, in the mitochondria, where apoptosis uncouples electron transport from ATP synthesis, leading to an increase in reactive oxygen species and a decrease in transmembrane potential. Changes at the plasma membrane lead to cell shrinkage and the formation of membrane protuberances that enclose cellular debris, become apoptotic bodies, and are subsequently engulfed by phagocytic cells.**

## I. CELLULAR EVENTS IN APOPTOSIS

Apoptotic cell death is crucial for both normal development and homeostasis of multicellular organisms. During embryonic development, apoptosis counteracts proliferation by removing unnecessary cells to ensure proper organogenesis. In the adult, apoptosis is important mainly in counteracting unrestricted (i.e., neoplastic) proliferation and in the cyclic involution of many endocrine-dependent tissues. Apoptosis is distinct from necrotic death in that (1) characteristic and specific morphological changes occur and (2) energy synthesis and protein synthesis are required in the dying apoptotic cell, to regulate specific genes and biochemical pathways.

The morphology of apoptosis involves changes within the nucleus, within specific organelles (most notably, the mitochondria), and within the plasma membrane. In what was once considered to be the hallmark of apoptosis, the chromatin condenses within the nucleus, as DNA is degraded first into large 30 to 50 kb fragments and then into smaller nucleosomal fragments of 180–200 bp. These nuclear alterations, however, are not a *sine qua non* of apoptosis, as their inhibition fails to block cell death.

Apoptosis leads to uncoupling of electron transport from ATP synthesis in the mitochondria, thereby leading to an increase in reactive oxygen species (ROS) and a decrease in transmembrane potential. These changes precede the nuclear changes described above and can occur in the absence of nuclear changes in apoptotic cells. Different members of the bcl-2 family of proteins oppose or promote cell survival under apoptotic conditions, as described in more detail below. The identification of bcl-2 family members in mitochondrial membranes suggests that mitochondrial changes are not merely the end result of apoptosis but are involved in the apoptotic cascade itself.

Changes in the plasma membrane and cytoskeleton lead to cell shrinkage and to the formation of membrane protuberances or “blebs.” As apoptosis proceeds, these blebs of membrane enclosing cellular debris detach and become “apoptotic bodies,” which are then engulfed by neighboring phagocytic cells. These changes are relatively easy to observe with light microscopy. However, the rapid time course of the apoptotic process, which is complete within a few hours, makes it difficult to identify a significant number of apoptotic cells at any given time. This problem is further compounded *in vivo*, where apoptotic rates are probably even slower than under experimental conditions *in vitro* and where the close proximity of phagocytic cells within normal tissue facilitates the rapid clearance of apoptotic cells. The loss of membrane asymmetry causes translocation of the phospholipid phosphatidyl serine from the internal leaflet to the outer surface, where it serves as a recognition marker for apoptotic cells by phagocytes.

## II. DETECTION METHODS

DNA fragmentation, long considered a characteristic feature of apoptosis, is often determined by the identification of a 200 bp DNA ladder with gel electrophoresis and, more recently, by enzyme-linked immunosorbent assay kits based on antibodies to histone–DNA complexes. However, these methods are not quantitative (i.e., one cannot determine the percentage of cells actually undergoing apoptosis in the population being assayed) and give only a rough qualitative estimate, at best. The alternative quantitative method is to stain DNA in ethanol-fixed cells and then analyze the DNA content by flow cytometry, using fluorescent dyes, such as propidium iodide, that intercalate into DNA. In this approach, apoptotic cells are characterized by a reduced (sub-G1) DNA content as compared to cells in the G1 phase of the cell cycle.

This analysis must be done carefully, so that true apoptotic nuclei (with a DNA content within 1 log of cells in G1) will be distinguished from smaller apoptotic bodies. The method is presumed to give the ratio of normal cells to apoptotic cells. However, it has recently been shown that necrosis, particularly when induced by ROS, also results in DNA fragmentation. Thus, an accurate analysis of apoptosis requires excluding necrotic death by other means (e.g., morphological analysis). Another widely used technique detects breaks in the DNA. The deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling, or TUNEL, assay utilizes TdT to label the 3′-ends of breakpoints in double-stranded DNA and can be used both for flow cytometry and for *in situ* labeling. TUNEL is the preferred method for detecting apoptosis *in vivo*, primarily due to the lack of technically feasible alternatives.

As mitochondrial dysfunction is an early common event in the apoptotic cascade, a number of methods analyze mitochondrial function to detect apoptosis. A popular and simple assay is the MTT assay, which is based on the ability of mitochondria to convert soluble yellow tetrazolium salt into blue formazan. This enables the quantification of viable cells. The MTT assay does not distinguish between apoptosis and necrosis per se, but it can be effectively used to quantify cell death when apoptosis has been confirmed by other methods. However, it is not suitable for use *in situ* or *in vivo*. Another method is based upon the uptake in live cells of fluorescent mitochondrion-selective dyes, such as rhodamine 123 or $DiOC_6$, which is dependent on mitochondrial membrane potential. This method can be used both in flow cytometry and in fluorescence microscopy, where necrotic cells can be excluded with the use of plasma membrane nonpermeable DNA dyes. Various mitochondrion-selective dyes have recently been modified so that they can be fixed, thereby enabling *in situ* staining.

An alternative method detects the loss of asymmetry of phospholipids in the plasma membrane of apoptotic cells. Annexin V is a calcium-dependent phospholipid-binding protein that, when conjugated to a fluorescent dye, can be used to label apoptotic cells where it recognizes phosphatidyl serine moieties that have been translocated to the outer surface of the cell membrane. When propidium iodide is included in this assay, it can concurrently detect necrotic cells that have lost membrane integrity. Annexin V–FITC is an ideal assay to measure apoptosis in living cultured cells, as it simultaneously allows rapid and quantitative detection of normal, apoptotic, and necrotic cell populations. However, annexin V–FITC is nearly impossible to apply in an *in vivo* setting.

## III. BIOCHEMICAL ASPECTS

Elucidation of the biochemical processes involved in apoptosis has been facilitated by the genetic analysis of development in *Caenorhabditis elegans*. In this nematode, which is made up of 1090 adult cells, 131 specific cells undergo apoptosis during embryonic development. Genes that either promote apoptosis (such as ced-3) or prevent apoptosis (such as ced-9) in *C. elegans* have mammalian homologues that encode related gene families. The cloning of ced-3 led to the discovery of a novel class of the caspase proteases, which cleave proteins after aspartic acid residues and seem to operate in an enzymatic cascade. Some caspases are apically activated by so-called "death molecules" [e.g., caspase-8 activation by Fas and other tumor necrosis factor-related molecules and others act as downstream effectors of various apoptotic events, such as cleavage of poly(ADP ribose) polymerase, lamin, actin, and other molecules]. Ced-9 is related to the bcl-2 family of proteins. Current models of the regulation of apoptosis suggest that the balance between members of the Bcl-2 family plays a critical role in determining whether a cell survives or activates the cell death machinery. Several members of the Bcl-2 family (Bcl-2, Bcl-xL, MCL-1, A1, and BAG-1) appear to promote survival, whereas others (Bcl-xS, BAD, BAX, and BAK) promote apoptosis. The balance between hetero- and homodimerization of the various Bcl-2 family proteins is thought to be critical in determining cell fate.

Caspase proteins not only are evolutionarily conserved, but also seem to universally operate in a similar way in most cell types that have been studied. In contrast, the role played by other proteins in the apoptotic cascade is more restricted. Bcl-2, for example, is not required for embryonic development. Bcl-2 can block apoptosis induced by a wide variety of stimuli, including chemotherapeutic agents, $\gamma$ and UV radiation, and overexpression of *myc* or *p53*, but this protein plays little or no role in other apoptotic processes, such as the negative selection of thymocytes. Even in *C. elegans*, genes that are involved in the apoptotic pathway of specific subtypes of the 131 cells that are eliminated during embryogenesis, such as *egl-1*, which is involved in the apoptosis of hermaphrodite-specific neurons, have been cloned. By controlling the genes involved in the common steps of the apoptotic pathway in specific cell types, such a gene can control apoptosis within a specific cell type or differentiation step. The mammalian homologues of egl-1 contain Bcl-2 homology region 3 domains. These domains are involved in protein–protein interactions, but the specific role they play in the cellular control of apoptosis remains to be determined.

Within the endocrine system, cyclic growth and involution as well as homeostasis of hormone-dependent target organs require tight cellular control of susceptibility to apoptosis. For example, in the murine reproductive tract, gonadectomy induces apoptotic cell death, which is dependent upon Fas neo-expression and is preceded by down-regulation of BCL-2; ovarian follicle atresia results from granulosa cell apoptosis, which is preventable by estrogens and FSH; apoptotic regression of decidual zones in the implanted uterine is hormone dependent and correlates with an increase in bax and a decrease in bcl-2. The emerging paradigm is that hormones can act on target cells not only by stimulating proliferation, but also by preventing apoptosis. It is interesting to note that hormones with a high level of anti-apoptotic activity, such as insulin-like growth factor-I, epidermal growth factor, platelet-derived growth factor, and nerve growth factor, activate specific cognate receptors, yet these receptors share strikingly similar functional activity and downstream signal transduction pathways. The receptors for each of these hormones possess intrinsic tyrosine kinase activity. Upon ligand binding, these receptors become self-activated by autophosphorylation. Shortly thereafter, three major signal transduction pathways are activated, including phosphatidylinositol 3′-kinase (PI-3 kinase), mitogen-activated protein kinase, and the stress-activated protein kinases p38 and JNK. The PI-3 kinase pathway is considered to play a major role in the anti-apoptotic activity of tyrosine kinase receptors. Activation of these receptors induces phosphorylation of the 85 kDa regulatory subunit of PI-3 kinase, thereby recruiting it to the membrane. Phosphorylation of phosphoinositides within the membrane then recruits pleckstrin homology domain-containing proteins, such as AKT, a serine–threonine protein kinase, and phosphtatidylinositol(3,4,5)triphosphate-dependent kinases. The assembly of these proteins within the same microdomain facilitates the phosphorylation of AKT at the two sites, which results in activation of the AKT molecule. The activated AKT then phosphorylates the mitochondrial pro-apoptotic protein BAD on Ser136. Phosphorylated BAD then forms a complex with 14-3-3 protein, which sequesters BAD and prevents its pro-apoptotic actions.

Although our knowledge of a complex network of pathways regulating apoptosis is much more advanced than it was a decade ago, it is still far from complete. Mapping of the human genome and other genomes, as well as rapid development of modern technologies, such as microarray analysis, will dramatically improve our understanding of the mechanisms underlying apoptotic death.

## Glossary

**apoptosis** Decision by a cell to die.

**Bcl family of proteins** Some members of this protein family prevent apoptosis.

**caspase proteins** When activated, these proteins mediate cell death.

**programmed cell death** The process by which cells actually die.

## See Also the Following Articles

**Apoptosis Gene Knockouts • Apoptosis, Glucocorticoid-Induced • Cancer Cells and Progrowth/Prosurvival Signaling**

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# Apoptosis Gene Knockouts

JAMES K. PRU AND JONATHAN L. TILLY
*Massachusetts General Hospital and Harvard Medical School*

I. OVERVIEW OF APOPTOSIS
II. APOPTOSIS GENE KNOCKOUTS AND THE MAMMARY GLAND
III. APOPTOSIS GENE KNOCKOUTS AND THE OVARY

**The cell death pathway of apoptosis is fundamental to sex determination and the embryonic development of reproductive organs, as well as to the normal reproductive and endocrine functions of the gonads, reproductive tract, and accessory reproductive tissues in postnatal life. Recent investigations have documented that germ cells and somatic cells of female reproductive tissues possess an evolutionarily conserved cohort of "cell death genes" that ultimately determine cell fate in response to physiological or pathological stimuli. However, much of the current knowledge regarding the role of apoptosis in female reproductive tissue development and function stems from studies that characterize the expression of genes known to coordinate and execute apoptosis. Although it is important to continue to understand when, and in what circumstances, these genes are expressed, it is equally important to distinguish between those genes that are correlatively or redundantly expressed from those that are functionally required for apoptosis to occur. This article reviews what is currently known of the principal intracellular pathways responsible for initiating and executing apoptotic cell death in vertebrate species, then discusses several published reports in which gene inactivation was used to identify functional, or in some cases dispensable, components of the cell death machinery in somatic cells and germ cells of the female reproductive system.**

## I. OVERVIEW OF APOPTOSIS

In most cell types, the process of apoptosis can be broken down into six phases that are arguably characterized by a decreasing level of complexity as the cell becomes progressively more committed to apoptosis (Fig. 1). In the signal transduction phase (phase I), a physiological or pathological stimulus (or stimuli) for death is relayed across the plasma membrane of the cell. The signal(s) conveyed by these stimuli is amplified and integrated through alterations in the activity of a wide spectrum of second messengers and signal transduction networks, including the phosphatidylinositol 3′-kinase (PI-3K)/Akt, p53, sphingomyelin, and c-jun N-terminal kinase (JNK) pathways. This amplification step can be initiated, for example, by the binding of a "death ligand," such Fas ligand ($Fas_L$) or tumor necrosis factor α (TNFα), to its cognate "death receptor." Alternatively, an inadequate level of growth ("survival") factor support from the extracellular environment can serve as a potent stimulus for engaging the machinery of apoptosis in many cell types. Pro-apoptotic signal transduction cascades can also be set in motion by oxidative stress or other damage to the cell, usually at the level of the plasma membrane, cytoskeleton or DNA. In most situations, the signaling cascades of phase I then initiate phase II (Fig. 1), in which activation of one or more pro-apoptotic members of the Bcl-2 family (Table 1) containing only the Bcl-2 homology domain 3 ("BH3-only") motif occurs.

At least 14 BH3-only proteins (Bad, Bid, Bim, Hrk/DP5, Nix, Noxa, MAP-1, Blk, Bik/Nbk, Bmf, Nip3/Bnip3, PUMA/Bbc3, Bcl-$G_{short}$, 2′-5′oligoadenylate synthase) have been identified in mammalian species to date (Table 1). Interestingly, the involvement of any given BH3-only protein in apoptosis appears to be dictated primarily by the stimulus for cell death. For example, Bad is a target for phosphorylation by the growth factor-coupled PI-3K/Akt cell survival pathway, and in its phosphorylated state Bad lacks cell death-promoting activity. If cells are exposed to inadequate levels of growth factor support, the absence of PI-3K/Akt signal transduction (phase I) thus permits Bad to exist in a nonphosphorylated or "active" state (phase II; BH3-only activation). As a consequence, hypophosphorylated Bad translocates to mitochondria where it interferes with the function of anti-apoptotic Bcl-2 family members (see below and Table 1). Activation of Bid occurs by a completely different mechanism involving a two-step process. The first is proteolytic cleavage of the inactive cytosolic protein by caspase-8, a signal transduction component coupled to ligand-activated death receptors. Truncated Bid is then myristolated as a means to promote translocation of this now "activated" protein to mitochondria where it functions with other pro-apoptotic Bcl-2 family members (see below and Table 1) to induce cell death.

In addition to these examples of BH3-only protein activation by the absence or presence of endocrine

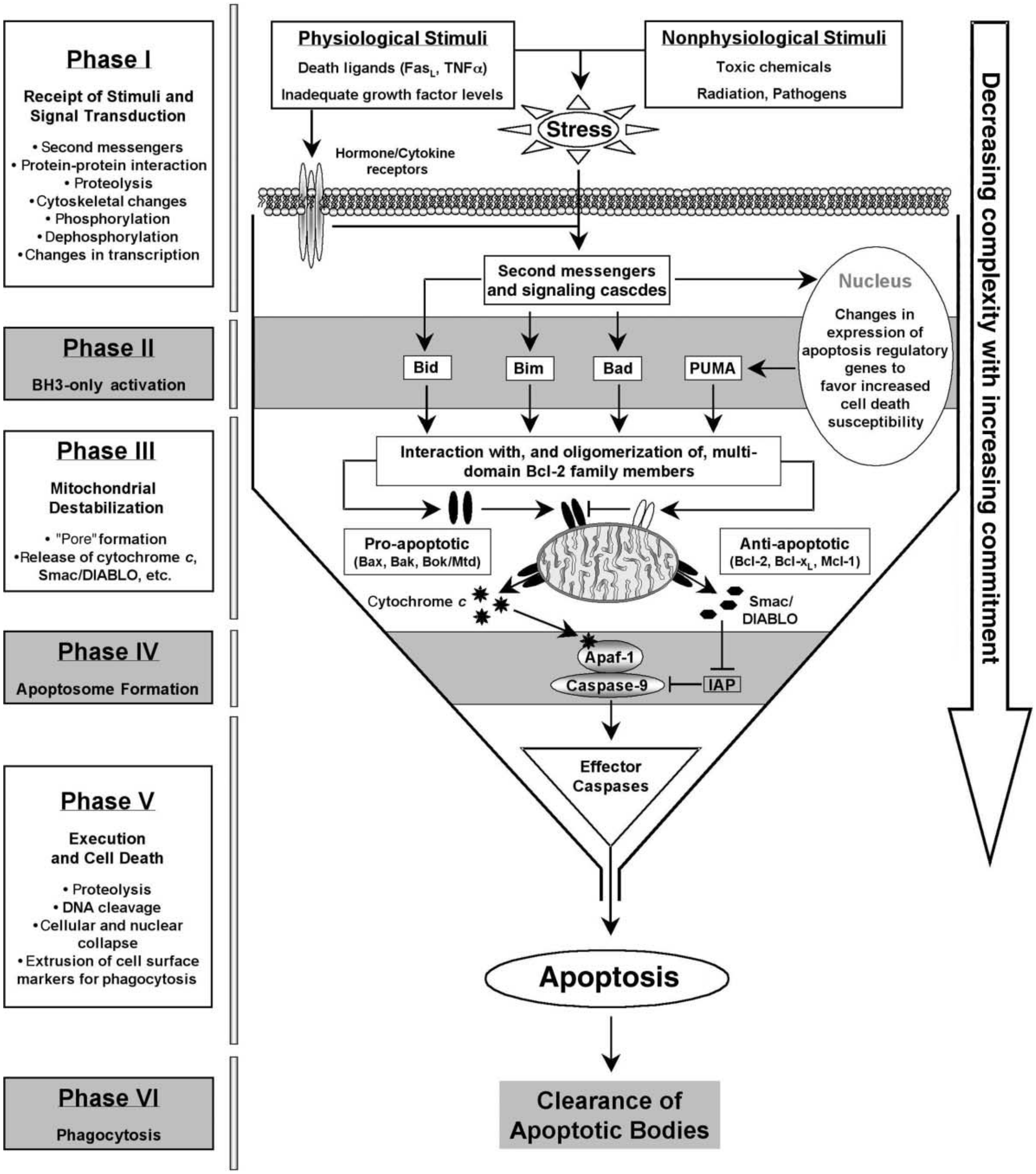
Phase I
Receipt of Stimuli and Signal Transduction
• Second messengers
• Protein-protein interaction
• Proteolysis
• Cytoskeletal changes
• Phosphorylation
• Dephosphorylation
• Changes in transcription
Phase II
BH3-only activation
Phase III
Mitochondrial Destabilization
• "Pore" formation
• Release of cytochrome c, Smac/DIABLO, etc.
Phase IV
Apoptosome Formation
Phase V
Execution and Cell Death
• Proteolysis
• DNA cleavage
• Cellular and nuclear collapse
• Extrusion of cell surface markers for phagocytosis
Phase VI
Phagocytosis
Physiological Stimuli
Death ligands ($\text{Fas}_L$, TNFα)
Inadequate growth factor levels
Nonphysiological Stimuli
Toxic chemicals
Radiation, Pathogens
Stress
Hormone/Cytokine receptors
Second messengers and signaling cascdes
Nucleus
Changes in expression of apoptosis regulatory genes to favor increased cell death susceptibility
Bid
Bim
Bad
PUMA
Interaction with, and oligomerization of, multi-domain Bcl-2 family members
Pro-apoptotic (Bax, Bak, Bok/Mtd)
Anti-apoptotic (Bcl-2, $\text{Bcl-x}_L$, Mcl-1)
Cytochrome c
Smac/ DIABLO
Apaf-1
Caspase-9
IAP
Effector Caspases
Apoptosis
Clearance of Apoptotic Bodies
Decreasing complexity with increasing commitment

FIGURE 1 Schematic diagram depicting the principal components of the six major phases of apoptosis in mammals. See text and bibliography for additional details.

signals, BH3-only proteins are recruited into action by a number of pathological insults, albeit by different mechanisms once again. For instance, DNA damage resulting from exposure of cells to ionizing radiation or various chemicals activates p53 and, if the genomic damage is irreparable, apoptosis. Recently, *noxa* and *puma/bbc3*, two transcriptionally regulated BH3-only members of the Bcl-2 family, were cloned as being p53-responsive genes involved in the apoptotic cell death response to DNA damage. As a final example of the diversity of mechanisms through which phase II of apoptosis can be set in motion, the Bim protein is normally sequestered to microtubules by its interaction with the LC8 component of the dynein motor complex. However, disorganization or disruption of the cytoskeleton by growth factor deprivation or chemical agents (e.g., Taxol) leads to a rapid release of Bim from the dynein motor complex into the cytoplasm. This activated form of Bim then translocates to mitochondria where the protein antagonizes the function of anti-apoptotic Bcl-2 family members (see below and Table 1).

Once activated, BH3-only proteins trigger phase III, or the mitochondrial destabilization phase, of apoptosis (Fig. 1) by facilitating the ability of pro-apoptotic Bcl-2 family members containing two or more Bcl-2-homology domains—the so-called "multidomain" pro-apoptotic members (e.g., Bax, Bak, and Bok/Mtd; Table 1)—to form "pores" in the outer mitochondrial membrane. In some cases (e.g., Bid), the destabilization of mitochondria can occur as a direct result of BH3-only proteins causing the oligomerization of pro-apoptotic multidomain members of the Bcl-2 family into pore-forming complexes. Alternatively, some BH3-only family members, such as Bad, Bim, and PUMA/Bbc3, function by direct interaction with anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-$x_{long}$, Mcl-1, and Bcl-w, anchored in the mitochondria. The latter scenario leads to a functional "sequestration" of anti-apoptotic Bcl-2 family members, allowing for pro-apoptotic multidomain family members to assume their pore-forming conformation. In either situation, a spectrum of cell death-promoting factors, including cytochrome *c*, Smac/DIABLO, apoptosis-inducing factor, and endonuclease-G, are released from mitochondria during phase III, presumably through pores created by pro-apoptotic Bcl-2 family members.

Importantly, the release of cytochrome *c* into the cytoplasm heralds the initiation of phase IV of apoptosis, during which time the protein functions as an essential co-factor in the formation of the "apoptosome" (Fig. 1). The apoptosome is a high-molecular-weight complex composed of many molecules of an adapter protein termed apoptotic protease-activating factor-1 (Apaf-1) and the pro-form of caspase-9. It is currently believed that the assembly of the apoptosome provides for an induced proximity model of autocatalytic or transcatalytic processing of procaspase-9 to the active enzyme. Interestingly, in nonapoptotic cells or in those cells not yet committed to die, a family of gene products referred to as inhibitor of apoptosis proteins (IAP) function to prevent premature or unwanted activation/activity of caspase-9. However, this survival checkpoint can be overridden by Smac/DIABLO released from the mitochondria during phase III, which dissociates IAP from caspase-9. Once freed from IAP-mediated constraint, caspase-9 rapidly activates phase V, or the execution phase (Fig. 1), of apoptotic cell death.

During this near-final phase of apoptosis, the pro-forms of a number of cell death effector caspases, such as caspase-2, -3, -6, and -7, are processed to form active enzymes by the apoptosome containing caspase-9 or by one another. These effector caspases then attack a wide spectrum of homeostatic and structural proteins within the cell, producing the morphological and biochemical characteristics of apoptotic cells and essentially sealing the cell's fate. Once cellular dissolution and fragmentation have occurred, phase VI of apoptosis is engaged. During this final step in the process, the membrane-bound vesicles containing the pieces of the dead cell are rapidly removed by phagocytosis to prevent an inflammatory response characteristic of pathological ("necrotic") cell death.

## II. APOPTOSIS GENE KNOCKOUTS AND THE MAMMARY GLAND

It has long been recognized that apoptosis occurs in the mammary gland of numerous mammalian species during weaning-induced involution of the tissue, and that hormones and cytokines play important roles as either survival factors (e.g., progesterone, glucocorticoids, and insulin-like growth factor-1/IGF-1) or death factors (e.g., transforming growth factor-β and $Fas_L$) in coordinating mammary epithelial cell fate. In addition, important roles for BH3-only proteins (Bad), multidomain pro-apoptotic members of the Bcl-2 family (Bax, Bak, and Bcl-$x_{short}$), multidomain anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-$x_{long}$, and Bcl-w), mitochondrial cytochrome *c* release, and caspases in controlling or executing mammary gland involution have been proposed. Unfortunately, many of these observations regarding the intracellular mediators of apoptosis in mammary epithelial cells remain correlative at present.

There are, however, four published examples of gene knockouts affecting mammary gland involution that have provided insight into functionally important components of at least phase I and phase III of apoptosis in this tissue. First, through the use of *lpr*/*lpr* mice, which are defective in the expression of the death receptor Fas, and *gld*/*gld* mice, which lack expression of $Fas_L$, in 2000, Song *et al.* demonstrated that both Fas and its ligand play important roles in apoptosis associated with the remodeling of post-lactational mammary tissue. At day 1 postweaning,

TABLE 1 Representative Members of the Mammalian BCL-2 Family

| Family member[a] | BH domains | Species studied[b] | Cellular localization in inactive state | Mechanism of BH3-only activation | Stimulus for BH3-only activation | Bcl-2-like interacting partner[c] |
|---|---|---|---|---|---|---|
| **Pro-apoptotic** | | | | | | |
| Multidomain | | | | | | |
| Bak | 1, 2, 3 | h, m, o, p, r, s | Mitochondria, ER[d] | NA | NA | Bid, Bax, Bcl-2, Bcl-X$_{long}$ |
| Bax | 1, 2, 3 | h, m, o, p, r, s | Mitochondria, ER, nucleus | NA | NA | Bid, Bax, Bcl-2, Bcl-X$_{long}$ |
| Bcl-G$_{long}$ | 2, 3 | h | Cytoplasm | NA | NA | NA |
| Bcl-rambo | 1, 2, 3, 4 | h | Mitochondria | NA | NA | NA |
| Bcl-x$_{short}$ | 2, 3, 4 | h, m, o, r | Mitochondria | NA | NA | Nrh |
| Bok/Mtd | 1, 2, 3 | h, m, r | Mitochondria | NA | NA | Mcl-1, Bcl-X$_{long}$, Bcl-2 |
| Diva/Boo[e] | 1, 2, 3, 4 | m | Perinuclear membranes | NA | NA | Apaf-1[f] |
| BH3-only | | | | | | |
| 2′-5′ Oligoadenylate synthase | 3 | h, m | Perinuclear | Transcription | Interferon-β | Bcl-2, Bcl-x$_{long}$ |
| Bad | 3 | b, h, m, p, r | Cytosol | Dephosphorylation | Growth factor deprivation | Bcl-x$_{long}$, Bcl-w |
| Bcl-G$_{short}$ | 3 | h | Mitochondria, heavy membranes | Unknown | Unknown | Bcl-2, Bcl-x$_{long}$ |
| Bid | 3 | h, m, p, r | Cytosol | Proteolysis and myristolation | Death receptor activation, calpains | Bax, Bak |
| Bik/NBK | 3 | h | Mitochondria | Phosphorylation | Unknown | Bcl-2, Bcl-w, Bcl-x$_{long}$ |
| Bim | 3 | d, h, m, p, r | Microtubules | Cytoskeletal alterations | Cytokine deprivation, γ-irradiation, Taxol | Bcl-2, Bcl-x$_{long}$ |
| Blk[g] | 3 | m, r | Mitochondria | Unknown | Unknown | Bcl-2, Bcl-x$_{long}$ |
| Bmf-1 | 3 | h, m | Microfilaments | Cytoskeletal alterations | Anoikis, DNA damage, cytokine deprivation | Mcl-1, Bcl-w, Bcl-2, Bcl-x$_{long}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| Hrk/DP5 | 3 | h, m, r | Mitochondria | Transcription | Calcium flux, DNA damage, cytokine deprivation | Bcl-2, Bcl-x$_{\text{long}}$ |
| Map-1 | 3 | h, m | Mitochondria | Unknown | Unknown | Bcl-2, Bax, Bcl-x$_{\text{long}}$ |
| Nip3/Bnip3 | 3 | h, m | Mitochondria | Transcription | Hypoxia | Bcl-2, Bcl-x$_{\text{long}}$, Nip3/Bnip3 |
| Nix | 3 | h, m | Mitochondria | Transcription | Hypoxia | Bcl-2, Bcl-x$_{\text{long}}$, Nix |
| Noxa | 3 | h, m | Mitochondria | Transcription | DNA damage | Bcl-x$_{\text{long}}$ |
| PUMA/Bbc3 | 3 | h, m | Mitochondria | Transcription | DNA damage, glucocorticoids, growth factor deprivation | Bcl-2, Bcl-x$_{\text{long}}$ |
| **Anti-apoptotic** | | | | | | |
| Multidomain | | | | | | |
| Bcl-2 | 1, 2, 3, 4 | h, m, o, p, r, s | Mitochondria, ER, nucleus | NA | NA | Hrk, Bcl-x$_{\text{long}}$, Bcl-2, Map-1 |
| Bcl-B | 1, 2, 3, 4 | h | Mitochondria, cytosol | NA | NA | NA |
| Bcl-x$_{\text{long}}$ | 1, 2, 3, 4 | h, m, o, p, r | Mitochondria | NA | NA | Bcl-G$_{\text{short}}$, Bcl-2, Noxa, Bax |
| Bcl-w | 1, 2, 3, 4 | h, m | Mitochondria | NA | NA | Bik, Bcl-x$_{\text{long}}$, Bcl-2 |
| Bfl-1/A1 | 1, 2, 3, 4 | h, m, p | Unknown | NA | NA | Unknown |
| Mcl-1 | 1, 2, 3, 4 | h, m, p, r | Mitochondria | NA | NA | Bmf-1, Bcl-w, Bcl-2, Bcl-x$_{\text{long}}$ |
| Nrh[h] | 1, 2, 3, 4 | h | Mitochondria, nuclear envelope | NA | NA | Bcl-x$_{\text{short}}$ |
| Boo/Diva[e] | 1, 2, 3, 4 | m | Perinuclear membranes | NA | NA | Apaf-1[f] |

[a] Except for Bcl-x and BCI-G, this list does not include numerous splice variants of various members.
[b] b, bovine; d, dog; h, human; m, mouse; o, ovine; p, nonhuman primate; r, rat; s, swine.
[c] Representative interactions, most of which were deduced from cell-free or yeast two-hybrid assays that may not be reflective of what occurs *in vivo*.
[d] ER, endoplasmic reticulum.
[e] Depending on the study and cell type, this member can be pro- or anti-apoptotic.
[f] Apaf-1 is a component of the apoptosome (phase IV).
[g] Blk (Bik-like killer) may be the mouse orthologue of human Bik/NBK.
[h] Nrh is a human orthologue of mouse Boo/Diva and avian NR-13.

mammary glands of wild-type mice showed high levels of expression of Fas, $Fas_L$, and the effector caspase, caspase-3, coincident with the initiation of apoptosis in the tubular and alveolar epithelia. In comparison, there were no apoptotic cells detected in mammary glands of either *lpr/lpr* or *gld/gld* mice examined in parallel at day 1 postweaning.

The next two examples of gene knockouts affecting cell death in the mammary gland underscore the importance of transcriptional events in the process. The first of these studies reported that a loss of p53 significantly delays involution of the mammary gland through the first 5 days postweaning, with the mammary epithelial area in mutant mice being as much as 60% larger than that of wild-type controls. Interestingly, however, by day 7 postweaning, there were no apparent differences in the mammary tissues of wild-type versus *p53*-null female mice, suggesting that the delay in epithelial cell apoptosis caused by p53 deficiency was eventually compensated for by as yet unknown mechanisms. The second of these studies utilized Cre recombinase–*lox* P technology to generate a conditional knockout of the gene encoding the transcription factor, signal transducer and activator of transcription 3 (Stat3), only in the mammary gland. In these animals, weaning-induced involution of mammary tissue was significantly delayed due to a marked decrease in epithelial cell apoptosis. In screening for potential targets of Stat3, in 1999 Chapman *et al.* noted no alteration in p53, $Bcl\text{-}x_{long}$, or Bax levels in the mammary glands of mutant mice. However, the induction of IGF-binding protein-5 (IGFBP5) expression that normally accompanies mammary gland involution was completely absent in *Stat3*-null mammary tissue analyzed in parallel. Given the likely role of IGF-1 as a survival factor for mammary epithelial cells, these findings suggest that the reduced incidence of mammary epithelial cell apoptosis in these mutant mice is due, at least in part, to increased or sustained availability of IGF-1 resulting from the lack of IGFBP5 expression.

In the fourth and final example of apoptosis gene knockouts affecting mammary gland involution, the Cre recombinase–*lox* P system was used to conditionally inactivate the gene encoding Bcl-x, a principal regulator of phase III of apoptosis, only in mammary epithelium. No differences were observed in the proliferation or differentiation of ductal and alveloar epithelial cells in mammary glands of wild-type versus *bcl-x*-conditional knockout mice during pregnancy or lactation. However, weaning-induced epithelial cell apoptosis and tissue remodeling were greatly accelerated in *bcl-x*-null mammary glands, highlighting the importance of this specific Bcl-2 family member in mammary epithelial cell survival. Interestingly, simultaneous deletion of the gene encoding Bax, a pro-apoptotic multidomain member of the Bcl-2 family, did not alter the accelerated rate of apoptosis caused by Bcl-x deficiency in postweaning mammary epithelial cells. Therefore, as predicted from *in vivo* studies of Bcl-2 family member interactions in mouse mammary tissue, it is likely that Bcl-x interacts with other pro-apoptotic multidomain members of the family, such as Bak, to regulate mammary epithelial cell fate.

## III. APOPTOSIS GENE KNOCKOUTS AND THE OVARY

The ovary is a hotbed of cell death activity, essentially from the point of its genesis during fetal life until the time of its senescence in adulthood. In the germ line, apoptosis claims up to 99.9% (depending upon the species) of the oocytes produced by gametogenesis, either directly or indirectly, following the apoptotic death of granulosa cells during atresia of maturing follicles. In addition, apoptosis of the ovarian surface epithelium has been implicated as a key step in the release of the egg at ovulation. Moreover, structural involution of the corpus luteum, at the end of each estrous cycle or pregnancy, clearly involves apoptosis. In light of this information, it is perhaps not surprising that the power of using apoptosis gene knockouts to study reproductive function is best exemplified in the ovary, where loss of phase I, III, and V components of the apoptosis pathway has been studied in detail. In addition, three cell populations—oocytes, granulosa cells, and luteal cells—have been examined with respect to the impact of a given apoptosis gene knockout on ovarian function. Thus, these studies have provided invaluable insight into not only the involvement of a specific apoptosis regulatory gene in the ovary but also the existence of cell lineage-specific pathways for executing ovarian cell death. As with the discussion of mammary epithelial cell death above, evaluation of the literature on apoptosis gene knockouts and the ovary will begin with phase I components and move progressively toward phase V.

Similar to the epithelium of the mammary gland, the Fas/$Fas_L$ system appears to play a key role in signaling apoptosis in multiple cell types in the ovary. Mutant mice with aberrant expression of Fas (*lpr/lpr*) or lacking expression of $Fas_L$ (*gld/gld*) exhibit a spectrum of ovarian abnormalities, including irregularities in follicle development. In particular, female *lpr/lpr* mice have increased numbers of secondary follicles and decreased numbers of antral follicles, as well as exhibit attenuated apoptosis in both oocytes and granulosa cells following Fas activation. Furthermore, reduced numbers of corpora lutea (*lpr/lpr*) and defective luteolysis (*gld/gld*) have been described in these mutants. Gene knockout mice lacking TNF receptor 1 (TNFR1), another death receptor, also show a spectrum of ovarian phenotypes. For example, prior to puberty *TNFR1*-null females have a heightened response to exogenous gonadotropin stimulation, as reflected by a significantly larger than normal ovulatory response to hormonal priming. With increasing age, however, reproductive performance declines precipitously in TNFR1-deficient females, with only 40% of mutant females exhibiting estrous cyclicity by 6 months of age. Unfortunately, given the pleiotropic nature of the actions of TNFα in various cell types, it remains to be established whether the alterations in female reproductive function resulting from TNFR1 deficiency are related, either directly or indirectly, to defective apoptosis.

Another component of phase I that has been examined in detail, at least in oocytes, by gene inactivation is the role of the ceramide-generating enzyme, acid sphingomyelinase (ASMase). Ceramide is a pro-apoptotic second messenger that, in many cell types, serves a crucial role in transducing stress stimuli. The mechanisms by which ceramide is believed to act are diverse and include superaggregation ("capping") of death receptors, activation of JNK, and facilitation of Bax-induced mitochondrial destabilization. Recent studies have shown that female *ASMase* gene knockout mice possess roughly twice as many oocytes at birth as wild-type sisters, suggesting that ASMase-generated ceramide regulates the death of oocytes during fetal ovarian development. This postulate was confirmed by studies with cultured fetal ovaries that showed that the high incidence of oocyte apoptosis in wild-type female gonads starved of hormonal support *ex vivo* was greatly attenuated in ASMase-deficient fetal ovaries cultured in parallel. Moreover, *ASMase*-null oocytes treated with the chemotherapeutic drug doxorubicin are resistant to apoptosis, providing evidence that the defect in apoptosis caused by ASMase deficiency is cell autonomous in nature. Unfortunately, there are no published reports showing whether granulosa or luteal cells lacking ASMase show similar defects in the signaling of apoptosis.

Despite the generation of several BH3-only family member knockouts, including mice lacking Bid and Bim, no studies have yet been published on ovarian phenotypes caused by deficiency of these or other phase II components. However, the ovarian "consequences" in mutant mice that do not express various multidomain Bcl-2 family members (phase III) have been described. The first of these studies reported that inactivation of the anti-apoptotic *bcl-2* gene caused a significant reduction in the number of oocyte-containing primordial follicles in the ovaries of young adult female mice. These data, which suggest that Bcl-2 serves an important survival function in the female germ line, have been supported by experiments to accomplish the opposite—that is, the generation of mice with increased expression of Bcl-2 in oocytes. Using two different promoters to drive *bcl-2* gene expression in oocytes, Morita *et al.* and others described a survival advantage for transgenic oocytes.

The second multidomain knockout studied in the context of ovarian development and function is the Bax-deficient mouse. In the first report on the generation of these mice, the investigators described "a marked accumulation of unusual atretic follicles" in the ovaries of *bax*-null females that "contained numerous atrophic granulosa cells that presumably failed to undergo apoptosis." Our laboratory has since confirmed that Bax-deficient granulosa cells are indeed defective in their capacity to undergo apoptosis (unpublished data). More importantly perhaps is the pronounced resistance of Bax-deficient oocytes to apoptosis induced by diverse stimuli. For example, postnatal loss of primordial and primary follicles, an event driven by death of the oocyte, is significantly reduced in *bax*-mutant female mice, leading to a dramatic prolongation of ovarian function into very advanced chronological age. In addition, a number of pathological stimuli known for their ovotoxic effects, including chemotherapeutic drugs and environmental toxicants, fail to trigger oocyte depletion and ovarian failure in the absence of functional Bax protein. Finally, an interesting story is emerging on the role of Bax in fetal ovarian germ cell death, with involvement of the protein in oocyte demise apparently dependent upon the stimulus for cell death.

This latter point can be prefaced by an overview of the third and final multi-domain mutant to be discussed, a *bcl-x* hypomorph. Conventional gene knockout of *bcl-x* leads to embryonic lethality at approximately day 12. This hurdle in studying the functional importance of Bcl-x to ovarian development was overcome somewhat serendipitously following the insertion of a neomycin cassette into the *bcl-x* promoter at a site important for germ cell-specific expression of the gene. Consequently, in mutant mice with two hypomorphic alleles, *bcl-x* expression was reduced in the germ line at embryonic day 12.5. Subsequent germ cell development was considerably impaired, leading to the birth of females with significantly reduced numbers of primordial and primary follicles in their ovarian reserves. Interestingly, simultaneous inactivation of the *bax* gene in *bcl-x* hypomorphic females rescued germ cell development and restored a normal level of follicle endowment, providing clear evidence that a cell death "rheostat" based on the opposing actions of anti- and pro-apoptotic Bcl-2 family members exists *in vivo*. However, despite these and other data implicating Bax as a key determinant of female germ cell death in developing fetal ovaries, Bax deficiency does not rescue fetal oocytes from death due to meiotic defects. As such, more than one cell death pathway must exist for the deletion of female germ cells.

At present, there are no published data indicating how a loss of apoptosome function, resulting from either Apaf-1 or caspase-9 deficiency, impacts on apoptosis in the ovary. Studies of mice lacking various phase V components have, however, provided evidence that a divergence in the apoptosis-signaling pathway occurs in ovarian germ cells and somatic cells at this step. In oocytes, knockout of the *caspase-2* gene provides oocytes with resistance to death induced by both physiological (e.g., cytokine starvation) and pathological (e.g., chemotherapy) stimuli. On the other hand, granulosa cell death and maturing follicle atresia occur normally in the absence of caspase-2. By comparison, mutant mice lacking caspase-3 show a dramatic defect in the ability of granulosa cells to execute apoptosis, both *in vivo* and *in vitro*, without a corresponding defect in oocyte death driven by either developmental cues or pathological insults. As such, these data suggest the existence of cell lineage-selective, if not cell lineage-specific, pathways of apoptosis execution in the ovary. Of additional note, a very recent study has reported that caspase-3 is required for cell death during luteal regression, underscoring the probable conservation of caspase-3 function in the granulosa cell lineage even after the process of luteinization.

As a final point of discussion and, to some degree, integration, a novel apoptosis-signaling pathway that utilizes gene transcription to kill oocytes has been recently identified. It was determined that treatment of oocytes with 9,10-dimethylbenz[*a*]anthracene (DMBA), a toxic chemical found in tobacco smoke and in the environment as a by-product of fossil fuel combustion, induces *bax* gene transcription as well as an accumulation of *bax* mRNA and Bax protein. Importantly, the induction of *bax* expression, and the resultant initiation of apoptosis, in oocytes involves DMBA-driven activation of the aromatic hydrocarbon receptor (AHR), a transcription factor of the *Per–Arnt–Sim* gene family. Furthermore, oocytes of mutant mice lacking either the AHR or Bax are completely resistant to DMBA-induced apoptosis. Given this information, it is not surprising that *Ahr*-null female mice are born with approximately twofold more primordial follicles than normal, suggesting that the AHR serves as a key phase I regulator of oocyte death in both physiological and pathological situations.

If one considers, however, the principles discussed herein regarding the coordinated and stepwise progression of apoptosis through phases I–V, it is unlikely that the up-regulation of a single gene, such as *bax*, by the DMBA-activated AHR triggers oocyte death. In fact, in such a paradigm of transcription-dependent cell death, one would envisage wholesale, modular changes in the expression of genes that function in the various phases of apoptosis. For example, because of the supple nature of the genome (i.e., genomic plasticity), could exposure of oocytes to DMBA increase the expression of genes encoding ASMase (phase I), Apaf-1 or caspase-9 (phase IV), and death effector caspases (phase V), in addition to multiple Bcl-2 family members (phases II and III)? An understanding of such complex events may one day help facilitate the development of therapeutic strategies to preserve female germ cells, regulate the endocrine function of the ovaries, and, perhaps, improve the quality of life for women.

## Glossary

**apoptosis** A form of physiological ("programmed") cell death characterized at the light and electron microscopic levels by separation of the cell from its neighboring cells and/or its extracellular matrix, a loss of cell volume, chromatin condensation and margination along the nuclear envelope (pyknosis), and the final budding and fragmentation of the cell into plasma membrane-bound vesicles that are cleared by phagocytosis.

**Bcl-2 family** A group of structurally and/or functionally related proteins that can either promote or prevent cell death and are considered to be the primary regulators of apoptosis via their actions at the level of intracellular organelles, particularly mitochondria.

**caspases** A cohort of cysteine aspartic acid-specific proteases that function either as initiators (e.g., caspase-8 and -9) or as executioners (e.g., caspase-2, -3, -6, and -7) of the apoptotic cell death program in vertebrates.

**gene knockout** A mutant mouse line generated by targeted disruption (inactivation) of a specific gene, generally through homologous recombination, to examine the functional significance of that gene product in cell, tissue, or organ function.

## See Also the Following Articles

**Apoptosis • Apoptosis, Glucocorticoid-Induced • Estrogen Receptor Biology and Lessons from Knockout Mice • Knockout of Gonadotropins and Their Receptor Genes • Placental Development**

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# Apoptosis, Glucocorticoid-Induced

RHEEM D. MEDH[*], E. BRAD THOMPSON[†], AND
E. AUBREY THOMPSON[†]

[*]*California State University, Northridge* • [†]*University of Texas Medical Branch, Galveston*

I. MECHANISM OF GLUCOCORTICOID ACTION
II. GC-EVOKED APOPTOSIS IN REPRODUCTIVE TISSUES
III. EFFECTS OF GCs ON BONE TURNOVER
IV. GC-MEDIATED REGULATION OF THYMOCYTE SELECTION AND LEUKOCYTE APOPTOSIS
V. MECHANISMS OF GC-EVOKED THYMOCYTE APOPTOSIS
VI. SUMMARY

**Glucocorticoids (GCs) play a vital role in maintaining normal metabolism, regulating various physiological processes, and overcoming several forms of stress. Because of their diverse actions, naturally occurring as well as synthetic glucocorticoids are commonly used as therapeutic agents for various disorders. In a number of physiological and therapeutic instances, induction of or protection from apoptosis is a critical aspect of GC action. Apoptosis is a form of suicidal cell death that has now been recognized as being an integral part of physiological cell turnover and helps maintain the dynamic state of cellular homeostasis. It is an energy-requiring process**

**caspases** A cohort of cysteine aspartic acid-specific proteases that function either as initiators (e.g., caspase-8 and -9) or as executioners (e.g., caspase-2, -3, -6, and -7) of the apoptotic cell death program in vertebrates.

**gene knockout** A mutant mouse line generated by targeted disruption (inactivation) of a specific gene, generally through homologous recombination, to examine the functional significance of that gene product in cell, tissue, or organ function.

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# Apoptosis, Glucocorticoid-Induced

RHEEM D. MEDH*, E. BRAD THOMPSON†, AND E. AUBREY THOMPSON†

*California State University, Northridge • †University of Texas Medical Branch, Galveston

I. MECHANISM OF GLUCOCORTICOID ACTION
II. GC-EVOKED APOPTOSIS IN REPRODUCTIVE TISSUES
III. EFFECTS OF GCs ON BONE TURNOVER
IV. GC-MEDIATED REGULATION OF THYMOCYTE SELECTION AND LEUKOCYTE APOPTOSIS
V. MECHANISMS OF GC-EVOKED THYMOCYTE APOPTOSIS
VI. SUMMARY

**Glucocorticoids (GCs) play a vital role in maintaining normal metabolism, regulating various physiological processes, and overcoming several forms of stress. Because of their diverse actions, naturally occurring as well as synthetic glucocorticoids are commonly used as therapeutic agents for various disorders. In a number of physiological and therapeutic instances, induction of or protection from apoptosis is a critical aspect of GC action. Apoptosis is a form of suicidal cell death that has now been recognized as being an integral part of physiological cell turnover and helps maintain the dynamic state of cellular homeostasis. It is an energy-requiring process**

**typically involving altered expression or function of key cell proliferation/death genes. Excessive or defective apoptosis has been implicated in a number of diseases, provoking the development of therapeutic strategies targeting any physiological imbalance in the process. In this article, we briefly outline the mechanism of action of GCs, identify specific physiological targets of GC-evoked apoptosis, and cite examples of therapeutic applications in which GC regulation of apoptosis is relevant to its therapeutic efficacy. Finally, we present a concise review of the mechanisms of GC-evoked apoptosis, based on the extensive literature from studies on thymocytes and leukemic lymphoblasts.**

## I. MECHANISM OF GLUCOCORTICOID ACTION

Glucocorticoids (GCs) modulate their actions via binding to a specific intracellular GC receptor (GR), which upon ligand binding is released from the complex of cytoplasmic proteins to which it is bound in the inactive state. The GR has been shown to be essential for GC-evoked apoptosis. The classical model of GR action postulates that the activated GR is translocated to the nucleus, where it regulates transcription of a finite set of genes via GC-response elements (GREs) on target genes. Recent studies have demonstrated that this model is rather simplistic, and GR-dependent transcriptional regulation is modulated by precise interactions of the GR with "co-regulatory" proteins and involves the formation of a "transcriptosome" complex, which includes basal transcription factors (such as TBP and TFIID) and either co-activators (such as CBP and other histone acetylases) or co-repressors (such as NcoR and other histone deacetylases). Most natural GR target genes contain complex GREs, termed GC-response units, that bind multiple transcription factors either cooperatively or competitively. GR also modulates physiological processes by cross talk with other signal transduction pathways and secondary transcriptional and posttranscriptional effects.

## II. GC-EVOKED APOPTOSIS IN REPRODUCTIVE TISSUES

GCs impact on normal cell turnover in several other tissues, as evidenced in experimental rodent models, in cell cultures, and in humans. In the prostate, glucocorticoids, in conjunction with androgens, stimulate glandular epithelial cell proliferation and prevent apoptosis. The relevance of GCs in the process is apparent in castration (androgen withdrawal)-induced prostate involution, which is inhibited by high doses of GCs. By a process that involves the GR, GCs prevent a castration-induced increase in the apoptotic genes TRPM-2, c-*fos*, and hsp 70. Testicular germ cells are prone to stress-induced apoptosis, mediated by increased secretion of GCs and resulting in suppression of testosterone levels. GCs modulate the cyclic pattern of epithelial cell proliferation and involution in the mammary gland in synergy with estrogens, progesterone, prolactin, and insulin. Using immortalized mammary cell culture models, GC deprivation has been shown to trigger apoptosis. Involution of postlactation mammary gland is caused by apoptotic loss of epithelial cells owing to a fall in levels of lactogenic hormones, including GCs.

## III. EFFECTS OF GCs ON BONE TURNOVER

In the skeletal system, GCs influence the production and function of paracrine and autocrine factors including hormones and cytokines secreted by bone cells, the net effect being increased bone resorption and decreased bone volume and density. The primary target of GC action in the bone seem to be osteoblasts, which modulate bone formation and mineralization and eventually become osteocytes and undergo apoptosis. GCs have been shown to induce osteoblast apoptosis and repress osteocalcin, insulin-like growth factor-1, and type I collagen synthesis, all implicated in preosteoblast differentiation and proliferation. Impaired osteoblastogenesis secondarily represses osteoclastogenesis, thereby resulting in diminished bone turnover and remodeling. In addition, GCs promote osteocyte apoptosis, a primary cause of osteonecrosis (a misnomer, since the cells are actually dying via apoptosis rather than necrosis) in isolated portions of bone after administration of pharmacological doses of GCs. Indeed GC-induced bone resorption is a major cause of osteoporosis, often manifested following GC administration for inflammatory disease or disorders of immune function. Paradoxically, GCs are effective therapeutic agents for chronic inflammatory diseases of the bone, such as rheumatoid arthritis. Rheumatoid arthritis is associated with neutrophil activation, infiltration, and apoptosis at the inflamed joint. GCs prevent bone erosion by inhibiting pro-inflammatory neutrophil function including apoptosis.

## IV. GC-MEDIATED REGULATION OF THYMOCYTE SELECTION AND LEUKOCYTE APOPTOSIS

Early studies demonstrated that GCs evoke thymic involution and affect the development of a normal immune system. During development, immature T-cells are subjected to a rigorous selection process in the thymus, allowing survival of cells with precisely defined properties. Double-positive, immature thymocytes that do not express functional T-cell receptors apoptose via a default pathway mediated by GCs. T-cells that do express functional T-cell receptors escape this pathway but are further subjected to negative selection if their receptors recognize self-antigen/MHC complexes with high avidity. GCs influence the TCR avidity “threshold” that distinguishes between cells that survive (low avidity) and those that die (high avidity). Indeed, in experimental models, apoptosis triggered by receptor-mediated T-cell activation is antagonized by GCs; deprivation of GC lowers the threshold of avidity beyond which negative selection ensues. GCs also affect the death of circulating leukocytes including neutrophils, eosinophils, and T- and B-lymphoid cells by mechanisms that are not clearly understood.

Because of their apoptotic actions, GCs are powerful therapeutic agents for a number of disorders, including autoimmune disorders, allergies, asthma, inflammatory diseases, and several forms of leukemia. GCs are effective components of multiagent combination therapy for ALL and CLL, where therapeutic efficacy is linked to the presence of functional GC. Eosinophil infiltration of bronchial mucosa causes many of the pathological features of asthma, including blockage of lung airways. GC-evoked apoptosis of eosinophils is instrumental in their ability to facilitate clearance of lung airways and reduce inflammation in asthma. Similarly, eosinophilia-associated allergic inflammation is also alleviated by GC-mediated eosinophil apoptosis. In autoimmune diseases, such as rheumatoid arthritis and lupus, GCs are effective therapeutic agents because of their ability to reduce the numbers of $CD4^+$ and $CD8^+$ thymocytes via apoptosis (see Fig. 1).

## V. MECHANISMS OF GC-EVOKED THYMOCYTE APOPTOSIS

GC-evoked thymocyte apoptosis has been extensively studied and has facilitated a better understanding of the molecular pathway for apoptosis. Morphological

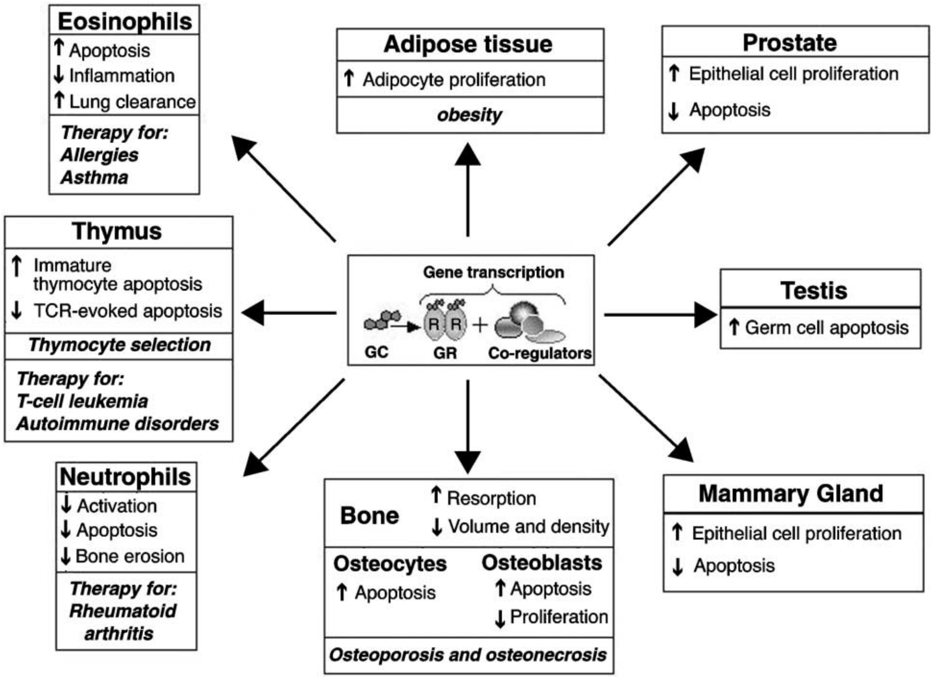
Eosinophils
↑ Apoptosis
↓ Inflammation
↑ Lung clearance
Therapy for:
Allergies
Asthma
Adipose tissue
↑ Adipocyte proliferation
obesity
Prostate
↑ Epithelial cell proliferation
↓ Apoptosis
Thymus
↑ Immature thymocyte apoptosis
↓ TCR-evoked apoptosis
Thymocyte selection
Therapy for:
T-cell leukemia
Autoimmune disorders
Gene transcription
R R
GC
GR
Co-regulators
Testis
↑ Germ cell apoptosis
Neutrophils
↓ Activation
↓ Apoptosis
↓ Bone erosion
Therapy for:
Rheumatoid arthritis
Bone
↑ Resorption
↓ Volume and density
Osteocytes
↑ Apoptosis
Osteoblasts
↑ Apoptosis
↓ Proliferation
Osteoporosis and osteonecrosis
Mammary Gland
↑ Epithelial cell proliferation
↓ Apoptosis

FIGURE 1 Targets of GC-evoked apoptosis and examples of therapeutic applications in which GC regulation of apoptosis occurs.

and biochemical changes associated with GC-evoked apoptosis include cell shrinkage, chromatin condensation, membrane flipping and blebbing, DNA fragmentation, and formation of apoptotic bodies. Researchers have been studying the molecular changes that precede these irreversible manifestations of apoptosis. It has become apparent that several overlapping or redundant pathways are simultaneously activated by GCs, all of which eventually culminate in protease activation, degradation of target substrates, and subsequent cell death (Fig. 2).

## A. Alterations in Gene Expression

GC-mediated regulation of certain genes seems to play a vital role in evoking thymocyte/lymphoid cell apoptosis. One of the early effects is the down-regulation of expression of the proto-oncogene c-*myc*, which has been implicated as a decisive event triggering the apoptotic machinery. In GC-sensitive leukemic CEM-C7 cells constitutively expressing ectopic c-*myc*, apoptosis is significantly delayed. c-*myc* levels are not repressed in GC-resistant lymphoid cell lines or in GC-resistant thymocytes from NOD mice, providing a strong correlation between GC-evoked apoptosis and c-*myc* suppression. In murine P1798 cells, however, GC-evoked c-*myc* suppression is not sufficient to trigger an apoptotic response; a simultaneous depletion of the G1 cyclin, cyclin D3, is necessary. Indeed, GC-evoked apoptosis of leukemic cell lines is accompanied by growth arrest in the G1 phase of the cell cycle, which is thought to require regulation of cyclin–cyclin-dependent kinase (cdk) complex formation. GC-mediated lymphoid cell growth arrest/apoptosis is associated with up-regulation of the cdk inhibitor p27$^{\text{kipI}}$ and down-regulation of various cyclins, effectively blocking cdk activity.

The proto-oncogene c-*jun*, a component of the transcription factor AP-1, is up-regulated in association with GC-evoked apoptosis of lymphoid cells and may alter GR function via AP-1-mediated cross talk. AP-1 is also known to activate protein kinase C isoforms, some of which have been implicated as being pro-apoptotic. Another second messenger, cAMP, promotes GC-mediated thymocyte apoptosis by as yet unidentified mechanisms The classic path of cAMP signaling is through activation of protein kinase A, leading to modulation of gene transcription via phosphorylation of cAMP-response element-binding protein (CREB) and recruitment of CREB-binding protein (CBP) to alter gene expression of cAMP-responsive genes. Both CREB and CBP are known to interact with GR as components of the GR co-regulatory complex.

## B. GC-Mediated Activation of Signaling Pathways

Induction of genes mentioned in the previous section is also accompanied by activation of multiple signal transduction pathways, including those activated via

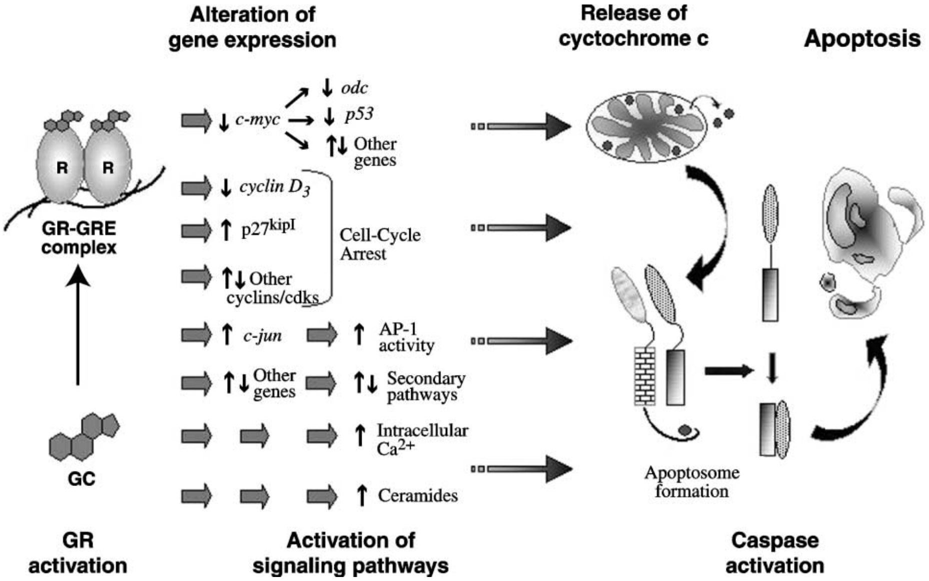
Alteration of gene expression
Release of cyctochrome c
Apoptosis
↓ c-myc
↓ odc
↓ p53
↑↓ Other genes
R
R
GR-GRE complex
↓ cyclin D3
↑ p27kipI
↑↓ Other cyclins/cdks
Cell-Cycle Arrest
↑ c-jun
↑ AP-1 activity
↑↓ Other genes
↑↓ Secondary pathways
↑ Intracellular Ca2+
↑ Ceramides
GC
Apoptosome formation
GR activation
Activation of signaling pathways
Caspase activation

FIGURE 2 Signaling pathways that are activated by GCs, eventually leading to protease activation, degradation of target substrates, and subsequent apoptosis.

protein kinases C and A. GCs elevate cytosolic $Ca^{2+}$ levels by depleting intracellular stores and trigger the activation of nuclear $Ca^{2+}$-dependent endonuclease(s), facilitating the accompanying DNA fragmentation. $Ca^{2+}$ may also exert its effects on thymocyte apoptosis via calmodulin-mediated activation of the $Ca^{2+}$-dependent protein phosphatase, calcineurin. GC-evoked apoptosis has been shown to generate ceramides via the sequential production or activation of the phosphoinosotide-specific phospholipase C, diacyl glycerol (DAG), protein kinase C, and acidic sphingomyelinase (aSMase). This process is dependent on GR, since RU 38486 can block the induction of aSMase production by dexamethasone (a synthetic glucocorticoid) in correlation with inhibition of apoptosis. This pathway is also linked to activation of the JNK/SAPK cascade, as demonstrated in stress-induced apoptosis of leukemic U937 cells. aSMase activation appears to mediate downstream activation of caspases, since inhibition of intermediate steps in the pathway blocks caspase activation in parallel with the prevention of apoptosis.

### C. Protease Activation

Proteases are believed to be the ultimate mediators of irreversible changes in apoptosis. Members of the caspase family of aspartate-specific cysteine proteases have been implicated as the executioners of apoptosis in the lymphoid system. Caspase activity can be detected in immature double-positive thymocytes that are subject to apoptotic selection, but not in double-negative or mature thymocyte populations that are generally resistant to GC-evoked apoptosis, indicating that caspases act as apoptotic effectors. In recent years, mice generated by targeted disruption of individual caspase genes have provided important, although preliminary, information on their role in normal thymocyte development and selection. Mice deficient in caspase 1, 2, 3, or 9 exhibit normal development and distribution of thymocyte subpopulations, suggesting either that caspase activation is not essential for thymocyte selection or that there is redundancy in their action. Thymocytes from caspase 3 knockout mice are normal, whereas those from caspase 9 knockout mice exhibit a delayed death response to dexamethasone. In CEM cells, GC-evoked apoptosis is dependent on the activity of a member of the caspase 3 subfamily, but not on caspase 1 or its homologues, which are involved in Fas/Fas L-mediated apoptosis. Cell-surface differences in the recruitment of individual caspases have been reported in different subsets of isolated normal mouse T-lymphocytes and T-cell lines when triggered to undergo apoptosis by the same stimulus. Thus, great diversity exists in the recruitment of individual caspases by various apoptotic pathways. Other proteases, such as calpain, granzyme A, and proteosomes, also mediate apoptosis; however, details of their role in the process have not been forthcoming.

### D. Mitochondrial Events

Loss of mitochondrial transmembrane potential and release of cytochrome c have been implicated as early events in apoptosis triggered by various agents; however, the precise mechanisms by which these events occur are still being debated. Also, in the case of GC-evoked lymphoid cell apoptosis, mitochondrial changes seem to be important for the activation of caspases; however, there is little information on the upstream events that trigger membrane permeability transition and loss of mitochondrial membrane potential. The release of cytochrome c is thought to recruit pro-caspases and adapter proteins to form an apoptosome, which activates initiator caspases via autocatalysis, setting on the caspase cascade that goes on to activate effector caspases, such as caspase 3, the final executioner in the process of cell death. The anti-apoptotic protein, Bcl-2, which acts via binding to the outer mitochondrial membrane and preventing cytochrome c release, is able to delay but not prevent GC-evoked lymphoid cell apoptosis, again suggesting that independent pathways may eventually lead to cell death.

## VI. SUMMARY

Glucocorticoid hormones play an important role in normal tissue turnover and cellular homeostasis, in addition to their widely recognized contribution to the regulation of metabolic processes, ion transport, and stress responses. GC-induced cytostatic or lytic action in several tissues has been exploited for therapeutic intervention in diseases such as autoimmune disorders and leukemia. Paradoxically, this aspect of GC action in tissues such as bone is a contraindication for high-dose GC therapy. Studies on the lymphoid cell system have provided insights into the mechanisms of GC-evoked apoptosis. In modulating their action, GCs engage GRs, which may alter the expression of key genes either autonomously or via cross talk with other signaling pathways. GC-evoked apoptosis appears to require suppression of proliferative genes, such as c-*myc* and *cyclin D3*, and up-regulation of proteins, such as p27$^{\mathrm{kipI}}$ and c-Jun. DAG-, Ca$^{2+}$-, ceramide-, and aSMase-mediated signaling processes are activated and are believed to contribute to the apoptosis process. Indeed, there is increasing evidence that GCs trigger multiple, seemingly independent pro-apoptotic pathways in parallel, ultimately culminating in protease activation and the collapse of cellular machinery, which brings about irreversible apoptotic cell death. Much needs to be learned about the precise sequence of events that modulate GC-evoked apoptosis in lymphoid and other systems.

## Glossary

**apoptosis** A genetically programmed process of cellular suicide characterized by cell shrinkage, membrane blebbing, nuclear condensation, and formation of membrane-bound cell fragments or "apoptotic bodies" that are engulfed by phagocytic cells.

**avidity** The total strength of an interaction between two multivalent surfaces.

**caspases** Family of aspartate-specific cysteine proteases that have been implicated in apoptosis.

**glucocorticoid receptor** Intracellular protein that binds with high specificity to glucocorticoids to form a hormone–receptor complex that is translocated to the nucleus and interacts with specific DNA sequences on target genes to elicit a transcriptional response.

**major histocompatability complex (MHC)** A set of membrane glycoproteins that present intracellular peptides to T-cells. MHC molecules are also involved in antigen processing and host defense.

**osteoblasts** Bone-forming cells derived from stromal mesenchymal cells and rich in alkaline phosphatase, collagens I and V, and other bone-specific proteins.

**osteoporosis** A progressive systemic skeletal disease characterized by low bone mass and deterioration of bone tissue, causing increased bone fragility and susceptibility to fracture.

**T-cell selection** The process that T-cells undergo during cell proliferation that eliminates potentially self-reactive cells and favors the survival of those cells that can recognize foreign antigens.

## See Also the Following Articles

**Apoptosis • Apoptosis Gene Knockouts • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Placental Development**

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# Appetite Regulation, Neuronal Control

Suzanne M. Appleyard
*Oregon Health and Science University, Portland*

I. BRAIN REGIONS INVOLVED IN APPETITE REGULATION
II. LEPTIN AND INSULIN
III. NPY AND MELANOCORTIN SYSTEM
IV. OREXIGENIC FACTORS
V. ANOREXIGENIC FACTORS
VI. SUMMARY

**Obesity is a major health problem in the United States today. Twenty to thirty percent of Americans are obese, leading to a rapid increase in diseases associated with obesity, such as heart disease, diabetes, and stroke. The development of obesity is due to an upset in energy homeostasis, the balance between energy intake and energy expenditure. There has been an explosion of research in this field over the past decade, leading to the discovery of many new hormones and neurotransmitters involved in the regulation of food intake. This article provides a brief overview of the various brain regions implicated in the regulation of food intake; the main focus will be on the hypothalamic peptides and hormonal regulation of food intake. However, the regulation of food intake is a complex process and the final output is likely to be due to an integration of the effects of each of the hormones and transmitters, involving all of the brain regions discussed in this article.**

## I. BRAIN REGIONS INVOLVED IN APPETITE REGULATION

### A. Hypothalamus

The hypothalamus is the primary brain region involved in the regulation of food intake (see Fig. 1). Lesions to the ventromedial hypothalamus (VMH) including the arcuate nucleus lead to an animal that is severely obese and hyperphagic, whereas lesions to the lateral hypothalamus (LH) cause a lean and hypophagic phenotype. Conversely, electrical stimulation of the VMH inhibits food intake, whereas electrical stimulation of the LH stimulates food intake. These experiments led to the dual-center model suggested by Stellar in the 1950s, which stated that the lateral hypothalamus served as a feeding center and the ventromedial nucleus as a satiety center. Extensive research in this area has refined this hypothesis from specific "centers" of the brain controlling food intake to the notion of discrete neuronal pathways capable of integrating satiety signals and responding to changing fuel needs. Multiple hypothalamic nuclei play a role in this complex regulation of food intake and energy homeostasis (Fig. 1).

The arcuate nucleus of the hypothalamus (ARC) is located near the median eminence, where the blood–brain barrier is weakest and can therefore "sense" circulating levels of peripheral hormones and the levels of nutrients in the blood. Lesions to the arcuate nucleus lead to hyperphagia and obesity, suggesting that this region is critical for the appropriate regulation of food intake and appetite. Neurons with cell bodies in this region are sensitive to the levels of circulating regulators of energy homeostasis, such as leptin, insulin, and glucose. Many peptides and other neuroregulators are expressed in the arcuate nucleus. Two of the most widely studied groups of neurons in the ARC are the leptin-sensitive neuropeptide

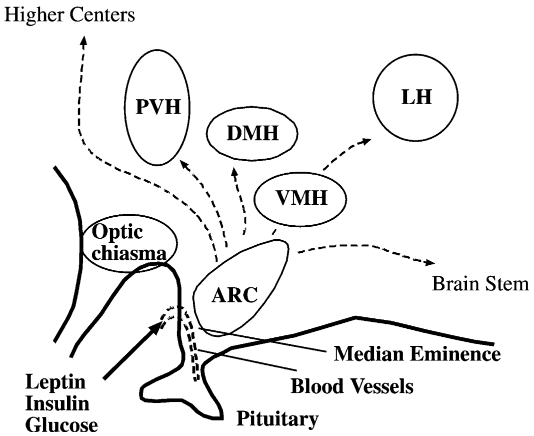

FIGURE 1 Schematic diagram of the role of the hypothalamus in appetite regulation. The arcuate nucleus lies directly above the median eminence, with its weak blood–brain barrier. Circulating satiety factors enter through the median eminence and regulate the activity of neurons within the arcuate nucleus. These neurons send projections to other hypothalamic nuclei as well as other brain regions involved in the regulation of appetite. The different hypothalamic nuclei are extensively interconnected, including projections back to the arcuate nucleus from its downstream targets. Therefore, hypothalamic regulation of food intake involves integration of the activity of numerous neuronal populations within the different nuclei. ARC, arcuate nucleus; PVH, paraventricular hypothalamus; DMH, dorsal medial hypothalamus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus.

Y (NPY) and pro-opiomelanocortin (POMC) neurons. The ARC has reciprocal connections with many regions of the hypothalamus, including the dorsomedial hypothalamus (DMH), VMH, LH, and paraventricular hypothalamus (PVH). ARC neurons also project to other brain regions involved in energy homeostasis, including the nucleus of the solitary tract (NTS), central nucleus of the amygdala, nucleus accumbens, and cerebral cortex.

The LH also plays a major role in the regulation of food intake and energy homeostasis. Lesions to the lateral hypothalamus cause hypophagia and weight loss, whereas stimulation leads to hyperphagia. The LH receives inputs from the arcuate nucleus and other regions implicated in the regulation of appetite. The LH also sends extensive projections throughout the brain, including monosynaptic projections to the cerebral cortex and NTS. The main transmitters expressed in the lateral hypothalamus are melanin-concentrating hormone (MCH), orexins (hypocretins), and the endogenous opioid dynorphin. The LH receives inputs from the leptin-sensitive NPY and POMC neurons of the ARC. However, leptin receptors are expressed in the LH, suggesting that leptin may also have direct effects. In addition, many neurons in the LH are directly sensitive to changes in glucose levels.

The DMH receives inputs from both the lateral hypothalamus and the ventromedial hypothalamus and sends projections to the PVH, and is therefore a potentially important relay pathway for the neuroendocrine functions. Leptin and insulin receptors are also located in the DMH, indicating that this region may be involved in mediating the feedback effects of these hormones. However, rats with lesions to the DMH show normal body composition and weight, respond normally to body weight-regulatory challenges, and have normal efficiency of food utilization.

The PVH is also involved in the regulation of food intake. The PVH receives extensive projections from many regions including the ARC, LH, DMH, and NTS. Injections of many appetite-regulating neurotransmitters have potent effects when injected into the PVH, including NPY, α-melanocortin-stimulating hormone (αMSH), galanin, monoamines, and opioids. Neurons located in the PVH express oxytocin, vasopressin, thyroid-releasing hormone (TRH), and corticotropin-releasing hormone (CRH).

The VMH is also thought to act as a satiety center. As outlined above, lesions to the VMH lead to hyperphagia and weight gain, whereas stimulation inhibits feeding. The VMH also has reciprocal connections with the PVH, LH, and DMH and has a high level of leptin receptor expression.

## B. Nucleus of the Solitary Tract

The NTS is a brainstem nucleus that is an important relay site for information from peripheral satiety signals to central sites. It also functions as a pathway relaying compensatory signals from the brain to peripheral targets. Many of these signals lead to changes in energy homeostasis, such as changes in autonomic functions. Vagal afferents from both the gastrointestinal tract and the abdominal viscera terminate in the NTS along with gustatory information from the oral cavity. The NTS also receives projections from the hypothalamus, amygdala, and nucleus accumbens, regions of the brain integral in the regulation of feeding. The NTS and the adjacent region, the area postrema, have weak blood–brain barriers and have access to circulating hormones and other regulators of feeding in much the same way as the arcuate nucleus of the hypothalamus. The NTS is also an important site of action of satiety factors released following a meal, such as cholecystokinin (CCK), that act to induce meal termination. Some of the inhibitory effects of the gastrointestinal tract on food intake do not require hypothalamic influences, as food intake is reduced even when connections between the forebrain and the hindbrain are severed.

## C. Other Brain Regions

The hypothalamus and brainstem are thought to be the primary integration sites for peripheral satiety signals; however, other "higher" centers are proposed to be important for the appropriate control of food intake and appetite. The amygdala, nucleus accumbens, caudate putamen (dorsal striatum), and cortex all receive and/or send inputs from and/or to the hypothalamus. Lesions to the amygdala result in hyperphagia and weight gain, although the nucleus and the mechanisms involved have not been definitively identified. The nucleus accumbens is thought to mediate some of the rewarding or "hedonic" aspects of food intake. The caudate putamen and cortex are also proposed to mediate some of the behavioral changes that regulate food intake through their connections with the hypothalamus.

## II. LEPTIN AND INSULIN

Leptin is a 15 kDa protein that is secreted by adipocytes. The finding that the profound obesity and hyperphagia of the ob/ob mouse were due to a mutation in the leptin gene led to the proposal that leptin acted as an inhibitor of food intake and promoted a negative energy balance. Injection of leptin into rodents potently inhibited food intake, and leptin was proposed to be the adiposity feedback signal that was released from the fat to signal the brain to decrease energy intake and reduce weight. Consistent with this hypothesis, plasma leptin levels are proportional to total fat content, and leptin enters the CNS at levels that correlate with circulating concentrations. Furthermore, leptin receptors are expressed in regions of the brain involved in energy homeostasis, and administration of leptin directly into the brain reduces food intake. However, the role of leptin in the regulation of food intake and energy homeostasis is more complicated than acting as a simple adiposity feedback signal. Leptin is also thought to indicate the nutritional health of the organism, as it is a required permissive signal for many endocrine functions including the development of puberty and normal fertility. Indeed, given the affinity of leptin for its receptor and the circulating levels in a normal rodent, a decrease in circulating leptin levels may be a more physiologically important signal than is an increase brought about by weight gain. The arcuate nucleus is a major site of action for the effects of leptin. Leptin receptors are highly expressed in this region and injections of leptin specifically into the arcuate potently inhibit food intake. Conversely, ablations of the arcuate attenuate the hypophagic effects of a generalized intracerebroventricular (icv) injection of leptin. Leptin receptors are found on the majority of arcuate NPY and POMC neurons, and these neurons are proposed to play a major role in transducing circulating leptin and insulin levels into a neuronal response.

Insulin is a pancreatic hormone that is released into the blood. In addition to its effects on glucose homeostasis, insulin inhibits food intake. It is thought that insulin reduces appetite by acting in the brain as icv injections of insulin inhibit food intake. The levels of insulin correlate with the degree of adiposity of the organism in a manner similar to the correlation shown by leptin. Furthermore, insulin receptors are expressed in regions of the brain involved in energy homeostasis, and, like leptin, insulin modulates the activity of hypothalamic and NTS neurons.

## III. NPY AND MELANOCORTIN SYSTEM

### A. Neuropeptide Y

NPY is one of the most potent stimulators of food intake known. The primary hypothalamic site of synthesis of NPY is arcuate neurons that project throughout the hypothalamus and the rest of the brain. In addition to increasing food intake, NPY promotes weight gain by reducing sympathetic nervous system outflow to brown adipose tissue, which increases energy efficiency, decreases the metabolic rate, and promotes energy saving. The effects of NPY therefore promote a positive energy balance and weight gain, and chronic central administration of NPY leads to obesity. Both the production and the release of NPY are altered by changes in energy balance, suggesting that the NPY system plays an important physiological role in the regulation of appetite and weight homeostasis. Interestingly, however, NPY knockout mice have normal growth, food intake, and refeeding following a fast. Furthermore, they have normal responses to diet-induced obesity, chemically induced obesity, and many genetic types of obesity. However, NPY deficiency partially decreases the obesity seen in ob/ob mice, suggesting that NPY is important for the weight phenotype seen in leptin-deficient mice. NPY-deficient mice are also more sensitive to the effects of leptin, suggesting that NPY may have a tonic inhibitory effect on leptin's actions.

NPY receptors are members of the G-protein-coupled family of receptors and five major subtypes have been described. Studies with antagonists suggest that NPY receptors 1 and 5 are the main receptors involved in the stimulation of food intake, although the putative autoreceptors NPY receptors 2 and 4 are also highly expressed in the hypothalamus, including on both arcuate NPY and POMC neurons. Paradoxically, mice lacking the NPY1, NPY2, or NPY5 receptors have mild obesity phenotypes and varying degrees of hyperphagia. The failure to see a lean phenotype after the removal of a potent stimulator of feeding such as NPY suggests that the pathways involved in the control of food intake, like other hypothalamic systems, are sufficiently redundant to compensate for the loss of a major signaling system.

### B. Melanocortins

The melanocortins αMSH and γMSH are the products of the pro-opioimelanocortin gene. POMC is a pro-hormone that is differentially processed into multiple bioactive peptides; in arcuate neurons, the main products of the POMC gene are αMSH, γMSH, and the endogenous opioid β-endorphin. αMSH is a potent inhibitor of food intake when administered centrally. There are five types of melanocortin receptors described to date, MCR1–5. The feeding effects of αMSH are mediated predominately by the MC4 receptor as an αMSH-like agonist does not decrease feeding in mice genetically engineered not to express the MC4 receptor. αMSH also increases sympathetic outflow to brown adipose tissue, thereby increasing energy expenditure, and together with decreased food intake leads to a negative energy balance and weight loss. The critical role of the melanocortins in the appropriate regulation of both food intake and energy balance is evident in mice with disrupted melanocortin signaling. Mice that either lack the MC4 receptor or overexpress an antagonist of the melanocortin receptor are severely hyperphagic and obese. Mice lacking the MC3 receptor display a milder obesity phenotype and have increased adiposity. However, these mice appear to be mildly hypophagic and the major phenotype of the mice is an alteration in the regulation of energy utilization. Interestingly, the anorexia/cachaxia associated with cancer is attenuated by melanocortin receptor antagonists and absent in MC4 knockout mice, providing evidence that the melanocortins may mediate the decreased appetite seen in chronic illnesses. The essential role of the melanocortins is also apparent in humans. Independent studies have demonstrated that approximately 5% of childhood-onset obesity is associated with mutations in the MC4 receptor, making it the most common monogenic form of human obesity described to date. Mutations in the *POMC* gene also lead to a severe early onset form of human obesity.

## C. Pro-Opiomelanocortin and Neuropeptide Y Neurons

Two of the main groups of neurons with their cell bodies in the arcuate nucleus are POMC and NPY neurons. These neurons are thought to act as the primary integration site of signals from peripherally circulating hormones and central signals involved in energy homeostasis. POMC neurons co-express cocaine- and amphetamine-regulated transcript (CART), whereas NPY neurons co-express agouti gene-related peptide (AGRP), an endogenous antagonist of the melanocortin receptors. NPY and POMC neurons project to many of the same regions, including the PVH, LH, and NTS. In addition to their opposing effects on food intake, NPY and αMSH have opposing effects on downstream target neurons, and the NPY and POMC neurons have been proposed to be the primary site of action of both leptin and insulin on food intake and energy homeostasis. Leptin is proposed to lead to a negative energy balance and decreased food intake by increasing the activity of POMC neurons and inhibiting NPY neurons (see Fig. 2). Conversely, decreasing the activity of the POMC neurons and increasing the activity of the NPY neurons lead to positive energy balance and increased food intake. Cross talk is thought to occur between the POMC and the NPY neurons. One mechanism of interaction functions via the endogenous antagonist of melanocortin receptors, AGRP, which is co-expressed in NPY neurons. AGRP is a potent activator of food intake and its actions are thought to occur primarily through the blockade of a basal inhibitory melanocortin tone. Therefore, the release of the NPY and AGRP peptides from NPY neurons has direct effects on downstream targets through the actions of NPY and indirect effects through inhibition of effects of any MSH released from POMC neurons. NPY and POMC neurons are also thought to have direct effects on each other's activities, which would also modulate the release of peptides from the opposing neuron. NPY and POMC neurons both express receptors for leptin; however, different responses are produced. Leptin turns on the expression of the immediate-early genes, c-fos and soc3, in POMC neurons. In contrast, leptin activates only socs3 expression in NPY neurons. Activation of c-fos is generally associated with an excitation of neurons, whereas soc3 expression is turned on

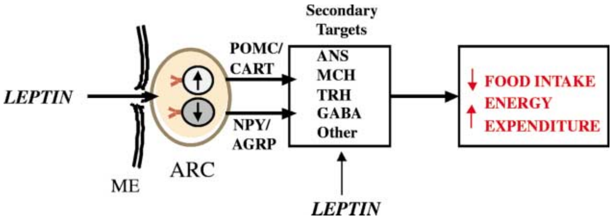

FIGURE 2 Leptin-regulated melanocortin and NPY circuits influence food intake and body weight. Leptin produces its effects on food intake and energy homeostasis in part through activation of the POMC and inhibition of the NPY neurons in the ARC. Potential downstream targets of these neurons include TRH and GABA neurons in the PVH, MCH neurons in the LH, and other targets in the hypothalamus, brainstem, and other brain regions. The ultimate consequence of activation of POMC neurons is a decrease in food intake. As NPY and AGRP tend to stimulate food intake and energy homeostasis, the ultimate consequence of inhibition of the NPY neurons will again be inhibition of food intake and increased energy expenditure. POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y; AGRP, agouti gene-related peptide; ANS, autonomic nervous system; MCH, melanin-concentrating hormone; TRH, thyroid-releasing hormone; GABA, gamma-aminobutyric acid; ME, median eminence; ARC, arcuate nucleus.

following cytokine receptor activation regardless of whether the effects on the neuron are excitatory or inhibitory. The immediate-early gene expression therefore suggests that leptin activates POMC neurons and inhibits NPY neurons. Indeed, electrophysiological recordings in the ARC have shown that leptin increases the activity of POMC neurons and decreases the activity of NPY neurons. Therefore, leptin acts to decrease food intake by both decreasing the release of the orexegenic peptides NPY and AGRP and increasing the release of the anorexegenic peptide αMSH. The downstream targets of the POMC and NPY neurons are less well understood. However, several possible targets are emerging. Both NPY and melanocortins have been shown to produce their effects on food intake and energy homeostasis following injections into the PVN. TRH neurons in the PVN express MC4 receptors and TRH expression is regulated by melanocortins. Furthermore, leptin's effects on TRH expression are mediated at least in part by the melanocortin system, although leptin has also been shown to have some direct effects on TRH expression. Regulation of TRH and other PVN neurons would regulate both the pituitary and the thyroid axes and provide one mechanism by which leptin and the melanocortin system may modulate both food intake and energy homeostasis. Other potential downstream targets of both the POMC and the NPY neurons are the MCH and orexin neurons discussed below. POMC and NPY neurons have many other projection sites including those involved in the regulation of autonomic functions, and it is likely that these multiple downstream targets act in concert to mediate the effects of both melanocortins and NPY on appetite.

## IV. OREXIGENIC FACTORS

### A. Melanin-Concentrating Hormone

MCH is expressed exclusively in the lateral hypothalamus, which sends projections to other brain regions involved in appetite regulation, including monosynaptic connections throughout the cortex and NTS. MCH is a potent stimulator of food intake following intracerebroventricular injection. Fasting or removal of leptin increases MCH mRNA levels and these are restored to normal levels following food intake or leptin administration. Targeted disruption of the MCH gene leads to mice that are hypophagic and lean, demonstrating a critical role for MCH in both food intake and weight homeostasis. Conversely, overexpression of MCH in transgenic mice leads to increased food intake and promotes obesity. MCH neurons have been proposed to be part of a secondary relay system for the actions of leptin and insulin and are hypothesized to be targets of the NPY and melanocortin neurons. MCH has also been shown to suppress the release of TRH from downstream target neurons. The finding that mice lacking MCH are lean contrasts with the lack of an obesity phenotype observed in the NPY-deficient mice and suggests a lack of redundancy at the level of the MCH neurons in the regulation of food intake and energy homeostasis.

### B. Orexins (Hypocretins)

Orexin A and B, or hypocretin 1 and 2, named by the two groups who simultaneously discovered them, are expressed in neurons with cell bodies in the LH and DMH. Orexin-positive neurons send projections to regions involved in appetite regulation, including projections that synapse on NPY neurons in the ARC. Conversely, ARC NPY neurons send projections that synapse on orexin-positive cells in the LH. Orexins have been shown to increase food intake when administered centrally. However, orexins also increase general arousal, and targeted deletion of the orexin gene in mice causes narcolepsy. The effects of the orexins on food intake may therefore be due to indirect effects on general arousal rather than a direct effect on food intake. Further studies are required to determine the exact role of the orexins in appetite regulation.

### C. Galanin

Galanin neurons are located as several discrete populations in the ARC, DMH, and PVN and innervate multiple areas of the hypothalamus and other brain regions involved in food intake. Central injections of galanin peptide into the PVN, LH, VMH, NTS, and central nucleus of the amygdala all stimulate feeding in rats. Galanin neurons have synaptic links with both NPY and POMC neurons, and galanin gene expression is increased by fasting, suggesting a role for this neuropeptide in appetite regulation. However, the food intake stimulated by galanin is much less robust than that stimulated by NPY and no obvious weight phenotype has been reported in galanin knockout mice.

### D. Endogenous Opioids

Several members of the endogenous opioids have been described, along with three main types of opioid receptor, the μ, δ, and κ receptors. β-endorphin is processed from the POMC pro-hormone and is therefore predicted to be released from the POMC neurons along with the melanocortins at many regions critical for the regulation of food intake and energy homeostasis. β-endorphin has affinity for all three opioid receptor subtypes. Dynorphin is an endogenous opioid with high affinity for the κ opioid receptor and affinity for the μ opioid receptor. Dynorphin is highly expressed in the lateral hypothalamus where it is co-localized in orexin neurons. Met- and Leu-enkephalin have the highest affinity for δ opioids and μ opioid receptors. Met- and Leu-enkephalin are expressed in the hypothalamus as well as other brain regions involved in the regulation of appetite, including the nucleus accumbens, the amygdala, and the NTS. Injections of opioid agonists, including β-endorphin and dynorphin, stimulate food intake, predominately through the μ and κ opioid receptors. Conversely, opioid antagonists inhibit food intake, suggesting that there is a basal tone of endogenous opioids that stimulates food intake. Furthermore, opioid receptor antagonists attenuate NPY-induced, galanin-induced, and fast-induced food intake. However, given the promiscuous pharmacology of the endogenous ligands, it is hard to determine which of the endogenous opioids mediates these effects. In addition to potentially having direct effects on food intake, endogenous opioids are thought to mediate some of the rewarding aspects of food intake. However, mice that lack dynorphin or enkephalin have not been reported to have abnormal body weights and mice lacking β-endorphin have an unexpected mild increase in body weight.

## E. Ghrelin

Ghrelin was first identified as an endogenous ligand for the growth hormone secretagogue receptor; however, it has recently been found to have orexegenic properties as well and is thought to act as an integrator of growth and energy needs. Ghrelin is produced in the stomach and is proposed to stimulate food intake via the hypothalamus when an increase in energy intake is needed. Consistent with this hypothesis, ghrelin stimulates food intake when administered both peripherally and centrally. The central injection of ghrelin produces a more robust and sustained food intake than a peripheral injection, and ghrelin has been proposed to have a central mode of action. As predicted for an energy deficiency signal, mRNA levels of ghrelin in the stomach, and circulating blood levels, are increased by fasting and decreased by feeding. Interestingly, caloric intake, and not stomach expansion, appears to decrease circulating levels of ghrelin. The effects of ghrelin can be long term, as subcutaneous injections of ghrelin twice daily for 2 weeks stimulated body weight gain and increased fat mass with no change in lean mass. Anti-ghrelin antibodies reduce fast-induced food intake when administered either peripherally or centrally, suggesting a tonic ghrelin tone.

The GHS or ghrelin receptor is highly expressed in the arcuate nucleus and the ventromedial hypothalamus, and ghrelin stimulates arcuate neurons and the expression of immediate-early gene products such as c-fos in the arcuate nucleus. Fifty percent of the arcuate neurons expressing the GHS receptor are NPY/AGRP neurons; ghrelin treatment increases both NPY and AGRP expression but the mRNA levels of POMC are unchanged. Pretreatment with NPY1 receptor antagonists attenuates the effects of ghrelin on food intake, consistent with the orexigenic effect of ghrelin being mediated by NPY/AGRP neurons. Ghrelin still has effects on feeding in NPY-deficient mice, suggesting that other transmitters (potentially AGRP) also contribute to the orexigenic effects of ghrelin. Leptin and ghrelin appear to have antagonistic actions on the regulation of food intake and weight homeostasis, and co-administration of ghrelin attenuates the effects of leptin.

## F. Endogenous Cannabinoids

The endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol, are found in the hypothalamus, and injections of cannabinoid agonists stimulate food intake, suggesting a role for cannabinoids in the regulation of appetite. Pharmacological studies suggest that the orexigenic effects of cannabinoids are mediated by the centrally expressed $CB_1$ receptor. Furthermore, chronic treatment with a $CB_1$ receptor antagonist inhibits food intake and decreases weight, although these effects last for less than a week. Consistent with the pharmacology, mice lacking the $CB_1$ receptor have reduced food intake following fasting. However, nonfasted $CB_1$ receptor knockout mice have normal food intake. Acute leptin administration reduces the levels of the endogenous cannabinoids. Conversely, the levels of the endogenous cannabinoids are increased in the hypothalamus in ob/ob and db/db mice and Zucker rats, animal models with defective leptin signaling, suggesting that the cannabinoids may mediate some of the effects of leptin.

### G. Dopamine

Dopamine has been proposed to contribute to the rewarding aspects of food intake. Both pharmacological depletion and genetic depletion of dopamine result in profound feeding deficits. However, dopamine deficiency also results in impaired movement and locomotion, and therefore, interpretation of these results is complicated. More recent studies specifically restoring dopamine in the caudate putamen of dopamine-deficient mice restored feeding. In contrast, rescue of dopamine specifically in the nucleus accumbens of dopamine-deficient mice restored exploratory behavior, but not feeding. This study separates the locomotive and feeding effects of the dopamine-deficient mice and suggests that depletion of dopamine in the caudate putamen is primarily responsible for the hypophagia phenotype in dopamine-deficient mice. This was somewhat of a surprise given the role of the nucleus accumbens in mediating the rewarding aspects of food intake. The mechanisms underlying the effects of dopamine on food intake remain to be established.

### H. Other Orexigenic Factors

Glucocorticoids stimulate an increase in food intake. Furthermore, many of the hypothalamic effects of leptin and insulin are opposed by glucocorticoids. Thyroid hormone and growth hormone also stimulate food intake. However, the mechanism by which the increase in food intake occurs is not known and may be indirect. Thyroid hormone effects may be indirect due to compensation for increased metabolic rate, whereas the effects of growth hormone may occur through growth hormone-releasing hormone.

## V. ANOREXIGENIC FACTORS

### A. Glucagon-like Peptide 1

Glucagon-like peptide 1 (GLP-1) is a gastrointestinal peptide that is released in response to food intake. GLP-1 plays an important role in glucose homeostasis and augments glucose-induced insulin secretion and inhibits glucagon secretion. However, GLP-1 is also proposed to act as a satiety factor. Consistent with this hypothesis, peripheral administration of GLP-1 inhibits food intake. In addition to being produced in the intestine, GLP-1 is expressed at hypothalamic sites, the caudal portion of the nucleus of the solitary tract, and the forebrain. Central administration of GLP-1 inhibits food intake through actions in the hypothalamus, including the PVH. Conversely GLP-1 antagonists stimulate feeding in satiated rats. GLP-1 antagonists also attenuate the effects of both leptin and CCK to inhibit food intake. Leptin has been shown to increase hypothalamic GLP-1 levels and to activate GLP-1 neurons in the NTS, providing further evidence of a role for GLP-1 in food intake and energy homeostasis. However, mice lacking the GLP-1 receptor do not show any feeding abnormalities.

### B. Cocaine- and Amphetamine-Regulated Transcript

Cocaine- and amphetamine-regulated transcript is a recently discovered hypothalamic peptide that is coexpressed in POMC neurons, although its expression is not restricted to these neurons. CART neurons are known to project to many sites important for appetite regulation. Furthermore, injections of CART inhibit food intake, and CART expression is regulated by changes in leptin levels, suggesting a role for this peptide in the regulation of energy homeostasis. However, mice deficient in CART have minimal changes in food intake and energy homeostasis, suggesting that although CART has effects on food intake and energy homeostasis, its effects are redundant in that the system appears to be able to compensate for the absence of CART.

### C. Corticotropin-Releasing Hormone

Cell bodies of CRH are expressed predominately in the PVN, a major site of action for appetite-regulating drugs. CRH has diverse effects and is the primary hypothalamic hormone that stimulates ACTH release from the pituitary, ultimately leading to the release of corticosterone from the adrenal gland. Injections of CRH centrally decrease food intake, including NPY-induced food intake. CRH also promotes a negative energy balance by stimulating energy expenditure, by increasing thermogenesis via sympathetic pathways. CRH levels are regulated by leptin, suggesting a role for CRH in mediating the effects of leptin. Another member of the CRH family is urocortin, which is more potent at suppressing food intake than CRH. The effects of both urocortin and CRH are mediated by CRHR1 or CRHR2 receptors. However, the weights and basal food intake of both CRHR1 and CRHR2 knockout mice are normal.

### D. Serotonin

Serotonin (5HT)-expressing neurons have their cell bodies in the brainstem and project widely in the brain including the hypothalamus. The 5HT or serotonin system is the primary target of drugs currently used to treat obesity. Dexfenfluramine and other diet drugs increase 5HT signaling and inhibit food intake. Serotonin turnover is increased by leptin, and mice lacking the 5HT2c receptor have increased food intake and increased weight. The obesity seen in these mice is mild, however, and leptin still inhibits food intake, suggesting that this receptor signaling pathway is not critical for the actions of leptin. More studies are required to determine the exact role of the serotonin system in weight homeostasis.

### E. Noradrenaline

The PVH receives a dense catecholaminergic innervation from the caudal medulla relaying predominately visceral sensory information. Many of the catecholaminergic neurons from the NTS co-express NPY. Injections of noradrenaline (NE) into the PVH stimulate food intake, supporting a role for this transmitter in the regulation of appetite. Furthermore, NE levels are regulated by changes in leptin levels and therefore appear to be sensitive to changes in energy state.

### F. Cholecystokinin

CCK was the first gut peptide identified to be a satiety factor. Injections of CCK produce a rapid onset inhibition of food intake that is short-lived. The satiety effects of peripherally administered CCK require an intact vagal nerve and are believed to be mediated through the NTS. Physiologically, CCK is secreted by the gut after a meal, and its release stimulates CCK receptors located on the vagal nerve. Vagal afferents terminate on neurons in the NTS believed to regulate appetite and energy homeostasis. CCK has synergistic effects with leptin on both food intake and c-fos expression in the NTS, area postrema, and PVH. Two subtypes of CCK receptors have been identified: $CCK_A$ and $CCK_B$. $CCK_A$ receptor-specific antagonists block the anorexic effects of CCK, suggesting that it is mediated primarily through the $CCK_A$ receptor. Furthermore, $CCK_A$ receptor antagonists have been shown to attenuate refeeding following food deprivation. Interestingly, rats that lack the $CCK_A$ receptor have a mild type of obesity, whereas $CCK_A$ receptor knockout mice do not. CCK is also expressed in the brain, suggesting that there are direct actions of CCK centrally as well as through modulation of the vagal afferents. Indeed, injections of CCK directly into either the NTS or the hypothalamus inhibit food intake.

### G. Other Anorexigenic Factors

Cytokines such as interleukins [interleukin-1 (IL-1) and interleukin-6 (IL-6)], tumor necrosis factor-α, ciliary neurotrophic factor, and leukemia inhibitory factor have all been shown to inhibit food intake. The mechanism remains to be established but may involve actions on insulin and leptin sensitivity and regulation of the melanocortin system. Interestingly, IL-6 knockout mice have a late onset obesity, although the cause is not yet established. The peptide bombesin reduces food intake in rodents, and mice lacking the bombesin-3 receptor become obese, suggesting that this peptide may also be important in regulating food intake. Furthermore, histamine is also thought to be an appetite suppressor through actions at the $H_1$ receptor. Enterostatin is a pentapeptide, produced in the gut, that also reduces food intake in rodents and reduces hunger ratings in humans. Interestingly, it appears to selectively reduce fat intake.

## VI. SUMMARY

The regulation of appetite and food intake is a complex process. It involves a coordinated response to many orexigenic and anorexigenic factors in multiple brain regions (Table 1). Significant progress has occurred over the past decade, including the discovery of the adipostatic hormone leptin and some of the pathways required for its actions. Leptin produces its effects, in part, by a coordinated action

TABLE 1 The Effects of Appetite Factors on Food Intake and the Regulation of Their Mrna/Protein Levels by Fasting

| Neuroregulator | Food | Regulation of mRNA levels by fasting |
|---|---|---|
| AGRP | + | + |
| Cannabinoids | + | + |
| CART | − | − |
| CRH | − | ? |
| Galanin | + | ? |
| Ghrelin | + | + |
| GLP-1 | − | − |
| Insulin | − | − |
| Leptin | − | − |
| MCH | + | + |
| MSH | − | ? |
| NE | − | − |
| NPY | + | + |
| Opioids | + | ? |
| Orexin/hypocretin | + | + |
| 5HT | − | − |

to simultaneously activate POMC and inhibit NPY neurons in the ARC to alter food intake and energy homeostasis. Downstream targets of the NPY and POMC neurons include specific neurons in the PVN, LH, NTS, and other brain regions. However, there are still many "black boxes" and it is likely that some critical appetite factors remain to be discovered. Furthermore, as is the case with other functions regulated by the hypothalamus, there appears to be some redundancy between these appetite factors.

Given the tremendous health impact of obesity in the world, elucidating the pathways and mechanisms involved in the regulation of energy homeostasis is essential to understanding more about this problem and for the development of novel therapeutic tools. Disruptions of these pathways clearly have a large impact on food intake and energy homeostasis, as illustrated by monogenic mutations resulting in severe obesity. However, the epidemic of obesity in recent years probably reflects more changes in lifestyle, particularly in food intake and exercise. This illustrates the importance of emotional and other influences on food intake that may modulate the pathways involved in energy homeostasis discussed in this article.

## Glossary

**anorexigenic** A compound that inhibits or decreases food intake.

**cachexia** A state of malnutrition associated with disease and consisting of a combination of anorexia, increased metabolic rate, and wasting of lean body mass.

**db/db mice** Mice that are deficient in the leptin (ob) receptor.

**knockout** A mouse with a gene that has been specifically targeted (or "knocked out") so that the protein is no longer made.

**ob/ob mice** Mice that are deficient in the hormone leptin.

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**thermogenesis** Process that occurs in the brown adipose tissue of rodents. Heat is generated and energy expended. It is a major determinant of metabolic rate in rodents.

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# Aromatase and Estrogen Insufficiency

Evan R. Simpson

*Monash Medical Centre, Australia*

I. INTRODUCTION
II. AROMATASE DEFICIENCY IN HUMANS
III. THE AROMATASE KNOCKOUT MOUSE
IV. CONCLUSIONS

to simultaneously activate POMC and inhibit NPY neurons in the ARC to alter food intake and energy homeostasis. Downstream targets of the NPY and POMC neurons include specific neurons in the PVN, LH, NTS, and other brain regions. However, there are still many "black boxes" and it is likely that some critical appetite factors remain to be discovered. Furthermore, as is the case with other functions regulated by the hypothalamus, there appears to be some redundancy between these appetite factors.

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# Aromatase and Estrogen Insufficiency

Evan R. Simpson
*Monash Medical Centre, Australia*

I. INTRODUCTION
II. AROMATASE DEFICIENCY IN HUMANS
III. THE AROMATASE KNOCKOUT MOUSE
IV. CONCLUSIONS

**Endogenous estrogens are formed from androgenic steroids by the action of the enzyme aromatase, which is a member of the cytochrome P450 superfamily, and encoded by the CYP19 gene. In humans, aromatase expression and hence estrogen biosynthesis occur in a number of tissue sites including ovary, placenta, fetal liver, adipose, bone, and brain. Analysis of the phenotypes of humans with natural mutations of the aromatase gene, as well as mice in which the gene has been inactivated by targeted disruption, has revealed a number of new and unexpected roles for estrogens in both males and females. These include roles in bone mineralization, lipid and carbohydrate metabolism, and spermatogenesis in the male. This article will briefly summarize the present and sometimes contradictory state of knowledge regarding the diverse roles that are played by the products of the aromatase reaction and point to future directions that may help to clarify these issues.**

## I. INTRODUCTION

Models of estrogen insufficiency have revealed new and often unexpected roles for estrogens in both males and females. These models include natural mutations of the aromatase gene in humans and one man with a mutation in the estrogen receptor-α (ER-α) gene, as well as the various mouse knockout (KO) models, namely, the ERαKO and ERβKO mice, the ERα/ERβ double KO mouse, as well as the aromatase knockout (ArKO) mouse.

Some of these roles for estrogen are nonsexually dimorphic and apply equally to males and females, for example, the role of estrogens in bone metabolism as well as in carbohydrate and lipid metabolism. Indeed, in the context of its role in male germ cell development, estradiol can appropriately be considered an androgen.

Estrogens are synthesized from C19 androgenic precursors by means of the aromatase enzyme, which is the product of the CYP19 gene. Aromatase is a member of the P450 gene superfamily, which has over 600 members in approximately 100 families; CYP19 is the sole member of family 19.

The human CYP19 gene was cloned some years ago when it was shown that the coding region spans nine exons beginning with exon II. Upstream of exon II are a number of alternative exons I that are spliced in the 5′-untranslated region of the transcript in a tissue-specific fashion (Fig. 1). Thus, placental transcripts contain at their 5′-end a distal exon, I.1, which is localized at least 40 kb upstream from the start of translation in exon II. This is because placental expression is driven by a powerful distal promoter upstream of exon I.1. On the other hand, transcripts in ovary and testes contain at their 5′-end a sequence that is immediately upstream of the translational start site. This is because expression of the gene in the gonads utilizes a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain yet another distal exon located at least 20 kb upstream of the start of translation, exon I.4. Splicing of these untranslated exons to form the mature transcript occurs at a common 3′-splice junction that is upstream of the translational start site. This means that although transcripts in different tissues have different 5′-termini, the coding region and thus the protein expressed in these various tissue sites are always the same. However, the promoter regions upstream of each of the several untranslated first

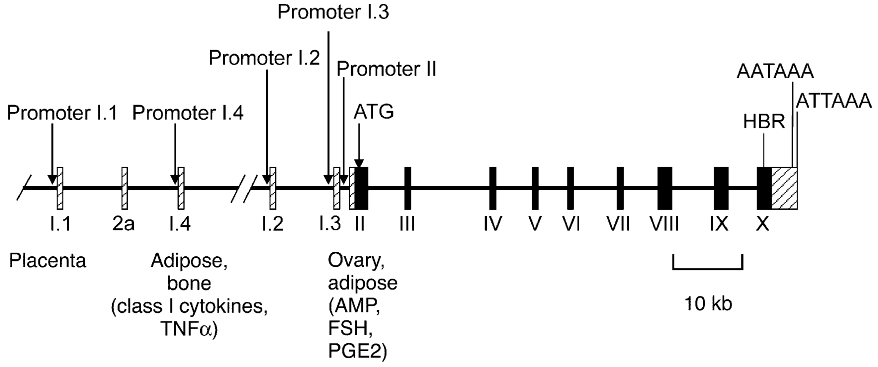


FIGURE 1 Diagram of the structure of the human aromatase gene showing the arrangement of the various untranslated first exons and their associated promoters.

exons have different cohorts of response elements, and so regulation of aromatase expression in each tissue that synthesizes estrogens is different. Thus, the gonadal promoter binds the transcription factors $Ca^{2+}$/cyclic AMP-response element-binding protein and Sp-1, and so aromatase expression in gonads is regulated by cyclic AMP and gonadotropins. On the other hand, adipose promoter I.4 is regulated by class I cytokines, such as interleukin-6 (IL-6), IL-11, and oncostatin M, as well as by tumor necrosis factor α. Thus, the regulation of estrogen biosynthesis in each tissue site of expression is unique.

## II. AROMATASE DEFICIENCY IN HUMANS

Aromatase deficiency appears to be a rare condition and there have been 10 mutations reported to date, affecting seven females and three males, one of whom is a child (Fig. 2). The phenotypes of these individuals will be summarized in this article as will the findings on the phenotype of ArKO mice created by disruption of the CYP19 gene, which encodes aromatase.

With the exception of the first reported case, namely, the Japanese patient, all of the mutations thus far identified are single base-pair changes giving rise to single amino acid substitutions and in one case a premature stop codon (Fig. 2). The mutation in the Japanese patient is a single base change that destroyed an exon–intron splice junction, giving rise to continued read-through of an extra 87 bases to a cryptic intron–exon splice junction within the intron, resulting in an in-frame insertion of 29 amino acids within the coding region. It should be pointed out that both siblings in the New York family were homozygous for the condition, which would suggest that the mutation does not cause any diminished likelihood of implantation or any serious problem with embryonic or fetal development. Apart from the Californian patient and the Swiss patient, who are compound heterozygotes, all of the subjects are homozygous for the mutation in question and are the products of consanguineous relationships. In most cases, it was the mother who presented, during the third trimester, complaining of virilization resulting in facial hair and acne. These symptoms subsided after delivery. In the cases of female newborns, they present with pseudo-hermaphroditism with clitoromegaly and hypospadias in varying degrees of severity. This virilization of both mother and fetus is a consequence of the inability of dehydroepiandrosterone of fetal adrenal origin to be converted to estrogens by the placenta, with its consequent peripheral conversion to androgens. These individuals then present again at the time of puberty with primary amenorrhea, failure of breast development, hypergonadotropic–hypogonadism, and cystic ovaries. Subsequent estrogen supplementation leads to regression of these symptoms.

In addition, two men have been reported with this condition. In each case, childhood development was uneventful. Each presented in their late twenties with tall stature due to sustained linear growth through puberty as a consequence of failure of epiphyseal fusion. They also had severely delayed bone age, resulting in osteopenia and undermineralization.

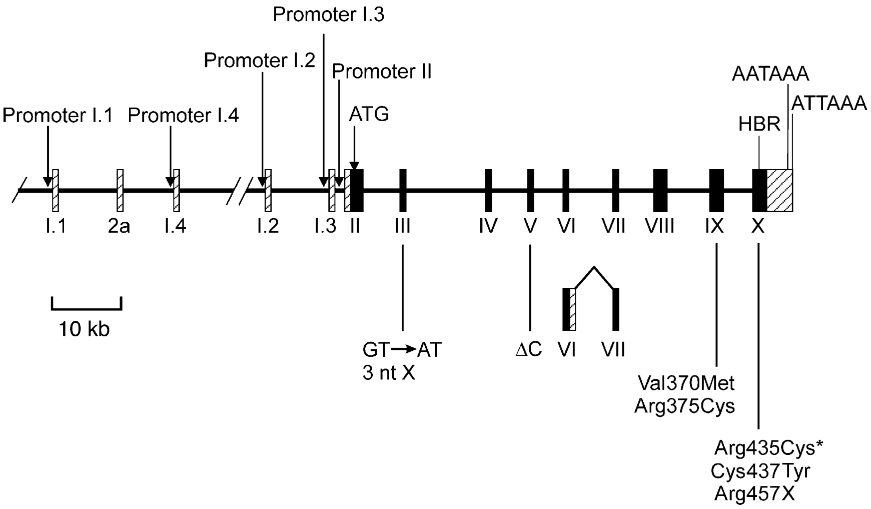


**FIGURE 2** Known mutations in the CYP19 gene that give rise to estrogen insufficiency in humans. The mutations in the Swiss compound heterozygote case are not included.

**TABLE 1 Plasma Hormone Levels in the Male Patient with CYP19 Deficiency**

| | | | |
|---|---|---|---|
| $\Delta^4$A | 335 | ng/dl | 30–263 |
| T | 2015 | ng/dl | 200–1200 |
| 5α-DHT | 125 | ng/dl | 30–85 |
| E1 | <7 | pg/ml | 10–50 |
| E2 | <7 | pg/ml | 10–50 |
| FSH | 28.3 | mU/ml | 5.0–9.9 |
| LH | 26.1 | mU/ml | 2.0–9.9 |
| Glucose | 70 | mg/dl | 70–105 |
| Insulin | 52 | μU/ml | 5–25 |
| GH | <0.5 | ng/ml | 0.5–4.2 |
| IGF-I | 203 | ng/ml | 182–780 |

Analysis of the plasma hormone levels of the New York male patient (Table 1) revealed undetectable estrogens and very high circulating androgens. Circulating follicle-stimulating hormone and luteinizing hormone (LH) were also elevated, indicative of an important role for estrogens in the negative feedback regulation of gonadotropins in males, as in females. Presumably, in the case of males, this estrogen is normally derived from local aromatization of testosterone within brain sites. The patient was 204 cm tall at the age of 24 years and had testes that were 35 ml in volume. Unfortunately, no semen sample was available so it was impossible to gauge his sperm count or sperm viability. He was unmarried and had no offspring. However, his testicular volume and hormonal profile would indicate the likelihood that he had functioning testes as far as their capacity to synthesize steroids was concerned.

The fetal testes synthesize anti-Müllerian hormone and testosterone, the former causing regression of the Müllerian structures and the latter causing virilization of the male external genitalia (Fig. 3). Classically this action of testosterone is thought to be achieved by conversion to 5α-dihydrotestosterone due to the activity of 5α-reductase. However, more recently it has become evident that there are several sites in the testes that are capable of converting testosterone to estrogens. This is because the enzyme aromatase, responsible for the conversion of androgens to estrogens, has been reported to be present at a number of sites within the testes including the Leydig cell and also germ cells at various stages of development. Additionally, both the α and the β isoforms of the estrogen receptor have been reported to be present at several sites. Furthermore, the levels of estradiol in the semen and rete fluid are greater than those circulating in the plasma of females. These considerations give rise to the concept that estrogens play a role in the function of the testes.

The second male patient was from a family in southern Italy and presented at the age of 28 years with tall stature, infertility, and skeletal pain. He was found to have open epiphyses and a bone age of 14.8 years. Treatment with testosterone for 8 months failed to result in any improvement of his condition, whereas treatment with transdermal estradiol for 6 months restored his bone density to within the normal range and eliminated his other symptoms. In contrast to the New York patient, this individual had a testicular volume of 8 ml and was infertile. Testicular biopsy revealed that his seminiferous tubules had little or no sperm present and gamete development appeared to be arrested at the spermatocyte level. Again in contrast to the New York patient, his circulating testosterone levels were not elevated and were reduced dramatically on administration of estradiol. These results might suggest a relationship between aromatase deficiency and testicular dysfunction. Unfortunately for this concept though, the individual has a brother with azoospermia but who is homozygous for the normal active aromatase gene. These observations suggest that the fertility problem in these men may be independent of the aromatase deficiency in this family. Thus, the study of these two men with aromatase deficiency has failed to elucidate the role of estrogens in male fertility and suggests that, at the very least, there is variable penetrance of the phenotype. The phenotypes of these men may be compared with that of the one known male with a mutation in the estrogen receptor-α isoform, who also presented with a failure of epiphyseal closure and undermineralized bones, but who has reduced sperm count and sperm motility.

In addition to the bone and fertility phenotypes outlined above, individuals with aromatase deficiency have a lipid and carbohydrate phenotype, namely, truncal obesity, hypertriglyceridemia, elevated low-density lipoprotein levels, and insulin resistance. These symptoms all subside on treatment with estrogen (Table 1).

## III. THE AROMATASE KNOCKOUT MOUSE

To gain further insight into the physiological role of estrogens, a mouse was created in which the aromatase gene was disrupted by homologous recombination (Fig. 4). Studies of these mice are indicative of a dramatic bone phenotype, with shortened femur length and reduction in all of the indices of bone mineralization. Because circulating

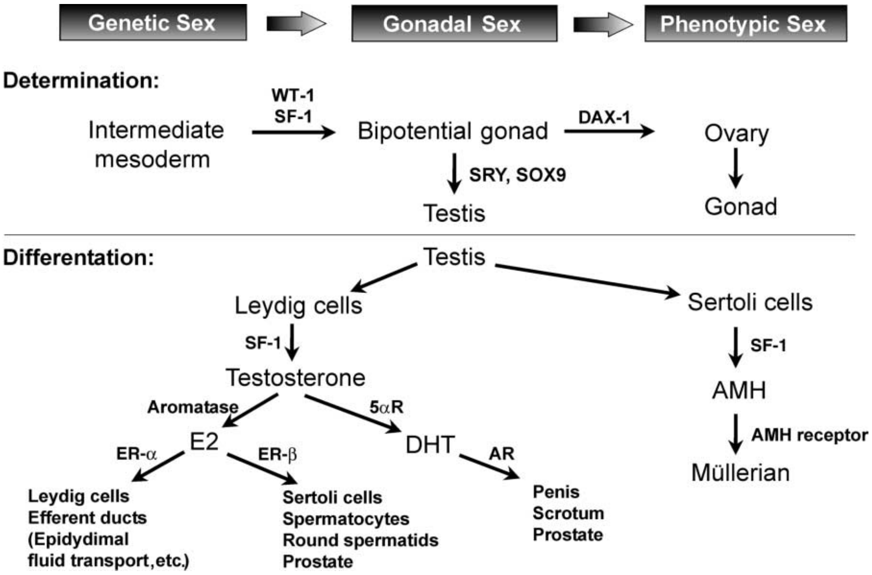
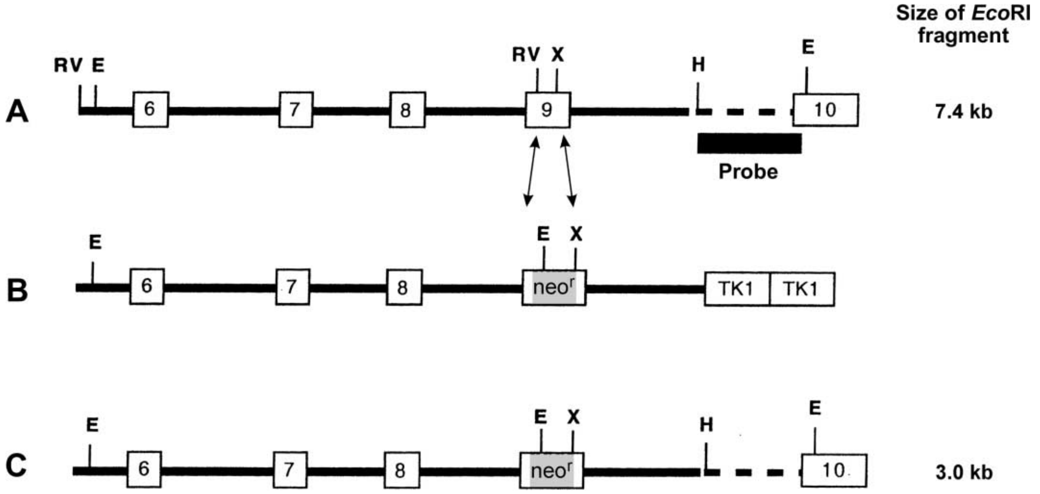

Genetic Sex
Gonadal Sex
Phenotypic Sex
Determination:
Intermediate mesoderm
WT-1
SF-1
Bipotential gonad
DAX-1
Ovary
SRY, SOX9
Testis
Gonad
Differentation:
Testis
Leydig cells
Sertoli cells
SF-1
Testosterone
SF-1
AMH
Aromatase
5αR
E2
ER-α
ER-β
DHT
AR
AMH receptor
Müllerian
Leydig cells
Efferent ducts
(Epidydimal
fluid transport,etc.)
Sertoli cells
Spermatocytes
Round spermatids
Prostate
Penis
Scrotum
Prostate

**FIGURE 3** Factors involved in testicular differentiation.

estradiol levels in wild-type as well as ArKO male mice are below the level of detection, this would indicate that the source of estrogens maintaining bone mineralization in the wild-type mice is likely to be local production within the bone itself or at other extragonadal sites.

Examination of the morphology of the ovaries of mice at the age of 10–12 weeks (Fig. 5) revealed a striking phenotype in that the ovaries contained a number of follicles that showed signs of antral information but appeared arrested prior to ovulation. No corpora lutea were present but the follicles had abundant granulosa cells displaying some mitotic figures. The stroma was hyperplastic, indicative of the high circulating LH levels and some pyknotic figures were present, perhaps indicative of follicular atresia. At later ages, cystic hemorrhagic follicles were present, coincident with an infiltration of macrophages and collagen deposition in the interstitium. By 1 year of age, there were no secondary or antral follicles in ArKO ovaries and atresia was widespread in the remaining primary follicles.


Size of EcoRI fragment
A
RV E
6
7
8
RV X
9
H
E
10
7.4 kb
Probe
B
E
6
7
8
E X
neo^r
TK1 TK1
C
E
6
7
8
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3.0 kb

**FIGURE 4** Diagram of part of the structure of the wild-type mouse CYP19 gene (A), the targeting vector (B), and the disrupted gene (C).

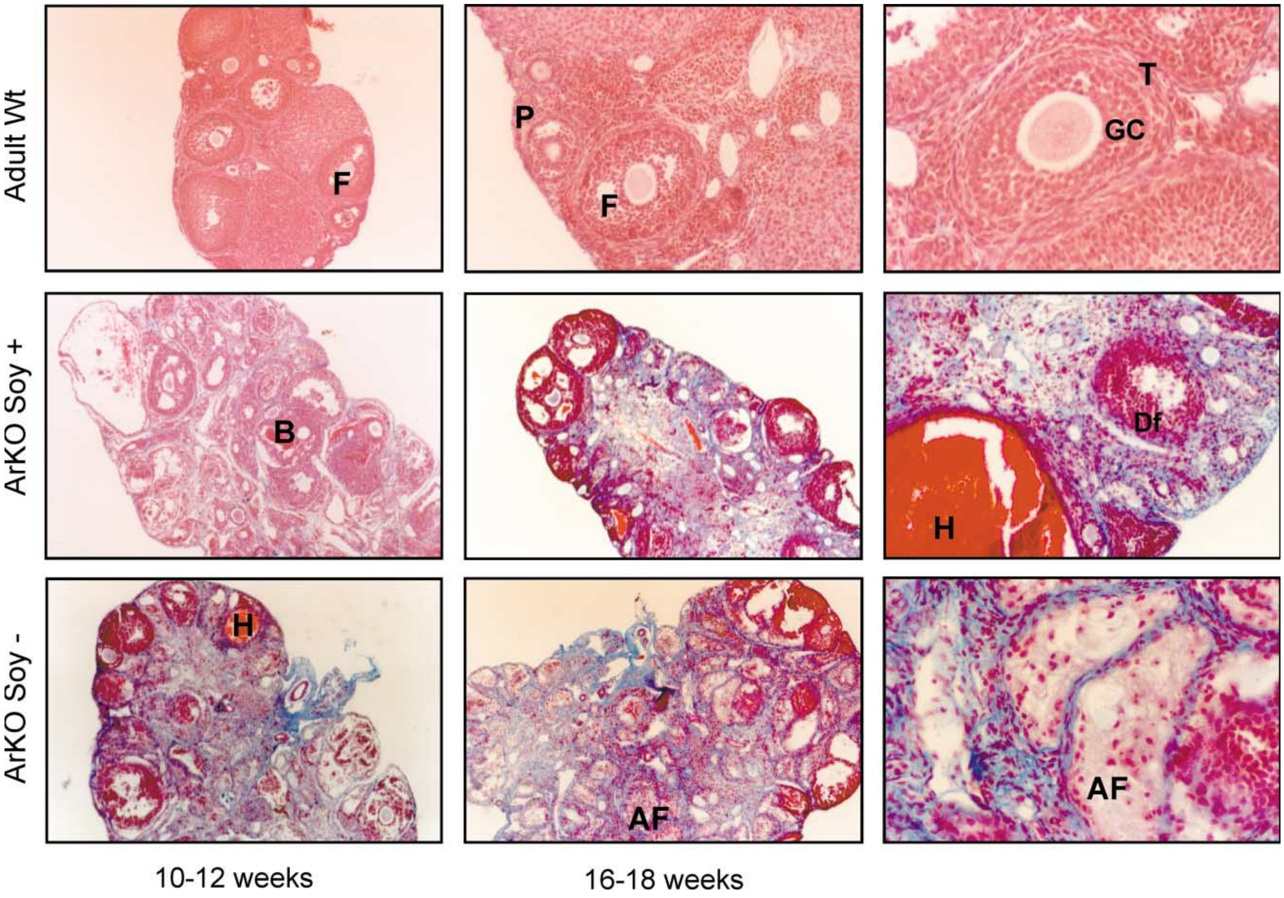

**FIGURE 5** Optical micrographs of ovaries of adult wild-type mice (top row), ArKO mice on a regular chow diet (middle row), and ArKO mice on a soy-free diet (bottom row). The right-hand panels of the ArKO ovaries are higher magnifications of parts of the central panels to show details. F, follicle; P, primordial follicle; T, theca; GC, granulosa cells; H, hemorrhagic cyst; Df, dying follicle; AF, abnormal follicle.

Examination of the testes of male mice at a similar age showed no obvious phenotypes and these males were fertile (data not shown). However, with increasing age, the numbers of litters they sired diminished compared with their wild-type littermates. Disruptions to spermatogenesis commenced at 4–5 months and became more progressive with advancing age. Spermatogenesis was primarily arrested at early spermiogenic stages, as characterized by an increase in apoptosis and the appearance of multinucleated cells, and there was a significant reduction in round and elongated spermatids, but no changes in Sertoli cells and earlier germ cells. In addition, Leydig cell hyperplasia/hypertrophy was evident, presumably as a consequence of increased circulating LH. These findings suggest that local expression of aromatase, at least in mice, is essential for spermatogenesis and indicate a direct action of estrogen on male germ cell development and, thus, fertility. These results are in contrast to those of ERKO mice in which the seminiferous tubules had grossly distended lumens with no sign of sperm. This is apparently due to failure of fluid transfer across the epithelium of the efferent ductules, resulting in increased pressure and back-up of fluid into the seminiferous tubules. By contrast, the ER-β knockout mice display no detectable testicular phenotype. These differences in phenotype of the various models of lack of estrogen representation are not readily explicable in terms of the current level of understanding. These are several possibilities, including the presence of endogenous estrogenic substances other than the products of the aromatase reaction non-ligand-mediated pathways of estrogen receptor activation and pathways of estrogen action other than via the known receptors.

Another phenotype developed by these mice, similar to the situation with humans carrying a natural mutation in the CYP19 gene, is a lipid and carbohydrate phenotype. ArKO mice, both male and female, develop a progressive increase in abdominal adiposity, involving both the gonadal and the infrarenal fat deposits. This is associated with increased plasma triglycerides and cholesterol, insulin resistance, and hyperleptinemia. Hepatic steatosis is also present. Interestingly, the increased adiposity is not accompanied by a marked increase in body weight. This is because there is a corresponding decrease in lean body mass, most likely skeletal muscle, which is associated with a decrease in activity. This adiposity phenotype is reversed dramatically within 3 weeks on administration of estradiol in the form of implants. A similar phenotype has been reported for the ERαKO mice, so these two models are in accordance as far as this phenotype is concerned.

## IV. CONCLUSIONS

Models of estrogen insufficiency, whether they be natural mutations in humans or targeted gene disruptions in mice, are revealing new and often unsuspected roles for estrogens in both males and females. In some cases, consistency is observed between the animal models, such as the adipose phenotype of the ArKO and ERαKO mice. On the other hand, the studies conducted so far on aromatase-deficient males have not served to throw light on the role of estrogens in male reproduction. This is because of the conflicting phenotypes of the two men with aromatase deficiency, as well as the apparent discrepancies between the phenotype of the ArKO mice and that of the ERαKO mice. This issue can be resolved only by more extensive characterization of the phenotypes of these animals at the cellular and molecular levels. There is also a pressing need to identify other men with aromatase deficiency.

## Acknowledgments

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## Glossary

**aromatase** A microsomal enzyme complex that catalyzes the conversion of testosterone to estradiol.

**exon** The portion of the DNA sequence in a gene that contains the codons that specify the sequence of amino acids in a polypeptide chain, as well as the beginning and end of the coding sequence.

**phenotype** The appearance or other characteristics of an organism, resulting from the interaction of its genetic constitution with the environment, as opposed to its underlying hereditary determinants, or genotype.

**pseudo-hermaphroditism** A condition in which an individual has sexual organs of only one sex but has either genital openings or external tissue exhibiting one or more traits of the opposite sex.

**spermatogenesis** The process by which undifferentiated male germ cells form into mature spermatozoa.

## See Also the Following Articles

**Estrogen and Spermatogenesis • Estrogen in the Male: Nature, Sources, and Biological Effects • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Biology and Lessons from Knockout Mice • Spermatogenesis, Hormonal Control of**

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# Auxin

Lawrence J. Hobbie
*Adelphi University, Garden City, New York*

I. HISTORY, STRUCTURES, AND APPLICATIONS
II. BIOCHEMISTRY
III. POLAR AUXIN TRANSPORT
IV. PHYSIOLOGICAL AND DEVELOPMENTAL ROLES
V. AUXIN SIGNAL TRANSDUCTION
VI. SUMMARY

**Auxins are a class of plant hormones that display a common set of biological activities. As originally defined, an auxin was a substance that could promote elongation in certain tissues. Now a spectrum of auxin effects are known, including promotion of lateral root formation, induction of cell division in callus tissue in conjunction with cytokinins, and induction of ethylene biosynthesis. A variety of natural and synthetic compounds possessing these activities have been identified.**

## I. HISTORY, STRUCTURES, AND APPLICATIONS

The existence of a mobile signal that controlled a plant's response to gravity was first postulated by Ciesielski in 1872. Charles Darwin and his son Francis suggested in 1881 that a transported substance was important for plants' response to light. In the 1920s, Went devised a method of capturing this substance and quantifying its biological activity, which led to the determination of the chemical structure of the first known auxin in the 1930s. Subsequent studies of auxins have focused on their physiological roles, biosynthesis and degradation, and cellular mechanism of action. Auxins are found in all vascular plants and have also been identified in green and brown algae, making it likely that they have been used as signaling molecules throughout plant evolution.

The major naturally occurring auxin is indole-3-acetic acid (IAA; IUPAC: indol-3-yl-acetic acid), a weak acid with a $pK_a$ of 4.75. Several other naturally occurring compounds with auxin activity have also been identified in various plant species, including 4-chloroindole-3-acetic acid and indole-3-butyric acid (Fig. 1). These other compounds have a high level of auxin activity, but it is unclear whether they have intrinsic auxin activity or are active only after their enzymatic conversion to IAA. Auxins are localized primarily to the cytoplasm and the chloroplasts in plant cells.

A large number of synthetic compounds with auxin activity have been produced (Fig. 2), some of which are more stable than IAA, which is subject to

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# Auxin

Lawrence J. Hobbie
*Adelphi University, Garden City, New York*

I. HISTORY, STRUCTURES, AND APPLICATIONS
II. BIOCHEMISTRY
III. POLAR AUXIN TRANSPORT
IV. PHYSIOLOGICAL AND DEVELOPMENTAL ROLES
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VI. SUMMARY

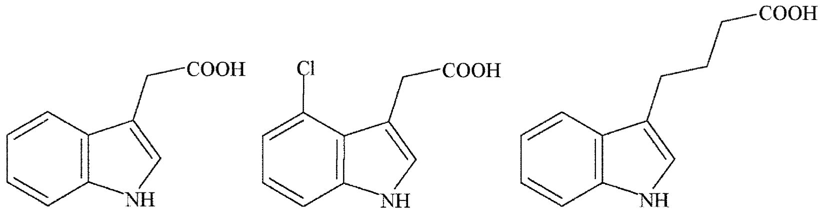
**Auxins are a class of plant hormones that display a common set of biological activities. As originally defined, an auxin was a substance that could promote elongation in certain tissues. Now a spectrum of auxin effects are known, including promotion of lateral root formation, induction of cell division in callus tissue in conjunction with cytokinins, and induction of ethylene biosynthesis. A variety of natural and synthetic compounds possessing these activities have been identified.**

## I. HISTORY, STRUCTURES, AND APPLICATIONS

The existence of a mobile signal that controlled a plant's response to gravity was first postulated by Ciesielski in 1872. Charles Darwin and his son Francis suggested in 1881 that a transported substance was important for plants' response to light. In the 1920s, Went devised a method of capturing this substance and quantifying its biological activity, which led to the determination of the chemical structure of the first known auxin in the 1930s. Subsequent studies of auxins have focused on their physiological roles, biosynthesis and degradation, and cellular mechanism of action. Auxins are found in all vascular plants and have also been identified in green and brown algae, making it likely that they have been used as signaling molecules throughout plant evolution.

The major naturally occurring auxin is indole-3-acetic acid (IAA; IUPAC: indol-3-yl-acetic acid), a weak acid with a p$K_{\mathrm{a}}$ of 4.75. Several other naturally occurring compounds with auxin activity have also been identified in various plant species, including 4-chloroindole-3-acetic acid and indole-3-butyric acid (Fig. 1). These other compounds have a high level of auxin activity, but it is unclear whether they have intrinsic auxin activity or are active only after their enzymatic conversion to IAA. Auxins are localized primarily to the cytoplasm and the chloroplasts in plant cells.

A large number of synthetic compounds with auxin activity have been produced (Fig. 2), some of which are more stable than IAA, which is subject to



Indole-3-acetic acid (IAA)

4-Chloroindole-3-acetic acid

Indole-3-butyric acid

**FIGURE 1** Structure of three naturally occurring auxins.

degradation by light and by plant enzymes. Among the synthetic auxins, a few have been widely used as herbicides, including picloram, 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T), and 2,4-dichlorophenoxy-acetic acid (2,4-D). Auxins are also used in horticulture to promote fruit set and root development of cuttings. In assays involving exogenous application to plants, "active" auxins generally show effects at submicromolar levels. The common structural requirement for auxin activity, based on a comparison of active and inactive auxin analogues, seems to be a negatively charged carboxyl group, linked at an approximate distance of 0.5 nm to a planar aromatic ring that possesses a fractional positive charge.

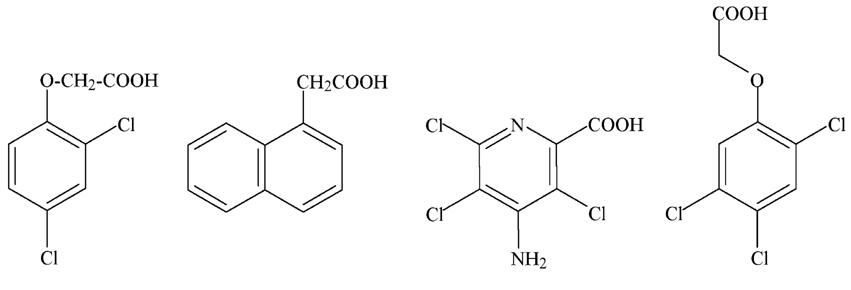
## II. BIOCHEMISTRY

### A. Techniques

For decades, the only methods available for quantifying auxin were bioassays, with the most widely used bioassay being the curvature test. In this assay, an agar block containing a sample is placed on one side of an *Avena sativa* coleoptile from which the tip has been excised, with the resulting curvature being measured. Comparison with the effects of known amounts of IAA allowed the auxin activity in the sample to be quantified. More recently, quantitative chemical analysis has become standard: Gas chromatography–mass spectrometry techniques incorporating stable isotope-labeled internal standards permit precise determination of IAA levels present in samples and correction for losses during purification.

### B. IAA Biosynthesis

IAA biosynthesis studies are challenging because IAA concentrations are generally very low (tens to hundreds of nanograms per gram of tissue) compared to other compounds, such as tryptophan, that can be converted to IAA by nonenzymatic processes or by



2,4-Dichlorophenoxyacetic acid (2,4-D)

1-Naphthaleneacetic acid (1-NAA)

4-Amino-3,5,6-trichloropicolinic acid (picloram)

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)

**FIGURE 2** Structures of four synthetic auxins. 2,4-D and 2,4,5-T were the major components of Agent Orange, used as a defoliant by American forces during the Vietnam War.

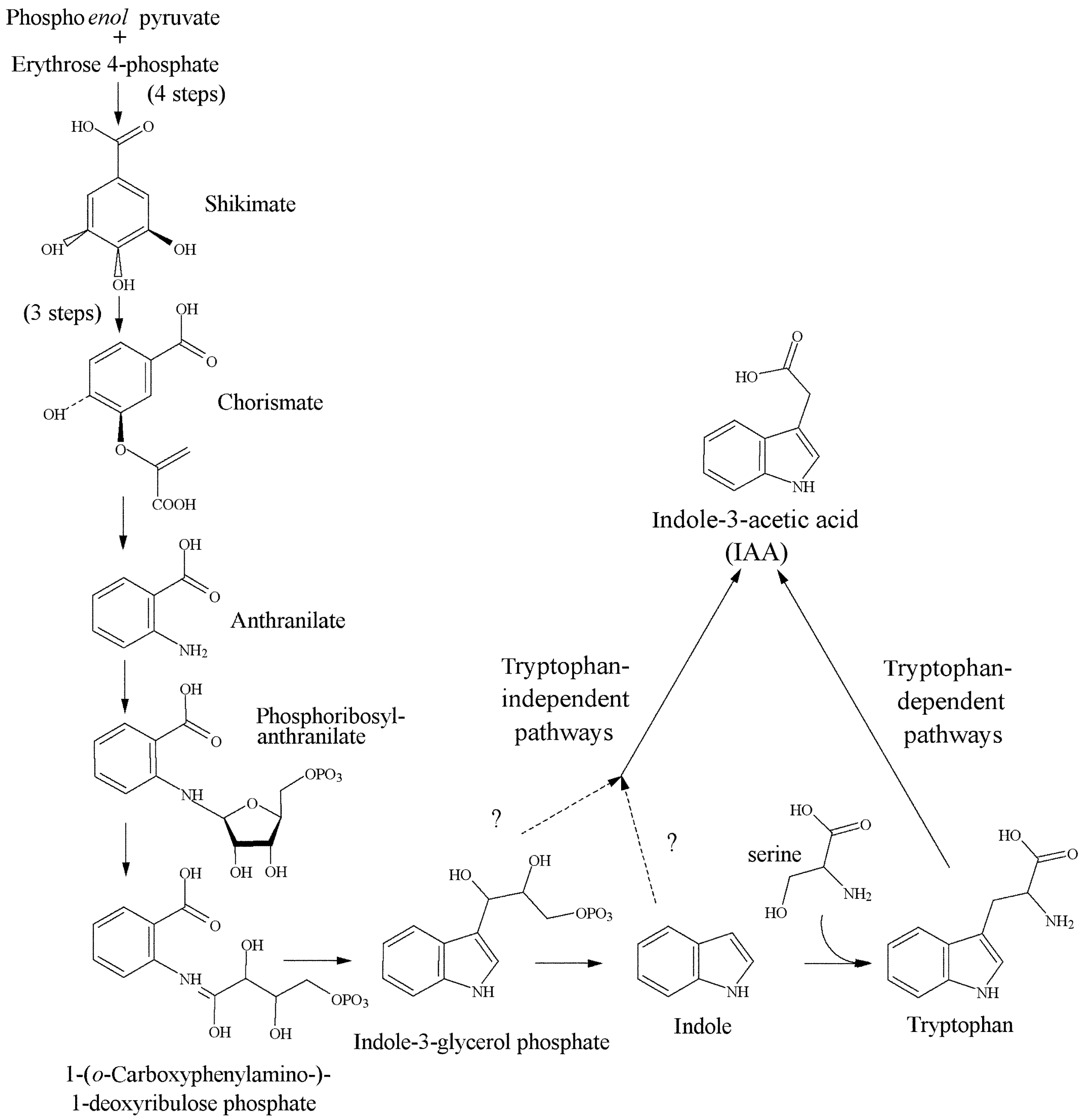

Phosphoenol pyruvate
+
Erythrose 4-phosphate
(4 steps)
Shikimate
(3 steps)
Chorismate
Anthranilate
Phosphoribosyl-anthranilate
1-(o-Carboxyphenylamino-)-1-deoxyribulose phosphate
Indole-3-glycerol phosphate
Indole
serine
Tryptophan
Indole-3-acetic acid (IAA)
Tryptophan-independent pathways
Tryptophan-dependent pathways
?
?

FIGURE 3 Overview of the pathways of IAA biosynthesis in plants. The enzymes catalyzing the reactions leading to tryptophan biosynthesis are found in the chloroplast. From Buchanan, B.B., Gruissem, W., and Jones, R.L. 2000. "Biochemistry and Molecular Biology of Plants." Copyright American Society of Plant Physiologists.

bacterial enzymes. Furthermore, extraction destroys enzyme compartmentation, which may also cause nonphysiological reactions to occur. Because of this, the final several steps leading to IAA biosynthesis are still not known with certainty.

IAA biosynthesis is believed to occur predominantly in the growing shoot tip and in the young leaves. The biosynthesis of IAA appears to proceed through several different routes, broadly divided into tryptophan-dependent and tryptophan-independent

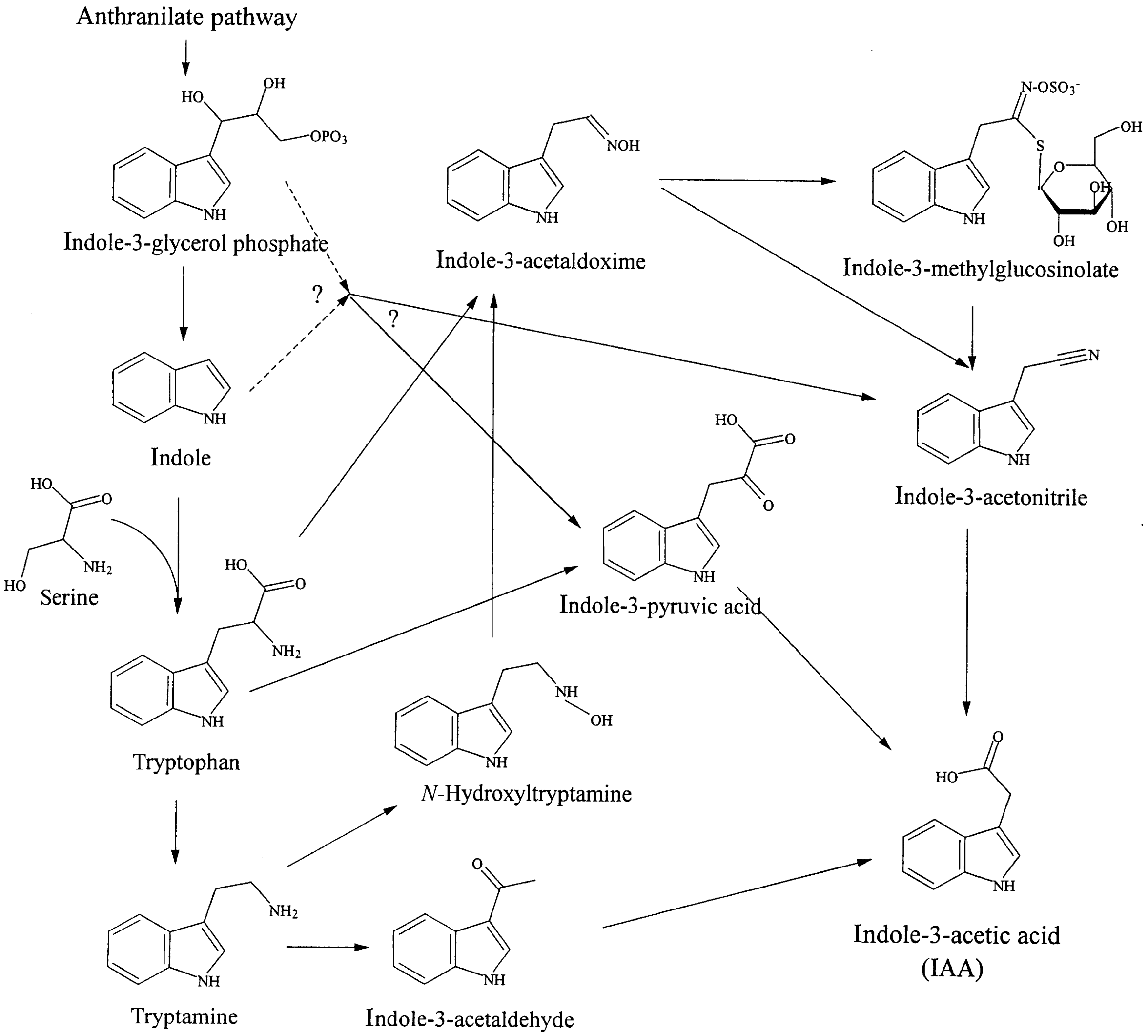

Anthranilate pathway
HO
OH
OPO3
NH
Indole-3-glycerol phosphate
?
?
Indole
HO
O
NH2
HO
Serine
Tryptophan
Tryptamine
NOH
Indole-3-acetaldoxime
N-OSO3-
S
O
OH
Indole-3-methylglucosinolate
N
Indole-3-acetonitrile
Indole-3-pyruvic acid
N-Hydroxyltryptamine
Indole-3-acetaldehyde
Indole-3-acetic acid
(IAA)

**FIGURE 4** Possible pathways of tryptophan-dependent and tryptophan-independent biosynthesis in plants. It is unclear whether indole or indole-3-glycerol phosphate is the IAA precursor in tryptophan-independent biosynthesis. Reprinted with permission from the *Annual Review of Plant Physiology & Plant Molecular Biology*, Volume 48, ©1997 by Annual Reviews, www.AnnualReviews.org.

pathways. The ultimate precursor is chorismate, which is converted into anthranilate and then subsequently into indole, tryptophan, and related molecules (Fig. 3). Tryptophan can be converted to IAA through one of three possible pathways (Fig. 4). One pathway is via indole-3-pyruvic acid and indole-3-acetaldehyde (likely to be important in tomato shoots). In a second pathway, tryptamine is the intermediate between tryptophan and indole-3-acetaldehyde; alternatively, in *Arabidopsis thaliana*, one pathway may proceed from tryptamine through *N*-hydroxyltryptamine and then to indole-3-acetaldoxime and eventually to IAA. A third pathway (important in only a subset of plants, including the mustards, grasses, and bananas) leads from tryptophan to indole-3-acetaldoxime to indole-3-acetonitrile and then to IAA. A branch of this third pathway proceeds from indole-3-acetaldoxime to indole-3-methylglucosinolate and then to indole-3-acetonitrile.

IAA biosynthesis independent of tryptophan has been demonstrated in plants using mutants of corn (*orange pericarp*) and *Arabidopsis* (*trp2* and *trp3*) blocked in tryptophan synthesis. Possible pathways are shown in Fig. 4. Probable precursors are indole-3-glycerol phosphate or indole, which could be converted to either indole-3-pyruvic acid or indole-3-acetonitrile and then to IAA. It is likely that different IAA biosynthetic pathways are important in different species, in different tissues, and at different developmental stages. For example, carrot cultures, maize, and *Arabidopsis* seedlings may use both tryptophan-dependent and -independent pathways. *Phaseolus vulgaris* seedlings appear to use primarily tryptophan-dependent pathways, whereas tryptophan-independent pathways are important in *Lemna gibba* (duckweed), maize endosperm, and oat coleoptiles.

### C. IAA Degradation and Inactivation

IAA is most likely degraded by oxidation, either of free IAA or of its conjugates. In maize, IAA degradation proceeds via conversion to oxindole-3-acetic acid, which is then further oxidized and conjugated to glucose. In contrast, in maize root tips, oxidative decarboxylation (removal of the carboxyl side chain) appears to be an important route of IAA degradation. In tomato and *Vicia faba*, IAA–aspartate is the target of oxidation and is conjugated to sugars as a means of deactivation.

Peroxidases were long thought to be the primary enzymes involved in IAA degradation. Recent evidence suggests that, although peroxidases are powerful IAA-degrading enzymes *in vitro*, they are unlikely to play a role *in vivo*, perhaps because of compartmentation.

### D. IAA Conjugation

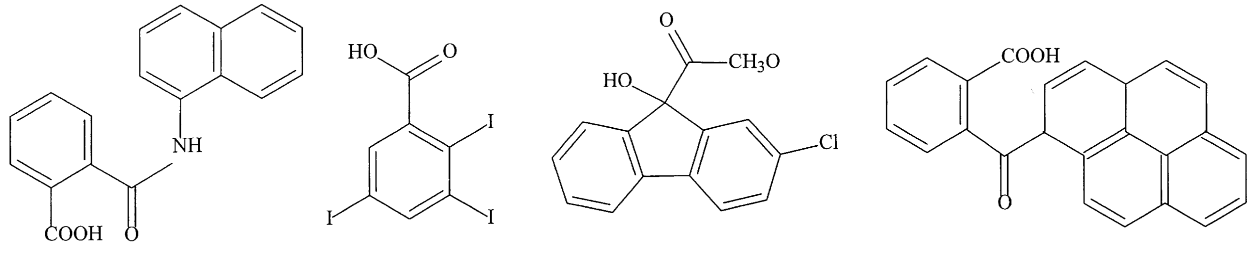
Most of the IAA is conjugated to other molecules, either via ester linkages to *myo*-inositol or sugars (such as glucose) or via amide linkages to amino acids or peptides. For example, maize endosperm, one of the best-studied tissues in this regard, contains IAA–glucose, IAA–*myo*-inositol, IAA–*myo*-inositol-galactose, and IAA–*myo*-inositol-arabinose, among other conjugates. IAA conjugates are believed to possess auxin activity only after hydrolysis, which releases free IAA. IAA conjugates have at least three functions. First, they may serve as a storage form of IAA. In maize seeds, for example, IAA conjugates stored in the endosperm move to the shoot following germination and are deconjugated to release free IAA. Second, conjugates are important in the regulation of IAA levels. For example, many mutant and transgenic plants that overproduce IAA have greatly increased levels of conjugates, thereby moderating the increase in levels of free IAA. Third, production of the conjugate IAA–aspartate is likely to be the first step in the inactivation and degradation of IAA in a variety of plants. Enzymes that carry out the conjugation and deconjugation reactions are beginning to be identified.

## III. POLAR AUXIN TRANSPORT

IAA is transported from its sites of synthesis and storage to other tissues throughout the plant. Auxin transport can occur either via the phloem or by a cell-to-cell pathway called polar auxin transport. Polar auxin transport requires energy and proceeds at a rate of 5 to 20 mm/h. Two routes of polar auxin transport are known. In the first, found in the shoot and central cylinder of the root, auxin travels down the shoot and into the root through parenchyma cells associated with the vascular tissue. In the second, auxin is transported from the root tip back to the root elongation zone in the epidermal and cortical tissues. Both routes are believed to use a similar mechanism and similar protein transporters.

The mechanism of polar auxin transport is explained by the chemiosmotic hypothesis, first proposed in the 1970s. According to this model, polar IAA transport is driven by chemical and electrical gradients across the cell membrane and results primarily from directional IAA efflux out of the basal side of cells. A further feature of the model is that IAA enters cells through two mechanisms: active uptake, probably by co-transport with protons, and diffusion. IAA that enters cells deprotonates in the neutral environment of the cytoplasm and, as a charged molecule, can no longer diffuse across the plasma membrane. $IAA^-$ will therefore tend to accumulate in cells. Efflux carriers localized to the basal side of the cell enable $IAA^-$ to move down the electrochemical gradient and out of the cell. It then enters the next cell below it and similarly effluxes out through the basal side. This polar efflux causes IAA to move unidirectionally down a file of cells.

Genetic approaches using *Arabidopsis* have led to the identification of transmembrane proteins likely to be important for auxin transport; their properties thus far are consistent with the chemiosmotic hypothesis. The AUX1 protein, a putative IAA uptake carrier, acts primarily in the root. The protein sequence shows similarity to amino acid transporters and consists of 485 amino acids, predominantly



1-*N*-Naphthylphthalamic acid (NPA)

2,3,5-Triiodo-benzoic acid (TIBA)

Methyl-2-chloro-9-hydroxy-fluorene-9-carboxylate

2-(1-Pyrenoyl)benzoic acid (PBA)

**FIGURE 5** Compounds that specifically inhibit polar auxin transport.

hydrophobic, with up to 11 transmembrane domains. Genes for three other proteins with significant similarity to AUX1 have been identified in *Arabidopsis*. Proteins of the PIN family (at least 14 family members in *Arabidopsis*) are strong candidates for the efflux carriers. PIN1 is a highly hydrophobic, multiple-membrane-spanning protein. It is localized to the basal side of xylem parenchyma cells, which is consistent with the chemisosmotic hypothesis and with a role in apical-to-basal polar auxin transport. The PIN2/EIR1/AGR1/WAV6 protein is localized on the upper membrane of epidermal and cortical cells in the root elongation zone, where it could function in IAA redistribution during root gravitropic response. The asymmetric cellular distribution of the PIN1 protein requires a functional membrane trafficking system; disruption of vesicle traffic with inhibitory chemicals or mutations in the *GNOM/EMB30* gene, believed to encode a key component of trafficking, destroys the localization pattern of PIN1 protein.

Many compounds that specifically inhibit polar auxin transport have been identified (Fig. 5). Most of these inhibitors of polar auxin transport have diverse effects on plants, including blocking gravitropic responses and inhibiting growth. The best-characterized such inhibitors are 1-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA). These compounds have been shown to block auxin efflux; TIBA may compete directly with IAA at the efflux carrier, whereas NPA and related compounds (called phytotropins) are postulated to bind to a regulatory subunit of the efflux carrier. In either case, inhibition of auxin efflux increases cytoplasmic auxin concentrations. Recently, it has been suggested that phytotropins could act by blocking general vesicle trafficking, thereby disrupting the dynamic basal localization of the efflux carrier. A number of endogenous flavonoids act similarly to NPA, suggesting that plants may use flavonoids to regulate polar auxin transport.

Polar auxin transport has been shown to regulate many developmental processes including inhibition of growth of axillary buds in shoots, promotion of growth of lateral roots, regulation of embryonic polarity, regulation of shoot meristem function and organization, and regulation of vascular tissue development.

## IV. PHYSIOLOGICAL AND DEVELOPMENTAL ROLES

### A. Auxin and Embryogenesis

Auxin plays a key role in several aspects of plant embryogenesis. For example, inhibitors of polar auxin transport prevent normal embryo development in carrot somatic embryos and block separation of the cotyledons in cultured *Brassica juncea* embryos. Treatment of cultured *B. juncea* embryos with exogenous auxin causes various developmental abnormalities, including nonseparated cotyledons and arrested ball-shaped embryos. Expression patterns of the PIN1 putative auxin efflux carrier in *Arabidopsis* embryos suggest that auxin is channeled to the central provascular cells and to the presumptive root pole. Indeed, localized activation of an auxin-responsive promoter has been found in the future root region of early *Arabidopsis* embryos, suggesting that there is in fact a high auxin concentration in these cells. Three *Arabidopsis* mutants affected in auxin response (*monopteros*, *bodenlos*, and *auxin-resistant6*) have defects in early embryogenesis, leading to similar defects affecting development of the hypocotyl, root, and cotyledons. Thus, genetic, physiological, and molecular evidence indicates that local patterns of auxin transport and accumulation are likely to regulate the establishment of bilateral symmetry and development of the root, hypocotyl, and vascular tissue.

### B. Auxin and Vascular Development

Auxin is believed to be a critical determinant of the differentiation and connectivity of vascular tissue. The auxin canalization hypothesis states that auxin flow through provascular tissue promotes its differentiation into vascular tissue, which increases auxin flow through these cells. The positive feedback between auxin flow and vascular development results in channeling of auxin flow to a small set of connected cells, ensuring the continuity necessary for proper function of the vascular system. This hypothesis is supported by the effects of experimental perturbation of auxin flow by wounding or auxin transport inhibitors. Further support comes from defects in vascular tissue formation and alignment seen in various auxin-related mutants of *Arabidopsis*. A direct correlation between vascular development and IAA concentration has been observed in the trunks of Scots pine and hybrid aspen, where IAA concentration is highest in the vascular cambium.

### C. Auxin and Root Development

Auxin produced in the shoot and transported to the root has been suggested to be essential for proper root development. A high concentration of auxin has been localized to the cells around the root tip in maize and *Arabidopsis* seedlings. Blockage of auxin accumulation by either genetic or chemical means causes changes in the patterning of cells in the root. Auxin may regulate the cell cycle in cells of the root quiescent center through actions on ascorbic acid and ascorbic acid oxidase.

### D. Auxin and Lateral Roots

Lateral roots are produced when cells in the pericycle, the layer of cells surrounding the central vascular cylinder, begin to divide, form additional cell layers that push through the outer cell layers of the primary root, and ultimately organize a second root meristem. Many lines of evidence indicate that lateral root development is promoted by transported auxin. Increasing the auxin concentration in roots causes increased lateral root formation. Approaches that have been used to increase auxin levels include mutations, transgene expression, and exogenous application of auxin to the entire root or to the stump of a severed root. Conversely, reducing auxin levels or response in the primary root leads to a decreased number of lateral roots. This is accomplished by inhibition of polar auxin transport at the root–shoot junction or by mutation-induced reductions in auxin response. Auxin appears to stimulate the division of the pericycle cells that initiate lateral root development and may also be required at the later stage of outgrowth.

### E. Auxin and Apical Meristem Function

Auxin is produced in the apical region of the growing shoot and seems likely to play an important role in the proper functioning of this organ. Reduced auxin transport out of the *Arabidopsis* shoot apex due to treatment with auxin transport inhibitors or genetic defects that reduce auxin transport or response (*pin1*, *pid*, and *mp* mutants) all result in pin-shaped inflorescence meristems lacking any floral organs. Mutations in the *ETTIN* gene, an auxin-response factor (see below), affect floral patterning. These observations indicate that normal auxin transport and response in the apical meristem area are essential for proper floral development.

Auxin has also been found to affect the proper initiation and arrangement of leaves on a shoot, called phyllotaxy. Auxin may play a role in determining the species-specific phyllotactic patterns, perhaps via auxin transport patterns. Characteristic pathways of auxin transport in the meristem could lead to local auxin maxima in specific positions relative to the meristem and existing organs. Evidence in support of this model includes the observation that application of IAA to tomato and *Arabidopsis* apices can induce initiation of lateral organs and that defects in phyllotaxy can result from disruptions in auxin transport.

### F. Auxin and Control of Branching

An axillary bud, the precursor of a branch or lateral shoot, is formed at the junction between a leaf and the stem. The shoot apex tends to inhibit outgrowth of axillary buds, a phenomenon called apical dominance. Removal of the shoot tip leads to growth of these buds to produce lateral shoots. Application of auxin to a decapitated shoot inhibits bud outgrowth, but this auxin effect can be blocked by inhibitors of polar auxin transport. These observations led to the model that auxin produced by the shoot apex and transported in a polar manner down the shoot inhibits the development and outgrowth of lateral shoots. However, this theory remains controversial. Decreased auxin levels in axillary buds would have been expected after decapitation, but were not in fact observed in several species. In contrast, many auxin-related mutants display changes in the degree of shoot branching. Mutants with reduced auxin response frequently have increased numbers of secondary shoots or of lateral branches, consistent with the involvement of auxin in apical dominance. It is likely that auxin regulates outgrowth of axillary buds indirectly rather than directly, perhaps by affecting levels of another growth regulator.

## G. Auxin and Fruit Development

Fertilization and fruit development seem to involve auxin at many steps. For example, pollen, seed endosperm, and embryos all produce auxin. In many species, including tomato and pineapple, auxin can induce fruit development in the absence of fertilization (parthenocarpy). Expression of bacterial auxin biosynthetic genes induces parthenocarpy in transgenic eggplant, tomato, and tobacco.

## H. Auxin and Elongation Growth

Application of exogenous auxin has long been known to induce elongation of excised stem or coleoptile sections. The initial rapid phase of this elongation is thought to be due to acid-induced growth (see Section V.D). A later phase of slower elongation probably proceeds by a different mechanism involving new protein synthesis. The importance of auxin in *in vivo* elongation growth has been supported by the dwarf phenotypes of many mutants with reduced auxin responses and transgenic plants with reduced auxin levels. In addition, application of auxin to intact pea plants induces stem elongation *in vivo*.

## I. Auxin and Tropisms

IAA was originally discovered using an assay based on plant responses to light (phototropism). Auxin has since been implicated in response to gravity (gravitropism) as well. The general mechanism proposed for auxin's involvement in tropistic responses is called the Cholodny–Went model. Under uniform illumination, auxin is distributed evenly across the growing region of the shoot. When a shoot is exposed to light coming from only one side, auxin becomes asymmetrically distributed across the growing region of the shoot and accumulates to higher concentrations on the side away from the light, causing the cells on the dark side to elongate more than those on the light side. This results in the shoot bending toward the light, thus producing the phototropic response.

The mechanism for shoot gravitropic responses is postulated to be similar to that described for phototropism. In roots, where auxin tends to inhibit cell elongation, auxin accumulates on the lower side in roots oriented perpendicularly to gravity, causing these cells to elongate less and the root to bend toward gravity.

Studies of cell growth, auxin distribution, and auxin transport in shoots and roots generally support the Cholodny–Went model, although some aspects remain controversial or poorly understood. Mutations affecting auxin response or auxin transport pathways frequently cause defects in responses to light and gravity, as does treatment with auxin transport inhibitors, confirming auxin's role in these tropisms.

The mechanisms by which tropic stimuli are transduced into asymmetric auxin distributions are not yet understood. Perception of the initial stimulus is presumed to result in lateral auxin transport and/or localized destruction, synthesis, or breakdown of IAA conjugates. In the root, both lateral auxin transport and the root tip-to-elongation zone transport pathway through the epidermal cells are likely to be important, because auxin transport inhibitors block normal root gravitropism. The PIN2 putative efflux carrier and the AUX1 putative influx transporter are likely participants in gravistimulus-induced lateral redistribution of auxin in roots. Both proteins localize to the epidermal and cortical cells in the root elongation zone, to which transported auxin from the root apex would be directed, and defects in the respective genes encoding either protein cause agravitropic roots in *Arabidopsis*.

### J. IAA Interactions with Other Hormones

Auxin interacts with other known plant hormones in often complex ways to regulate many physiological and developmental processes. One of the best understood interactions is the induction of ethylene biosynthesis by auxin, which occurs because auxin induces expression of the enzyme involved in the rate-limiting step in ethylene biosynthesis, ACC synthase. Auxin-induced ethylene triggers a wide variety of effects, including promoting abscission (the shedding of leaves, flowers, and fruit), inhibition of bud growth, and inhibition or promotion of flowering (depending on the species). Ethylene in turn has been observed to inhibit auxin transport.

A second class of hormones with which auxin interacts is the cytokinins. In some processes, the interactions are antagonistic. For example, the ratio of cytokinins to auxins in plant tissue culture media controls whether roots or shoots are produced by callus tissue (a high auxin ratio leads to root growth, and a high cytokinin ratio leads to shoot growth). An antagonism between auxin and cytokinin also seems to control growth of axillary buds (lateral shoots): Auxin inhibits and cytokinin promotes this outgrowth. In other processes, the interactions are synergistic. For example, both hormones seem to be required to produce cell division in tissue cultures of many plant species.

### K. Involvement of IAA in Microorganisms' Association with Plants

Many microorganisms whose life cycles include growth on plants have evolved to synthesize IAA as a way of manipulating their hosts. The best characterized are a number of bacteria that infect plants, including *Pseudomonas syringae* pv *savastonoi*, *Agrobacterium tumefaciens* (the cause of crown gall), *Agrobacterium rhizogenes* (the cause of hairy root disease), and *Erwinia herbicola* pv *gypsophila*. Bacteria most commonly synthesize IAA via the intermediates indole-3-acetamide and indole-3-pyruvate. The *iaaM* gene (from *Ag. tumefaciens*) encodes tryptophan monooxygenase, which converts tryptophan to indole-3-acetamide (IAM). IAM can then be converted, by the product of the *Ag. tumefaciens iaaH* gene or by endogenous plant hydrolases, into IAA. Various genes transferred from *Ag. rhizogenes* to the host plants also alter auxin metabolism and response.

*Rhizobium* and related species form nitrogen-fixing nodules on the roots of plants of the Leguminosae. Induction of the root cortical cell divisions that lead to nodule formation may involve IAA. Treatment of host plant roots with inhibitors of polar auxin transport, in the absence of bacteria, induces formation of empty nodules. Signaling molecules produced by the bacteria, called lipo-chitin oligosaccharides, have been found to produce local accumulation of auxin in roots of clover, probably by local inhibition of auxin transport.

## V. AUXIN SIGNAL TRANSDUCTION

Cellular responses to auxin include cell division (e.g., in the root pericycle when lateral roots are initiated), cell differentiation (e.g., in provascular cells that become vascular tissue), and cell elongation (e.g., in tropic responses). The molecular mechanisms of cellular auxin response are still being elucidated, but several key themes have emerged. The importance of transcriptional control proteins and the involvement of ubiquitin-mediated protein degradation in auxin response now seem clear. Evidence for the involvement of phosphorylation events and membrane signaling in auxin response is also growing.

### A. Auxin-Binding Proteins

Auxin signaling is presumed to begin with auxin binding to a receptor. Although a number of auxin-binding proteins have been identified, there is still no undisputed auxin receptor with a well-understood mechanism of action. The best-characterized auxin-binding protein is called auxin-binding protein 1 (ABP1), which has been identified and cloned in maize, *Arabidopsis,* and many other species. ABP1 is a protein of approximately 22 kDa that is initially synthesized with a signal sequence, indicating that it is co-translationally inserted into the endoplasmic reticulum. Following cleavage of the signal sequence, most of the protein is retained in the lumen of the endoplasmic reticulum via its C-terminal Lys-Asp-Glu-Leu (KDEL) sequence. A small amount of the protein may reach the plasma membrane and cell wall. ABP1 specifically binds IAA and active auxin analogues with relatively high affinity ($K_d$ for IAA $= 3.2\ \mu M$). Many lines of evidence support ABP1's role as an auxin receptor. For example, anti-ABP1 antibodies inhibit or mimic auxin-induced electrophysiological changes in protoplasts.

ABP1-derived peptides mimic auxin-induced electrophysiological changes. Overexpression of ABP1 induces auxin-dependent cell expansion in transgenic tobacco. ABP1 knockout mutants in *Arabidopsis* are lethal, with the homozygotes arresting at the early embryo stage, lacking the normal patterns of cell elongation. However, skepticism persists about ABP1's relevance as an auxin receptor because the protein lacks any obvious functional domains by which it might transmit a signal. Although it seems clear that ABP1 is important for auxin-regulated cell elongation, there may well be other auxin receptors involved in mediating other auxin responses.

## B. Auxin-Induced Genes: The Aux/IAA Genes

Auxin application to plants alters the expression of many genes. At least four families of genes whose expression is rapidly induced by auxin have been identified in plants: the Small Auxin Up RNAs (SAURs), the GH3 family, the ACC synthase family, and the Aux/IAA family. Of these, the Aux/IAA genes have the best-characterized role in auxin response. The Aux/IAA proteins are encoded by large multigene families in both monocots and dicots. Transcription of most of the Aux/IAA genes is specifically induced in response to auxin and not to other hormones. In most cases, induction is detectable within 4 to 30 min after auxin treatment, although some of the genes are induced more slowly. The proteins encoded by these genes share four small domains of homology separated by variable regions that differ greatly in size, accounting for the 19 to 39 kDa range of predicted protein sizes. Aux/IAA proteins share two key characteristics of many regulatory proteins: They are localized to the nucleus and are short-lived. Direct tests show that some Aux/IAA proteins act as repressors of gene expression from auxin-inducible promoters. However, the Aux/IAA proteins most likely act not by binding DNA directly, but by interacting with other proteins (primarily auxin regulatory factors, or ARFs; see below) that bind DNA. This interaction occurs through domains 3 and 4 of the proteins, which are found in both the Aux/IAA proteins and the ARFs. Many auxin-response mutants in *Arabidopsis* have been found to have mutations in the Aux/IAA genes, confirming the significance of the Aux/IAA genes in auxin response. The Aux/IAA proteins may also play an important role in plants' responses to light.

## C. Auxin-Response Factors

Analysis of the promoters of auxin-induced genes led to identification of conserved DNA sequence motifs that mediate auxin induction, called auxin-responsive elements or AuxREs. The minimal AuxRE identified in the promoter of the GH3 gene is 11 bp in length and includes the sequence TGTCTC. In the promoters of Aux/IAA genes, two conserved elements were identified, one of which contains the essential sequence TGTCCCAT.

The protein auxin-response factor 1 (ARF1) was first isolated by its ability to bind to and activate expression at a highly auxin-responsive synthetic promoter. ARF1 contains an N-terminal DNA-binding domain and two short C-terminal domains that are homologous to domains 3 and 4 of the Aux/IAA proteins. Subsequently, other ARFs were identified, and it is now known that the ARFs comprise a multigene family. The ARF proteins can dimerize with themselves, with other ARFs, or with Aux/IAA proteins, through binding of domains 3 and 4. *In vivo* evidence for the significance of the ARFs in auxin response has come from the phenotypes of mutants in three of the ARF genes. Mutants in the *MONOPTEROS/ARF5* gene are affected in auxin-related processes: Strong *mp* alleles are homozygous lethal, lacking a root and hypocotyl after germination and having severe defects in vascular development. Weak *mp* alleles also show defective vasculature combined with reduced levels of auxin transport in inflorescence stems. *ARF3* has been identified as *ETTIN*, mutations in which lead to defects in floral development. Mutations in *ARF7/NPH4/MSG1* alter tropic responses in the hypocotyl and reduce auxin sensitivity in the leaf and hypocotyl. It has been proposed that ARFs regulate a variety of downstream genes that are important in auxin-regulated processes such as vascular differentiation. DNA binding by ARFs can either activate or repress transcription from auxin-regulated promoters, depending on the ARF. ARF activity could be controlled by dimerization with other ARFs or with Aux/IAA proteins, which might act to prevent ARFs from binding to promoters.

## D. Auxin and Membranes

Auxin has dramatic effects on the electrophysiology of plant cells. Both intact cells and protoplasts (plant cells with the cell walls removed) hyperpolarize (develop a more negative membrane potential) within 10–30 min after treatment with active auxins. This response is primarily due to rapid activation of the plasma membrane $H^+$-ATPase. This protein pumps protons out of the cytoplasm and into the cell wall and extracellular space, resulting in acidification of the cell wall and alkalinization of the cytoplasm. Auxin-induced increases in $H^+$-ATPase activity appear to involve both activation of existing transporters and induction of increased expression of the $H^+$-ATPase gene. According to the acid growth hypothesis, this auxin-induced cell wall acidification is the primary mechanism by which auxin induces cell elongation. Cell wall acidification leads to cell wall "loosening," perhaps through activation of cell wall-modifying enzymes. Turgor pressure then stretches the cell wall and the cell expands, producing the rapid elongation as seen, for example, in stem or coleoptile sections immersed in auxin solutions.

Auxin affects membrane physiology in other ways as well. The effects on potassium channels seem to be especially important. In *V. faba* guard cells, which respond to auxin by an increase in turgor and subsequent stomatal opening, active auxins at concentrations up to 10 μM rapidly (within less than a second) produce an increased inward flow of potassium through potassium channels. Outward-directed potassium channels are also activated by auxins. Effects on ion channels have been demonstrated at the level of gene expression also. In maize coleoptiles, for example, auxin increases expression of an inward-directed potassium channel that appears to be necessary for continued elongation growth.

Ions and lipids known to be involved in other signal transduction pathways have also been implicated in auxin action, including calcium, phosphatidylinositols, and the products of phospholipase $A_2$. Definitive evaluation of the importance of these for auxin signal transduction awaits further studies.

## E. Ubiquitin Pathway of Protein Degradation

Regulated protein degradation is important in many aspects of cell physiology, including progression through the cell cycle. Studies of *Arabidopsis* mutants with altered auxin responses have implicated the ubiquitin pathway in auxin response.

Ubiquitin is a highly conserved, 76-amino-acid protein that is found in all eukaryotes. It is covalently attached to target proteins to mark these proteins for degradation. Ubiquitin-activating enzyme (E1) activates ubiquitin and then transfers it to ubiquitin-conjugating enzyme (E2), which together with ubiquitin ligase (E3) attaches ubiquitin to lysines on specific target proteins. Multiubiquitin chains are formed on target proteins through continued action of the pathway. Ubiquitinated proteins are then, most commonly, targeted to the 26S proteasome, a large multisubunit cytoplasmic protease that degrades the target proteins and recycles the ubiquitins. Of the several known types of E3 ubiquitin ligases, one in particular has been implicated in auxin action. This is the multisubunit $SCF^{TIR1}$ complex, composed of the proteins SKP1 (ASK1 in *Arabidopsis*), cullin/Cdc53, RBX1, and the F-box protein TIR1. The protein cullin is itself modified by attachment of a ubiquitin-like protein called RUB1; this modification is necessary for cullin activity. RUB1 is activated and then attached by the action of at least three enzymes: A dimer called AXR1/ ECR1 activates RUB1, and then the RUB-Conjugating Enzyme RCE1 attaches RUB1 to cullin. *Arabidopsis* plants with mutations in components of $SCF^{TIR1}$ and in the RUB1 activation and conjugation pathway have been identified, and all of them confer altered auxin responses on the plants. Likely targets for auxin-regulated ubiquitin-mediated degradation are the rapidly turned-over proteins of the AUX/IAA family. Altered stability of these proteins could lead to changes in activity of the ARF transcription factors and thereby to changes in gene expression. Although it is unlikely that only auxin signal transduction requires this pathway, it does seem to play a critical role in auxin response.

## F. Protein Kinases and Auxin Signal Transduction

Several lines of evidence suggest that protein kinases may be involved in auxin signaling, but as yet the pathway is unclear. In experiments using transiently transfected maize protoplasts, expression of a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK) called NPK1 blocked auxin induction of an auxin-regulated promoter. Further experiments in *Arabidopsis* suggested the MAPKKK may normally function in stress response. Thus, the effects of auxin signal transduction may be opposed by stress-induced pathways. A MAP kinase activity that is very rapidly induced by auxin treatment has been identified in *Arabidopsis* root culture. The *RCN1* gene, which regulates auxin transport, encodes a protein phosphatase subunit, and the *PINOID* gene, which when mutated causes defects in auxin transport, is a serine/threonine kinase. One model suggests that phosphorylation and dephosphorylation activities controlled by auxin may regulate degradation of the AUX/IAA proteins by the ubiquitin pathway.

## VI. SUMMARY

Decades of research have shown that auxin is important in almost every aspect of plant development and physiology. Levels of free auxin are regulated by the interplay of pathways of biosynthesis, degradation, and conjugation. Distribution of auxin throughout the plant also requires polar auxin transport, whose molecular basis is at last becoming clear. Cellular auxin responses depend at least in part on transcriptional regulatory proteins that are encoded by multigene families and that show significant posttranslational regulation of activity via degradation and dimerization. Modern approaches combining many techniques are finally yielding insights into the complexity of auxin biochemistry, physiology, and cell biology.

## Glossary

***Arabidopsis thaliana*** A small member of the mustard family (Brassicaceae), commonly used as a model system for plant genetics, physiology, and molecular biology.

**coleoptile** The outermost sheathing leaf of a grass seedling, which serves as a protective sheath for the leaf plumule and shoot apical meristem.

**hypocotyl** The stem like axis between the seed leaf or leaves and the root in an embryo or seedling.

**protein kinase** An enzyme that adds a phosphate group to a target, usually as a means to control the activity of the target.

**signal transduction** The series of biochemical reactions that transform a signal (such as hormone binding to a receptor) into a cellular response (such as cell elongation or cell division).

**tropism** A growth response that is directionally oriented with respect to the stimulus.

## See Also the Following Articles

**Abscisic Acid • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Jasmonates • Salicylic Acid**

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# AVP

**See *Vasopressin***

# Bombesin-like Peptides

Eliot R. Spindel
*Oregon National Primate Research Center*

I. INTRODUCTION
II. AMPHIBIAN BOMBESIN-LIKE PEPTIDES
III. RECEPTORS FOR BOMBESIN-LIKE PEPTIDES
IV. GASTRIN-RELEASING PEPTIDE
V. NEUROMEDIN B
VI. BRS-3
VII. SUMMARY

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**The entire bombesin-like peptide family includes the gastrin-releasing peptide subfamily, the neuromedin B subfamily, and the bombesin-related peptide family. Three bombesin-like peptide receptors are known to function in mammals. In conjunction with their peptide ligands, these receptors play roles in a wide array of activities in the brain, lungs, esophagus, and intestinal tract.**

## I. INTRODUCTION

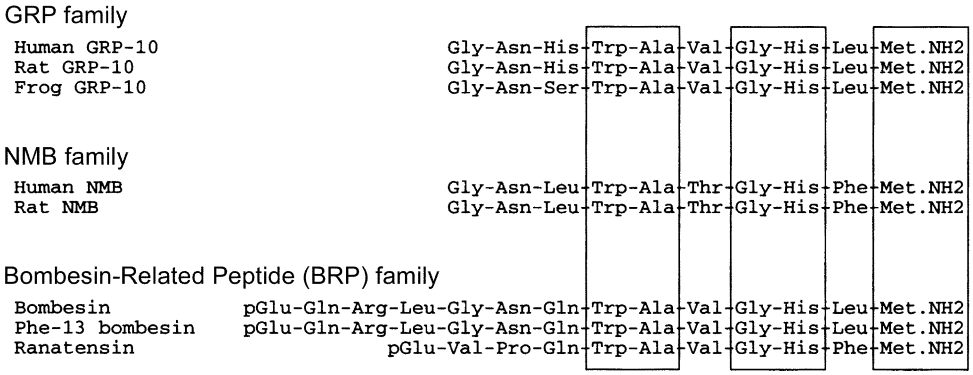
The bombesin-like peptides were initially characterized in frog skin; then subsequently found to be widely distributed in mammals. Bombesin occurs in frogs of the *Bombina* species, and related peptides such as ranatensin occur in other amphibian species. Soon after its isolation, bombesin was found to have many potent central nervous system (CNS) effects in mammals, including effects on temperature regulation, grooming and scratching behaviors, appetite, heart rate, and gastric acidity regulation. Outside of the CNS, bombesin is a potent secretagogue for gastrin and other gastrointestinal hormones, a secretagogue for pancreatic exocrine secretion, and a stimulus for gastrointestinal (GI) growth. Early studies of bombesin used radioimmunoassays to establish that bombesin-like immunoreactivity (BLI) is widespread in brain, lung, and GI tract tissues. This prompted a search for mammalian homologues of bombesin; in 1979, McDonald *et al.*, using gastrin release as a bioassay, isolated a 27-amino-acid peptide from porcine gut. This peptide and bombesin had a similar C-terminus, thus the peptide was named gastrin-releasing peptide (GRP). In 1983, a second mammalian bombesin-like peptide, neuromedin B (NMB), was isolated from porcine spinal cord by Minamino *et al.* (Fig. 1). GRP was initially considered the mammalian homologue of bombesin, but when it was discovered that frogs have both GRP and bombesin it became clear that GRP and bombesin are in different subfamilies of the bombesin-like peptides. The bombesin-like peptides can be divided into three subfamilies—the GRP family, the NMB family, and the bombesin-related peptide (BRP) family (Fig. 1). At present, the BRP subfamily has been characterized only in amphibians but also likely occurs in mammals.

The two mammalian bombesin-like peptides, GRP and NMB, each have their own cognate receptors. The GRP and NMB receptors belong to the same seven-membrane-spanning domain superfamily and are highly homologous. BB4, a receptor for one form of frog bombesin (Phe-13 bombesin), has been cloned and is phylogenetically distinct from the GRP and NMB receptors. BRS-3, a third receptor for bombesin-like peptides, was cloned by Battey and co-workers from rat testes and by Gorbulev *et al.*, from pregnant guinea pig uterus. The affinity of this receptor for the known bombesin-like peptides is very weak, indicating that its endogenous ligand remains to be characterized. This receptor is most homologous to the frog BB4 receptor, suggesting that the ligand for the BRS-3 receptor will belong to the BRP subfamily.

Physiologically, the key functions of the bombesin-like peptides involve autonomic regulation, GI function, appetite control, and growth regulation. Bombesin-like peptides are also expressed in a variety of cancers and may be autocrine growth factors for cancers.

## II. AMPHIBIAN BOMBESIN-LIKE PEPTIDES

The initial characterization of the bombesin-like peptides in frog skin and later detection in mammals are similar to the discovery of sauvagine, a corticotropin-releasing hormone (CRH)-like compound found in frog skin; the characterization of sauvagine led to the characterization of urocortin. Peptides in frog skin are located in cutaneous granular glands (Fig. 2) that are surrounded by smooth muscle. α-Noradrenergic stimulation causes contraction of the smooth muscle, resulting in secretion of the contents within the glands. These glands also contain multiple other peptides and nonpeptide compounds



| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **GRP family** | | | | | | | |
| Human GRP-10 | Gly-Asn-His | Trp-Ala | Val | Gly-His | Leu | Met.NH2 |
| Rat GRP-10 | Gly-Asn-His | Trp-Ala | Val | Gly-His | Leu | Met.NH2 |
| Frog GRP-10 | Gly-Asn-Ser | Trp-Ala | Val | Gly-His | Leu | Met.NH2 |
| **NMB family** | | | | | | |
| Human NMB | Gly-Asn-Leu | Trp-Ala | Thr | Gly-His | Phe | Met.NH2 |
| Rat NMB | Gly-Asn-Leu | Trp-Ala | Thr | Gly-His | Phe | Met.NH2 |
| **Bombesin-Related Peptide (BRP) family** | | | | | | |
| Bombesin | pGlu-Gln-Arg-Leu-Gly-Asn-Gln | Trp-Ala | Val | Gly-His | Leu | Met.NH2 |
| Phe-13 bombesin | pGlu-Gln-Arg-Leu-Gly-Asn-Gln | Trp-Ala | Val | Gly-His | Leu | Met.NH2 |
| Ranatensin | pGlu-Val-Pro-Gln | Trp-Ala | Val | Gly-His | Phe | Met.NH2 |

**FIGURE 1** Representative members of each of the three subfamilies of bombesin-like peptides. Bombesin and ranatensin are amphibian; gastrin-releasing peptide (GRP) and neuromedin B (NMB) are mammalian. GRP-10 is the C-terminal decapeptide of GRP and contains the full biological activity of GRP. The grouping into families is based on phylogenetic analyses of prohormones.

that have been the sources of many pharmaceuticals. For example, the compound epibatidine, a highly potent nicotinic agonist, is found in these glands.

Different species of frogs have slightly different variants of bombesin-like peptides. For example, the peptide ranatensin (Fig. 1) comes from the frog *Rana pipiens*, whereas bombesin comes from the frog *Bombina orientalis*. Phylogenetic analysis of the mRNAs and prohormones that encode the peptides, however, shows that all the frog skin peptides are related and can be grouped in the same subfamily. Frogs also contain in their brain and gut GRP-like and NMB-like peptides that are distinct from the bombesin-related peptides in their skin. Within a given frog species, multiple forms of bombesin-related peptides occur in skin. For example, in *B. orientalis*, there are three forms of bombesin in skin, differing only by one or two amino acids but

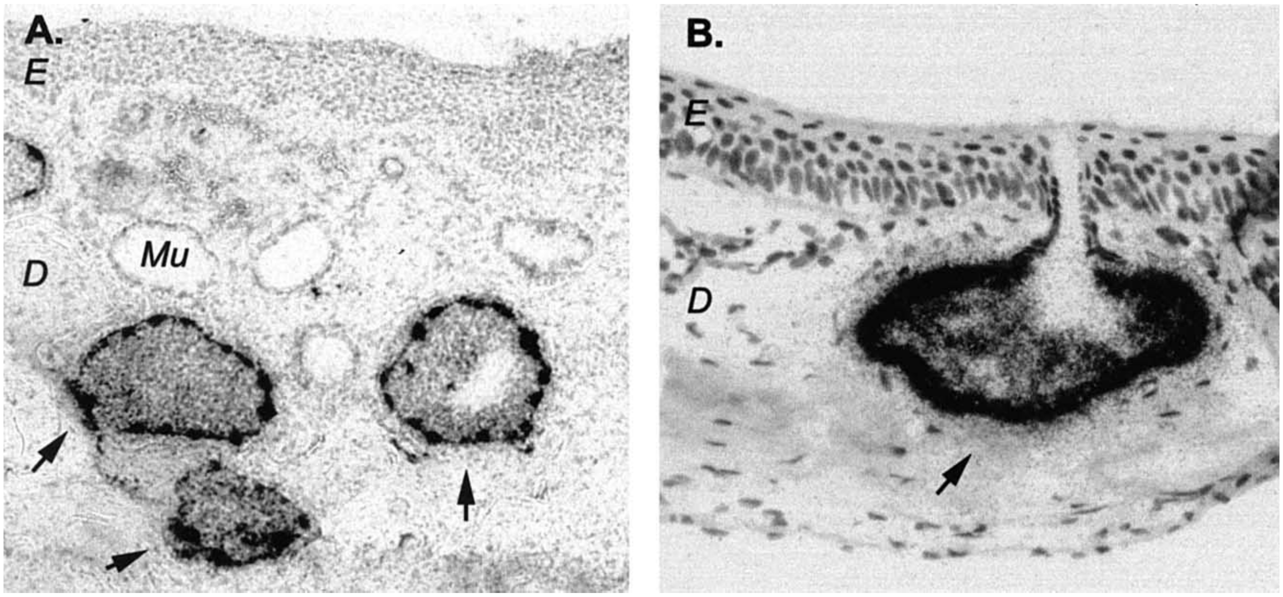


**FIGURE 2** Bombesin-like peptide mRNA expression in frog skin. (A) Low-power view showing ranatensin mRNA expression in cutaneous granular glands in the skin of *Rana pipiens* (100 × ). (B) Higher power view showing bombesin expression in the skin of *Bombina orientalis* (200 × ). E, Epidermis; D, dermis; Mu, mucus gland; arrows point to peptide-containing glands.

arising from distinct genes. The frog *Phyllomedusa sauvagei* has two bombesin-related skin peptides, Phe-8 phyllolitorin and Leu-8 phyllolitorin, which differ by only a single amino acid. Interestingly, these two different peptides arise by RNA editing of a single gene transcript. The presence of multiple forms of bombesin-related peptides in frog skin is significant because it suggests that if these peptides occur in humans, they may similarly occur in multiple forms.

The three families of bombesin-like peptides in frogs all have distinct receptors. The frog GRP receptor is related to the mammalian GRP receptor and the frog NMB receptor is related to the mammalian NMB receptor; the BB4 class of receptors for the frog skin bombesin-related peptides has been cloned. It appears that there are multiple subtypes of BB4 receptors in frogs corresponding to the multiple subtypes of bombesin-related peptides in frogs. Phylogenetic analysis shows that the amphibian BB4 receptor is closest to the mammalian BRS-3 receptor. This raises the possibility that the BRS-3 receptor is the mammalian homologue of the BB4 receptor; if so, when the ligand for the BRS-3 receptor is discovered, it will be the mammalian counterpart of the frog skin bombesin-related peptides.

## III. RECEPTORS FOR BOMBESIN-LIKE PEPTIDES

The receptors for the bombesin-like peptides are highly homologous seven-transmembrane, G-protein-linked receptors sharing an overall homology of 50–60% at the amino acid level. Each bombesin-like peptide has its own cognate receptor, but to date multiple subtypes for each receptor have not been identified. The most commonly used nomenclature for the bombesin-like peptide receptors is the NMB receptor, GRP receptor, BRS-3 receptor, and BB4 receptor. These receptors have also been assigned numbers and can also be, respectively, called the BB1, BB2, BB3, and BB4 receptors. The distributions of the specific receptors are related to the functions and distributions of their ligands (see later).

The GRP receptor, cloned in 1989 by Spindel and co-workers and by Battey and co-workers, was the first bombesin-like peptide receptor identified; subsequently, the remaining receptors were cloned, and careful pharmacology by Jensen and co-workers laid out the affinity of the receptors. Each receptor has the highest affinity for its cognate ligand in the range of 1–5 n*M* and has varying degrees of affinity for the other receptors. The BB4 receptor is least selective and the BRS-3 receptor is most selective. The approximate affinities of each receptor for the canonical bombesin-like peptides is shown in Table 1. Mutational analysis has shown that ligand affinity is controlled by a small number of key amino acid residues in each receptor. For example, in the GRP receptor, selectivity for GRP over NMB is controlled by two amino acid residues in the third extracellular domain. Highly specific antagonists such as [D-F$_5$-Phe$^6$,D-Ala$^{11}$]Bn(6–13)O-Me (BIM26226), 3-phenylpropanoyl-His$^{20}$,D-Ala$^{24}$, Pro$^{26}$, Psi$^{26,27}$, Phe$^{27}$ GRP(20–27) (BW2258U89), and [D-Phe$^6$]bombesin(6–14) ethylamide (readily available from commercial sources) have been developed for the GRP receptor, and selective antagonists for the NMB receptor, such as PD168368, are beginning to be developed. No selective antagonists for the BRS-3 and BB4 receptor have yet been described. Agonists are less selective, although design of a selective agonist for the BRS-3 receptor, [D-Tyr$^6$,(*R*)-Apa$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14), has recently been described.

The bombesin receptors are all G-protein linked utilizing $G_{\alpha q}$ and βγ to activate phospholipase C-β to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to for diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP$_3$). DAG, in turn, activates protein kinase C (PKC), and InsP$_3$ leads to increased

**TABLE 1** Affinities of Bombesin-like Peptides for the Four Bombesin Receptor Subtypes[a]

| Receptor | GRP | NMB | Bombesin | Phe-13 Bn | Nle-Bn(6–14) |
|---|---|---|---|---|---|
| GRP | 2.5[b] | 200 | 2.5 | 1 | 1 |
| NMB | 150 | 2[b] | 25 | 1 | 2.5 |
| Human BRS-3 | >10,000 | 5000 | >10,000 | 7000 | 2.5 |
| Frog BB4 | 25 | 25 | 10 | 1[b] | 1 |

[a] Nle-Bn(6–14), [D-Phe$^6$, β-Ala$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14). Phe-13 Bn, Phe-13 bombesin. Affinities averaged from data of Nagalla *et al.* (1995), Mantey *et al.* (1997), and Katsuno *et al.* (1999).

[b] Cognate ligand–receptor interactions.

intracellular calcium. Activation of PKC and the increase in intracellular calcium lead to increases in gene transcription and cell growth. Activation of all of the bombesin-like receptors leads to cell growth and hence to the role of bombesin-like peptides as autocrine growth factors. (The role of bombesin-like peptides in neoplastic growth is discussed further in Section IV). The bombesin receptors also lead to activation of the small GTPases such as Rho and Rac, which provides another pathway for gene activation. The three mammalian bombesin receptors have all been knocked out, which has helped clarify the specific physiologic roles of each peptide.

## IV. GASTRIN-RELEASING PEPTIDE

Gastrin-releasing peptide was the first mammalian bombesin-like peptide discovered. After it was discovered that amphibian bombesin stimulates the release of gastrin in mammals, gastrin release was used as a bioassay and a 27-amino-acid peptide was isolated from porcine stomach by McDonald and co-workers (Fig. 3). Subsequently, GRP has been isolated from multiple species, ranging from sharks, frogs, and mice to humans. The C-terminus of GRP is highly conserved across all species, the amino terminus less so. GRP circulates in two forms, a large form of 27 to 29 amino acids and a truncated 10-amino-acid form (GRP-10; Fig. 3). Both forms have roughly similar affinities for the receptor and the significance of the two forms is not clear, although GRP may be more resistant to proteolytic cleavage.

Like most neuropeptide hormones, GRP is processed from a larger prohormone. The amino acid sequence of GRP immediately follows the signal peptide and following the sequence of GRP is a C-terminal extension peptide of unknown function. GRP is cleaved from the prohormone by prohormone convertases and the C-terminus is amidated by the action of peptidylglycine α-amidating monooxygenase (PAM). Cleavage of GRP to GRP-10 is adjacent to a single arginine residue that is conserved in all known GRPs. Cleavage at the C-terminus of GRP is adjacent to two basic amino acids in all sequenced GRP prohormones.

### A. Distribution

GRP is widely distributed in the brain, lung, and GI tract. In humans, the highest levels of GRP are found in fetal lung and in small-cell lung carcinoma (SCLC). In the developing lung, GRP is found in pulmonary neuroendocrine cells (PNECs) and the highest levels occur from 14 to 30 weeks of gestation. GRP can be detected in PNECs as early as 10 weeks into gestation, and RNA levels in 20-week fetal lungs are more than 200-fold higher than in full-term lungs. In humans, increased levels of GRP are also found in bronchopulmonary dysplasia and chronic obstructive pulmonary disease (COPD). In the gut, GRP is located primarily in intrinsic neurons, where it regulates gastrointestinal hormone release, pancreatic enzyme secretion, and smooth muscle contraction. In the brain, GRP is widely distributed. Highest levels of GRP are found in the hypothalamus (with highest levels in the suprachiasmatic nucleus and medial preoptic nucleus), the brain stem (with highest levels in the nucleus of the lateral solitary tract and the parabrachial nucleus), and the hippocampus, amygdala, and dentate gyrus. The distribution of the GRP receptor follows the terminal fields of GRP-containing neurons in the brain and closely tracks that of GRP.

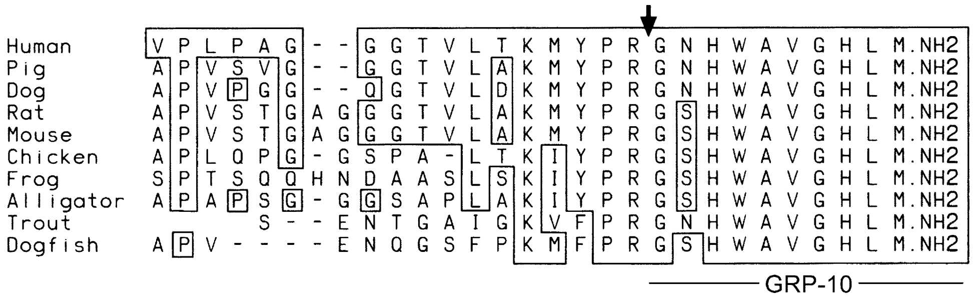

Human V P L P A G - - G G T V L T K M Y P R G N H W A V G H L M.NH2
Pig A P V S V G - - G G T V L A K M Y P R G N H W A V G H L M.NH2
Dog A P V P G G - - Q G T V L D K M Y P R G N H W A V G H L M.NH2
Rat A P V S T G A G G G T V L A K M Y P R G S H W A V G H L M.NH2
Mouse A P V S T G A G G G T V L A K M Y P R G S H W A V G H L M.NH2
Chicken A P L Q P G - G S P A - L T K I Y P R G S H W A V G H L M.NH2
Frog S P T S Q Q H N D A A S L S K I Y P R G S H W A V G H L M.NH2
Alligator A P A P S G - G G S A P L A K I Y P R G S H W A V G H L M.NH2
Trout S - - E N T G A I G K V F P R G N H W A V G H L M.NH2
Dogfish A P V - - - - E N Q G S F P K M F P R G S H W A V G H L M.NH2
GRP-10

**FIGURE 3** Sequences of gastrin-releasing peptides from various species. Amino acid identities with human gastrin-releasing peptide (GRP) are boxed. The GRP-10 sequence is underlined, and the arrow indicates the monobasic cleavage site for generation of GRP-10.

## B. Central and Peripheral Functions of GRP

GRP in the brain plays a key role in autonomic function, having regulatory effects on thermoregulation, heart rate, activity, acid secretion, appetite, and energy balance. GRP stimulates acid secretion through a complex mix of central and peripheral effects, although peripheral mechanisms dominate. Consistent with this, both central and peripheral administration of GRP antagonists will lower acid secretion in both animals and humans. In the GI tract, GRP is released from intrinsic neurons, acting as a paracrine hormone to stimulate the release of many different GI hormones and to control GI motility. For example, GRP released from neurons in the stomach stimulates release of gastrin and somatostatin and GRP released in the pancreas stimulates release of exocrine enzymes. The effect of GRP on gastric smooth muscle contraction is abolished in the GRP knockout, showing this is mediated specifically by the GRP receptor.

In analyzing the central actions of GRP, given the overlapping affinities of ligands for the GRP and NMB receptor, the use of receptor knockout animals has been particularly important. Bombesin-like peptides have profound effects on thermoregulation, leading to decreases in body temperature when animals are injected with GRP or NMB and placed in the cold. Analysis with GRP and NMB knockout animals shows that the response to GRP is abolished in GRP receptor knockout animals and that much of the response to NMB is also blocked in GRP receptor knockout animals. This indicates that most of the effect of bombesin-like peptides on thermoregulation is mediated by the GRP receptor, although a small portion is mediated by the NMB receptor. GRP receptor (GRP-R) knockout mice also show significant changes in behavior, with increased motor activity in the knockouts and increased aggressive social behaviors, suggesting that GRP circuits affect these behaviors.

GRP also appears to play a key role in regulating development of memory for fear. Studies by Shumyatsky and co-workers have shown high levels of GRP in the amygdala and have demonstrated that development of memory to adverse conditioned stimuli is significantly increased in GRP knockout mice. This has significant implications for the possible role of GRP in anxiety and in mental disorders.

## C. GRP and Appetite

Administration of bombesin-like peptides leads to decreased food intake. This is observed after both central and peripheral administration of the peptides, although peptides are more potent when administered into the brain, suggesting a central mechanism. Animals given bombesin cease eating sooner than normal and ingest smaller amounts of offered nutrients, suggesting an effect on satiety. Humans given bombesin similarly experience less hunger and an earlier feeling of fullness. This effect is mediated by the GRP receptor, because the effect of bombesin or GRP on appetite is eliminated in GRP receptor knockout animals and is unchanged in NMB receptor knockout mice. Chronic administration of bombesin to mice does not cause weight loss, perhaps because of development of tolerance. The effect of chronic administration of bombesin or GRP on human weight has not been reported.

## D. GRP in Normal and Neoplastic Lung

GRP is expressed in high levels in developing primate lung tissue. GRP is localized in pulmonary neuroendocrine cells and GRP receptors are expressed in airway epithelial cells. GRP stimulates mitogenesis in airway epithelial cells, thus GRP is a paracrine growth factor for the developing lung. GRP also stimulates surfactant expression in the developing lung, thus it also plays a role in lung maturation. At the same time, excessive GRP secretion may play a role in the pathogenesis of bronchopulmonary dysplasia (BPD), because levels of GRP are increased in lungs from BPD patients and GRP is increased in urine in infants preceding the onset of BPD. Preliminary data from a baboon model of the disease suggest that antibodies against bombesin may prevent development of the most severe symptoms of BPD. The mechanism linking GRP to BPD is unknown, but likely involves stimulation of inappropriate and disordered lung growth.

In 1985, Minna and co-workers made the key observation that many small-cell lung carcinomas express GRP, express GRP receptors, and respond to GRP with growth. Thus, it was proposed that GRP is an autocrine growth factor for small-cell lung carcinoma. Consistent with this hypothesis, it was demonstrated that in a number of models, GRP antagonists or monoclonal antibodies to bombesin could slow tumor growth. Although GRP and GRP receptor expression is most common in SCLC, expression is also seen in non-small-cell lung carcinomas (NSCLCs). Clinical trials with a1nti-GRP antibodies and antagonists have been performed but have only proved helpful to a very limited patient population. Clinical studies with broad-spectrum neuropeptide antagonists that block GRP receptors as well as other neuropeptide receptors are ongoing and may yield new therapeutic approaches. Other cancers, such as pancreatic and colon cancer, express GRP receptors and thus may also be targets for therapies that target the GRP receptor. Because a relatively large percentage of SCLC express GRP, measurement of GRP prohormone in blood has been used as tumor marker for SCLC.

## V. NEUROMEDIN B

Neuromedin B was initially isolated by Minamino and co-workers in 1983 from porcine spinal cord. Neuromedin B circulates in both a large 32-amino-acid form (NMB-32) and a nonapeptide form (NMB; Fig. 1). The relation between NMB-32 and NMB is similar to that of GRP and GRP-10, with the sequence of NMB-32 immediately following the signal peptide in the NMB prohormone and a single monobasic cleavage for the cleavage of NMB from NMB-32. In all species analyzed to date, the sequence of the nonapeptide form of NMB is identical.

The receptor for NMB is highly homologous to the GRP receptor, with only a few amino acids conferring selectivity for NMB over GRP in the receptor. Signal transduction mechanisms for the NMB receptor are the same as for the GRP receptor. The original evidence for a distinct NMB receptor came from studies of ligand binding in the esophagus by Jensen and co-workers, which showed clearly different affinities for GRP and NMB. The esophagus remains one of the few peripheral tissues with a distinct role for NMB. Specific nonpeptide NMB receptor antagonists have been recently developed (PD16838 and PD165929).

### A. Distribution

In the brain, NMB has a distribution distinct from that of GRP. Highest levels are found in the olfactory cortex, substantia nigra, and somatosensory regions, including the principal sensory nucleus of the brain stem, reticular formation, and central raphe. High levels are also found in the trigeminal nucleus and dorsal root ganglion. NMB is also present in the pituitary and is found in nerves innervating the esophagus and intestines. Like GRP, NMB occurs in some small-cell lung carcinomas.

### B. Central and Peripheral Functions of NMB

Analyses of GRP and NMB receptor knockout mice have revealed remarkably few functions for NMB. Most effects of injected NMB are abolished in the GRP receptor knockout mouse, indicating that most effects of NMB are mediated by NMB cross-reacting with the GRP receptor. These studies have demonstrated that NMB appears to have no specific effect on appetite or contraction of the gut and only a small effect on thermoregulation. In fact, the NMB receptor knockout mouse shows remarkably little phenotype, with no changes in activity, appetite, or social behavior, as is observed in the GRP knockout. The NMB knockout does show mild changes in stress responses mediated by serotoninergic neurons, consistent with localization of NMB in dorsal raphe neurons. Characterized distinct functions for NMB to date are primarily localized to the esophagus, where NMB affects contractility and motility, and to sensory functions in the peripheral nervous system. In the pituitary, NMB may modulate thyroid-stimulating hormone (TSH) secretion. In the subset of SCLCs that express NMB, NMB also appears to be an autocrine growth factor for SCLCs, although expression of NMB by SCLCs occurs less frequently compared to GRP expression.

## VI. BRS-3

The BRS-3 receptor remains the intriguing mystery of the bombesin-like peptide family. The BRS-3 receptor was cloned by Battey and co-workers from a testes library and by Gorbulev and co-workers from a uterus library prepared from pregnant guinea pigs. The endogenous ligand for the BRS-3 receptor is unknown. Although none of the known natural bombesin-like peptides have much affinity for the BRS-3 receptor, the BRS-3 receptor is clearly a receptor for bombesin-like peptides based on its high homology to the other bombesin-like peptide receptors. This relationship is further confirmed by the fact that mutation of four amino acids in the BRS-3 receptor increases affinity for GRP 100-fold. Signal transduction mechanisms for the BRS-3 receptor are similar to those for the other bombesin-like peptide receptors.

Although the endogenous ligand for the BRS-3 receptor is unknown, the synthetic bombesin-like peptide [D-Tyr$^6$, Ala$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14) has nanomolar affinity for the BRS-3 receptor (Table I). [D-Tyr$^6$, Ala$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14) also has nanomolar affinities for all of the known

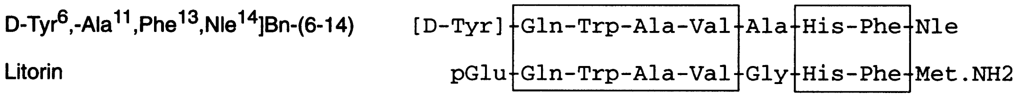

D-Tyr6,-Ala11,Phe13,Nle14]Bn-(6-14)
[D-Tyr]-Gln-Trp-Ala-Val-Ala-His-Phe-Nle
Litorin
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met.NH2

**FIGURE 4** Relationship between a synthetic BRS-3 agonist and the frog bombesin-like peptide litorin. Boxed residues show conservation between the two peptides. Nle, Norleucine.

bombesin-like peptides. [D-Tyr$^6$, -Ala$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14) shows significant homology to the frog skin peptide litorin, which suggests that the BRS-3 ligand resembles one of the known frog skin bombesin-like peptides (Fig. 4). Derivatives of [D-Tyr$^6$, Ala$^{11}$, Phe$^{13}$, Nle$^{14}$]Bn(6–14) that are more selective for BRS-3 have been developed and will be valuable tools in defining the function of the BRS-3 system.

The BRS-3 receptor has a highly limited distribution; it is expressed in the uterus, in the testes, and in the hypothalamus. Levels of expression in the uterus increase during pregnancy. In the hypothalamus, BRS-3 expression is found in the paraventricular, arcuate, striohypothalamic, and dorsal hypothalamic nuclei and in the lateral preoptic areas and the lateral and posterior hypothalamic nuclei.

### A. Central and Peripheral Functions of BRS-3

What makes the BRS-3 receptor so interesting is that, despite the limited distribution of BRS-3, BRS-3 knockout mice show metabolic defects and become obese. In contrast to the GRP knockouts, which show defects in appetite control and satiety, the BRS-3 knockout shows abnormalities in energy regulation. BRS-3 knockout mice develop mild obesity, mild hypertension, decreased glucose tolerance, elevated levels of insulin, and reduced metabolic rate. BRS-3 mice also show increased levels of leptin. Exact details of how the BRS-3 receptor mediates these changes remain to be determined, although it has been proposed that the changes may be secondary to changes in glucose metabolism. Clearly, investigation into this pathway will be facilitated by identification of the BRS-3 ligand.

## VII. SUMMARY

There are three known mammalian bombesin-like peptide receptors, the GRP receptor, NMB receptor, and the BRS-3 receptor. GRP is distributed in the brain, gut, and lung and regulates autonomic function, appetite, lung development, and GI hormone secretion. GRP also affects both normal and neoplastic lung growth. NMB is distributed in the brain and esophagus, but the functions of NMB remain poorly characterized. The endogenous ligand for the BRS-3 receptor has not yet been characterized, but may be related to the bombesin-like peptides that occur in frog skin. The BRS-3 receptor is involved in energy balance and its further study may provide new therapeutic approaches to obesity.

## Glossary

**bombesin-related peptides** Members of the bombesin-like peptide subfamily; phylogenetically related to amphibian bombesin and to date have been found only in frogs.

**bronchopulmonary dysplasia** Chronic lung disease that can occur in premature infants; characterized by abnormal lung structure, tachypnea, and continuing need for supplemental oxygen.

**peptidylglycine $\alpha$-amidating monooxygenase** Enzyme that synthesizes the amino-terminal amide group on neuropeptides through use of the nitrogen from the adjacent glycine residue.

**prohormone** Protein precursor to the active peptide hormone; must be proteolytically cleaved and enzymatically processed to yield the active hormone.

**pulmonary neuroendocrine cells** Airway epithelial cells that secrete a variety of neuropeptides, growth factors, and amines; may also be involved in oxygen sensing.

**RNA editing** Process of making posttranscriptional changes in the RNA sequence by enzymatic mechanisms; in a classic example, this process is involved in the formation of different forms of the glutamate receptor.

## See Also the Following Articles

**Gastrin • Peptide YY • Vagal Regulation of Gastric Functions by Brain Neuropeptides**

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# Bone Morphogenetic Proteins

DI CHEN, MING ZHAO, AND GREGORY R. MUNDY
*University of Texas Health Science Center*

I. DISCOVERY OF BMPS
II. CHEMICAL STRUCTURE OF BMPS
III. RECEPTORS FOR BMPS
IV. SIGNAL TRANSDUCTION PATHWAY
V. DISORDERS ASSOCIATED WITH BMPS
VI. NULL MUTATIONS OF BMP LIGANDS AND RECEPTORS
VII. PHYSIOLOGIC ROLES OF BMPS
VIII. BMPS AS POTENTIAL THERAPIES

**Bone morphogenetic proteins, composed of ~400–525 amino acids, comprise one subset of the transforming growth factor-β superfamily. They are closely related structurally to other subsets, but are distinguished by a capacity to stimulate ectopic bone formation in rodents. After binding to their receptors, they play a role in bone and cartilage development.**

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily. BMPs were originally identified from bone matrix using an ectopic bone formation assay. The activity of BMPs was first identified in the late 1950s, but the proteins responsible for bone induction remained unknown until the purification of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and -4 in the late 1980s. The purification of BMPs was completed using the rat ectopic bone formation assay. This assay involves combining the sample containing the unknown protein to be assayed with demineralized rat bone matrix, which has been treated with dissociative agents such as guanidine and urea to remove all of the endogenous BMP activity. This combination is then implanted subcutaneously in rats, and after 1–2 weeks, formation of new cartilage and bone is detected histologically. Using this bioassay, BMPs were purified and sequenced. Purifying growth regulatory factors present in trace amounts in bone tissue to homogeneity using a slow and cumbersome *in vivo* bioassay was an amazing feat.

To determine the biological activities of each individual BMP member, a cloning approach was

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To determine the biological activities of each individual BMP member, a cloning approach was taken to obtain molecular clones corresponding to each of the proteins present in the purified extract. Based on the BMP sequence information, oligonucleotide probes were synthesized and used to screen bovine genomic libraries. Once bovine genes corresponding to the proteins were obtained, they were then used to obtain human cDNA clones encoding each protein within the extract. A large number of clones were identified by this strategy. All of the BMPs have homologous amino acid sequences. To date, around 20 BMP family members have been identified and characterized.

## II. CHEMICAL STRUCTURE OF BMPS

BMPs are 30- to 38-kDa homodimers that are synthesized as prepropeptides of approximately 400–525 amino acids. The mature C-terminal region of 100–140 amino acid residues is released from a propeptide region by cleavage at an Arg-X-X-Arg sequence. The secretion of the C-terminal mature segment is as a dimer, sometimes disulfide linked. Although homodimers are considered the standard form, there are natural heterodimers with similar bioactivity. It has been reported that a heterodimer composed of BMP-4 and BMP-7 is a potent inducer of mesoderm. All BMPs have similar sequences, including seven similarly spaced cysteine residues located in the mature region of the proteins. Six of seven cysteine residues on each BMP subunit form three intrachain disulfide bonds, with the remaining residue forming an interchain bridge to create the dimer. Many of the BMPs are glycosylated in both the mature and the propeptide regions, as determined by biochemical characterization and the presence of appropriate carbohydrate addition sites in the presumed amino acid sequence. Unlike TGF-β, secreted BMP proforms apparently do not form latent complexes with their mature counterparts. There is considerable cross-species bioactivity for the BMPs. The BMPs have been grouped into subsets based on amino acid sequence homology. The groupings are suggested to be as follows: (1) BMP-2 and BMP-4, (2) BMP-3 and BMP-3b, (3) BMP-5, BMP-6, BMP-7, and BMP-8, (4) BMP-9 and BMP-10, (5) BMP-12, BMP-13, and BMP-14, and (6) BMP-11 and growth/differentiation factor 8 (GDF-8).

The production of BMPs by recombinant methods has allowed the activities of each molecule to be investigated *in vitro* and *in vivo*. These recombinant BMPs have been produced using both a mammalian cell expression system and an *Escherichia coli* expression system. BMPs have been implicated in a variety of functions. BMPs induce the formation of both cartilage and bone. During the process of bone formation, BMPs create a rudimentary environment that is conducive to the development of functional bone marrow. In addition, BMPs play a role in a number of nonosteogenic developmental processes. BMP-2 can direct the development of neural crest cells into neuronal phenotypes, and BMP-4 and BMP-7 specifically induce a sympathetic adrenergic phenotype. BMP-4 and GDF-8 give direction to somite development by inhibiting the process of myogenesis. BMPs also appear to be responsible for normal dorsal/ventral patterning. BMP-4 specifies the development of ventral structures (e.g., skin from ectoderm and connective tissue/blood from mesoderm). Dorsal structures (nervous system and muscle) apparently appear when BMP-4 signals are interrupted through the activities of binding proteins. In the limb bud, and as part of the fibroblast growth factor 4 (FGF-4) and sonic hedgehog (Shh) interaction, BMP-2 apparently inhibits limb bud expansion and induces the formation of chondrocyte and osteoblast precursors.

## III. RECEPTORS FOR BMPS

BMPs signal through serine/threonine kinase receptors that are composed of type I and type II subtypes. Three type I receptors have been shown to bind BMP ligands, including type IA and IB BMP receptors [BMPR-IA (ALK-3) and BMPR-IB (ALK-6)] and type IA activin receptor (ActRIA or ALK-2). Three type II receptors for BMPs have also been identified and they include type II BMP receptor (BMPR-II) and types II and IIB activin receptors (ActRII and ActRIIB). Whereas BMPR-IA, BMPR-IB, BMPR-II, and ActRIA are specific to BMPs, ActRII and ActRIIB are also signaling receptors for activins. These receptors are expressed differentially in various tissues. Ligand–receptor cross-interactions are observed among the BMP, activin, and TGF-β receptor family members.

## IV. SIGNAL TRANSDUCTION PATHWAY

For the TGF-β family, ligands bind to type II receptors in the absence of type I receptors. Type I receptors can bind ligands only in the presence of type II receptors. However, BMPs bind weakly to the type I receptors in the absence of type II receptors. In the presence of type II receptors, the binding of BMPs to type I receptors is accelerated. The type II BMP receptor kinase transphosphorylates the $G_s$ domain in the type I receptor, which leads to activation of the type I receptor kinase. Type II receptor is a primary binding protein for ligands, and the type I receptor acts as an effector in signal transduction. This notion is supported by the observation that mutation of glutamine to aspartic acid in the $G_s$ domain of the type I BMP receptor results in a receptor with a constitutively activated kinase. In these mutants, signals are transduced from the type I BMP receptor in the absence of ligand and type II BMP receptor.

The type I BMP receptor substrates include a recently identified protein family, the Smad proteins, which play a central role in the relay of BMP signals from the receptor to target genes in the nucleus. Smad1, Smad5, and Smad8 are phosphorylated by BMP receptors in a ligand-dependent manner. After release from the receptor, Smad proteins associate with the related protein Smad4, which acts as a shared partner. This complex translocates into the nucleus and participates in gene transcription with other transcription factors.

Activation of specific genes by Smads is conducted by interaction with specific DNA-binding proteins. One of the transcription factors that interact with Smad1 has been identified as a homeodomain DNA-binding protein, Hoxc-8. Hoxc-8 serves as a transcriptional repressor for osteopontin gene transcription. Interaction of Smad1 with Hoxc-8 relieves the repressive activity of Hoxc-8 and activates osteopontin gene transcription. Another protein, which interacts with Smad1 and Smad5 in the nucleus, is core-binding factor α1 (Cbfα1). Cbfα1 is an osteoblast-specific transcription factor and plays a central role in osteoblast differentiation and bone formation. Targeted disruption of Cbfα1 in mice reveals that Cbfα1 expression is absolutely required for bone development *in vivo*. A complete lack of both endochondral and intramembranous ossification, with an absence of mature osteoblasts throughout the body, is observed in homozygous Cbfα1-deficient mice.

## V. DISORDERS ASSOCIATED WITH BMPS

Studies of naturally occurring mutations of BMPs and BMP receptors have shown that BMPs also play important roles in several inherited diseases. Disruption of the gene for BMP-5 in mice results in short ears and a wide range of skeletal defects, including reductions in long bone width and the size of several vertebral processes and an overall lower body mass. Mutations in growth/differentiation factor-5 (GDF-5; also named CDMP-1 and BMP-11) genes result in brachypodism in mice and chondrodysplasia in humans. The BMP-5 and GDF-5 genes are localized to chromosome 2 in mice and to chromosome 20 in humans. GDF-5 has been shown to bind to BMPR-IB specifically, and null mutations in the BMPR-IB gene cause a skeletal phenotype similar to that observed in GDF-5 mutant mice.

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare and disabling genetic disorder characterized by congenital malformations of the great toes and by progressive heterotopic endochondral ossification in predictable anatomical patterns. Mutations in the *noggin* gene, which encodes a BMP-binding protein, have been found in FOP patients and linked to the FOP disease. In addition, ectopic expression of BMP-4 is also found in FOP patients. Familial primary pulmonary hypertension is a rare autosomal dominant disorder that has been mapped to chromosome 2q33. Monoclonal plexiform lesions of proliferating endothelial cells in pulmonary arterioles are the characteristic phenotype of this disease. These lesions lead to elevated pulmonary artery pressure, right ventricular failure, and death. Genotyping multiple families with this disorder has shown that BMPR-II mutations are found in these patients. Mutations in GDF-9 and GDF-9b genes have been found in patients with premature ovarian failure and polycystic ovary syndrome. Overexpression of BMP-2, -4, -5, and BMPR-IA is associated with malignancy of the oral epithelium, and overexpression of BMP-3 has been described in prostate cancer cells. Mutations in the BMPR-IB gene are associated with increased ovulation rates in Booroola Mérino ewes and in sheep. Female mice deficient in BMPR-IB are infertile due to a constellation of defects, including irregular estrous cyclicity, impaired pseudopregnancy response, severe defects in cumulus cell expansion, and insufficient uterine endometrial gland development.

## VI. NULL MUTATIONS OF BMP LIGANDS AND RECEPTORS

To understand the roles of BMP ligands and BMP receptor signaling in embryonic development and in postnatal life, null mutations of BMP ligands and BMP receptors have been created and phenotypic changes in these animal models have been extensively studied. Mice deficient for BMP-2 and BMP-4 are nonviable. Homozygous BMP-2 mutant embryos die between days 7.0 and 10.5 of gestation and have defects in cardiac development, manifested by the abnormal development of the heart in the exocoelomic cavity. Homozygous BMP-4 mutant embryos die between days 6.5 and 9.5 and show little or no mesodermal differentiation. BMP-7-deficient mice die shortly after birth because of poor kidney development. Histological analysis of mutant embryos at several stages of development reveals that metanephric mesenchymal cells fail to differentiate, resulting in a virtual absence of glomerulus in newborn kidneys. In addition, BMP-7-deficient mice have eye defects that appear to originate during lens induction. BMP-6-deficient mice are viable and fertile and show no overt defects in tissues known to express BMP-6 mRNA. BMP-6 is mainly expressed in hypertrophic cartilage. Because BMP-2 and BMP-6 are coexpressed in this tissue, BMP-2 may functionally compensate in BMP-6 null mice. Growth/differentiation factor-8 (GDF-8, myostatin) is expressed specifically in developing and adult skeletal muscle. During early stages of embryogenesis, GDF-8 expression is restricted to the myotome compartment of developing somites. At later stages and in adult animals, GDF-8 is expressed in many different muscles throughout the body. GDF-8 null mutant mice are significantly larger compared to wild-type mice and show a large and widespread increase in skeletal muscle mass.

Null mutation of the BMPR-IA gene causes embryonic lethality in mice. Animals die at embryonic day 9.5. Homozygous mutants with morphological defects are first detected at day 7.0. No mesoderm forms in the mutant embryos, suggesting that BMPR-IA is essential for the inductive events that lead to the formation of mesoderm during gastrulation. Mice lacking BMPR-IB are viable and exhibit defects in the appendicular skeleton. In BMPR-IB-deficient mice, proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region are markedly reduced. In adult mutant mice, the proximal interphalangeal joint is absent and the phalanges are replaced by a single rudimentary element, but the distal phalanges are unaffected. The lengths of the radius, ulna, and tibia are normal, but the metacarpals/metatarsals are reduced. The appendicular defects in BMPR-IB mutant mice resemble those seen in mice homozygous for the GDF-$5^{\text{bp-j}}$ null allele of the GDF-5 locus. Because GDF-5 has been shown to play a critical role in cartilage formation and binds BMPR-IB with high affinity, these results suggest that BMPR-IB plays a nonredundant role in cartilage formation *in vivo*. BMP ligands may utilize multiple type I receptors to mediate their signaling during bone formation. This hypothesis is supported by observations in BMPR-IB and BMP-7 double mutant mice. In the double mutant, severe appendicular skeletal defects have been observed in the forelimbs and hindlimbs. The ulna is nearly absent and the radius is shortened. Because BMP-7 binds efficiently to both BMPR-IB and ActRIA, it is conceivable that BMPR-IB and ActRIA play important synergistic or overlapping roles in bone formation *in vivo*.

## VII. PHYSIOLOGIC ROLES OF BMPS

Physiologic roles of BMPs and BMP receptor signaling on normal bone formation have also been studied. Injection of BMP-2 locally over the surface of calvariae of mice induces periosteal new bone formation on the surface of calvariae of mice without a prior cartilage phase. Systemic administration of BMP-6 increases trabecular bone volume and bone formation rates in mice. In the transgenic mice that express a dominant-negative truncated BMPR-IB driven by the osteoblast-specific type I collagen promoter, the bone mineral density, the static bone volume, and the dynamic bone formation rates are decreased, suggesting that BMP receptor signaling plays an important role in normal postnatal bone formation.

## VIII. BMPS AS POTENTIAL THERAPIES

The osteoinductive capacity of BMPs has been demonstrated in preclinical models, and the efficacy of BMPs for the treatment of orthopedic and dental patients is now being evaluated in clinical trials. The clinical applications of recombinant BMP-2 and BMP-7 are being studied most extensively. Many of the animal models used to evaluate the capacity of BMPs to heal bone defects have utilized critical-sized defects. In these animal models, bone defects are large enough that they will not heal without a therapeutic intervention. This setting facilitates analysis of the ability of a BMP to induce bone. Recombinant human BMP-2 combined with a variety of matrices has been shown to heal defects in a canine mandibular defect model. Healing of long bone critical-sized defects by BMP-2 has been demonstrated in several species, including rats, rabbits, dogs, sheep, and nonhuman primates. The implantation of BMP-7 containing bovine collagen matrix preparations restored large diaphyseal segmental defects in monkeys, rabbits, and dogs, leading to the regeneration of new bone that is fully functional biologically and biomechanically. Osteochondral defects are the common end point for several types of joint diseases, such as degenerative arthritis, infection, and trauma. Preparations of BMP-7 in conjunction with a type I collagen carrier induces autologous cells and repairs iatrogenic osteochondral defects in rabbits. These results suggest that BMP-7 may be useful in articular cartilage repair. Both BMP-2 and BMP-7 have been shown to induce new dentine formation and have a potential application as a substitute for root canal surgery.

## Glossary

**bone formation** Comprises distinct processes involving osteoblast proliferation, differentiation, and mineralization at sites of prior resorption. Two types of bone are formed in humans: bone formed immediately after a cartilage phase in growing long bones at epiphyses (endochondral bone formation) and bone formed on periosteal surfaces in calvarial and scapular bones (intramembranous bone formation). Cellular events in bone formation are regulated by ambient concentrations of transforming growth factor-β superfamily members as well as by other growth regulatory factors.

**bone morphogenetic protein receptors** Serine/threonine kinase receptor complexes of type II and type I components, responsible primarily for ligand binding and signal transduction, respectively.

**Smads** Signal transduction molecules in the bone morphogenetic protein signal transduction pathway; responsible for transmitting the signal generated by ligand binding to the receptor through the cytoplasm to the nucleus in order to activate specific gene expression.

**transforming growth factor-*β* superfamily** Large group of structurally related peptides present in the bone matrix and other tissues.

## See Also the Following Articles

**Activins • Osteogenic Proteins • Vitamin D and Cartilage • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone**

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Devin K. Binder
*University of California, San Francisco*

I. INTRODUCTION
II. STRUCTURE
III. SIGNALING
IV. LOCALIZATION, TRANSPORT, AND RELEASE
V. BDNF AND DEVELOPMENT

diaphyseal segmental defects in monkeys, rabbits, and dogs, leading to the regeneration of new bone that is fully functional biologically and biomechanically. Osteochondral defects are the common end point for several types of joint diseases, such as degenerative arthritis, infection, and trauma. Preparations of BMP-7 in conjunction with a type I collagen carrier induces autologous cells and repairs iatrogenic osteochondral defects in rabbits. These results suggest that BMP-7 may be useful in articular cartilage repair. Both BMP-2 and BMP-7 have been shown to induce new dentine formation and have a potential application as a substitute for root canal surgery.

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I. INTRODUCTION
II. STRUCTURE
III. SIGNALING
IV. LOCALIZATION, TRANSPORT, AND RELEASE
V. BDNF AND DEVELOPMENT

VI. BDNF GENE REGULATION
VII. BDNF AND SYNAPTIC PLASTICITY
VIII. BEHAVIORAL AND DISEASE ASSOCIATIONS
IX. SUMMARY

**Brain-derived neurotrophic factor is a protein that is active in components of the central nervous system, particularly the hippocampus. It is important in maintaining viability and function of hippocampal and cerebral cortical neurons, axons, and dendrites. Because of its role in synaptic transmissions, brain-derived neurotrophic factor is of great interest for possible therapeutic applications in neurodegenerative and neuropsychiatric disorders.**

## I. INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of neurotrophic factors. Originally purified from pig brain, it was shown to have survival-promoting action on a subpopulation of dorsal root ganglion neurons. There is a strong homology between the amino acid sequences of BDNF and nerve growth factor (NGF), the first neurotrophin described for its trophic (survival- and growth-promoting) effects on sensory and sympathetic neurons. Since the discovery of NGF in the early 1950s by Rita Levi-Montalcini and Viktor Hamburger and the discovery of BDNF by Yves Barde and colleagues in 1982, other members of the neurotrophin family have been discovered, including neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Each factor appears to have a distinct profile of trophic effects on subpopulations of peripheral nervous system (PNS) and central nervous system (CNS) neurons.

## II. STRUCTURE

The mature form of human BDNF has been mapped to chromosome 11 and shares about 50% amino acid identity with human NGF, NT-3, and NT-4/5. The structure of each neurotrophin contains three elements: (1) a signal peptide following the initiation codon, (2) a pro-region containing an N-linked glycosylation site and a proteolytic cleavage site for furin-like pro-protein convertases, followed by the mature sequence, and (3) a distinctive three-dimensional structure containing two pairs of antiparallel β-strands and cysteine residues in a cystine knot motif. Mature neurotrophins are noncovalently linked homodimers with molecular mass about 28 kDa. Dimerization appears essential for NT receptor activation.

## III. SIGNALING

Each neurotrophin binds one or more high-affinity receptors [the trk receptors] ($K_d \sim 10^{-11}$ *M*). The trk proteins are transmembrane receptor tyrosine kinases (RTKs) homologous to other RTKs, such as the epidermal growth factor (EGF) receptor and insulin receptor family. Signaling by receptor tyrosine kinases involves ligand-induced receptor dimerization and dimerization-induced trans-autophosphorylation. Receptor autophosphorylation on multiple tyrosine residues creates specific binding sites for intracellular target proteins, which bind to the activated receptor via SH2 domains. For the neurotrophin family, these target proteins have been shown to include PLC-γ1 (phospholipase C), p85 (the noncatalytic subunit of phosphatidylinositol 3-kinase), and Shc (SH2-containing sequence); activation of these target proteins can then lead to a variety of intracellular signaling cascades such as the Ras–mitogen-activated protein (MAP) kinase cascade and phosphorylation of the cyclic AMP response element binding protein (CREB). Binding specificity is conferred via the juxtamembrane immunoglobulin (Ig)-like domain of the extracellular portion of the receptor in the following pattern: trkA is the high-affinity receptor for NGF (with low-affinity binding by NT-3 in some systems), trkB is the high-affinity receptor for BDNF and NT-4/5 (with lower affinity binding by NT-3), and trkC is the high-affinity receptor for NT-3.

All of the neurotrophins bind to the low-affinity neurotrophin receptor, designated $p75^{NTR}$ ($K_d \sim 10^{-9}$ *M*). $p75^{NTR}$ has a glycosylated extracellular region involved in ligand binding, a transmembrane region, and a short cytoplasmic sequence lacking intrinsic catalytic activity. It is related to proteins of the tumor necrosis factor receptor (TNFR) superfamily. Neurotrophin binding to $p75^{NTR}$ is linked to several intracellular signal transduction pathways, including nuclear factor-κB (NF-κB), Jun kinase, and sphingomyelin hydrolysis. $p75^{NTR}$ signaling mediates biologic actions distinct from those of the high-affinity trk receptors, notably the initiation of programmed cell death (apoptosis).

## IV. LOCALIZATION, TRANSPORT, AND RELEASE

BDNF mRNA as well as the mRNA encoding the high-affinity receptor for BDNF (trkB) have a widespread distribution in the central nervous system. In parallel, BDNF protein immunoreactivity is also widespread, and is localized in neuronal cell bodies, axons, and dendrites. Like BDNF mRNA, constitutive BDNF protein expression is particularly high in the hippocampus, where the mossy fiber axons of dentate granule cells display intense BDNF immunoreactivity.

Unlike the classical target-derived trophic factor model in which neurotrophins such as NGF are retrogradely transported, there is now abundant evidence that BDNF is also anterogradely transported in the brain. Biochemical studies demonstrate that endogenous BDNF may be packaged in a releasable vesicular pool, and recent evidence indicates that neurotrophins are released acutely following neuronal depolarization.

## V. BDNF AND DEVELOPMENT

The classical view of neurotrophin function, derived initially from studies of NGF, includes effects on growth and survival of neurons, and indeed BDNF has been shown to be necessary for the survival of some neurons during vertebrate development. Certain peripheral sensory neurons, especially those in vestibular and nodose-petrosal ganglia, depend on the presence of BDNF, as demonstrated by the loss of these sensory neurons in BDNF knockout mice (lacking both alleles for BDNF). Unlike NGF effects, with BDNF knockout, sympathetic neurons are not affected, nor are motor neuron pools. BDNF knockout mice fail to thrive, demonstrate lack of proper coordination of movement and balance, and ultimately die by 3 weeks of age. Conversely, provision of BDNF or other neurotrophins to peripheral nerves during development enhances outgrowth.

BDNF is abundantly expressed in the central nervous system, especially in the hippocampal formation, cerebral cortex, and amygdaloid complex. Its expression increases in the early postnatal period and then stays high into adulthood, consistent with a continuous role in the mature CNS. *In vitro* and *in vivo* studies have demonstrated that BDNF has survival- and growth-promoting actions on a variety of CNS neurons, including hippocampal and cortical neurons. Lack or blockade of BDNF leads to death of certain identified forebrain neurons (thalamic neurons).

## VI. BDNF GENE REGULATION

Many stimuli have been shown to alter BDNF gene expression in both physiologic and pathologic states. Physiologic stimuli are known to increase BDNF mRNA content. For example, light stimulation increases BDNF mRNA in the visual cortex, osmotic stimulation increases BDNF mRNA in the paraventricular hypothalamic nucleus, and whisker stimulation increases BDNF mRNA expression in the somatosensory barrel cortex. Electrical stimuli that induce long-term potentiation (LTP) in the hippocampus, a cellular model of learning and memory, increase BDNF and NGF expression. Even physical exercise has been shown to increase NGF and BDNF expression in hippocampus.

This physiologic alteration in BDNF gene expression may be very important in the development of the brain. For example, there is an exciting body of work implicating BDNF in activity-dependent development of the visual cortex. Provision of excess BDNF or blockade of BDNF signaling leads to abnormal patterning of ocular dominance columns during a critical period of visual cortex development. This suggests a role for BDNF in the patterning of axonal arborizations from the lateral geniculate nucleus (LGN; a part of the thalamus) to the visual cortex during development. BDNF expression can also be regulated by neurotransmitters and hormones. For example, glutamate receptor agonists induce, whereas $\gamma$-aminobutyric acid type A ($GABA_A$) receptor agonists inhibit, BDNF expression.

Pathologic states are also associated with alteration in BDNF gene expression. For example, seizures dramatically up-regulate BDNF mRNA. Increased BDNF expression is seen after hypoxia/ischemia and hypoglycemic coma, whereas reduced BDNF expression is associated with stress.

## VII. BDNF AND SYNAPTIC PLASTICITY

A great deal of evidence now indicates that BDNF and its high-affinity receptor trkB, in addition to modulating neuronal survival and differentiation, are also critically involved in modulation of synaptic transmission. Recent studies have demonstrated that BDNF is transported anterogradely in CNS neurons, is released on neuron depolarization, and triggers rapid intracellular signals and action potentials in central neurons. Direct activity-dependent pre- to postsynaptic transneuronal transfer of BDNF has recently been demonstrated using fluorescently labeled BDNF.

BDNF has an enormous range of physiologic actions at both developing and mature synapses, enhancing or reducing transmission at excitatory and inhibitory synapses by both pre- and postsynaptic mechanisms. Overall, the evidence favors a role for BDNF causing increased excitability both by strengthening of excitatory (glutamatergic) synapses and by weakening of inhibitory (GABAergic) synapses. Application of BDNF potentiates synaptic transmission both *in vitro* and *in vivo*. Incubation of hippocampal or visual cortical slices with trkB inhibitors inhibits LTP, and hippocampal slices from BDNF knockout animals exhibit impaired LTP induction that is restored by reintroduction of BDNF.

## VIII. BEHAVIORAL AND DISEASE ASSOCIATIONS

### A. Learning and Memory

Learning and memory depend on persistent selective modification of synapses between CNS neurons. Because BDNF appears to be critically involved in activity-dependent synaptic strengthening of the sort observed in the long-term potentiation model, there is great interest in its role as a molecular mechanism of learning and memory. The hippocampus, which is required for many forms of long-term memory in humans and animals, appears to be an important site of BDNF action. Indeed, rapid and selective induction of BDNF expression in the hippocampus during contextual learning has been demonstrated, and function-blocking antibodies to BDNF and/or knockout of forebrain trkB signaling in mice impair spatial learning.

### B. Neurodegenerative Diseases

The idea that degenerative diseases of the nervous system may result from insufficient supply of neurotrophic factors has generated great interest in BDNF as a potential therapeutic agent. Many reports have documented evidence of decreased expression of BDNF in cases of Alzheimer's and Parkinson's diseases. Selective reduction of BDNF mRNA in the hippocampus has been reported in Alzheimer's disease specimens and decreased BDNF protein has been demonstrated in the substantia nigra in Parkinson's disease, areas that degenerate in these diseases. BDNF promotes survival of all major neuronal types affected in Alzheimer's and Parkinson's diseases, such as hippocampal and neocortical neurons, cholinergic septal and basal forebrain neurons, and nigral dopaminergic neurons. Interestingly, recent work has implicated BDNF in Huntington's disease as well. Huntingtin, the protein mutated in Huntington's disease, up-regulates BDNF transcription, and loss of huntingtin-mediated BDNF transcription leads to loss of trophic support to striatal neurons, which subsequently degenerate in the hallmark pathology of the disorder. In all of these disorders, provision of BDNF or increasing endogenous BDNF production may conceivably be therapeutic if applied in the appropriate spatiotemporal context.

### C. Epilepsy

The discovery that limbic seizures increase mRNA levels for nerve growth factor led to the idea that seizure-induced expression of neurotrophic factors may contribute to the lasting structural and functional changes underlying epileptogenesis. Recent *in vitro* and *in vivo* findings implicate BDNF in the cascade of electrophysiologic and behavioral changes underlying the epileptic state. BDNF mRNA and protein are markedly up-regulated in the hippocampus by seizure activity in animal models, and infusion of anti-BDNF agents or use of BDNF knockout mice inhibits epilepsy in animal models. Conversely, overexpression of BDNF in transgenic mice leads to spontaneous seizures. The hippocampus and closely associated limbic structures are thought to be particularly important in the pro-epileptogenic effects of BDNF, and, indeed, increased BDNF expression in the hippocampus is found in specimens from patients with temporal lobe epilepsy. It is hoped that understanding of the hyperexcitability associated with BDNF in epilepsy animal models may lead to novel anticonvulsant or antiepileptic therapies.

### D. Pain

BDNF also may play an important neuromodulatory role in pain transduction. BDNF is synthesized by spinal cord dorsal horn neurons and is markedly up-regulated (along with NGF) in inflammatory injury to peripheral nerves. Application of BDNF in experimental studies acutely sensitizes nociceptive afferents and elicits hyperalgesia. Central pain sensitization is an activity-dependent increase in excitability of dorsal horn neurons leading to a clinically intractable condition termed "neuropathic pain" in which normally nonpainful somatosensory stimuli (touch and pressure) become exquisitely painful (allodynia). Electrophysiological and behavioral data demonstrate that inhibition of BDNF signal transduction inhibits central pain sensitization.

### E. Depression

BDNF signaling may also be involved in affective behaviors. Environmental stresses (e.g., immobilization) that induce depression also decrease BDNF mRNA. Conversely, physical exercise is associated with decreased depression and increased BDNF mRNA. Existing treatments for depression are thought to work primarily by increasing endogenous monoaminergic (i.e., serotonergic and noradrenergic) synaptic transmission, and recent studies have shown that effective antidepressants increase BDNF mRNA in the brain. Exogenous delivery of BDNF promotes the function and sprouting of serotonergic neurons in adult rat brains. Thus, new pharmacologic strategies are focused on the potential antidepressant role of BDNF.

## IX. SUMMARY

Since the purification of BDNF in 1982, a great deal of evidence has pointed to its central roles in neuronal morphology, development, physiology, and pathology. Aside from its importance in neural development and regeneration, BDNF appears essential to molecular mechanisms of synaptic plasticity. Basic activity-related changes in the central nervous system are thought to depend on BDNF modification of synaptic transmission, especially in the hippocampus and neocortex. Pathologic levels of BDNF-dependent synaptic plasticity may contribute to conditions such as epilepsy and chronic pain sensitization; conversely, application of the trophic properties of BDNF may lead to novel therapeutic options in neurodegenerative diseases and perhaps even in neuropsychiatric disorders.

## Glossary

**$\gamma$-aminobutyric acid** Amino acid neurotransmitter; the major inhibitory neurotransmitter in the central nervous system.

**glutamate** Amino acid neurotransmitter; the major excitatory neurotransmitter in the central nervous system.

**hippocampus** Curved, elongated ridge [(Gk) "sea horse"] in the medial temporal lobe of the brain; thought to be responsible for consolidation of short-term memories and their conversion to long-term memories; also involved in spatial learning. Hippocampal morphology is markedly altered in epilepsy (in which certain hippocampal neurons die and others sprout aberrant connections) and Alzheimer's disease (in which there is degeneration of hippocampal neurons).

**long-term potentiation** Cellular model of learning and memory that uses stereotyped *in vitro* or *in vivo* stimulation of defined neural pathways to elicit long-term increases in synaptic strength; thought to be a general model for how brain synapses are altered by experience and learning.

**neurotrophin** One of a family of growth factors that includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5 (NT-4/5), neurotrophin-6, and neurotrophin-7. All have been shown to have growth- or survival-promoting actions on subpopulations of peripheral or central nervous system neurons.

**$p75^{NTR}$** Low-affinity receptor for all neurotrophins; linked to distinct intracellular signal transduction pathways; may modulate neurotrophin binding to trk receptors.

**synaptic plasticity** General term for a variety of alterations in synapses, both strengthening and weakening; may involve alterations in neurotransmitter receptors, neurotransmitter release, or propensity for activation of intracellular second messengers. Brain-derived neurotrophic factor has particularly strong effects on synaptic plasticity in a variety of systems.

**thalamus** Deep brain structure involved in processing and transmitting peripheral sensory information to the cerebral cortex. The lateral geniculate nucleus of thalamus receives visual input from the retina and transmits it to the visual cortex in the occipital lobe.

**tropomyosin receptor like kinases (trk)** High-affinity receptors for neurotrophins, including trkA (binds nerve growth factor and neurotrophin-3 with lower affinity), trkB (binds brain-derived neurotrophic factor, neurotrophin-4/5, and neurotrophin-3 with lower affinity), and trkC (binds neurotrophin-3); linked to a variety of intracellular signal transduction pathways.

## See Also the Following Articles

**Nerve Growth Factor (NGF) • Neurotrophins • Placental Development • Protein Kinases**

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# Brassinosteroids

JIANMING LI
*University of Michigan, Ann Arbor*

I. INTRODUCTION
II. STRUCTURAL FEATURES AND PHYSIOLOGICAL ACTIVITIES
III. BIOSYNTHESIS AND METABOLISM
IV. SIGNAL PERCEPTION AND MOLECULAR RESPONSES

**Brassinosteroids are a group of polyhydroxylated plant steroids that are ubiquitous in the plant kingdom. These compounds elicit a variety of responses that affect plant growth and development and are now widely recognized as a new class of major plant hormones.**

## I. INTRODUCTION

Brassinosteroids, first isolated from *Brassica napus* L., are the focus of extensive biochemical studies. Cell cultures of *Catharanthus roseus* (periwinkle) have been used to examine brassinosteroid (BR) biosynthesis; data from those studies, coupled with the recent discovery and molecular genetic/ metabolic characterization of BR-deficient and BR-insensitive dwarf mutants in *Arabidopsis*, pea, and tomato plants, have provided strong evidence for essential roles of BRs in plant growth and development.

## II. STRUCTURAL FEATURES AND PHYSIOLOGICAL ACTIVITIES

Brassinolide, a potent plant growth stimulator, was the first BR isolated; it was discovered in rape (*B. napus*) pollen in 1979, and its structure was determined by X-ray crystallography and spectroscopic analysis to be (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-*B*-homo-7-oxa-5α-cholestan-6-one (Fig. 1). So far, more than 40 brassinolide analogues, collectively known as BRs, have been identified and characterized from many different plant species, including 37 angiosperms, 5 gymnosperms, a pteridophyte (*Equisetum arvense*), and an alga (*Hydrodictyon reticulatum*). BRs have been isolated from almost every plant tissue, although immature seeds and pollen contain the highest concentrations of the steroids. When applied exogenously to intact plants or to explants at nanomolar to micromolar concentrations, BRs can induce a variety of physiological responses, including seed germination, pollen tube growth, stem elongation, leaf unrolling and bending, root growth inhibition, vascular differentiation, microtubule reorientation, proton pump activation, induction of ethylene biosynthesis, altered gene expression, and stress response modulation.

All naturally occurring BRs have a common 5α-cholestane skeleton with different substitutions in the A/B rings and the side chain (Fig. 1). Whereas the variations in the A ring mainly come from the presence and/or orientations of hydroxyl groups at the C-1, C-2, and C-3 positions, the modifications of the B ring are produced by C-6 oxidation, giving rise to 6-deoxo, 6-oxo, or 7-oxalactone steroids. Based on the degree of alkylation of the side chain, BRs can also be grouped into $C_{27}$, $C_{28}$, and $C_{29}$ steroids.

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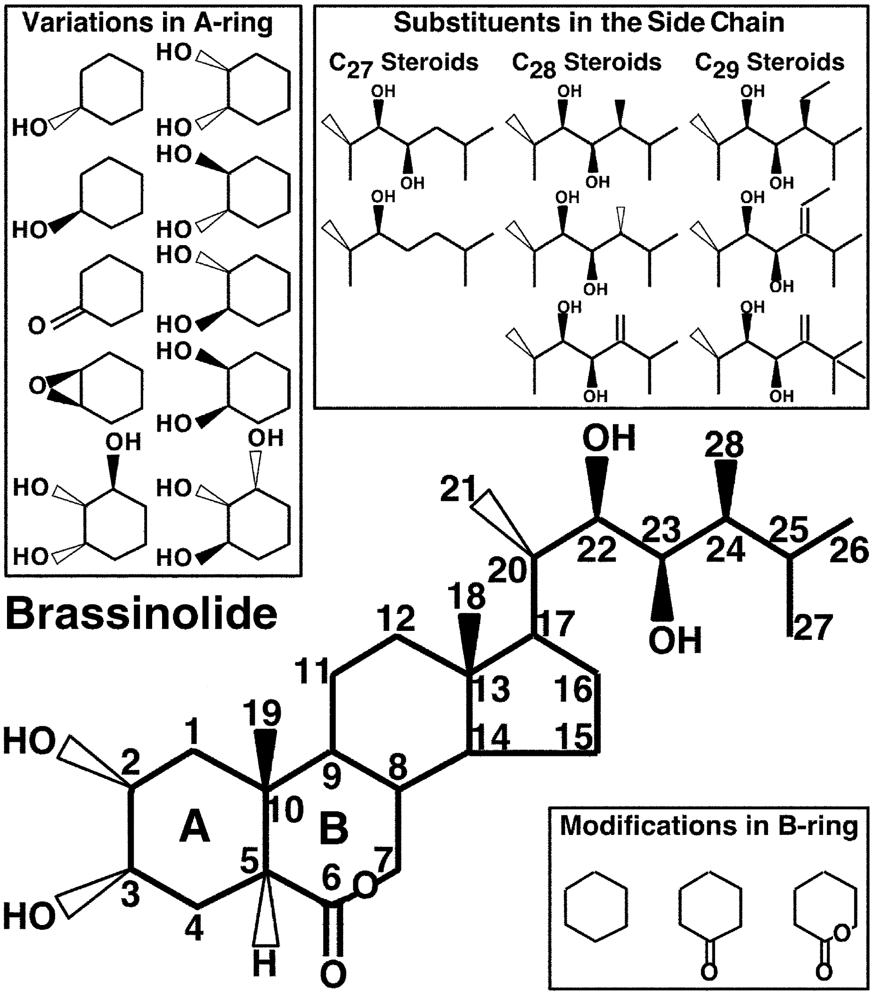

FIGURE 1 Brassinolide structure and structural variations.

Most BRs isolated so far contain a 24*R*-methyl group, but steroids containing a 24*S*-methyl, 24-methylene, 24*R*-ethyl, 24-ethylidene, 24-methylene-25-methyl, or no substitution at C-24 also exist in nature. Almost all natural BRs contain *R*-oriented vicinal hydroxyls at positions C-22 and C-23. Structure-activity evaluation of various synthetic brassinolide analogues concluded that the biological activities of BRs depend on the presence of the following functional groups: (1) A/B trans-fused ring junction, (2) α-oriented vicinal hydroxyls at C-2 and C-3, (3) 6-oxo or 7-oxalactone functionality in ring B, (4) alkylation at the C-24 position, and (5) a 22*R*,23*R*-vicinal diol moiety. Among the natural BRs, brassinolide and castasterone, two $C_{28}$ steroids with a 24*R*-methyl group, are the most widely distributed steroids in the plant kingdom and have the strongest biological activities.

## III. BIOSYNTHESIS AND METABOLISM

Brassinolide is synthesized from campesterol, a common plant sterol that has the same carbon skeleton as that found in brassinolide (Fig. 2). The first step of the reaction is conversion of campesterol to campestanol via three intermediates; 24*R*-methylcholest-4-en-3β-ol, 24*R*-methylcholest-4-en-3-one, and 24*R*-methyl-5α-cholestan-3-one. The resulting campestanol is then converted to castasterone through either of two alternative routes: the early and late C-6 oxidation pathways, depending on whether the oxidation at C-6 occurs before or after

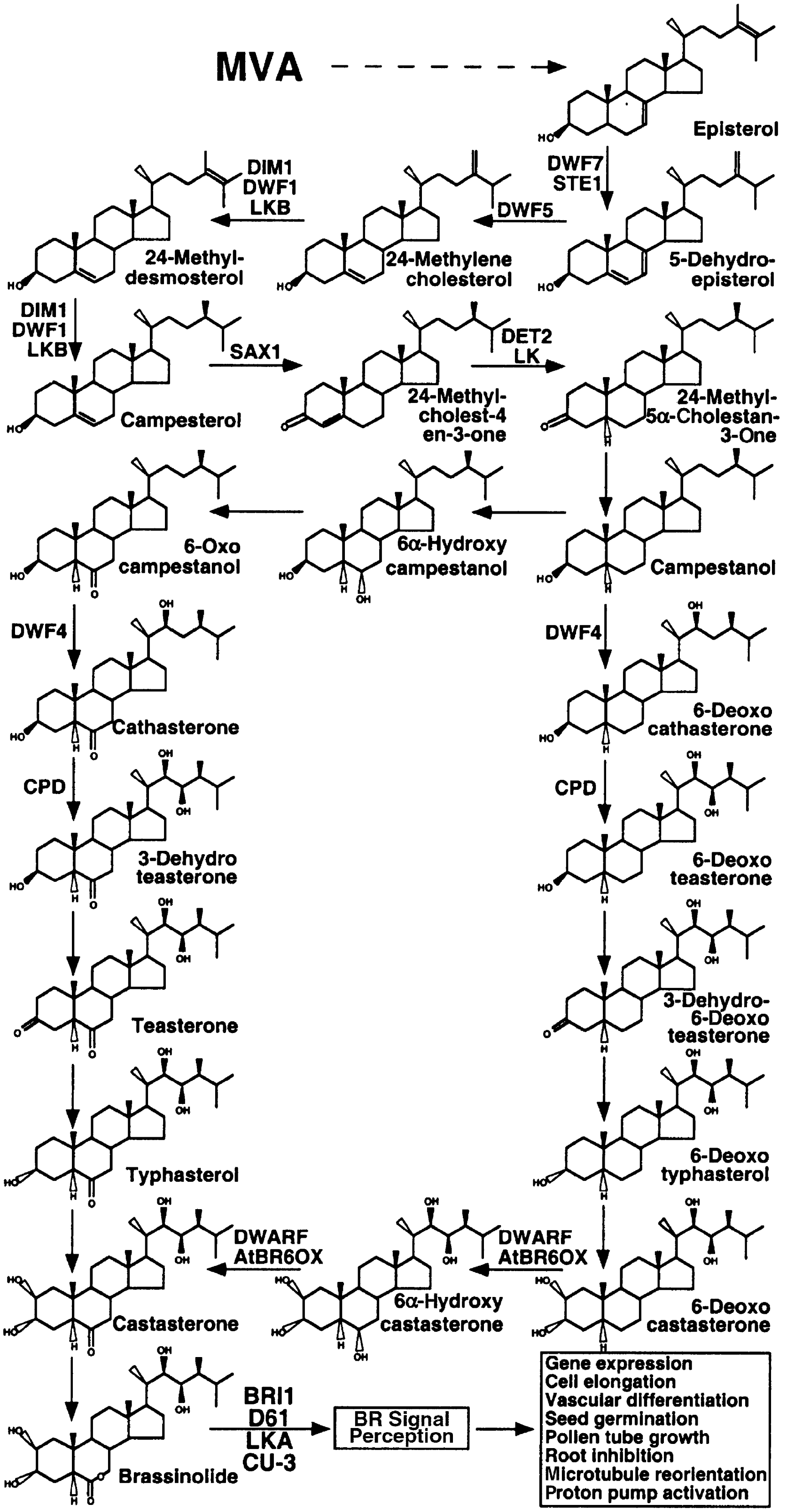
MVA
Episterol
DWF7
STE1
5-Dehydro-episterol
DWF5
24-Methylene cholesterol
DIM1
DWF1
LKB
24-Methyl-desmosterol
DIM1
DWF1
LKB
Campesterol
SAX1
24-Methyl-cholest-4 en-3-one
DET2
LK
24-Methyl-5α-Cholestan-3-One
Campestanol
6α-Hydroxy campestanol
6-Oxo campestanol
DWF4
Cathasterone
CPD
3-Dehydro teasterone
Teasterone
Typhasterol
DWF4
6-Deoxo cathasterone
CPD
6-Deoxo teasterone
3-Dehydro-6-Deoxo teasterone
6-Deoxo typhasterol
DWARF
AtBR6OX
6-Deoxo castasterone
DWARF
AtBR6OX
6α-Hydroxy castasterone
Castasterone
Brassinolide
BRI1
D61
LKA
CU-3
BR Signal Perception
Gene expression
Cell elongation
Vascular differentiation
Seed germination
Pollen tube growth
Root inhibition
Microtubule reorientation
Proton pump activation
HO
OH
H
O

FIGURE 2 Proposed biosynthetic pathways of brassinolide and proteins involved in brassinosteroid biosynthesis and signal perception. Genes involved are shown alongside the reaction arrows. MVA, mevalonic acid.

the introduction of vicinal hydroxyls at C-22 and C-23 of the side chain. In the early C-6 oxidation pathway, campestanol is oxidized at the C-6 position to produce 6α-hydroxycampestanol and 6-oxocampestanol; the latter is then modified by two consecutive hydroxylation reactions at positions C-22 and C-23, yielding cathasterone and teasterone, respectively. Teasterone undergoes 3-epimerization to become typhasterol, involving an inversion from 3β-hydroxyl to 3α-hydroxyl via a 3-oxo intermediate. The introduction of an additional hydroxyl group at the C-2α position of typhasterol leads to the formation of castasterone. In the late C-6 oxidation pathway, the reactions from campestanol to 6-deoxocastasterone exactly mirror those between 6-oxocampestanol and castasterone in the early C-6 oxidation pathway. 6-Deoxocastasterone is then oxidized at the C-6 position to yield castasterone, via 6-hydroxycastasterone, to connect the two parallel routes. Finally, the 6-oxo group of castasterone is further oxidized via a Baeyer-Villiger-type oxidation to produce a lactone group, yielding brassinolide. Although the initial metabolic study of BR biosynthesis was conducted using cultured cells of *C. roseus*, which can produce high levels of BRs, various intermediates of the proposed pathways have also been identified in many other plant species. Gas chromatography–mass spectrometry analyses of metabolites of *C. roseus* and *Arabidopsis* plant cells fed with deuterium-labeled intermediates have confirmed most of the enzymatic steps.

Further support for the proposed BR biosynthetic pathways has come from molecular genetic and/or biochemical studies of many BR-deficient *Arabidopsis*, pea, and tomato mutants. These mutants are characterized by dwarf stature, darker green coloration with short petioles and rounder, epinastic leaves, reduced male fertility, delayed flowering and senescence, and varying degrees of de-etiolation in the dark; these mutants can be normalized to wild-type phenotypes by exogenous application of BRs, and their characteristics can be mimicked by treating normal plants with brassinazole, a highly specific BR biosynthesis inhibitor. The cabbage-like dwarfs have furnished indisputable evidence that BRs are essential for normal plant growth and also have provided much needed materials for testing the hypothesized BR biosynthetic pathways and a powerful means for identifying BR biosynthetic enzymes.

The *sax1* mutants of *Arabidopsis* are defective in the oxidation and isomerization of 3β-hydroxy-$\Delta^{5,6}$ precursors to 3-oxo-$\Delta^{4,5}$ steroids, and the *SAX1* gene is postulated to encode a 3β-hydroxyl steroid dehydrogenase that catalyzes the first step of the campesterol to campestanol conversion. Both the *Arabidopsis det2* mutant and the pea *lk* mutant are defective in the formation of campestanol. Careful metabolic analysis has indicated that the reduction of 24-methylcholest-4-en-3-one to 24-methylcholestan-3-one is blocked in the *det2* and *lk* mutants. The cloned *DET2* gene was found to encode a plant homologue of mammalian steroid 5α-reductases and the *det2* mutation could be complemented by transgenic expression of human steroid 5α-reductase genes. The *Arabidopsis dwf4* mutant is defective in 22α-hydroxylation, whereas the *Arabidopsis cpd* and the tomato *dumpy* mutants are blocked in the 23α-hydroxylation steps, resulting in strong dwarf phenotypes that can be rescued only by 22α- and 23α-hydroxylated BRs, respectively. These results provide strong genetic support for previous physiological data indicating that the vicinal hydroxyls are essential for the biological activities of BRs. The *DWF4* and *CPD* genes both encode cytochrome P450 monooxygenases that share significant sequence homology to mammalian steroid hydroxylases. The tomato *dwarf* mutant is defective in the formation of castasterone, resulting in dwarfism as a consequence of inhibited stem elongation and leaf expansion. The *DWARF* gene and its *Arabidopsis* orthologue, *AtBR6ox*, encode cytochrome P450 enzymes that can catalyze the C-6 oxidation of 6-deoxocastasterone, yielding castasterone, and may also catalyze the conversion of other 6-deoxo BRs to the corresponding 6-oxo BRs, thus linking the late C-6 oxidation pathway to the early C-6 oxidation pathway at multiple positions.

In addition to genes specifically involved in BR biosynthesis, several other genes are involved in the synthesis of sterol precursors to BR biosynthesis (Fig. 2), including *DWF7/STE1*, *DWF5*, and *DIM1/DWF1* from *Arabidopsis* and *LKB* from pea plants. Mutations in these genes impart a weak BR-deficient dwarf phenotype that can be rescued by exogenous BR application. Biochemical analysis of endogenous sterols from the mutants revealed that *dwf7/ste1*, *dwf5*, and *dim1SQ4/dwf1/lkb* mutants are blocked in the dehydrogenation of episterol to 5-dehydroepisterol, the conversion of 5-dehydroepisterol to 24-methylenecholesterol, and the reduction of the $\Delta^{24(28)}$ double bond, respectively. All three *Arabidopsis* genes have been cloned and found to encode a sterol C-5-desaturase, a sterol

$\Delta^7$-reductase, and a FAD-dependent oxidoreductase, respectively.

Like many other plant hormones, the activities of BRs are often modulated by further metabolic reactions. O-Glucosylation at the existing hydroxyl groups and/or new hydroxylation are important mechanisms for deactivating plant steroids. Natural 23-O-β-glucopyranosyl and 3-O-β-glucopyranosyl BRs have been identified. Exogenous BRs, when fed into tomato cell cultures, are converted to 25β-D-glucopyranosyloxy and 26β-D-glucopyranosyloxy BRs in a process involving hydroxylation and subsequent glucosylation at C-25 and C-26, respectively. An activation tagging screening involving transferred DNA (T-DNA, derived from *Agrobacterium tumefaciens*) identified an *Arabidopsis* cytochrome P450 gene, *BAS1*; elevated expression of *BAS1* led to an increased production of 26-hydroxybrassinolide with reduced accumulation of brassinolide, giving a phenotype similar to BR-deficient mutants. This is the first genetic evidence that further hydroxylation on the side chain could serve as an inactivation mechanism to regulate BR activities. Plants also contain other mechanisms for deactivating BRs, including sulfonation of the C-22 hydroxyl group by a sulfotransferase and catabolic side chain degradation by a multistep metabolic process. BRs can also be conjugated with fatty acids. Exogenous BRs are esterified at the 3β position with lauric acid, myristic acid, or palmitic acid, and two natural fatty-acid-conjugated BRs have been identified in lily anthers. It is thought that these acylated BRs might function as storage forms of BRs, which would be released as free BRs in response to developmental and environmental signals.

## IV. SIGNAL PERCEPTION AND MOLECULAR RESPONSES

In animals and insects, steroid action is mediated mainly by intracellular steroid receptors; these receptors function as ligand-dependent transcriptional factors that regulate gene expression. Although BRs are structurally similar to animal/insect steroids and are known to affect gene expression, no similar nuclear receptor has been found in plant systems. The completely sequenced *Arabidopsis* genome did not yield a single gene that encodes a homologue of animal and insect intracellular steroid receptors. It is thought that plants might use a different receptor system to detect the presence of steroid hormones.

Several genetic screens for BR signaling mutants have led to the identification of the *Arabidopsis* gene *BRASSINOSTEROID-INSENSITIVE1* (*BRI1*), which encodes a protein with sequence homology to leucine-rich-repeat (LRR) receptor-like kinases. Mutations in the *BRI1* gene result in a phenotype that is indistinguishable from the severe BR-deficient phenotype, except that it cannot be rescued by BR application. Interestingly, *bri1* mutants accumulate high levels of brassinolide and brassinolide biosynthetic precursors, with the most severely affected alleles promoting the highest levels of brassinolide, suggesting that *BRI1* is necessary for homeostasis of steroid hormones. BRI1 consists of an extracellular domain with 25 tandem LRRs disrupted by a 70-amino-acid island between the 21st and 22nd LRRs, a single transmembrane α-helix, and a cytoplasmic domain having the serine/threonine kinase signatures. Sequence analysis of many *bri1* mutant alleles shows that both the LRR-containing extracellular domain, especially the 70-amino-acid island, and the cytoplasmic kinase are essential for BR signaling. The BR-insensitive dwarf mutants have also been identified in other plant species, including tomatoes (*cu-3*), peas (*lka*), and rice (*d61*). The *lka* and *d61* mutants have been found to contain mutations in their respective *BRI1* homologues.

The *Arabidopsis* BRI1 protein is localized in the plasma membrane and can function as a serine/threonine kinase *in vitro*. In addition, the extracellular domain of BRI1 can confer dose-dependent BR responsiveness to Xa21, a similar LRR-containing receptor kinase of rice involved in disease resistance. Moreover, transgenic *Arabidopsis* plants overexpressing the *BRI1* gene show higher BR sensitivity and stronger brassinolide binding activity that can be co-immunoprecipitated with the receptor kinase. Interestingly, brassinolide treatment stimulates autophosphorylation of BRI1 proteins. This finding led to a hypothesis that BR binding to BRI1, either directly or indirectly, stimulates the kinase activity of the BRI1 protein, which in turn activates a signaling cascade to control a variety of BR-regulated processes, including gene expression and cell elongation.

The first BR-regulated gene, *BRU1*, to be isolated from BR-treated elongating soybean epicotyls encodes a protein exhibiting 48–74% sequence identity to various xyloglucan endotransglycosylases (XETs), which are implicated in cell wall growth. An increase in *BRU1* message levels correlates with the extent of stem elongation and with increases in plastic extensibility of the cell wall in response to BR treatment, suggesting that BRs may promote cell elongation by regulating gene expression and/or activities of cell-wall-modifying enzymes. Such a hypothesis is supported by the identification of similar BR-regulated *XET* genes in *Arabidopsis*, rice, and tomato plants. BRs can also exert their effects on cell elongation by regulating the orientations of cortical microtubules. Genes encoding β-tubulins, a component of microtubules, and an actin effector protein, which controls polymerization of actins that associate with microtubules, have been found to be regulated by the plant steroids.

BR-regulated gene expression may also control other BR-regulated physiological processes. The BRI1-mediated feedback inhibition of *CPD* gene expression is involved in homeostasis of the steroid hormones. BRs are also known to induce the expression of an aminocyclopropane-1-carboxylate synthase gene, which might be responsible for BR-stimulated ethylene biosynthesis. Other BR-regulated genes include *CDC2b*, an *Arabidopsis* cyclin-dependent kinase gene involved in cell division, a tomato invertase gene that plays a role in controlling source/sink balance and carbohydrate metabolism, and several *B. napus* genes that encode heat-shock proteins, which are implicated in thermotolerance.

Despite intensive studies of BRI1 and identification of many BR-regulated genes, virtually nothing is known about how the BR signal, once transmitted to a cell via BRI1, is transduced in the cytosol and reaches the nucleus, where it affects gene regulation. It also remains to be determined whether all known BR-regulated processes require a BR-regulated gene expression event.

## Glossary

**leucine-rich repeats** Tandem repeats of a versatile protei–protein interaction domain that often consists of 24 residues with a consensus sequence, LxxLxxLxx-LxLxxNxxxGxIPxx, where x represents any amino acid.

**T-DNA activation tagging** A novel mutagenesis method utilizing transferred DNA (T-DNA). T-DNA is derived from *Agrobacterium tumefaciens* and is used to introduce multiple copies of a viral transcriptional enhancer element randomly into a plant genome, resulting in transcriptional activation of a plant gene that is near the introduced T-DNA.

**xyloglugan endotransglycosylase** A cell-wall-modifying enzyme that cleaves xyloglucan polymers internally and then religates the newly generated ends to other xyloglucan ends.

## See Also the Following Articles

**Abscisic Acid • Auxin • Cytokinins • Ethylene • Gibberellins • Jasmonates • Salicylic Acid**

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# Bulimia

See *Eating Disorders*

# Calcitonin

David M. Findlay[*] and Patrick M. Sexton[†]

[*]*University of Adelaide, Australia* • [†]*Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne*

I. INTRODUCTION
II. CHEMISTRY
III. BIOSYNTHESIS
IV. METABOLISM
V. BIOLOGICAL ACTIONS
VI. MECHANISMS OF ACTION
VII. SUMMARY

**Calcitonin is a protein that is synthesized in the mammalian thyroid gland. Both calcitonin and its receptors are found in many mammalian cell types and tissues, and calcitonin is a potent inhibitor of osteoclast-mediated bone resorption. Understanding the chemistry and mechanisms of action of calcitonin led to its use in treatment of various bone disorders, and further analysis of its diverse roles may lead to additional clinical applications.**

## I. INTRODUCTION

Calcitonin (CT) is a 32-amino-acid peptide synthesized in mammals by the C cells of the thyroid gland. CT was discovered because of its ability to lower the concentration of plasma calcium. This hypocalcemic response was found to be due principally to a potent inhibitory action of CT on osteoclast-mediated bone resorption and it is this action that underlies its use clinically in the treatment of bone disorders, including Paget's disease, osteoporosis, and hypercalcemia due to malignancy. Receptors for CT were subsequently identified as members of the large family of seven-membrane-spanning, G-protein-coupled receptors. Since the initial discovery of CT, expression of CT and its receptors has been demonstrated in many cell types and tissues other than bone. This suggests additional, biologically diverse roles for CT, including functions in brain and kidney and in cell differentiation and tissue morphogenesis.

## II. CHEMISTRY

CT belongs to a family of molecules that are structurally related but biologically diverse, comprising calcitonins of various species, calcitonin gene-related peptides (CGRPs), amylin, and adrenomedullin. The CT sequence has been determined for many species and shows considerable divergence, with the common features being that all sequences contain 32 amino acids, a carboxy-terminal proline amide, and a disulfide bridge between cysteine residues at positions 1 and 7 (Fig. 1). Based on their amino acid s]equence homologies, CTs from different species are classified into three groups. The first is the artiodactyl group, which includes porcine, bovine, and ovine CT; within this group each CT differs by four amino acids. The second group is the primate/rodent group, which includes human and rat CT, and these differ within the group by two amino acids. The third group is the teleost/avian group, which includes salmon, eel, goldfish, and chicken CT, each differing within the group by four amino acids. The order of biological potency of the CTs, with respect to a hypocalcemic response, is teleost ≥ artiodactyl ≥ human, although absolute biological activities vary considerably among different animal species.

Studies of substituted, deleted, and otherwise modified CTs have provided considerable information regarding structure/activity relationships of the CT molecule. For example, stabilization of the N-terminal ring structure, by substitution of the disulfide bridge with an ethylene linkage or an acetylenic bridge, leads to greatly improved stability of the salmon calcitonin (sCT) molecule and retention of biological potency. Despite this, analogues of sCT that lack the ring structure can retain full potency, in terms of hypocalcemic activity. However, the N-terminal amino acids are critical for the agonist activity of CT. Progressive truncation of this region of sCT leads to generation of, first, weak or partial agonists and, then, with deletion of six or seven amino acids, to peptides with antagonist activity. Deamidation of the amino terminus can increase the potency of CT peptides *in vivo*, perhaps by increasing their resistance to degrading endopeptidases. Modifications of CT peptide length from the C-terminal end, either by deletion or elongation, are poorly tolerated. Additionally, the C-terminal amide is required, with the COOH-form having markedly reduced activity. The lower *in vivo* potency of human CT (hCT), compared with teleost CTs, is due partly to

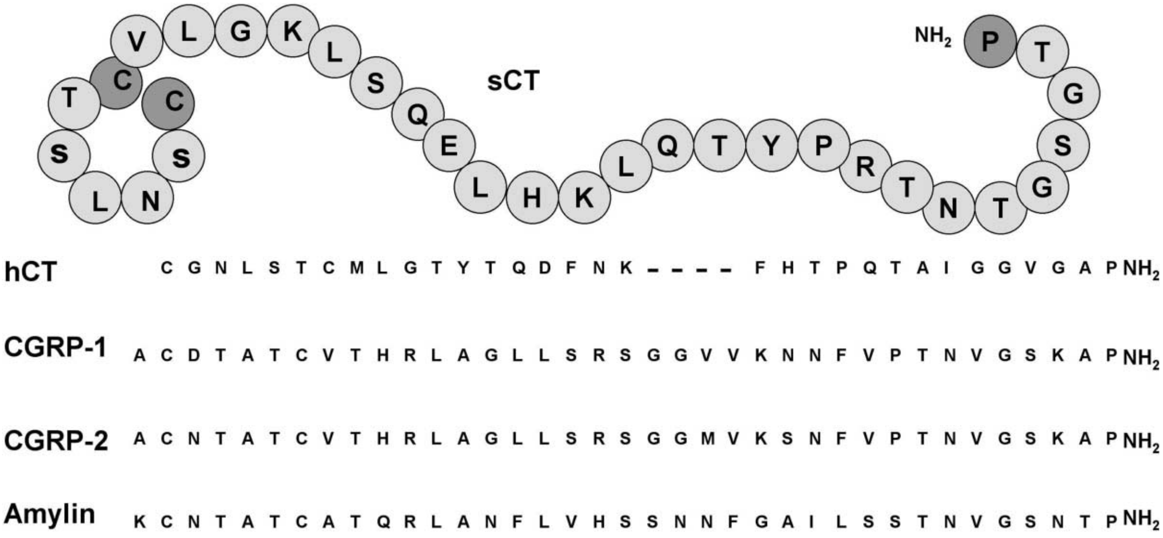
sCT
C S N L S T C V L G K L S Q E L H K L Q T Y P R T N T G S G T P
NH2
hCT C G N L S T C M L G T Y T Q D F N K - - - - F H T P Q T A I G G V G A P NH2
CGRP-1 A C D T A T C V T H R L A G L L S R S G G V V K N N F V P T N V G S K A P NH2
CGRP-2 A C N T A T C V T H R L A G L L S R S G G M V K S N F V P T N V G S K A P NH2
Amylin K C N T A T C A T Q R L A N F L V H S S N N F G A I L S S T N V G S N T P NH2

**FIGURE 1** Primary amino acid sequence of salmon calcitonin (sCT), human calcitonin (hCT), calcitonin-related gene peptide-1 and -2 (CGRP-1 and -2), and amylin. Note the homologies between the peptides, and the cysteine (C) residues, which form the disulfide-bonded ring structure at the N-terminus of each molecule. The C-terminal proline residue in each case is amidated, which is important for activity.

its increased rate of metabolic degradation and clearance, contributed to by the methionine in position 8, which is susceptible to oxidation.

Circular dichroism studies indicate that sCT exhibits considerable secondary structure in the presence of lipid, and predicts the generation of an amphipathic α-helix between residues 8 and 22, in proximity to the cell membrane. The less potent human CT has reduced propensity to form this secondary structure. In addition, amino acid substitutions that enhance the formation of an α-helix between residues 8 and 16 also increase the hypocalcemic potency of the peptide. However, modifications of residues in the 8–22 sequence that alter the ability of sCT or hCT to form a helical secondary structure have yielded conflicting results in terms of the biological significance of this structure, which may be explicable on the basis of differences between CT receptor (CTR) subtypes. In rat tissues, two CTR subtypes have been described: the first, designated CT-H (for helical), displays high-affinity interaction only for peptides with a strong potential to form helical secondary structure; the other, designated CT-L (for linear), is capable of high-affinity interaction with both helical and nonhelical peptides. Following cloning of the rat C1a and C1b CTR isoforms, it was found that the CT-L and CT-H subtypes corresponded to the C1a and C1b receptors, respectively (see Section VI).

## III. BIOSYNTHESIS

### A. Sites of Production

Elevation of circulating calcium concentrations stimulates CT release from the C cells of the thyroid, and low serum calcium levels inhibit release. Sensitivity by thyroidal C cells to circulating calcium levels appears to be mediated by the same extracellular calcium-sensing receptor that is found in parathyroid cells. The basal concentration of hCT in human blood in normal individuals is very low (<10 pg/ml). Size fractionation analysis of CT extracted from plasma and tissues demonstrates the presence of multiple immunoreactive forms of CT. In addition to the CT monomer (~3500 Da), a number of high-molecular-weight forms exist in certain situations. These forms probably correspond to precursor molecules and dimers and other aggregates of the CT molecule and are largely inactive biologically. In several pathological states, such as CT-secreting tumors, circulating levels of CT are increased. Conditions of stress, such as some chronic lung problems, burns, acute pancreatitis, and certain infections/inflammatory conditions, are characterized by high circulating levels of large molecular-weight forms of CT.

CT synthesis also occurs in extrathyroidal sites. For example, assays using specific anti-hCT serum have detected hCT-like immunoreactivity (hCT-i) in the blood and urine of patients after total thyroidectomy. In fact, significant amounts of CT have been found in many human tissues. Thyroid and prostate gland contain the highest levels of hCT-i and significant amounts are also extractable from the gastrointestinal tract, thymus, bladder, lung, and central nervous system (CNS). The physiological role of CT in these extraskeletal tissues is not known. However, CT-producing cells in extrathyroidal sites do not appear to respond to elevations in serum calcium, suggesting that CT produced in these sites is not involved in calcium homeostasis.

Both hCT-i and salmon CT-i (sCT-i) have been found in the CNS of vertebrate and invertebrate species, the latter again consistent with roles for CT unrelated to osteoclast inhibition. hCT-i is detectable in human cerebrospinal fluid, and in extracts of postmortem human brain. In addition, low levels of material immunologically similar to sCT-i are found in the hypothalamus, which also contains high densities of CT receptors. Release of a sCT-like peptide from cultured rat anterior pituitary cells has been described, and a cell line derived from a mouse pituitary carcinoma produces abundant sCT-i activity. Calcitonin receptors are present in the intermediate pituitary, thus calcitonin-like material present at this site may act as a paracrine regulator in this tissue. In accord with this, both anti-sCT and anti-hCT antisera were shown to significantly increase the release of prolactin from cultured anterior pituitary cells, presumably by neutralizing the effects of endogenously produced CT.

### B. Calcitonin Gene Products

The human calcitonin gene is located in the p14 *qter* region of chromosome 11. Like several other small peptide hormones, CT is synthesized as part of a larger precursor protein, of 136 amino acids. The DNA sequence of the human calcitonin gene predicts that the hormone is flanked in the precursor by N- and C-terminal peptides. Two forms of the C-terminal flanking peptide (CCP-I and CCP-II), which differ in their last eight amino acids, are produced by alternative splicing of the human CT mRNA primary transcript, such that part of exon 4 splices to exon 5. The alternative donor site is absent in rat, rabbit, chicken, and sCT mRNA sequences. CCP-I is the form found predominantly, although CCP-II is also present in the plasma and thyroidal tissues of both normal and medullary thyroid carcinoma (MTC) patients. CCP-I, also termed “katacalcin,” does not influence serum calcium levels and has no known bioactivity. However, measurement of serum katacalcin levels, together with CT and CGRP concentrations, is a useful marker for diagnosis of MTC. The N-terminal flanking peptide of procalcitonin contains 57 amino acids; although a stimulatory action on osteoblast proliferation has been proposed for this peptide, this claim is controversial.

It has been found that the calcitonin gene transcript actually encodes two distinct peptides that arise by tissue-specific alternative splicing of the primary mRNA transcript. The primary mRNA transcript is spliced almost exclusively to calcitonin mRNA in the thyroid, and to a mRNA encoding a calcitonin gene-related peptide in the nervous system. However, aberrant expression of CGRP is commonly seen in medullary thyroid carcinoma. The calcitonin/CGRP gene was one of the first recognized examples of a cellular gene exhibiting alternative, tissue-specific processing and has served as an important paradigm to study the molecular mechanisms of RNA splicing. Processing of the premessenger RNA to the calcitonin mRNA transcript involves usage of exon 4 as a $3'$ terminal exon, with concomitant polyadenylation at the end of exon 4. Processing to produce the CGRP mRNA involves the exclusion of exon 4 and direct ligation of exon 3 to exon 5, with polyadenylation at the end of exon 6.

The CT gene family consists of four known genes, CALC-I, CALC-II, CALC-III, and CALC-IV, that contain nucleotide sequence homologies. CALC-I is the only gene that produces CT. CALC-I and CALC-II genes produce two different forms of CGRP, CGRP-1 and CGRP-2, respectively, which differ from each other by three amino acids. CALC-III is thought to be a pseudogene, producing neither peptide, and CALC-IV produces another molecule of the CT-related peptide family, amylin.

## IV. METABOLISM

Calcitonin is rapidly cleared from the circulation at a rate that depends on the species of calcitonin, the route of administration, and the preparation of administered peptide. This clearance has implications for the use of CT clinically, and CT is usually injected intramuscularly in vehicles that maintain useful serum levels for 4–8 h after injection. Degradation of the CT molecule into inactive fragments occurs in various organs, with the kinetics and sites of inactivation being different for different species of CT. The hypocalcemic activity of CT is reduced by incubation *in vitro* in serum at 37°C, with relative rates of loss in the following order: porcine CT $>$ human CT $=$ rat CT $>$ salmon CT $\gg$ chicken CT. For salmon CT, the half-life of elimination from plasma *in vivo* is about 90 min and the metabolic clearance rate is around 200 ml/min. Incubation of CT with extracts of liver, kidney, or spleen showed that hypocalcemic activity was lost most rapidly in liver and kidney extracts due to a thermolabile degrading factor, with different rates of degradation for different CTs. The primary sites of CT degradation are extravascular, mainly in the liver for porcine CT and mainly in the kidney for human and salmon CT. Impaired renal function results in delayed elimination of human and salmon CT.

## V. BIOLOGICAL ACTIONS

### A. Bone Actions of Calcitonin

The first discovered and best understood physiological role of CT is its ability to potently inhibit bone resorption. This role is probably most relevant at times of stress on skeletal calcium conservation, such as pregnancy, lactation, and growth, when bone remodeling by osteoclasts and consequent release of calcium stores in bone need to be tightly regulated to prevent unnecessary bone loss. Evidence for a role of CT during growth, when the rate of bone turnover is high, is that the calcium-lowering effect of CT in the rat is strongly evident in young animals and becomes less marked with increasing age of the animals. In normal adult humans, even quite large doses of CT have little effect on serum calcium levels, because the process of bone resorption is a slow one in maturity. However, in pathologies characterized by increased bone turnover—for example, thyrotoxicosis, metastatic bone disease, or Paget's disease—CT treatment acutely inhibits bone resorption, which results in a lowering of plasma calcium. The lack of overt effects on serum calcium levels in maturity may indicate that the role of CT in bone is that of a regulator of the bone resorptive process, whatever the rate of resorption. Thus, CT probably should not be regarded primarily as a "calcium-regulating hormone," but as a regulator of bone resorption. CT is the only hormone known to be capable of inhibiting resorption by a direct action on bone. Evidence in support of an important physiological role for endogenous CT in protecting against bone loss comes from experiments showing that treatment with parathyroid hormone in parathyroidectomized (CT-deficient) rats caused more loss of cancellous bone than in intact (CT-replete) rats.

In addition to the hormonal activity of CT in regulating bone resorption, it is clear that many factors produced locally in bone also regulate this process. Key molecules that have central roles in bone resorption have recently been identified. Osteoclast formation and resorptive function both depend on the interaction of a molecule that is a receptor activator of NF-κB ligand (RANKL) with its cognate receptor, RANK. RANK is located on the cell surface of osteoclast precursors and mature osteoclasts. A soluble receptor-like molecule, termed osteoprotegerin (OPG), is synthesized in bone, and is a natural antagonist of RANKL. OPG can interfere with the RANKL–RANK interaction to inhibit both osteoclast formation and bone resorption. Overexpression of OPG in mice results in an osteopetrotic phenotype, due to reduced osteoclast numbers. Conversely, OPG knockout mice develop extensive osteoporosis associated with an increased number of osteoclasts. Because injection of RANKL into mice produces rapid increases in serum calcium levels, it appears that OPG tonically modulates the rate of osteoclast activity. As yet, the relative roles of CT and OPG in bone resorption are not known.

The inhibition of bone resorption by CT can be explained by a direct action on osteoclasts. Calcitonin treatment of resorbing osteoclasts has a profound effect on cell morphology, resulting in rapid loss of osteoclast ruffled borders, characteristically present at the resorption site, reduced cytoplasmic spreading, and decreased release of lysosomal enzymes. CT infused into rats results in an immediate reduction in the rate of excretion of hydroxyproline, consistent with the action of the hormone to inhibit the breakdown of bone collagen. The other means by which CT could inhibit resorption is through inhibition of osteoclast formation. However, the experimental data relating to such an action are conflicting, with several reports showing CT inhibition of osteoclast-like cell formation in bone marrow cultures of human, baboon, and mouse origin, and other studies, using more physiological concentrations of CT, showing no reduction in osteoclast formation.

### B. Renal Actions of Calcitonin

CT has been reported to have several effects in the kidney. These include an increase in the urinary excretion rate of sodium, potassium, phosphorus, chloride, and magnesium. Salmon CT was much more potent than porcine or human CT when administered subcutaneously into rats. A transient increase in calcium excretion, due probably to inhibition of renal tubular calcium reabsorption, has not usually been regarded as an important effect of CT, although recent observations link it to the calcium-lowering effect of CT in hypercalcemic patients with metastatic bone disease, in addition to inhibition of osteolysis by CT. Calcitonin receptors have been demonstrated clearly in the kidney, and a further action on the kidney is to enhance 1-hydroxylation of 25-hydroxyvitamin D in the proximal straight tubule of the kidney by stimulating the expression of 25-hydroxyvitamin D 1α-hydroxylase. These results suggest that CT is involved in the regulation of vitamin D production.

## C. Central Actions of Calcitonin

In addition to their presence in kidney and bone, CTRs are also abundant in the central nervous system (CNS). Central administration of CT generates potent effects that include analgesia and inhibition of appetite and gastric acid secretion. The centrally mediated actions of CT correlate well with the location of CT binding sites. The periaqueductal gray is important in central regulation of pain, and CT binding within this region is likely to be involved in CT-induced analgesia, although other brain regions have also been implicated. The recent identification of mouse CTR mRNA in serotonergic neurons, which are known to project into the spinal cord, forming a descending inhibitory system against pain transmission, also strongly supports a central analgesic role for CT. Administration of CT in clinical situations of bone pain can be very effective in ameliorating the pain symptoms. The hypothalamic binding parallels the multiple hypothalamic actions of CT, which include modulation of hormone release, decreased appetite, gastric acid secretion, and intestinal motility. There are also high densities of receptors in the subfornical organ (SFO), the vascular organ of the lamina terminalis (VOLT), and the area postrema, which, as circumventricular organs, are directly accessible to thyroidally derived, blood-borne CT. The area postrema is likely to contribute similarly to the anorexia of peripherally administered CT. The SFO and VOLT are involved in fluid and electrolyte homeostasis and thus are potential targets for CT-induced alteration in drinking behavior. Salmon calcitonin, when injected into the lateral part of the paraventricular nucleus of the hypothalamus, significantly reduced sleep duration and profoundly affected sleep cycles, producing prolonged insomnia, major reduction of slow-wave sleep, and a long period of alternation of rapid eye movement (REM) sleep and wakening.

## D. Other Actions of Calcitonin

Using various experimental approaches, CT and its receptors have been identified in a large number of other cell types and tissue sites, suggesting multiple roles for the CT/CTR. CTR binding sites include kidney, brain, pituitary, testis, prostate, spermatozoa, lung, and lymphocytes, as well as cancer-derived cells from lung, breast, pituitary, bone (osteoclastoma, osteogenic sarcoma), and embryonal carcinoma. There is evidence consistent with the involvement of CT in cell growth and differentiation and in tissue development and remodeling. CT appears to be important both for blastocyst implantation and for development of the early blastocyst. In addition, embryonic expression of the mouse CTR, and of a lacZ construct driven by the CTR promoter, suggest that CTRs may play important roles in morphogenesis. Reports that CT treatment of human breast cancer cells inhibited cell proliferation, together with the reported mitogenic action of CT in certain prostate cancer cell lines, support a role for CT in the modulation of cell proliferation. Recently, CT was found to inhibit profoundly the growth of cells overexpressing human CTR, causing an accumulation of cells in the $G_2$ phase of the cell cycle, which is associated with a prolonged increase in $p21^{WAF1/CIP1}$ expression and a sustained activation of the p42/44 mitogen-activated protein (MAP) kinase proteins. CT may also have a role in cell survival, and has been shown to be protective of drug-induced apoptosis in osteoblast-like and osteocyte-like cells and prostate cancer cell lines, and to promote the survival of osteoclasts. On the other hand, CT has been found to potentiate neuronal death due to oxygen and glucose deprivation.

As already stated, specific high-affinity receptors for CT have been demonstrated in a number of cancer cell lines and CT has been found to be a potent inhibitor of the growth of human breast cancer cells *in vitro*. A study of CTR in surgically obtained human breast cancers identified receptor mRNA production in all cases examined. CTR expression by tumors possibly represents a recapitulation of fetal expression, and it was recently reported that the CTR is expressed in several fetal tissues in the mouse, including the developing mammary gland, although it is absent in the same tissues postnatally. There are no data on the developmental expression of the CTR in humans, but it is reasonable to speculate that CTR expression in certain tumors represents a reappearance of fetal expression.

## VI. MECHANISMS OF ACTION

### A. Receptors

CT is known to act by binding to receptors on the plasma membrane of responsive cells. Our knowledge of the molecular basis of CT action, both in terms of ligand-binding and post-binding events, has been greatly assisted by the cloning of the CTR gene from several species. The first receptor cloned, in 1991, was the porcine CTR, which was isolated by expression cloning from a cDNA library derived from a renal epithelial cell line. Subsequently, human, rat, mouse, rabbit, and guinea pig CTR cDNAs were isolated. Analysis of the proteins translated from the CTR DNA sequences revealed that these receptors comprise approximately 500 amino acids and belong to the class II (family B) subclass of G-protein-coupled receptors, which also includes the receptors for other peptide hormones such as secretin, parathyroid hormone (PTH), glucagon, glucagon-like peptide-1, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating peptide, and gastric inhibitory peptide. The human CTR gene is located on chromosome 7 at 7q21.3. In the mouse it is in the proximal region of chromosome 6, and in chromosomal band 9q11–q12 in the pig, which is homologous to the 7q location in humans. The CTR gene, like the genes for other class II G-protein-coupled receptors, is complex, comprising at least 14 exons with introns ranging in size from 78 nucleotides to >20,000 nucleotides. The total receptor gene is estimated to exceed 70 kb in length. In organization and size, the human CTR gene is similar to the pig CTR gene, although some interspecies differences in organization do occur. For instance, in the pig, the intracellular domain 1 (I1) insert is generated by selective use of alternate splice sites located in exon 8, whereas in humans the I1 insert occurs on a separate exon.

### B. Receptor Isoforms

Receptor cloning shows the existence of multiple receptor isoforms that arise from alternative splicing of the CTR gene. The human receptor has been most extensively studied and at least five splice variants have been described. Differential splicing at the amino terminus of the coding sequence leads to either a potential additional upstream ATG translation start site or a deletion of the first 47 amino acids. However, the two most common human CTR variants arise by alternative splicing in the sequence encoding intracellular domain 1 (Fig. 2). Of these, the most common of the I1 variants leads to a 16-amino-acid insert in the first intracellular loop of the human CTR ($I_{1+}$). An alternative small exon may also be spliced into this region, leading to termination of the protein in this domain ($I_{1ter}$). Alternate splicing to yield a 16-amino-acid insert form of I1 also occurs in the pig CTR. However, splicing of the pig CTR primary transcript differs slightly from that in the human transcript, in that the insert arises from alternate splicing within exon 8 of the pig gene, whereas the insert is encoded by a separate exon in the human. Evidence exists for the presence of the I1 truncated form of the receptor only in the human CTR.

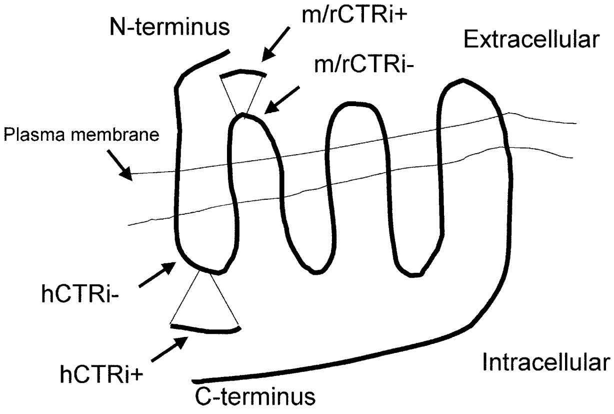

**FIGURE 2** Diagrammatic representation of the calcitonin receptor (CTR). The receptor is shown spanning the cell membrane, with the N-terminal domain extracellular and the C-terminal domain intracellular. The mouse and rat CTRs are expressed as two predominant isoforms that differ by the exclusion (m/rCTRi − ) or inclusion (m/rCTRi + ) of a 37-amino-acid insert in the first putative extracellular loop of the receptor. The human CTR is expressed as two predominant isoforms that differ by the exclusion (hCTRi − ) or inclusion (hCTRi + ) of a 16-amino-acid insert in the first putative intracellular loop of the receptor.

Different splice variants have been identified in the CTRs of other species. In rodents, alternate splicing leads to two receptor isoforms (termed C1a and C1b); these differ by the presence (C1b; $E_{2+}$) or absence (C1a) of an additional 37 amino acids in the second extracellular (E) domain (Fig. 2). In rabbits, alternate splicing can lead to deletion of the exon encoding most of the seventh transmembrane domain. In the case of the human, rodent and rabbit receptors, the insert-negative form of the CTR is the predominant isoform.

Alternative splicing of the CTRs has consequences for receptor function. For example, C1b variants show a loss of affinity for CT peptides that have a low propensity to form an α-helical secondary structure, such as hCT, whereas peptides with strong helical secondary structure, such as sCT, maintain high affinity at this receptor. In the case of the human CTRs, the receptor isoform containing a 16-amino-acid insert displays similar affinity for peptides to the $I_{1-}$ variant but has dramatically reduced ability to signal intracellularly. Presence of the 16-amino-acid insert leads to complete loss of CT-induced intracellular calcium mobilization and profoundly reduced stimulation of cAMP production in response to CT.

The physiological significance of the multiple CTR isoforms remains to be established. However, receptor isoforms are differentially expressed in different cell types, suggesting that alternative splicing is highly regulated and that receptor variants do have physiological roles. For example, the level of expression of the $I_{1+}$ isoform is relatively high in human ovary and placenta, and the rodent C1b receptor isoform is widely expressed in the CNS. The C1b receptor has only very weak affinity for the thyroidally derived form of CT (rat or human) and thus may act as a receptor for endogenous sCT-like peptides that have been found in neural tissues. Alternate splicing thus produces CTR variants with altered function.

It has recently been found that changes in receptor function can also be achieved by interaction between the CTR and proteins of a newly recognized family called receptor activity-modifying proteins (RAMPs). To date, three RAMPs, RAMP1, RAMP2, and RAMP3, have been identified. These proteins are single-transmembrane-spanning proteins that act to modify receptor processing, glycosylation, and presentation on the cell surface. Interactions between CTRs, and several closely related receptors, and RAMPs can alter ligand specificity. For example, in the case of the human CTR, which alone binds amylin with low affinity, RAMP3 enables these receptors to interact with amylin with greatly increased affinity (Fig. 3). This finding is of great interest because it has not been possible to identify the amylin receptor as a

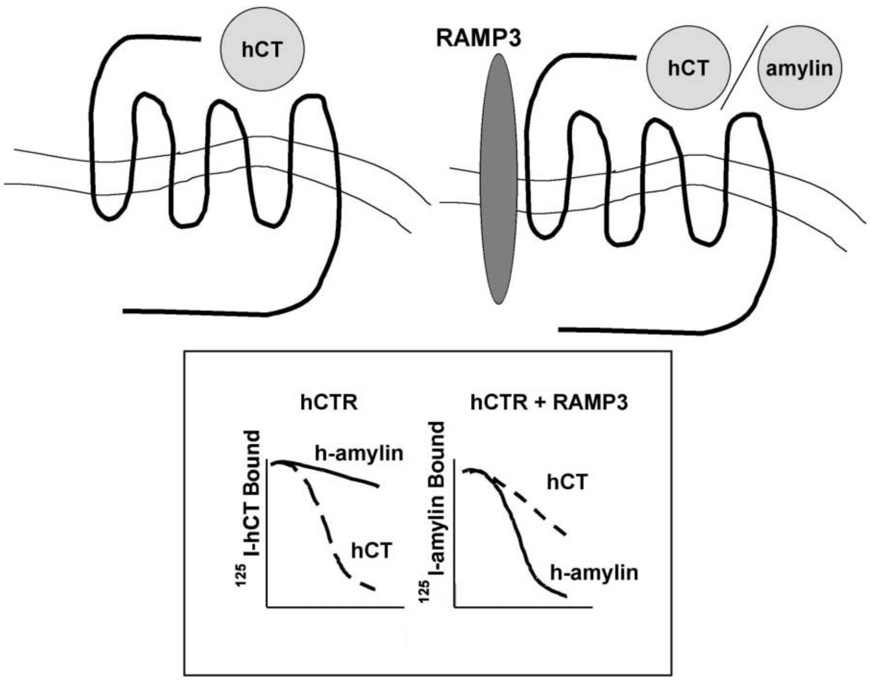

FIGURE 3 Schematic model of altered ligand recognition by the calcitonin receptor due to receptor interaction with RAMP protein. The model shows that expression of the human CTR (hCTR) alone leads to high-affinity interaction with human calcitonin (hCT) and poor interaction with amylin, as shown by the relative ability of these peptides to compete for binding of $^{125}$I-labeled hCT (lower panel). The co-expression of RAMP3 with hCTR leads to an amylin receptor "phenotype" that can bind either hCT or amylin, as shown by the competitive binding of $^{125}$I-labeled amylin and unlabeled amylin.

unique gene product. Thus, RAMPs present a novel mechanism for alteration or generation of receptor phenotype, and regulation of RAMPs may be important in the regulation of the physiological response to the CT family of peptides.

## C. CTR Signaling

The molecular mechanisms by which CTRs signal inside cells to produce cellular effects are still being elucidated. However, the signaling pathways appear to depend on the cell type as well as on the animal species. As with most other G-protein-coupled receptors, the CTR can couple to multiple members of the guanosine triphosphate (GTP)-regulated G-protein family (Fig. 4). In many cell types, activation of the CTR results in its ability to interact with $G_s$, which leads, in turn, to activation of adenylate cyclase and elevated intracellular levels of cyclic adenosine monophosphate (cAMP). The action of CT in osteoclasts to inhibit bone resorption is accompanied by increased cAMP levels. In addition, both forskolin, which directly activates adenylate cyclase, and dibutyryl cAMP, which elevates intracellular cAMP independent of adenylate cyclase action, inhibit bone resorption. Calcitonin also stimulates adenylate cyclase activity in kidney, with the pattern of CT responsiveness paralleling the distribution of CTRs in this tissue. Calcitonin induction of cAMP has now been documented in a large number of cultured CTR-bearing cells, including LLC-PK1 pig kidney cells, and in cancers of lung, breast, and bone. Receptor cloning and expression studies have confirmed that cAMP production is an important component of CTR-mediated signaling in many cell types.

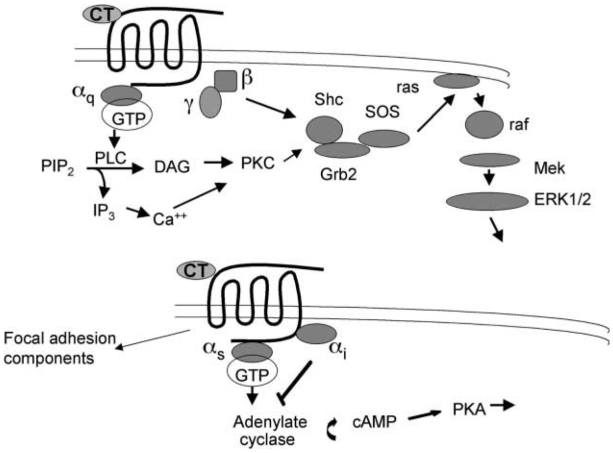

**FIGURE 4** Signaling capability of the CTR, showing some of the major intracellular signaling pathways that are activated on binding of CT to the CTR. Ligand binding causes a conformational change in the receptor that enables it to bind to (and to activate, by allowing the binding of GTP) a number of heterotrimeric G-proteins. These G-proteins then dissociate into α and βγ subunits, which act as adapter molecules that in turn bind and activate effector molecules, such as phospholipase C (PLC) and adenylate cyclase. Downstream pathways activated include those involving protein kinase C (PKC), protein kinase A (PKA), mitogen-activated protein kinase (extracellular signal-related kinase, ERK), and components of the focal adhesions.

Activation of the insert-negative isoforms of the CTR can also induce mobilization of intracellular calcium. In osteoclasts, there is evidence that signaling through both cAMP and intracellular calcium is important in CT action. Inhibition of osteoclast-mediated bone resorption by CT can be mimicked by dibutyryl cAMP and phorbol esters or blocked by protein kinase inhibitors. Thus, coupling of the CTR to different G-proteins can activate adenylate cyclase as well as phospholipase C. In the latter case, coupling of the CTR to $G_q$ can lead to increased intracellular triphosphate levels and thence to increased cytosolic calcium, which, together with co-liberated diacylglycerol, activates protein kinase C. In brain tissue, CT apparently couples primarily to G-proteins other than $G_s$, based on the limited evidence of activation of adenylate cyclase in neural tissue. In hepatocytes, CT-induced activation of adenylate cyclase has not been shown, but CT, even at very low concentrations, is capable of increasing cytosolic calcium, and CT-induced differentiation of early rat embryos is dependent on intracellular calcium mobilization. In LLC-PK1, pig kidney cells, CT can induce changes mediated by either cAMP or intracellular calcium, in a cell-cycle-dependent manner. Finally, expression of cloned receptors in several cell types has conclusively shown that CTRs of human, rat, and porcine origin are capable of signaling through both cAMP- and calcium-activated second-messenger systems. Study of cell lines expressing different CTR levels has suggested that the magnitude of this response is proportional to the receptor density, a common finding also in other receptor systems. This phenomenon has been described, for example, for the thyrotropin-releasing hormone and PTH receptors, so that relative receptor density in target tissues can influence the signaling pathways that are activated in particular cells.

An interesting "calcium-sensing" function of the CTR has been reported, whereby CTR-bearing cells are rendered sensitive to extracellular calcium in terms of increased cytosolic calcium. Thus, CT treatment of CTR-bearing cells, in the presence of extracellular calcium, initiates a sustained rise in intracellular calcium level, the extent of which is dependent on the concentration of the extracellular calcium. Because osteoclasts, which express high levels of CTR, are reportedly exposed to calcium concentrations as high as 26 m*M* during bone resorption, this phenomenon may have particular relevance for this cell type. In fact, CT and extracellular calcium can both cause intracellular calcium transients in isolated osteoclasts and, interestingly, CT and extracellular calcium greatly augment the signal produced by either agent alone.

In addition to cAMP- and $Ca^{2+}$-mediated signaling, CTR-mediated activation of the MAP kinase (MAPK) pathway was also recently described (Fig. 4). Rapid or sustained activation of MAPK has been observed under different circumstances, the former apparently involving both $G_i$ and $G_q$ mechanisms. Evidence for coupling of CTRs to $G_i$ was seen in cells overexpressing the C1b CTR isoform, in which pertussis toxin enhanced the cAMP response to CT. The means by which CTR activation leads to MAPK activation requires further examination, and, in particular, consideration of a possible role for βγ G-protein subunits, as is commonly the case for other G-protein-coupled receptors. There is also evidence that CT can influence cell attachment mechanisms by modulating components of focal adhesions and the cytoskeleton. CT can induce phosphorylation of the focal adhesion-associated protein, human enhancer of filamentum 1 (HEF 1), paxillin, and focal adhesion kinase (FAK), and the association of these latter two proteins with HEF 1. This effect of CT requires cell attachment and the integrity of the cytoskeleton, and involves c-Src. CT treatment of cells can thus result in complex effects on cell attachment mechanisms, although it is not yet clear how these *in vitro* results relate to the actions of CT *in vivo*.

## D. Receptor Regulation

The cell surface expression of receptors is, in general, tightly regulated, and both the absolute amount of cell surface receptor and the affinity of the receptor for ligand can be modulated. The CTR is subject to regulation both by CT (homologous regulation) and by other agents (heterologous regulation). Calcitonin-induced CTR down-regulation has been demonstrated in various transformed CTR-expressing cell lines, primary kidney cell cultures and osteoclasts. The CT-induced loss of CTRs in osteoclasts has been proposed as a mechanism to account for the well-known loss of responsiveness by patients to CT on repeated administration clinically. This refractoriness has been termed “escape.” Down-regulation of the CTR is mediated by specific loss of cell surface receptors, which occurs by an energy-dependent internalization of the ligand–receptor complex. Prevention of lysosomal degradation also prevents loss of cell surface receptors, indicating that the principal internalization pathway involves processing of the receptor–ligand complex into lysosomes and subsequent degradation of the receptor. Prevention of lysosomal recycling does not influence receptor levels, suggesting that the CTRs are unlikely to be recycled. Down-regulation of CTRs *in vivo* has been observed in the kidney, in animals chronically administered CT and in animals with CT-secreting tumors. Down-regulation of CTRs is accompanied by desensitization of responses to CT, in particular activation of adenylate cyclase. This presumably occurs via uncoupling of the CTR from G-proteins.

Acutely, down-regulation of receptors is not mimicked by activators of either protein kinase A (PKA) or protein kinase C (PKC), and may be dependent on G-protein receptor kinases. The intracellular C-terminal tail of the CTR is subject to phosphorylation by second messenger-dependent and second messenger-independent kinases following agonist activation of the receptor, and it has been shown that integrity of the C-terminal tail is important for cellular internalization of the CTR. However, the mechanisms of CTR expression by homologous down-regulation appear to be cell-type dependent. For example, in mouse or rat osteoclasts, a potent down-regulation of CTR mRNA appears to be mediated by a cAMP-dependent mechanism (Fig. 5), in addition to down-regulation of the receptor by internalization. The mechanism of CT-induced receptor mRNA loss in osteoclasts is due principally to destabilization of receptor mRNA. The 3′-untranslated regions of mouse and rat CTR mRNAs contain four AUUUA motifs, as well as other A/U-rich domains and a large number of poly(U) regions. Such motifs, commonly found in cytokines and oncogenes, function as signals for rapid mRNA inactivation. Interestingly, CTR regulation in human osteoclasts differs from that in mice in that receptor down-regulation and CT-induced receptor mRNA loss appear to be due to PKC-mediated events, rather than involving cAMP (Fig. 5). In nonosteoclastic cells, regulation of the CTR was found to be mechanistically distinct from that in osteoclasts. In a number of cell types, CTR down-regulation was cAMP-independent and did not involve reduced CTR

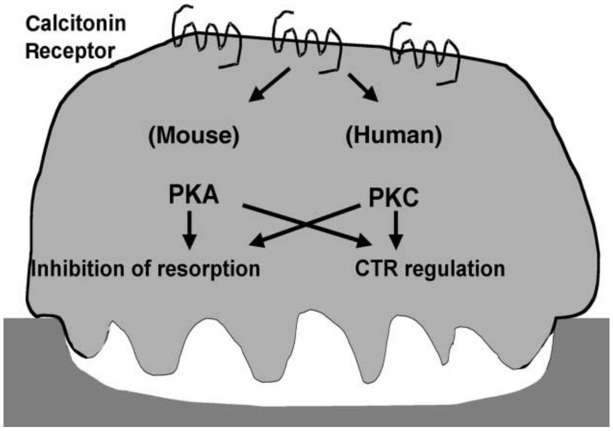

FIGURE 5 Species specificity of CTR-mediated signaling in osteoclasts. Mammalian osteoclasts express abundant CTRs and CT is a potent inhibitor of osteoclast resorptive activity. CT also causes rapid and prolonged down-regulation of the CTRs in osteoclasts. However, the signaling pathways activated in osteoclasts are species specific, with inhibition of osteoclast activity and CTR regulation due primarily to PKA activation in mouse osteoclasts and due primarily to activation of PKC in human osteoclasts.

mRNA levels. It has now been found that the CTR gene in the mouse has at least three promoters and that one of these, P3, appears specific for osteoclasts. Different promoter usage may provide a mechanism for the tissue-specific regulation of the CTR.

An additional interesting aspect of CT-induced CTR regulation in osteoclasts is that glucocorticoid treatment can substantially prevent the loss of CTRs. In the mouse, glucocorticoid treatment was shown by nuclear run-on analysis to increase transcription of the CTR gene. It is worth noting that clinical evidence suggests that glucocorticoids, when administered with CT, might prevent to some extent the CT-induced resistance to its own action. It is also worth noting that the degree of internalization of human CTRs appears to be isoform specific, with the $I_{1+}$ variant being resistant to internalization. Thus, the regulation of receptors, and consequently peptide responses, may also vary according to the level of specific receptor isoforms present in each tissue.

Heterologous regulation of the CTR by other agents has also been found. As previously indicated, in mouse and human osteoclast cultures, glucocorticoids increase the level of cell surface CTR expression following up-regulation of receptor mRNA levels; this is an effect mediated at the level of transcription. Similarly, the CT-mediated decrease in cell surface receptors and mRNAs is attenuated by dexamethasone. Increased production of CTRs in response to glucocorticoid stimulation also occurs in the human T47D breast cancer cell line, which requires cortisol for expression of CTRs, suggesting that this may be a common regulatory mechanism for induction of CTR expression. Transforming growth factor-β (TGF-β) also increases CTR levels in human blood monocyte cultures; this is perhaps related to induction of cellular differentiation toward the osteoclast lineage. As with homologous CTR regulation, responses to heterologous agents are cell-type specific. Thus, in UMR106-06 cells, TGF-β reduces the cell surface CTR levels. CTR expression is an early marker for differentiation of cells toward osteoclasts, and indeed is considered the most reliable marker for monitoring osteoclast differentiation. Thus, factors that induce osteoclasts induce CTRs as part of this process. A recent example of this is the combination of macrophage colony-stimulating factor (M-CSF) and the soluble osteoclast differentiation factor, RANK ligand, which can induce CTR expression in human or mouse monocytes or mouse spleen cells, secondary to promotion of osteoclast differentiation.

## VII. SUMMARY

There is much yet to learn about the actions and role of CT. It will be important to better understand the physicochemistry of CT–CTR interactions, and the pharmacokinetics of CT, so that small-molecule CT mimetics and more potent, differently delivered, longer acting forms of the molecule can be designed to control bone resorption and turnover. An understanding of the actions of CT in inflammation and stress, in the CNS, in blastocyst implantation and development, in cancer, and in cell growth and morphogenesis could well have interesting and unexpected consequences. The recent recognition that the CTR can also interact with other peptides by collaborating with RAMPs provides additional complexity to CT physiology. The study of calcitonin in and well beyond its role as a calcium-regulating and bone-sparing hormone will continue to provide insights of biological interest and of importance in our understanding of health and disease.

## Glossary

**calcitonin** A 32-amino-acid peptide hormone produced by the C cells of the thyroid. Immunologically similar molecules have also been identified in other tissues, suggesting additional local autocrine or paracrine actions of calcitonin.

**G-proteins** Family of intracellular proteins that act as linking molecules or adapters to link receptors to effector molecules. G-Proteins are heterotrimers of $\alpha\beta\gamma$ subunits and are activated by receptor binding, which enables them to bind GTP and to dissociate into $\alpha$ and $\beta\gamma$ subunits, which in turn activate molecules such as adenylate cyclase and phospholipase C. Activation is terminated by hydrolysis of GTP to GDP.

**isoforms** Different but related forms of protein molecules. Different isoforms of the calcitonin receptor arise by differential splicing of the primary mRNA transcript. This means that the CTR mRNA, which is transcribed from a single gene, can be further processed (spliced) to remove portions of mRNA that correspond to particular exons of the CTR gene. The different mRNA species then code for proteins that contain or exclude portions of the receptor, and this has functional consequences.

**osteoclasts** Large, multinucleated cells that are responsible for the resorption of bone in the cycle of bone turnover that comprises bone removal and bone formation. Osteoclasts are sensitive to CT, which rapidly and potently inhibits their bone-resorbing activity.

**receptor** A protein molecule that recognizes and binds its cognate ligand with high affinity and specificity. The calcitonin receptor is a cell surface receptor with extracellular domains that interact with the calcitonin molecule (ligand). The intracellular domains interact with intracellular signal transduction molecules to translate ligand binding into cellular actions.

## See Also the Following Articles

**Calcium Signaling • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Humoral Hypercalcemia of Malignancy • Osteoporosis: Hormonal Treatment • Parathyroid Hormone**

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# Calcium Signaling

STANKO S. STOJILKOVIC
*National Institute of Child Health and Human Development, Maryland*

I. INTRODUCTION
II. RECEPTOR-CONTROLLED CALCIUM MOBILIZATION
III. RECEPTOR-CONTROLLED VOLTAGE-SENSITIVE CALCIUM INFLUX
IV. CALCIUM MOBILIZATION THROUGH RYANODINE RECEPTOR CHANNELS
V. CALCIUM INFLUX THROUGH EXTRACELLULAR LIGAND-GATED CHANNELS
VI. CALCIUM SIGNALING PATTERNS
VII. CALCIUM-CONTROLLED CELLULAR MACROMOLECULES AND PROCESSES
VIII. CONCLUSIONS

**G-proteins** Family of intracellular proteins that act as linking molecules or adapters to link receptors to effector molecules. G-Proteins are heterotrimers of αβγ subunits and are activated by receptor binding, which enables them to bind GTP and to dissociate into α and βγ subunits, which in turn activate molecules such as adenylate cyclase and phospholipase C. Activation is terminated by hydrolysis of GTP to GDP.

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# Calcium Signaling

Stanko S. Stojilkovic

*National Institute of Child Health and Human Development, Maryland*

I. INTRODUCTION
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VI. CALCIUM SIGNALING PATTERNS
VII. CALCIUM-CONTROLLED CELLULAR MACROMOLECULES AND PROCESSES
VIII. CONCLUSIONS

**Calcium acts as both an extracellular (first) and an intracellular (second) messenger in biological systems. Intracellular calcium signals are generated by activation of ionic channels and pathways in plasma membrane, endoplasmic and sarcoplasmic reticula, mitochondria, and cell nuclei. Calcium signals vary with respect of their spatial and temporal characteristics and frequency and amplitude coding. Calcium functions are versatile and universal in living systems: triggering life at fertilization, controlling cell division and differentiation, modulating the subsequent activity of cells, and ultimately signaling apoptosis.**

## I. INTRODUCTION

Over 100 hormones and neurotransmitters, acting on their respective receptors and receptor channels, relay their signals by altering intracellular calcium concentrations ($[Ca^{2+}]_i$). For the generation of intracellular $Ca^{2+}$ signals, cells depend on pathways that facilitate calcium release (i.e., $Ca^{2+}$ mobilization) from the endoplasmic reticulum (ER) and the sarcoplasmic reticulum (SR) and/or calcium influx across the plasma membrane. Calcium mobilization occurs in both nonexcitable and excitable cells and is mediated through $Ca^{2+}$ channels that are associated with intracellular organelles. These channels belong to two major classes: inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive receptor channels ($InsP_3Rs$) and ryanodine-sensitive calcium release channels (RyRs). The phospholipase C-controlled production of $InsP_3$ is essential in the initiation of $Ca^{2+}$ release through $InsP_3Rs$, and this family of enzymes is controlled by several G-protein-coupled receptors as well as by some members of tyrosine kinase receptors. $InsP_3$-controlled $Ca^{2+}$ release is also commonly associated with $Ca^{2+}$ influx; $Ca^{2+}$ release predominates during the early phase of agonist stimulation, whereas $Ca^{2+}$ entry is essential during sustained agonist stimulation. In nonexcitable cells, $Ca^{2+}$ influx occurs through store-operated $Ca^{2+}$ channels. In excitable cells, these channels may also be expressed and act in conjunction with voltage-gated calcium channels (VGCCs). RyRs are expressed in the ER and SR membranes and are sensitive to the plant alkaloid ryanodine, after which they are named. At the present time, it is unknown whether they operate as intracellular ligand-gated channels. However, $InsP_3$-induced $Ca^{2+}$ release and action potential (AP)-driven $Ca^{2+}$ influx through VGCCs signal the release of intracellular $Ca^{2+}$ through RyRs, a process known as $Ca^{2+}$-induced $Ca^{2+}$ release.

Calcium influx can also occur independently of the status of calcium release pathways. There are two major mechanisms by which receptors control calcium influx: VGCCs and external ligand-gated channels. VGCCs are highly selective for $Ca^{2+}$ and are opened by a decrease in the electrical potential across the plasma membrane ($V_m$), which is the membrane depolarization. In excitable cells, this pathway is typified by the firing of $Ca^{2+}$-dependent APs. Cells can fire APs spontaneously and/or in response to receptor activation. Hormones can also inhibit spontaneous electrical activity and associated $Ca^{2+}$ signals. In general, receptors can modulate firing of APs directly, by $\beta\gamma$-subunits of G-proteins, or indirectly, by intracellular messengers activated by these receptors. Receptor-induced modulation of AP-driven $Ca^{2+}$ entry is characterized by changes in AP amplitude, duration, or firing frequency; all of which alter $Ca^{2+}$ influx. On the other hand, ligand-gated receptor channels, including nicotinic acetylcholine receptor channels, glutamate receptor channels, and adenosine 5′-triphosphate (ATP) receptor channels, require the binding of an extracellular ligand to open the channel pore. Many of these channels are permeable to $Ca^{2+}$, but also conduct other ions to control membrane excitability.

Spontaneous and agonist-induced $Ca^{2+}$ signals can exhibit different spatial and temporal patterns. Calcium signals can be localized within the cytoplasm, such as those driven by electrical activity, but it can also be propagated within the cell (intracellular $Ca^{2+}$ waves) or between cells (intercellular $Ca^{2+}$ waves) to produce more global $Ca^{2+}$ signals. Some cells respond to agonist stimulation with nonoscillatory rises in $[Ca^{2+}]_i$, whereas others respond with oscillatory increases in $[Ca^{2+}]_i$. The complexity of the temporal and spatial organization of $Ca^{2+}$ signals is in accord with the numerous intracellular $Ca^{2+}$ functions, including control of mitochondrial metabolism, "packaging" in the trans-Golgi network, exocytosis of secretory vesicles, and control of gene expression in the nucleus.

## II. RECEPTOR-CONTROLLED CALCIUM MOBILIZATION

In nonexcitable cells, receptor-controlled $Ca^{2+}$ mobilization is the major pathway for $Ca^{2+}$ signaling. This pathway is also operative in excitable cells. In both cell types, calcium-mobilizing receptors coupled to $G_q/G_{11}$ (gonadotropin-releasing hormone [GnRH], thyrotropin-releasing hormone [TRH], angiotensin II, endothelin, vasopressin receptors, etc.), as well as several receptors coupled to pertussis-toxin-sensitive $G_i$ (luteinizing hormone, dopamine D2, serotonin 5HTlc, muscarinic m2, formyl-methionyl-leucyl-phenylalanine receptors, etc.), activate phospholipase C-β, whereas tyrosine kinase receptors activate phospholipase C-γ. Both enzymes hydrolyze membrane-associated phosphatidylinositol 4,5-bisphosphate to increase the production of $InsP_3$ and diacylglycerol. Diacylglycerol remains in the plasma membrane, where it acts on protein kinase C, whereas $InsP_3$ rapidly diffuses into the cytosol to release $Ca^{2+}$ from a fraction of the nonmitochondrial stores containing $InsP_3Rs$.

Purification and functional reconstitution of $InsP_3Rs$ demonstrate that the binding sites for $InsP_3$ are on the same protein that makes up the $Ca^{2+}$ release channels. This channel is composed of four similar subunits forming a four-leaf clover-like structure, the center of which makes the $Ca^{2+}$-selective channel. Three subtypes of these receptor channels have been identified and most cells express multiple isoforms of $InsP_3Rs$. These channels are common for all cells and are localized in the ER membrane, nuclear membrane, and possibly the plasma membrane in some cell types. Functionally reconstituted purified $InsP_3Rs$ respond to $InsP_3$ with an increase in the probability of the open state due to a large conformational change. The release of $Ca^{2+}$ is electrically compensated by an inward potassium flux. The $InsP_3$ binding sites are located within the first 788 residues of the N-terminus of each subunit. In addition to $InsP_3$, cytosolic $Ca^{2+}$ is the major messenger controlling $InP_3R$ gating. Investigation of the single-channel function of type 1, type 2, and type 3 $InsP_3R$ has revealed isoform-specific properties in terms of isoform sensitivity to $InsP_3$ and $Ca^{2+}$. Several other factors also modulate the activity of $IP_3Rs$, including lumenal calcium, protein kinase A, protein kinase C, calcium/calmodulin-dependent protein kinase II, adenine nucleotides, and pH.

Activation of $InsP_3$-dependent $Ca^{2+}$ mobilization is associated with depletion of the ER calcium pool, and $Ca^{2+}$ entry through $Ca^{2+}$-conducting channels is essential to sustaining agonist-induced $Ca^{2+}$ spiking and refilling of intracellular $Ca^{2+}$ pools on termination of receptor activation. The mechanism of activation of voltage-insensitive $Ca^{2+}$ influx pathways has been termed "capacitative calcium entry," indicating that intracellular $Ca^{2+}$ stores prevent entry when they are charged (filled by $Ca^{2+}$), but promote entry as soon as the stored $Ca^{2+}$ is discharged (released). Because depletion of the ER $Ca^{2+}$ stores is followed by the influx of $Ca^{2+}$ into the cell, the channels involved in such influx are termed "store-operated $Ca^{2+}$ channels" (SOCCs). At the present time, the nature of these channels and the mechanism of their regulation in response to store depletion are unknown. The fruit fly *trp* gene and its mammalian counterparts have been considered to encode components of SOCCs. Others have proposed CaT1 protein as a constituent of the ion-conducting pore of SOCCs. According to one theory, a diffusible signal (calcium influx factor) is released from the ER during store depletion and binds to and activates SOCCs. The other model proposes that depletion of $Ca^{2+}$ stores leads to a conformation change in $InsP_3Rs$, which can then bind to SOCCs (Fig. 1).

In excitable cells, calcium-mobilizing receptors, including GnRH, bradykinin, opioid, substance P, ATP, adrenergic, TRH, and angiotensin II receptors, also stimulate or inhibit voltage-gated $Ca^{2+}$ influx by altering the $V_m$. During the initial receptor activation, agonist-induced release of $Ca^{2+}$ from the ER opens $Ca^{2+}$-activated $K^+$ or $Cl^-$ channels that hyperpolarize the membrane. This in turn inhibits AP firing and the associated $Ca^{2+}$ influx, preventing a lethal increase in $[Ca^{2+}]_i$. The depletion of the ER calcium pool terminates activation of these channels, frequently leading to depolarization of cells and activation of voltage-gated calcium influx. Several channels have been implicated in sustained receptor-induced depolarization of cells, including non-elective cationic channels and SOCCs, and M-type voltage-dependent $K^+$ channels (Fig. 1).

## III. RECEPTOR-CONTROLLED VOLTAGE-SENSITIVE CALCIUM INFLUX

Stimulation of $Ca^{2+}$ influx through VGCCs is the major pathway for $Ca^{2+}$ signaling in excitable cells. VGCCs are large multisubunit proteins that span the plasma membrane to provide a $Ca^{2+}$-selective pathway into the cytosol. Biophysical and pharmacological studies have identified multiple subtypes of VGCCs that can be distinguished by their ion selectivity, single-channel conductance, pharmacology, metabolic regulation, and tissue localization. Consistent with functional studies, molecular cloning has identified several genes that encode different VGCC subtypes. The first $Ca^{2+}$ channel was purified from skeletal muscle, a highly enriched source of L-type $Ca^{2+}$ channels. Purification of the channel identified five subunits, including a large $\alpha_1$ subunit and four smaller ancillary subunits, $\alpha_2$, β, γ, and δ.

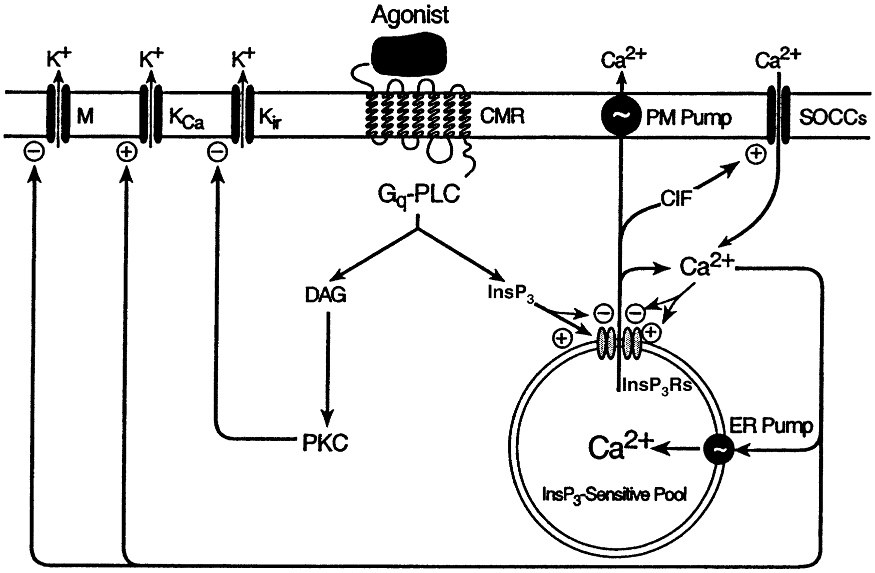

FIGURE 1 A calcium-mobilizing receptor (CMR) and calcium signaling. Activation of cell surface receptors by peptide hormones results in the production of inositol 1,4,5-trisphosphate (InsP$_3$) via G-protein and phospholipase C (PLC). InsP$_3$ binds to its receptors (InsP$_3$Rs) on the endoplasmic reticulum (ER), resulting in release of Ca$^{2+}$ and a diffusible signal, the calcium influx factor (CIF). Ca$^{2+}$ acts as a coagonist in the control of InsP$_3$R permeability. When the receptor is occupied by InsP$_3$, low concentrations of calcium facilitate Ca$^{2+}$ release from the ER, and subsequently the elevated [Ca$^{2+}$]$_i$ inhibits InsP$_3$Rs. During prolonged occupation of the receptor, InsP$_3$ may also inhibit InsP$_3$Rs. CIF binds to and activates store-operated calcium channels (SOCCs) on the plasma membrane (PM). An alternative model proposes that release of Ca$^{2+}$ from the ER leads to a conformation change in the InsP$_3$R, which can then bind to SOCCs (not shown). In excitable cells expressing CMRs, discharge of Ca$^{2+}$ from the ER activates Ca$^{2+}$-controlled potassium channels (K$_{Ca}$), leading to a transient hyperpolarization of cells. During sustained agonist stimulation, calcium inhibits M-type potassium channels, an action that depolarizes cells and facilitates voltage-gated Ca$^{2+}$ influx. Protein kinase C (PKC)-mediated inhibition of spontaneously active inward rectifier potassium channels (K$_{ir}$) has similar effects on voltage-gated Ca$^{2+}$ influx. Finally, in excitable cells expressing SOCCs, activation of these channels leads to depolarization of cells and facilitation of voltage-gated Ca$^{2+}$ influx. DAG, diacylglycerol.

The $\alpha_1$ subunit consists of four homologous repeats, each one composed of six transmembrane segments. Located within the $\alpha_1$ subuint are the voltage sensor, gating machinery, channel pore, and multiple protein kinase A- and C-dependent phosphorylation sites. Since the first $\alpha_1$ subunit was cloned from skeletal muscle, at least seven isoforms have been identified, including $\alpha_{1S}$, $\alpha_{1C}$, $\alpha_{1D}$, $\alpha_{1B}$, $\alpha_{1A}$, $\alpha_{1E}$, and $\alpha_{1G}$.

Voltage-gated Ca$^{2+}$ channels serve two major functions in excitable cells; one is to generate and/or shape action potentials and the other is to allow Ca$^{2+}$ influx during the transient depolarization. Membrane depolarization increases the probability that VGCCs will open, whereas membrane hyperpolarization decreases this probability. In some cells, VGCCs account entirely for the upstroke of an AP. In other cells, voltage-gated Na$^+$ and Ca$^{2+}$ channels contribute to the upstroke of the AP. In spontaneously quiescent cells, G-protein-coupled receptors can initiate AP firing by depolarizing cells. In spontaneously firing cells, G protein-coupled receptors can further depolarize cells and stimulate AP-driven Ca$^{2+}$ entry, as well as inhibit it by hyperpolarizing cells. Receptor-mediated stimulation and inhibition of AP firing can be regulated by direct actions on VGCCs and/or indirectly through other plasma membrane ionic channels. In general, G$_q$/G$_{11}$-coupled receptors can modulate voltage-gated Ca$^{2+}$ influx via [Ca$^{2+}$]$_i$/protein kinase C-dependent pathways; G$_i$/G$_o$-coupled receptors modulate via $\beta\gamma$ dimers and G$_s$-coupled receptors modulate via cAMP/protein kinase A signaling pathways (Fig. 2).

## A. Direct Modulation of Voltage-Gated Calcium Influx

Receptors modulate the gating properties of VGCCs by two pathways. Inhibition of VGGCs is controlled by the fast membrane-delimited pathway, in which the $\beta\gamma$ dimer of the G$_i$/G$_o$ protein is a direct

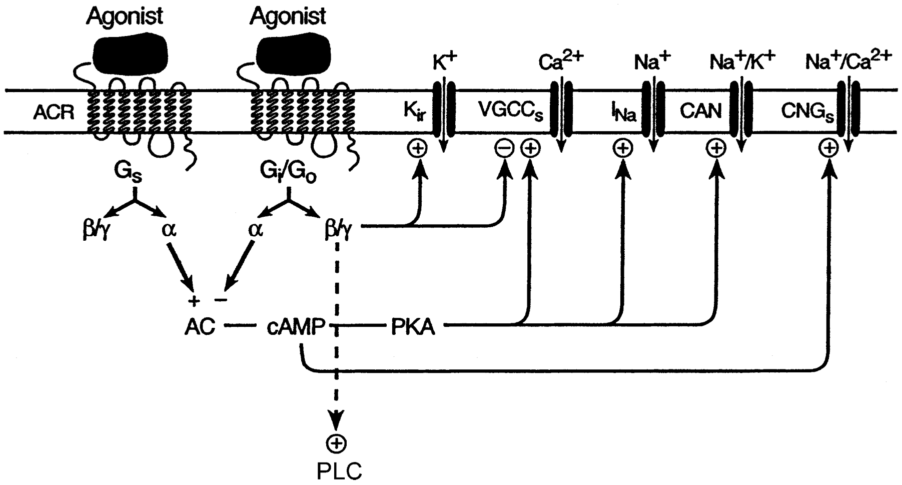

FIGURE 2 Adenylyl cyclase-coupled receptor (ACR) and calcium signaling. ACRs modulate voltage-gated $Ca^{2+}$ influx by the direct actions of G-protein subunits on plasma membrane inward rectifier potassium ($K_{ir}$) and voltage-gated calcium channels (VGCCs), as well as through protein kinase A (PKA)-dependent phosphorylation of VGCCs, tetrodotoxin-insensitive sodium channels ($I_{Na}$), and nonselective cationic channels. In cells expressing cyclic nucleotide-gated channels (CNGs), cAMP mediates the action of $G_s$-coupled receptors. In addition to "classical" $G_q/G_{11}$-coupled $Ca^{2+}$-mobilizing receptors, $G_i$-coupled receptors can also stimulate PLC, leading to production of inositol 1,4,5-trisphosphate and facilitation of $Ca^{2+}$ release from the endoplasmic reticulum (see Fig. 1).

intermediate between the plasma membrane receptor and VGCCs. In the second pathway, diffusion of cytoplasmic messengers mediates the stimulatory action of the G-protein-coupled receptor. The essential structural elements required for G-protein modulation are found in VGCCs, supporting the direct action of G-proteins. Inhibition of the $Ca^{2+}$ channel (N- and P/Q-types) mediated by βγ dimer is a time- and voltage-dependent process. A fraction of the total $Ca^{2+}$ channels open much more slowly and require larger depolarization to open; this produces a slowing of the activation kinetics and a reduction in the current amplitude. The G-protein-dependent inhibition of these channels can be reversed by hormones acting through the protein kinase C pathway. Facilitation of $Ca^{2+}$ influx through L-type VGCCs can occur by protein kinase A-dependent phosphorylation of the $\alpha_1$ and/or β subunits. On the other hand, protein kinase C-dependent phosphorylation leads to inhibition of voltage-gated calcium influx. Cytosolic calcium also inactivates L-type calcium channels and facilitates P/Q-type $Ca^{2+}$ channels, both in a calmodulin-dependent manner.

## B. Indirect Modulation of Voltage-Gated Calcium Influx

The most well-characterized mechanism by which G-protein-coupled receptors indirectly modulate voltage-gated $Ca^{2+}$ influx is that mediated through activation/inhibition of inward rectifier potassium ($K_{ir}$) channels. In general, inhibition of $K_{ir}$ depolarizes the membrane to increase excitability, whereas activation hyperpolarizes the membrane to decrease excitability. These channels are susceptible to modulation by a wide range of hormones and neurotransmitters that exert their actions via G-zproteins directly or via several different intracellular messenger pathways, including protein kinases A and C, intercellular ATP, and pH. In pituitary melanotrophs and lactotrophs, dopamine activates $K_{ir}$ channels via the fast membrane-delimited pathway to hyperpolarize the membrane, causing a cessation of AP firing and a decrease in $[Ca^{2+}]_i$ and hormone secretion. Like dopamine, somatostatin stimulates $K_{ir}$ channels via a βγ dimer to reduce membrane excitability and GH secretion in pituitary somatotrophs. On the other hand, in pituitary corticotrophs, corticotropin-releasing factor inhibits the $K_{ir}$ via protein kinase A-dependent pathways to increase AP frequency and associated $Ca^{2+}$ entry. TRH can also inhibit $K_{ir}$ channels in GH3 cells and pituitary lactotrophs, presumably via the protein kinase C pathway, leading to an increase in membrane excitability (Fig. 3). G-Protein-coupled receptors can also modulate voltage-gated $Ca^{2+}$ influx via tetrodotoxin (TTX)-sensitive and -insensitive $Na^+$ channels, $Na^+/Ca^{2+}$-conducting ligand-gated channels, and nonselective

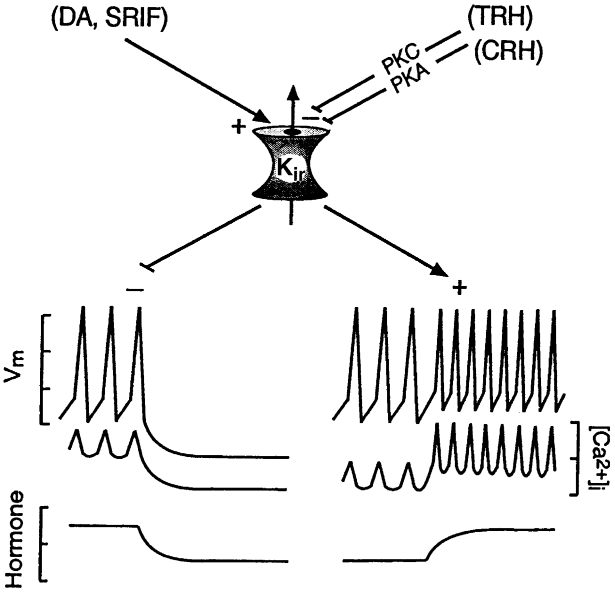

FIGURE 3 Receptor-controlled inward rectifier potassium current ($K_{ir}$) and membrane excitability. A number of receptors negatively coupled to adenylyl cyclase stimulate type 3 $K_{ir}$ channels, leading to membrane hyperpolarization and abolition of spontaneous electrical activity, a decrease in $[Ca^{2+}]_i$, and a concomitant decrease in hormone secretion. Calcium-mobilizing receptors and those positively coupled to adenylyl cyclase inhibit type 1 and type 2 $K_{ir}$ channels in a protein kinase C (PKC)- and protein kinase A (PKA)-dependent manner, leading to depolarization of cells and an increase in action potential frequency, which increases $[Ca^{2+}]_i$ and augments hormone secretion. DA, dopamine; SRIF, somatotropin release-inhibiting factor; TRH, thyrotropin-releasing hormone; CRH, corticotropin-releasing hormone.

cationic channels. For example, in pituitary somatotrophs growth hormone-releasing hormone (GHRH) activates TTX-insensitive $Na^+$ channels via a cAMP/protein kinase A signaling pathway, leading to cell membrane depolarization and an increase in AP frequency and $[Ca^{2+}]_i$.

## IV. CALCIUM MOBILIZATION THROUGH RYANODINE RECEPTOR CHANNELS

RyRs were originally identified as $Ca^{2+}$ release channels expressed in the SR of skeletal muscle fibers and cardiac myocytes, where they play a central role in excitation–contraction coupling. These channels are also expressed in neurons, chromaffin cells, sea urchin eggs, and several nonexcitable cell types. Mammalian tissues express three isoforms: $RyR_1$ is expressed predominantly in skeletal muscle, $RyR_2$ is expressed in cardiac muscle, and $RyR_3$ has a wide tissue distribution, including the endocrine cells. $RyR_1$ and $RyR_2$ channels display a 66% identity, whereas the $RyR_3$ channel is much shorter. Like the $InsP_3Rs$, RyRs are tetramers, with a large N-terminal region forming heads, and a C-terminal region that forms the $Ca^{2+}$-selective channel. Although these channels are frequently co-expressed with $InsP_3Rs$, the physiological importance of their co-expression and their variable density within the cells are still largely unclear.

Cytosolic $Ca^{2+}$ is a major regulator of RyRs; at low concentrations, $Ca^{2+}$ promotes release, whereas higher concentrations are inhibitory. However, inhibition of RyRs by high $[Ca^{2+}]_i$ is somewhat controversial, because it requires $[Ca^{2+}]_i$ to be in the millimolar concentration range, which is not reached under physiological conditions. The ability of $Ca^{2+}$ to stimulate its release from the ER/SR via RyRs is called "$Ca^{2+}$-induced $Ca^{2+}$ release." This process is of fundamental importance for coordinating the elementary $Ca^{2+}$ release events into $Ca^{2+}$ spikes and waves. Unlike $InsP_3Rs$, RyRs can release $Ca^{2+}$ in response to an increase in $[Ca^{2+}]_i$, without any other change in the concentration of second messengers. This is crucial for excitation–contraction coupling. For example, in cardiac cells, $Ca^{2+}$ entry through dihydropyridine-sensitive channels activates RyRs to induce a further increase in $[Ca^{2+}]_i$. In skeletal muscle cells, the dihydropyridine receptors act primarily as voltage sensors to activate RyRs directly in response to $V_m$ depolarization. In neuroendocrine and endocrine cells, activation of RyRs coincides with stimulation of phospholipase C signaling pathway, but also can be coupled with electrical activity, for example, in pancreatic beta cells. In addition to $Ca^{2+}$, there are numerous other endogenous modulators of RyRs. These include, ER/SR $Ca^{2+}$, cytosolic pH, $Mg^{2+}$ and other cations, $Cl^-$ and other anions, nucleotides, cyclic adenosine 5′-diphosphate ribose, several protein kinases, calmodulin, and other $Ca^{2+}$-binding proteins.

## V. CALCIUM INFLUX THROUGH EXTRACELLULAR LIGAND-GATED CHANNELS

Extracellular ligand-gated receptor channels differ from other $Ca^{2+}$-conducting plasma membrane channels in three respects. First, their activation depends on the delivery and binding of a ligand to the extracellular domain. Second, termination of their activities requires removal of the ligand, which is usually mediated by a specific pathway for ligand degradation and/or uptake. Third, they are not highly selective for $Ca^{2+}$, but conduct other ions as well. Because ligand-gated channels are generally activated by neurotransmitters, they are also known as neurotransmitter-controlled channels. However, some of these channels can be controlled through hormonal transmission, i.e., a ligand for a particular receptor channel can be colocalized with hormones in secretory vesicles and cosecreted on cell activation. Once released, the ligand can act in an autocrine and/or paracrine manner.

There are two classes of ligand-gated channels, the excitatory cation-selective receptor channels, operated by acetylcholine, glutamate, 5-hydroxytryptamine (5-HT), and ATP, and the inhibitory anion-selective receptor channels, activated by γ-aminobutyric acid (GABA) and glycine. Structural information obtained by cDNA cloning of ligand-gated receptor channels has led to the identification of several families of evolutionarily related proteins. The $5\text{-HT}_3$, GABA, and glycine receptor channels possess structural features similar to those of the nicotinic acetylcholine receptor channel, hence these receptors can be grouped as one family. These channels are composed of five subunits (pentamers), each of which contributes to the ionic pore. Each subunit has a large extracellular amino-terminal region followed by four hydrophobic putative membrane-spanning segments and an extracellular carboxyl terminus.

Glutamate receptor channels (GluRs) are traditionally divided into three major subtypes: (1) the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), (2) kainate (KA), and (3) *N*-methyl-D-asparate (NMDA) receptor channels. Molecular cloning revealed numerous subunits for each receptor group. For NMDA receptor channels, NR1 and NR2A–NR2D subunits have been established. For the non-NMDA receptor channels, GluR1–GluR4 denote the AMPA-sensitive family, whereas GluR5–GluR7, KA1, and KA2 denote the kinate subclass. The molecular diversity of GluRs is further increased by variants created by alternative splicing and RNA editing. All GluR subunits contain four hydrophobic segments, labeled as M1-M4, but their M2 segment forms a pore-loop structure, entering and exiting the cell membrane from the intracellular side. The M2 amino acids line the inner channel pore, and specific residues in this segment determine the ion selectivity of the channel. These receptor channels are expressed in neural cells and in neuroendocrine and excitable endocrine cells.

The ATP-gated channels, termed "P2X receptor channels" (P2XRs), have only two putative transmembrane domains, with the amino- and carboxyl-termini facing the cytoplasm. Seven cDNAs $P2X_1$–$P2X_7$, have been expressed individually in different expression systems. There is 35–50% identity and 50–65% similarity in amino acid sequences between pairs of P2XRs, whereas the recombinant receptors characterized differ among themselves with respect to the action of ATP analogues, the desensitization rate, and the effectiveness of antagonists. As with nicotinic and glutamate channels, functional diversity of P2X channels is generated by subunit multimerization. It is believed that functional channels are tetramers and that both hetero- and homotetramers account for the native receptor channels. In contrast to acetylcholine receptor channel and GluRs, P2XRs have been identified in both excitable and nonexcitable cells.

Because of their permeability to $Na^+$ and $Ca^{2+}$, the majority of ligand-gated channels act as a $Ca^{2+}$ influx pathway and as a modulator of AP-driven $Ca^{2+}$ influx. In pituitary gonadotrophs, for example, activation of P2XRs drives $Ca^{2+}$ into the cell to initiate AP firing in quiescent cells or to increase AP frequency and associated $Ca^{2+}$ entry in spontaneously active cells. Because ATP is cosecreted with other neurotransmitters or hormones, it may bind to its receptors to amplify agonist-induced $Ca^{2+}$ signals and secretory responses by further increasing AP-driven $Ca^{2+}$ entry. Activation of $GABA_A$ receptor channels in GnRH-secreting neurons also depolarizes the membrane to increase membrane excitability. Unlike P2XRs, however, activation of $GABA_A$ receptor channels increases the membrane permeability to chloride ions, which diffuse out of the cell to depolarize the membrane. In other cells, $GABA_A$ receptors decrease excitability due to hyperpolarization of the plasma membrane. The mechanisms of desensitization of ligand-gated channels and hormonal control of this process are incompletely characterized.

## VI. CALCIUM SIGNALING PATTERNS

### A. Localized Calcium Signals

Analysis of images obtained by confocal microscopy of individual cells loaded with $Ca^{2+}$-sensitive fluorescent dyes makes possible the visualization of localized events in $Ca^{2+}$ signaling. These events occur in the vicinity of a single channel or a small group of channels and consist of a brief opening of the channels and a concomitant rise in local $[Ca^{2+}]_i$ of about 2 $\mu$m in diameter; these are termed sparks (in cells expressing RyRs) and blips and puffs (in cells expressing $InsP_3Rs$). At subthreshold agonist concentrations, the two major factors for the generation of localized $Ca^{2+}$ signals are the site of action of the primary stimulus and the density of $Ca^{2+}$-mobilizing receptors in the plasma membrane. The focal and discrete injection of an agonist close to one region of the cell leads to a localized rise in $[Ca^{2+}]_i$ near the region of stimulation. Similarly, the initial rise of $[Ca^{2+}]_i$ in oocytes occurs at the point of sperm entry. In pancreatic acinar cells, the $InsP_3Rs$ are predominantly localized in a zone with secretory granules, providing localized $Ca^{2+}$ signals independent from the regions of stimulation by agonist. Depolarization-driven $Ca^{2+}$ entry also forms several types of localized increases in $[Ca^{2+}]_i$ close to the plasma membrane. At the mouth of an open VGCC, $[Ca^{2+}]_i$ can reach hundreds of micromoles, and such a signal is analogous to the blips generated by the activation of a single $InsP_3R$. These highly localized peaks are transient, with a time scale faster than that of $Ca^{2+}$ binding to its buffers, and are relevant for colocalized processes, such as exocytosis. Conversely, the synchronized activity of several VGCCs can generate bumps, or quantum emission domains, which are localized $Ca^{2+}$ signals that are analogous to sparks and puffs.

## B. Global Calcium Signals

The elementary calcium events may be arrested and remain local $Ca^{2+}$ signals, or they may trigger the spread of $Ca^{2+}$ throughout the cytoplasm to form global $Ca^{2+}$ signals. Both $Ca^{2+}$ release and $Ca^{2+}$ influx can make the transition from local to global signals. Depending on the cell type, $Ca^{2+}$ waves propagate at 5–100 $\mu$m/s. The pattern of $InsP_3Rs$ and RyRs distribution and the size of cells are critical for wave propagation. In $InsP_3R$-dependent wave propagation, the "priming" of cells with $InsP_3$ is required for wave initiation, whereas $Ca^{2+}$ serves as both a positive and a negative feedback element. Depolarization-induced global signals result form the synchronized activity of VGCCs and RyRs, whereby the opening of RyRs is tightly coupled to AP firing.

## C. Oscillatory vs Nonoscillatory Calcium Signals

There is an enormous heterogeneity of $[Ca^{2+}]_i$ signals from one cell type to another. Among cells operated by $Ca^{2+}$-mobilizing receptors, some respond to agonist stimulation by the generation of sinusoidal oscillations, in which agonist concentration regulates the amplitude but not the frequency of $Ca^{2+}$ transients. In other cells, discrete $[Ca^{2+}]_i$ transients or spikes can be observed above a constant baseline level, and this pattern of $[Ca^{2+}]_i$ oscillations is referred to as baseline spiking. Here, the agonist concentration regulates the frequency, but not the spike amplitude. In some cells, both oscillatory patterns are observed, depending on the agonist concentration. Other cells respond with monophasic or biphasic nonoscillatory $Ca^{2+}$ signals in response to agonist stimulation. Monophasic $[Ca^{2+}]_i$ responses are characterized by the generation of a single spike increase in $[Ca^{2+}]_i$. Conversely, biphasic $[Ca^{2+}]_i$ signals can be characterized by the generation of a single transient increase in $[Ca^{2+}]_i$ followed by a sustained increase in $[Ca^{2+}]_i$ of lower amplitude.

## D. Intraorganelle Calcium Signals

Except for the ER and SR, where the mechanism of $Ca^{2+}$ release into and uptake from the cytosol is quite well established, $Ca^{2+}$ fluxes across membranes of other organelles are much less understood. New results are emerging, especially on nuclear and mitochondrial $Ca^{2+}$ transport and regulation. The nuclear membranes share many features with that of the ER, including expression of $InsP_3Rs$ and $Ca^{2+}$-ATPase in the outer membrane, and $InsP_3$ and cADP-ribose-regulated channels in the inner nuclear membrane. Because the inositol lipid cycle also takes place in the nucleus, $Ca^{2+}$ can enter the nucleus directly from perinuclear stores, but can also diffuse from the cytosol through the nuclear pore complex, a large multiprotein structure that provides direct exchange between the cytoplasm and the nucleoplasm. Although research on the control of transport through the pore complex is far from complete, it seems clear that the opening of the pore is regulated by the state of depletion in the perinuclear $Ca^{2+}$ stores. Mitochondria possess their own sophisticated $Ca^{2+}$ efflux/influx systems that are unique compared to the mechanisms taking place at the membranes of the ER and nucleus. Calcium influx occurs via a $Ca^{2+}$ uniporter that is very fast and does not require energy for its operation. Calcium efflux from the mitochondria via two electroneutral antiporters, $2Na^+/Ca^{2+}$ and $2H^+/Ca^{2+}$, requires energy in the form of ATP hydrolysis. Sodium-dependent transport dominates in muscle and neuroendocrine tissues, and sodium-independent transport dominates in the liver and kidney. The mitochondrial system for handling $Ca^{2+}$ constitutes an active element in the formation of $Ca^{2+}$ waves and oscillations, and it can also regulate transient $[Ca^{2+}]_i$ changes, induced by either $V_m$-controlled or $Ca^{2+}$-mobilizing processes.

### E. Intercellular Calcium Waves

In addition to intracellular $Ca^{2+}$ signals, waves of $[Ca^{2+}]_i$ can flow through connected cells to coordinate the cellular activity of a tissue. Intercellular $Ca^{2+}$ waves are characterized by an increase in $[Ca^{2+}]_i$ that crosses cell borders into neighboring cells through direct or indirect contact with the stimulated cell. Intercellular $Ca^{2+}$ waves can be initiated *in vitro* by electrical or mechanical stimulation of a single cell or by focal application of hormones or neurotransmitters. Once initiated, waves travel complex routes for several hundred micrometers at rates of 15 to 25 $\mu$m/s. Intercellular $Ca^{2+}$ waves have been observed in dissociated cell and organotypic cultures of many tissues, including the heart, brain, pituitary, kidney, liver, mammary gland, and lens epithelium. They have also been observed *in situ* using glial cells in acutely isolated rat retinas. Two pathways mediate intercellular $Ca^{2+}$ waves: the movement of cytosolic messengers between connected cells via gap junctions and extracellular diffusion of messenger molecules, such as ATP.

## VII. CALCIUM-CONTROLLED CELLULAR MACROMOLECULES AND PROCESSES

### A. Calcium-Controlled Enzymes

Calcium can indirectly, by binding to specific intracellular proteins, control the activity of a number of enzymes. In all nonmuscle and smooth muscle cells, calmodulin is the predominant $Ca^{2+}$ binding protein. A related $Ca^{2+}$ binding protein, known as troponin C, dominates in skeletal muscle. The calcium–calmodulin complex controls over 20 intracellular enzymes that participate in regulation of several intracellular functions, such as cyclic nucleotide and glycogen metabolism, secretion, motility, $Ca^{2+}$ transport, and cell cycle. Many of these enzymes are inhibited in an intramolecular manner, and the calcium–calmodulin complex releases this inhibition. Activated calmodulin can bind directly to the effector protein and modulate its activity or can act indirectly through a family of calcium–calmodulin-dependent protein kinases and phosphatases. The kinase family includes myosin light chain kinase, phosphorylase kinase, calmodulin kinase I, calmodulin kinase II, extracellular factor-2 (EF-2) kinase, and calmodulin kinase IV. A number of other enzymes are directly controlled by cytosolic $Ca^{2+}$. For example, mammalian cells express a family of several closely related protein kinase C isoenzymes, many of which require $Ca^{2+}$ for activation. Cytosolic $Ca^{2+}$ also participates in the control of phospholipases C, D, and $A_2$. Several members of the adenylyl cyclase family of enzymes are also sensitive to changes in $[Ca^{2+}]_i$. Subtypes 5 and 6 of these enzymes are inhibited by elevation in $[Ca^{2+}]_i$ in a calmodulin-independent manner. The activity of three other members of this family of enzymes, subtypes 1, 3, and 8, is facilitated by the rise in $[Ca^{2+}]_i$ in a calmodulin-dependent manner. Several members of other enzyme families participating in cyclic nucleotide metabolism, i.e., nitric oxide synthase, guanylyl cyclases and phosphodiesterases, are also $[Ca^{2+}]_i$ sensitive.

### B. Calcium-Controlled Channels

A rise in $[Ca^{2+}]_i$ is an effective signal for activation and/or inhibition of several plasma and ER membrane calcium channels. The role of $Ca^{2+}$ in control of $InsP_3Rs$ and RyRs has already been discussed. SOCCs are also sensitive to $[Ca^{2+}]_i$, and both calcium-dependent activation and inactivation of these channels have been observed. The conductivity of L- and P/Q-types calcium channels is also altered by $[Ca^{2+}]_i$ in a calmodulin-dependent manner. In addition to $Ca^{2+}$-selective channels, other ionic channels are controlled or modulated by $[Ca^{2+}]_i$. These include several potassium, chloride, and nonspecific channels, all of which are known to be $Ca^{2+}$-activated channels.

### C. Fertilization, Development, and Apoptosis

Calcium plays important roles in fertilization and embryonic development. At fertilization, the sperm interacts with the egg to trigger $Ca^{2+}$ oscillations. These oscillations last for several hours and may be critical in triggering the enzymes involved in the cell division cycle. The mechanism of these oscillations is not well defined, but it is likely that $InsP_3$ is involved in their generation. Also, a prolonged period of $Ca^{2+}$ signaling, similar to that during fertilization, is an important growth signal for many cells, including normal immune cells and cancer cells exhibiting unlimited growth. During development, cells also use $Ca^{2+}$ signaling pathways to regulate development. For example, the rise in $[Ca^{2+}]_i$ contributes to body axis formation, organ development, cell migration, and formation of neuronal circuits. The finding that alterations in intracellular $Ca^{2+}$ homeostasis are commonly observed in necrosis and apoptosis is consistent with a view that elevated $[Ca^{2+}]_i$, especially if maintained for long periods, can be cytotoxic. Necrosis is usually accompanied by prolonged nonoscillatory elevations in $[Ca^{2+}]_i$. In contrast, apoptosis represents a more orderly program of cell death. A wide variety of candidate molecules could be involved in the $Ca^{2+}$-sensitive apoptotic process. These include calmodulin-dependent kinase/phosphatase, $Ca^{2+}$-sensitive proteases such as calpain and nuclear scaffold protease, $Ca^{2+}$-activated endonuclease, and transglutaminase.

### D. Exocytosis

In endocrine cells and neurons, regulated exocytosis is triggered by an elevation in $[Ca^{2+}]_i$. A rise in $[Ca^{2+}]_i$ is a critical step in the process of exocytosis, which is accomplished by rapid, $Ca^{2+}$-regulated fusion of neurotransmitter- or hormone-filled vesicles with the plasma membrane. Once the fusion is complete, the components of secretory vesicles are selectively recovered by endocytosis. Secretory vesicles are regenerated by a recycling pathway, and the final stages of this pathway include the recruitment, docking, and ATP-dependent priming of the secretory vesicles, in preparation for the next cycle of exocytosis. Distinct factors are required for sequential docking, ATP-dependent priming, and $Ca^{2+}$-dependent triggering (fusion) reactions. For example, phospholipids may contribute to the priming, but not to the fusion, whereas $Ca^{2+}$ is required for both steps in exocytosis. Although exocytosis is a highly specialized process, the molecules involved in this process are related to those that mediate the targeting and fusion of transport vesicles in other intracellular membrane trafficking pathways. Among the identified cytosolic $Ca^{2+}$ sensors, synaptotagmin I has been the most thoroughly characterized. Synaptotagmin I is a presynaptic vesicle protein that has two repeats of cytoplasmic calcium domain (C2A and C2B), each sharing homology with the C2 regulatory domain of protein kinase C. These domains mediate $Ca^{2+}$-regulated interaction with liposomes and the plasma membrane protein syntaxin, suggesting the underlying mechanism for synaptic vesicular docking and fusion to the plasma membrane. Over 50 C2 domain sequences have been identified, several of which have been implicated in exocytosis, including the synaptotagmin isoforms, rabphilin, doc2, and munc13-1.

### E. Nuclear Functions

The transcriptional activity of several genes is controlled by $[Ca^{2+}]_i$. Calcium can directly modulate transcriptional activity, but it can also act through individual $Ca^{2+}$-sensitive proteins that serve as molecular decoders of intracellular $Ca^{2+}$ signals. For example, both $Ca^{2+}$ and cAMP can be involved in the regulation of c-*fos* transcription via calcium- and cAMP-dependent phosphorylation of the dimeric transcription factor, $Ca^{2+}$/calmodulin response element binding protein (CREB). It is interesting that $Ca^{2+}$ exhibits stimulatory and inhibitory actions on c-*fos* expression that depend on $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ dose dependence of c-*fos* transcription and mRNA accumulation is bell-shaped, with facilitation at low $[Ca^{2+}]_i$ and inhibition at high $[Ca^{2+}]_i$. It is also likely that different genes require different $[Ca^{2+}]_i$ thresholds for their activation.

### F. Mitochondrial Functions

Calcium regulates several mitochondrial dehydrogenase enzymes involved in oxidative ATP synthesis, including pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutaric dehydrogenase. Calcium regulates these enzymes in the range of $0.2-2\ \mu M$ concentration. It has been suggested that increases and decreases in $[Ca^{2+}]_i$ are translated into parallel changes in the concentration of mitochondrial $Ca^{2+}$. Accordingly, $V_m$-dependent and calcium-mobilizing-dependent mitochondrial $[Ca^{2+}]$ spiking occurs in intact hepatocytes. Furthermore, each $Ca^{2+}$ spike in cytosol is sufficient to cause a transient increase in mitochondrial $[Ca^{2+}]$, which is then associated with a transient activation of $Ca^{2+}$-sensitive mitochondrial dehydrogenases. This action also triggers sustained activation of mitochondrial metabolism as long as the spiking frequency is higher than 0.5/min. In contrast, sustained low-amplitude $[Ca^{2+}]_i$ response and the slow, partial elevations in $[Ca^{2+}]_i$ are ineffective in increasing mitochondrial $[Ca^{2+}]$ and dehydrogenase activities. These observations are further supported by findings in chromaffin cells and isolated mitochondria, in which an increase in $[Ca^{2+}]_i$ to about 500 nM is required to activate calcium electrogenic uniport.

## VIII. CONCLUSIONS

Calcium is the most versatile and universal intracellular messenger. In contrast to other intracellular messengers, $Ca^{2+}$ signals can be generated by activation of many different ionic channels and other pathways expressed in the plasma membrane, ER/SR, mitochondria, and nucleus. Calcium can terminate and/facilitate its intracellular messenger functions by modulating the activity of several enzymes and channels participating in control of calcium influx and mobilization. Calcium can also modulate cellular function by activating enzymes involved in signal transduction, such as adenylyl and guanylyl cyclases, phosphodiesterases, and protein kinase C. In addition to intracellular messenger functions, $Ca^{2+}$ also acts as a first messenger, participating in cellular $Ca^{2+}$ homeostasis and several other processes, by controlling a number of extracellular $Ca^{2+}$-sensing receptors. This results in the incredible diversity and versatility of $Ca^{2+}$ signals, which differ in term of their spatial/temporal organization and amplitude coding. Spatial organization includes not only local and global $Ca^{2+}$ signals within the cytosol, but also the intraorganelle and intercellular $Ca^{2+}$ signals. Such a variety of $Ca^{2+}$ signals and patterns, and the pathways involved in their generation, provides a framework for understanding the universality of $Ca^{2+}$, the agent that signals for new life at the stage of fertilization and is used during embryonic development and cell differentiation. All differentiated cells can use the same $Ca^{2+}$ signaling patterns over and over again, but at any time point they preserve the ability to switch to a pattern of signaling that will activate the program that leads to cell death.

## Glossary

**adenylyl cyclases** Integral membrane protein enzymes that colocalize with regulatory $G_s$- and $G_i$-proteins and catalyze formation of cAMP at the inner surface of a membrane. cAMP acts as a messenger for protein kinase A, cyclic nucleotide channels, and phosphodiesterases.

**G-protein-coupled receptors** A large variety of hormones, neurotransmitters, and chemokines regulate cellular function via cell surface receptors that are coupled to guanine nucleotide-binding regulatory proteins (G-proteins). Heteromeric G-proteins regulate ion channels directly, by binding of their $\beta\gamma$ dimers to channels, or indirectly by activating several intracellular messengers, including calcium, protein kinases, cyclic nucleotides, and nitric oxide.

**ion channels** Macromolecular pores in cell membranes; play a central role in cell excitability, calcium signaling, and transport of small molecules. At rest, the channels remain closed, blocking ion entry into the cell. A group of voltage-gated channels can rapidly activate in response to a change in membrane potential, allowing ions to flow through the aqueous pore of the channel. The direction of ion flow is determined by an electrochemical gradient. After only a few milliseconds, or even as long as several hundred milliseconds, the channels inactivate and the flow of ions is again blocked. Inactivated channels cannot be activated to the conducting state until their inactivation block is removed. Another group of channels, ligand-gated receptor channels, require a ligand to become activated. Once activated, these channels desensitize, i.e., reduce or completely block ion conductance, although ligand is present, and removal of ligand is required for their return to resting state. Ligand-gated receptor-channels can be expressed in plasma membranes (extracellular), but also in the endoplasmic reticulum and the nuclear membranes (intracellular).

**phospholipase C** Inositol phospholipid-specific phospholipase C is the enzyme family that generates several phosphoinositide-derived messenger molecules, including inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ is a ligand for calcium receptor channels expressed in the endoplasmic reticulum and nuclear membranes ($InsP_3$ receptor channels). Diacylglycerol and/or calcium activate protein kinase C, a family of serine/threonine protein kinases.

## See Also the Following Articles

**Apoptosis • Calcitonin • Calmodulin • Corticotropin-Releasing Hormone Receptor Signaling • GPCR (G-Protein-Coupled Receptor) Structure • Protein Kinases**

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# Calmodulin

Marcia A. Kaetzel and John R. Dedman
*University of Cincinnati*

I. CALCIUM: A REGULATORY ION
II. DISCOVERY OF CALMODULIN
III. CALMODULIN GENOMICS
IV. $CA^{2+}$-MODULATED MOLECULAR ANATOMY
V. TARGET SITES FOR CALMODULIN BINDING
VI. DISCRIMINATION OF THE $CA^{2+}$–CALMODULIN SIGNAL
VII. $CA^{2+}$–CALMODULIN REGULATION OF ION CONDUCTANCES
VIII. $CA^{2+}$–CALMODULIN REGULATION OF TRANSCRIPTION
IX. SUMMARY

**Calmodulin is a major sensor and mediator of changes in intracellular $Ca^{2+}$ concentrations. In 1883, Sir Sidney Ringer provided the first reports relating tissue function to calcium. He demonstrated that calcium was necessary for normal contraction of the isolated frog heart. Calcium, therefore, was recognized as an essential component of physiological salines.**

## I. CALCIUM: A REGULATORY ION

The primary regulatory role of calcium in biological systems including fertilization, development, adhesion, secretion, growth, motility, chemotaxis, division, differentiation, and phagocytosis is well established. $Ca^{2+}$ has also been associated with a number of diseases, in particular, disorders of the muscular and nervous systems in which calcium plays an important role in contraction and neurotransmitter release. The understanding of calcium action has proven to be a complex topic and has required the inspiration and insight of many individuals from broadly distinct fields of expertise. Calcium is not like other metal ions involved in biological activities, such as $K^+$ and $Na^+$, which produce electrogenic membrane potentials, nor is it like manganese, magnesium, and zinc, which act as enzyme co-factors involved in the catalysis of metabolic intermediates.

Early studies characterizing intracellular $Ca^{2+}$ action used model systems such as the glycerinated muscle fiber. Calcium could be regulated and contraction and glycogenolysis monitored. It was found that calcium was active at micromolar levels and was rapidly sequestered from the cytosol. Calcium channels and pumps were identified using isolated enclosed vesicles through which the movement of $^{45}Ca^{2+}$ was monitored. Convincing evidence for the movement of calcium into and out of cells using the isotopic method proved difficult since the element is in a dynamic state of flux between the various intracellular organelles, the cytoplasm, and the extracellular fluids.

To monitor real-time $Ca^{2+}$ movements in living cells, calcium indicators were developed by Roger Tsien. Since the free acids of these fluorescent indicators are not membrane-permeable, they were made permeable by esterification of the free carboxyl acid groups. Once in the cytosol, multiple cellular esterases de-esterify the probe, trapping it within the cell. Fluorescent $Ca^{2+}$ indicators have provided valuable insights into the "calcium signal." The intracellular resting calcium level is approximately 100 nM free calcium. Cell stimulation by a variety of agents causes a transient rise in intracellular calcium (400–800 nM) lasting from milliseconds to a few minutes. The amplitude of the signal varies from tissue to tissue and may be related to the type of stimulation. Cells must convert this common regulator, calcium, into a multifunctional signal in order to achieve a variety of cellular functions. The sensing, mediation, and ultimate discrimination of the intracellular calcium signal at the molecular level will provide valuable insight into understanding the broader aspects of the role of calcium in organ, tissue, and cellular function.

## II. DISCOVERY OF CALMODULIN

The identification of calmodulin as a calcium-binding entity and mediator was a process that took several years, and thus, no single experiment can be singled out as the key in the discovery. In 1957, Hodgkin and Keynes postulated the existence of intracellular calcium receptors, due to the fact that $^{45}Ca^{2+}$ microinjected into the squid axon was not freely mobile in an electric field. In 1970, Cheung reported a putative protein activator of cyclic nucleotide phosphodiesterase. In the same year, Kakiuchi reported a calcium-activated phosphodiesterase and

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## III. CALMODULIN GENOMICS

From its early discovery, the most striking features of calmodulin were its ubiquitous occurrence in eukaryotes and structural conservation; calmodulin isolated from a coelenterate was an effective activator of human enzymes. Calmodulins from vertebrates, invertebrates, and plants have very few sequence differences. All plant CaM contains a cysteine at position 27; CaM from animal phyla lacks cysteines. Invertebrate CaM sequences are characterized by a serine at position 148 in place of alanine in vertebrates. All vertebrate species express an identical protein, making calmodulin one of the most highly conserved proteins known in nature. More interesting, mammals are unique in that they have three distinct CaM genes (I, II, and III), located on different chromosomes with unique promoters. These three genes have different intron–exon structures and different exon sequences and collectively produce six distinct mRNA species. This arrangement would suggest an evolutionary opportunity to permit differential expression of CaM isoforms in order to allow for discrete changes in enzyme regulation. All six unique CaM mRNAs, however, translate into identical proteins. This consequence suggests that the evolutionary pressure of a protein to appropriately sense and mediate the many actions of intracellular $Ca^{2+}$ disallowed any, even modest, changes in the sequence and structure of the protein. Each gene can, however, be dynamically regulated, producing six differentially expressed mRNAs. Differential message induction signals, stability, and cellular targeting contribute to the accurate expression of CaM as physiologically required.

## IV. $CA^{2+}$-MODULATED MOLECULAR ANATOMY

Parvalbumin was the first high-affinity $Ca^{2+}$-binding protein structure to be solved by X-ray crystallography. A helix-loop-helix ("EF-hand") composed of six precisely positioned amino acids in the 12-residue loop coordinate the $Ca^{2+}$ ion. This structural motif has been found in numerous proteins including troponin C, S-100, and CaM. The crystal analysis of $Ca^{2+}$–CaM revealed a "dumbbell" structure, that is, two globular domains linked by a flexible α-helix. In the absence of micromolar $Ca^{2+}$, the E-F α-helices are nearly parallel in a closed conformation. $Ca^{2+}$ binding to the 12-residue loop causes the E-F α-helices to move perpendicular to each other, creating an open conformation that promotes a flexible pocket to bind target proteins.

## V. TARGET SITES FOR CALMODULIN BINDING

The extreme structural conservation of CaM between phyla predicted a similar conservation in the binding site of the target protein. The CaM-binding component of MLCK was identified using limited proteolysis. It is a 17-residue amphipathic α-helical peptide. This peptide is an effective competitive inhibitor of CaM-activated enzymes. Analysis of co-crystals of $Ca^{2+}$–CaM and the MLCK peptide revealed a compact conformation. The globular domains individually grasped the binding peptide like a vice. As additional CaM-binding sites were identified from other target proteins, it became clear that sequence conservation was not rigidly maintained. The binding motif is typically hydrophobic and basic in nature. In many cases, large hydrophobic residues are precisely spaced at positions 1-16, 1-8-14, or 1-5-10. Isolation of high-affinity CaM-binding peptides using a bacteriophage display library identified peptides that contained a trytophan–proline combination, a binding motif not found in nature. Comparison of the 3-D structures of $Ca^{2+}$–CaM–MLCK peptide (1-8-14) and $Ca^{2+}$–CaM–CaMKII peptide (1-5-10) demonstrated that although CaM is highly conserved in sequence, it is structurally flexible. This adaptability is due to the linker α-helix and the high concentration of malleable methionines present on the binding surfaces.

The CaM-binding site of many target enzymes is adjacent to an autoinhibitory domain. $Ca^{2+}$–CaM binding derepresses the active site. This activation is reversed as intracellular levels fall below 800 nM $Ca^{2+}$. An additional class of CaM-binding site includes the "IQ motif." This motif begins with an isoleucine–asparagine followed by two precisely spaced arginines and a terminal hydrophobic anchor, IQxxxRxxxxRxxF/V. Many of these target proteins bind CaM in the absence of $Ca^{2+}$ and allow CaM to remain anchored during unstimulated periods. The highly adaptive nature of CaM binding suggests that caution should be exercised when identification of target proteins is based primarily on sequence motifs. Binding of CaM should change the activity or property of the putative target; CaM and the target should co-localize, and disruption of the binding motif in gene knock-in studies should modify cellular function.

## VI. DISCRIMINATION OF THE $CA^{2+}$–CALMODULIN SIGNAL

Major mechanisms of mediation of the intracellular $Ca^{2+}$ signal include protein modification through MLCK, phosphorylase kinase, $Ca^{2+}$–CaM-dependent protein kinases I, II, and IV, and calcineurin (protein phosphatase 2B). MLCK is a dedicated protein kinase and is the major mediator of smooth muscle contraction via thick filament regulation. Several kinases can reduce the affinity of $Ca^{2+}$–CaM for MLCK including protein kinase A (PKA), protein kinase C (PKC), CaMKII, and p21-activated kinase (PAK). In nonmuscle cells, MLCK regulates myosin II, which, in turn, modifies focal adhesion, cell morphology, contraction, cell locomotion, mitosis, and secretion.

CaM kinase II comprises a family of multifunctional kinases that are encoded by four genes, α, β, γ, and δ. Each can be alternatively spliced. Although it is found in all tissues, CaMKII is a major protein in neurons. $Ca^{2+}$–CaM binding causes catalytic activation including autophosphorylation at threonine-286. This phosphorylation markedly decreases the dissociation rate for $Ca^{2+}$–CaM and causes persistence of activity. Dephosphorylation occurs with protein phosphatase 1. Many cells exhibit oscillations in $Ca^{2+}$ levels. CaM kinase II is capable of decoding this frequency modulation. For example, efficiency of synaptic transmission is characteristic of long-term potentation (LTP), which is developed by repeated depolarization of postsynaptic spines associated with an influx of $Ca^{2+}$. CaMKII is localized in these dendritic spines and demonstrates changes in the level of threonine-286 phosphorylation depending on the duration, amplitude, and frequency of the nerve stimulation. Mutation of threonine-286 to alanine allows $Ca^{2+}$–CaM activation of the enzyme but not autophosphorylation. Mice containing this mutation are deficient in LTP and display learning disabilities. Primary substrates for CaM kinase II at the postsynaptic density are the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (potentiation of current), the *N*-methyl-D-aspartate (NMDA) receptor (increasing $Ca^{2+}$ entry), and neuronal nitric oxide synthase (inhibition). CaM kinase II also regulates $Ca^{2+}$ levels through the phosphorylation of the L-type $Ca^{2+}$ channel, inositol 1,4,5-trisphosphate ($IP_3$) receptor, phospholamban, and the $Ca^{2+}$ pump.

Calcineurin, protein phosphatase 2B, is a major $Ca^{2+}$–CaM-binding protein in neural tissues and is broadly distributed in other tissues. It is the only phosphatase regulated by $Ca^{2+}$–CaM. Calcineurin contains a binuclear $Fe^{3+}$–$Zn^{2+}$ metal center. Oxidation of the $Fe^{3+}$ causes catalytic inactivation even in the presence of $Ca^{2+}$–CaM. This activation is reversed by an associated enzyme, superoxide dismutase. This modulation allows for the coupling of $Ca^{2+}$ modulation to the redox state of cells. Substrate specificity is narrow and includes protein phosphatase inhibitor 1, PKA regulatory subunit, nuclear factor of activated T cells (NF-AT), neurogranin, synapsin 1, and phosphorylase kinase. Phosphatase activity is not inhibited by protein phosphatase inhibitor-1 or -2, okadaic acid, calyculin, or microcystin. Calcineurin, however, is a target for the immunosuppressive drugs FK506 and cyclosporin A (CsA). When bound to their respective binding proteins, FKBP12 and cyclophilin A, FK506 and CsA inhibit phosphatase activity.

## VII. $CA^{2+}$–CALMODULIN REGULATION OF ION CONDUCTANCES

Elegant studies by Kung and colleagues with swimming mutants of Paramecium first identified an *in vivo* role for CaM. Paramecia avoid undesirable environments by entering a "tumble" and changing the direction in which they are swimming. The ciliates were mutated and examined for their ability to respond to harmful stimuli. Many of the mutant animals displayed an exaggerated or a diminished response. These mutant paramecia showed no change in growth rate, secretion, or excretion. Electrophysiological measurements identified changes in $Ca^{2+}$-dependent $Na^+$ currents (underreactors) and $Ca^{2+}$-dependent $K^+$ currents (overreactors). Furthermore, reconstitution studies identified CaM as the defective gene product with individual single point mutations. $Na^+$ current mutants had substitutions of conserved amino acids in the N-terminal lobe of the CaM dumbbell, whereas the overreactors (defective $K^+$ currents) had substitutions in the C-terminal lobe. No mutations were found in the central helix. These studies established that each lobe has a distinct cellular role, which, in turn, modifies behavior. Recent reports have shown a unique regulatory mechanism for CaM. CaM binds individual, inactive $K^+$ channel subunits in the absence of $Ca^{2+}$ through an IQ motif. Membrane depolarization allows an influx of $Ca^{2+}$, which causes the CaM to dimerize the channel subunits and increase $K^+$ conductance. This process is reversed as $Ca^{2+}$ levels are reduced.

In 1978, Brehm and Eckert characterized a voltage-gated $Ca^{2+}$ channel in Paramecium. The channel demonstrated a rapid opening with a slow inactivating decay. This inactivation was $Ca^{2+}$ dependent. This conductance was observed in cardiac and smooth muscle and in neurons. Recently it was shown that this L-type channel was regulated by CaM. CaM is tethered to the channel via an IQ motif, a $Ca^{2+}$-independent interaction. Channel inactivation requires $Ca^{2+}$ binding to the CaM. The NMDA receptor, a ligand-gated $Ca^{2+}$ channel, also demonstrates $Ca^{2+}$–CaM-dependent inactivation. This negative feedback mechanism is very efficient. $Ca^{2+}$ enters the cell to activate many $Ca^{2+}$–CaM target proteins and then feeds back to terminate the stimulus.

## VIII. CA$^{2+}$–CALMODULIN REGULATION OF TRANSCRIPTION

The two primary pathways of regulating gene transcription by $Ca^{2+}$–CaM are through the phosphorylation and dephosphorylation of the transcription factors $Ca^{2+}$/cyclic AMP-response element (CRE)-binding protein (CREB), and NF-AT. CREB resides in the nucleus. Several kinases including RSK2, PKA, PKC, and CaM kinase I, II, and IV can phosphorylate CREB at serine-133. This modification causes dimerization, binding to CRE, and recruitment of the transcriptional co-activator, CREB-binding protein. This complex then cooperates with other transcription factors, such as ELK, which promote transcription. This assembly process can be prevented or reversed by dephosphorylation of serine-133 or phosphorylation at serine-142 by CaM kinase II. This is another example of feedback regulation of a $Ca^{2+}$–CaM-mediated pathway.

T lymphocytes produce cytokines [interleukin-2 (IL-2), IL-3, and IL-4] in response to antigen binding. Activation of T cells elevates intracellular $Ca^{2+}$ levels. The response is blocked by the immunosuppressive drugs FK506 and CsA. Activated T cells contain a transcription factor within the nucleus (NF-AT). In unstimulated cells, this protein resides in the cytosol in a phosphorylated state, yet remains transcriptionally competent. This stimulus transcription pathway is well defined. T-cell receptor binding elevates $IP_3$ levels, which elevate $Ca^{2+}$–CaM levels, which activate calcineurin. Dephosphorylation of NF-AT conceals a nuclear export signal (NES) and exposes a nuclear import signal. NF-AT enters the nucleus, combines with other transcription factors, and promotes gene transcription. Protein kinases including glycogen synthase kinase-3, JUN N-terminal kinase, and MEKK1 rephosphorylate NF-AT and expose the NES, and NF-AT exits the nucleus. NF-AT is a family of transcription factors that function in a similar manner in virtually all tissues including heart and neurons.

## IX. SUMMARY

Our knowledge of the many properties of the intracellular $Ca^{2+}$ signal continues to evolve. The resting intracellular ionized $Ca^{2+}$ level is approximately 100 nM. Cell stimulation by a variety of agents causes a transient rise in intracellular free $Ca^{2+}$ (400–800 nM); the increase is variable, lasting for milliseconds to minutes. The amplitude of the $Ca^{2+}$ spike also varies from tissue to tissue. The elevation in $Ca^{2+}$ may be uniform throughout a cell or a group of cells or may be highly localized to specific regions of individual cells. In many cellular systems, the $Ca^{2+}$ signal occurs as a wave, beginning at a discrete initiation site and then moving across the cell. $Ca^{2+}$ oscillations of various frequencies and amplitudes are influenced by cell stimuli.

TABLE 1 **Major Calmodulin Target Proteins**

| |
|---|
| CyclicAMP phosphodiesterase |
| Adenylate cyclase |
| Plasmalemma $Ca^{2+}$ pump |
| Nitric oxide synthase |
| NAD kinase |
| Phosphorylase kinase |
| Myosin light chain kinase |
| Nonmuscle myosins I, II, and V |
| Calmodulin-dependent protein kinases I–IV |
| Protein phosphatase 2B (calcineurin) |
| NMDA receptor |
| L-type $Ca^{2+}$ channel |
| SR $Ca^{2+}$ release channel (RYR1) |
| IP3 receptor |
| $Ca^{2+}$-activated $K^+$ channel |

The major sensor and mediator of intracellular $Ca^{2+}$ is a highly conserved protein, calmodulin. $Ca^{2+}$ binding causes conformational changes that allow this mediator to bind numerous target proteins. Major calmodulin target proteins are listed in Table 1. The diverse activities regulated by $Ca^{2+}$–CaM reflect the interplay of major cellular functions including cyclic nucleotide metabolism, redox state, transcription, membrane potential, nitric oxide synthesis, and contraction. Although the calcium–calmodulin pathway is common in all eukaryotic cells, the target proteins are the discriminators of the calcium regulatory signal. The localization, expression, and posttranslational modifications of the calmodulin target proteins dictate the ultimate cellular response to external stimuli. Isolation and functional characterization of individual target proteins will continue to expand the understanding of $Ca^{2+}$–CaM action in more precise terms.

## Glossary

**$Ca^{2+}$ signal** $Ca^{2+}$ indicator dyes have shown intracellular free $Ca^{2+}$ to be maintained at 100 nM. Stimulation of cells by hormones, cytokines, or membrane depolarization causes a transient elevation (400–800 nM) in cytosolic free $Ca^{2+}$. This $Ca^{2+}$ signal regulates numerous cellular functions.

**calcineurin (protein phosphatase 2B)** A calmodulin target protein that dephosphorylates proteins including enzymes and transcription factors in a $Ca^{2+}$-dependent manner.

**calmodulin** An intracellular $Ca^{2+}$-binding protein that acts as a sensor and mediator of the $Ca^{2+}$ signal by binding and regulating specific target proteins.

**calmodulin-regulated kinases** Enzymes that are activated by $Ca^{2+}$–calmodulin during the $Ca^{2+}$ signal.

**calmodulin target proteins** Enzymes, ion pumps, and channels that bind calmodulin and change their activity in a $Ca^{2+}$-dependent manner.

## See Also the Following Article

**Calcium Signaling**

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# Cancer Cells and Progrowth/Prosurvival Signaling

GIRISH V. SHAH AND MAURIZIO CHIRIVA-INTERNATI
*Texas Tech University Health Sciences Center*

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**Cancer cells represent a process of increased cell growth and decreased cell attrition. Changes in biological regulatory systems that lead to cancer and tumors are mediated by expression of mitogenic peptides and growth factors and/or by mutations in receptors or signal transduction pathway components. Elucidation of the signaling mechanisms that promote cancer cell growth is key to regulating and reversing tumorigenic processes.**

## I. INTRODUCTION

At a cellular level, cancer is characterized by several paradigms: unregulated proliferation, whereby mechanisms controlling the cell cycle and/or programmed cell death are lost; loss of differentiated phenotype; and acquisition of the ability to evade host defenses, to invade, and to metastasize. These changes allow cancer cells to grow unchecked in the absence of extracellular growth-stimulating signals and to overcome growth-inhibitory signals and host immune responses. It has been suggested that malignant transformation of a normal cell results from a stepwise accumulation of mutations in proto-oncogenes and tumor suppressor genes. These mutations are known to modulate oncogenic signals that stimulate anchorage-independent proliferation, rearrangement of the cytoskeleton, inhibition of apoptosis, and promotion of survival. Because cancer arises as a consequence of unregulated cell growth and survival, it is critical to understand the role of normal mitogens such as hormones and growth factors in growth and survival, and how they activate specific interconnecting signaling pathways that regulate cell growth and apoptosis.

## II. GROWTH FACTORS AND THEIR RECEPTORS

Usually, growth factors (GFs) are secreted locally in an organ to regulate the growth of target cells in a paracrine/autocrine fashion. GFs or their receptors are often overexpressed in many cancers, and these can continuously stimulate the growth of cancer cells. Likewise, overexpression of GF receptors can cause a cancer cell to become hyperresponsive to ambient GF levels that normally would not trigger cell proliferation. There is growing evidence for up-regulated epidermal growth factor receptor (EGFR) signaling in formation and progression of human cancers. For example, the epidermal GF receptor EGFR/erb B (or Her2/neu) is overexpressed in stomach, brain, ovarian, and breast cancers. Additionally, gross overexpression or structural alteration of GF receptors can elicit ligand-independent signaling. Truncated EGFRs, which are found in various human cancers, lack much of their cytoplasmic domain but are constitutively active. Extracellular matrix receptors or integrins also activate mitogenic signaling, and cancer cells are known to increase the expression of the types of integrins that transmit progrowth signals.

### A. GF Signaling

Normally, GF signaling pathways are activated on binding of a ligand to its receptor. The receptor undergoes ligand-induced dimerization and cross-phosphorylation (Fig. 1). Phosphorylation of tyrosine sites in the receptor leads to recruitment of intracellular docking proteins (such as Grb2, Shc, and Nck), followed by their formation of a complex with the guanine nucleotide exchange factor (GEF) Sos. These docking proteins create a molecular scaffold from which subsequent signals emanate. For example, the guanine nucleotide exchange factor Sos binds to Grb2, which interacts with Ras protein. Ras serves as a molecular switch in the plasma membrane that alternates between an inactive guanosine diphosphate (GDP)-bound state to an active guanosine triphosphate (GTP)-bound state. Binding of GTP to Ras leads to conformational changes and causes activation of downstream effectors such as

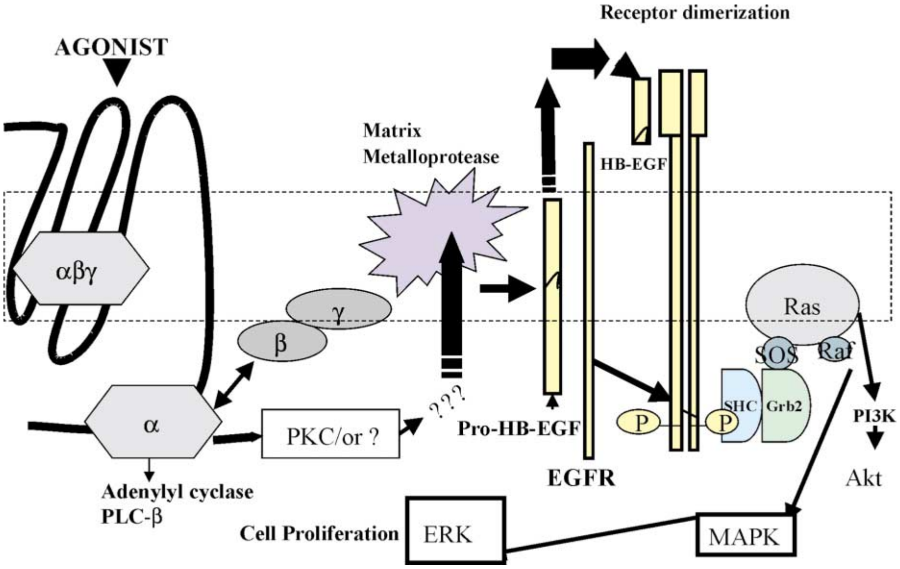

**FIGURE 1** G-Protein-coupled receptor-mediated cell proliferation via transactivation of epidermal growth factor receptor (EGFR). Several G-protein-coupled receptors, such as those for endothelin, thrombin, or muscarinic cholinergics, activate a matrix metalloprotease, which in turn cleaves pro-heparin-binding EGF (pro-HB-EGF), causing it to shed mature HB-EGF. The released HB-EGF binds to EGFR, activating the receptor tyrosine kinase-mediated Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. ERK, extracellular signal-related kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC-β, phospholipase C-β.

Raf and mitogen-activated protein kinases (MAPKs). In addition to activation of the Ras/Raf/MAPK pathway, GF receptor may also activate other pathways, perhaps involving PLC-γ, phosphatidylinositol 3-kinase (PI3K), Syp, and Src.

## III. PARACRINE/AUTOCRINE PEPTIDES

Recent studies suggest that solid tumors involved in breast, lung, prostate, and colorectal cancers synthesize and secrete numerous neuropeptides, such as thrombin, bombesin, calcitonin, bradykinin, substance P, endothelin, neurotensin, serotonin, gastrin, and cholecystokinin. Receptors for these peptides are also expressed in human cancers. Although these peptides serve as neurotransmitters or modulators in the central nervous system, they can serve as potent mitogens for poorly differentiated cancer cells. Current evidence supports the notion that growth of the tumors is also driven by multiple autocrine and paracrine loops involving the neuropeptides. These structurally diverse signaling peptides exert their effects on cell proliferation by binding to their specific G-protein-coupled receptors (GPCRs) on the surface of their target cells (Fig. 2).

### A. GPCR (Heptahelical) Receptor Family

The family of GPCR (heptahelical) receptors comprises over 1000 members and displays a common structural motif consisting of seven transmembrane domains. A diverse array of external stimuli, such as hormones, neurotransmitters, phospholipids, photons, odorants, taste ligands, and growth factors, can activate a specific receptor of this family to transduce signals in the cytoplasm of target cells. There is compelling evidence for the role of GPCRs in many human neoplasias, including small-cell lung carcinoma, thyroid carcinoma, colon carcinoma, prostate cancer, gastric hyperplasia and carcinoma, and pancreatic carcinoma. These carcinomas express wild-type GPCRs that are persistently stimulated by tumor-released agonists in an autocrine or paracrine fashion. In addition, activating mutations in GPCRs have been identified in various tumors. For example, *mas* oncogene, which encodes a putative GPCR, was initially cloned using standard transfection by virtue of its ability to induce tumors in mice. Subsequently, serotonin 1c; muscarinic m1, m3, and m5; and adrenergic α1 receptors were shown to transform contact-inhibited cultures of rodent fibroblasts when persistently activated. However, the mechanisms whereby GPCRs regulate cell proliferation are not completely understood.

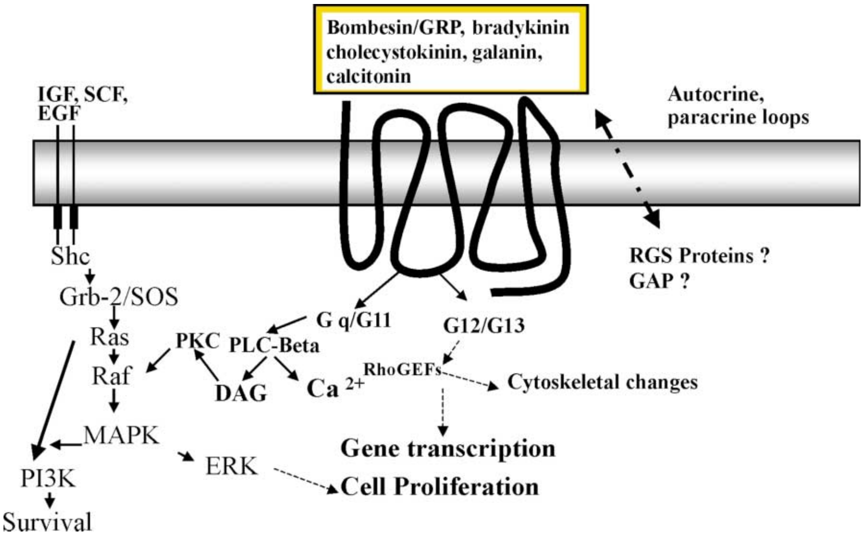

**FIGURE 2** Neuropeptide-induced cell proliferation. Activation of G-protein-coupled receptors by neuropeptide ligands leads to the activation of appropriate G-proteins. Activation of $G_q$- or $G_{11}$-coupled receptors activates downstream effectors such as protein kinase C (PKC). Protein kinase C can directly activate Raf/mitogen-activated protein kinase (MAPK) to stimulate cell proliferation or differentiation (depending on the cell types). On the other hand, activation of $G_{12}$- or $G_{13}$-coupled receptors causes the activation of Rho guanine nucleotide exchange factor (GEF) GTPases, which then activate the cascades that induce changes in the cytoskeleton. Activation of G-protein-coupled receptors can be modulated by membrane proteins such as regulators of G-protein signaling (RGS proteins) or GTPase-activating proteins (GAPs). Growth factors, on the other hand, activate Raf/MAPK through receptor tyrosine kinase-stimulated activation of Ras. Ras may also induce the survival pathway through the activation of phosphatidylinositol 3-kinase (PI3K). DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; IGF, insulin-like growth factor; PLC-β, phospholipase C-β; SCF, stem cell factor.

## IV. GTP-BINDING PROTEINS

Heterotrimeric GTP-binding proteins (G-proteins) are composed of three subunits, α, β, and γ. In a resting state, G-protein exists as the GDP-bound α-subunit complexed with the β- and γ-subunits. The β- and γ-subunits are tightly bound and are commonly referred to as the $G_{\beta,\gamma}$ subunit. In a typical G-protein-coupled signaling pathway, ligand-activated receptor catalyzes the exchange of GDP for GTP bound to the α-subunit, followed by the dissociation of the GTP-α and $G_{\beta,\gamma}$ subunits. The GTP-α subunit then activates its respective effector molecule–for example, stimulating or inhibiting adenylyl cyclase (in the case of αs or αi). The $G_{\beta,\gamma}$ subunit also activates the same or different effector molecules. Hydrolysis of bound GTP to GDP by intrinsic GTPase of the α-subunit leads to a conformational change, which terminates its interaction with the effector molecule. The GDP-α, thus freed, recombines with the $G_{\beta,\gamma}$ subunit and the newly formed GDP-αβγ heterotrimer reenters the signaling cycle. Modulation of this basic signaling pathway may be accomplished by signaling modulators such as GPCR kinases, regulators of G-protein signaling (RGS proteins), and effectors with intrinsic GTPase-activating protein (GAP) activity). Other signaling molecules, such as nonreceptor tyrosine kinases (RTKs), protein kinase C, ADP-ribosylases, and lipid transferases, also modulate this process by modifying GPCRs and/or G-proteins.

The subunits of G-proteins display a wide range of heterogeneity. To date, 17 α-subunits, 5 β-subunits, and 12 γ-subunits have been identified and cloned. The heterogeneity of α-subunits has been used to define and classify the G-proteins. The α-subunits with 50% or greater homogeneity are grouped into four distinct classes or subfamilies, i.e., $G_s$, $G_i$, $G_q$, and $G_{12}$. Although this classification is rather arbitrary, a functional similarity between the members of a subfamily is observed. For example, members of the $G_s$ subfamily stimulate adenylyl cyclase, whereas those of the $G_i$ subfamily inhibit this activity. Members of the $G_q$ family interact with phospholipase C-β, whereas those of $G_{12}$ regulate small GTPases. $G_s$ and $G_i$ proteins also regulate ion channels, certain phosphatases, and phosphodiesterases. In addition, $G_{\beta,\gamma}$ subunits regulate certain ion channels, phospholipase C, and phosphatidylinositol

3-kinases. Cells expressing the constitutively active form of $G_{\alpha s}$ display translocation of PKC-ζ molecules in membrane fractions, which suggests a role for $G_{\alpha s}$ in activation of this enzyme. Regulation of G-protein-mediated signal transduction is further complicated by the heterogeneity of effector molecules. For example, several isoforms of adenylyl cyclases and phospholipase C have been identified, and they are distributed in a cell-specific manner.

Although GPCRs have traditionally been linked to tissue-specific fully differentiated cell functions, increasing evidence suggests that GPCRs also play an important role in cell proliferation and cellular transformation. For example, activating mutations of subunits αs and αi are found in a subset of endocrine tumors. Furthermore, *gsp* oncogene, which mutationally activates the $G_{\alpha s}$ subunit, causes endocrine cell hyperplasia and induces tumors in human pituitary gland and thyroid. Activating mutations have also been identified in $G_{\alpha i2}$ (*gip*2 oncogene) in a subset of ovarian sex chord stromal tumors and adrenocortical tumors. Mutants of different α-subunit proteins, when expressed in diverse cell lines, demonstrate an increase in cell growth. For example, constitutive expression of $G_{\alpha s}$ causes a dramatic increase in the rate of prostate cancer cell proliferation and tumorigenicity. Similar actions of several other α-subunits, such as $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha o}$, $G_{\alpha 16}$, $G_{\alpha z}$, $G_{\alpha 12}$, and $G_{\alpha 13}$, have been demonstrated in other systems.

## V. MAJOR PROGROWTH/PROSURVIVAL SIGNALING PATHWAYS

A wide array of mitogenic GFs, hormones, and neuropeptides activate their specific receptors to induce survival and growth of their target cells. However, most of these receptors generally use one or multiple common intracellular signaling pathways to produce biological effects. Among existing signaling pathways, three pathways have taken a center stage in the transduction of progrowth/prosurvival signals: (1) the Ras/Raf/mitogen-activated protein kinase pathway, (2) the JAK/Stat pathway, and (3) the phosphoinositol 3-kinase/Akt pathway.

### A. Ras/Raf/MAPK Pathway

The Ras/Raf/MAPK pathway plays a dominant role in the processes of cell proliferation and differentiation and may also activate survival pathway in some cell types. The available evidence suggests that the Ras/Raf/MAPK-mediated growth signaling pathway is up-regulated in about 25% of all human tumors. As many as half of the cases of human colon carcinoma bear the mutant *ras* oncogene, which allows persistent release of mitogenic signals even in the absence of upstream regulators.

RTK receptors, GPCRs, and progrowth integrins stimulate cell proliferation by activating the Ras/Raf/MAPK pathway. Interestingly, the pathways of GPCR and RTK-mediated MAPK activation are convergent in many cases. It has been shown that stimulated GPCRs rapidly activate matrix metalloproteases, thus releasing heparin-binding EGF (HB-EGF) from its precursor protein (Fig. 1). These proteases are activated through either protein kinase C-dependent or -independent pathways, depending on the stimulus, and in a cell-specific manner. Shed HB-EGF then acts as a ligand to transactivate EGFR, which leads to RTK-mediated activation of the Ras/Raf/MAPK pathway. Metalloprotease inhibitor BB94 inhibits bombesin- and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced transactivation of EGFR in PC3 human prostate, breast, and colon carcinoma cell lines. Treatment of Rat1 cells with GPCR agonists such as endothelin-1, lysophosphatic acid, or thrombin rapidly phosphorylates EGFR or the Her2 receptor. Moreover, agonist-induced MAPK-activation in these cells can be attenuated by the addition of EGFR inhibitor tyrophostin AG1478 or the expression of a dominant-negative EGFR mutant. Activation of this pathway has been demonstrated in several cell types, including human keratinocytes, mouse astrocytes, PC12 cells, and vascular smooth muscle cells. GPCR-induced EGFR transactivation has been shown to occur via pertussis toxin (PTX)-sensitive and -insensitive pathways, and EGFR inhibition strongly attenuates $G_q$- and $G_i$-induced MAPK activation in COS-7 cells. $G_{13}$-Coupled receptors have also been shown to cause EGFR transactivation. Moreover, $G_{\beta,\gamma}$ dimers may also activate Ras proteins, but this pathway has not been completely delineated. Receptors coupled to $G_q$ or $G_{12}$ may activate the Raf/MAPK pathway in a Ras-independent manner by activating protein kinase C, which can directly activate Raf (Fig. 2). The Ras/Raf/MAPK mitogenic cascade is linked to other signaling pathways through cross-talking connections, which enable various mitogenic ligands to produce multiple biological effects.

Ligands that activate cell proliferation via the MAPK pathway also induce prosurvival signals in several cases. A direct interaction of Ras protein with PI3K may enable growth signals to evoke survival signals concurrently within the cells. For example, deletion of the cytoplasmic tail in granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor in BaF3 cells abrogates interlukin-2 (IL-2)-induced activation of the Ras/Raf/MAPK pathway. However, overexpression of activated Ras in these cells rescues this defect, supporting IL-3-induced survival. It is conceivable that Ras transformation may activate PI3K in this system. However, overexpression of downstream effectors of Ras does not affect PI3K activity, but suppresses cell death. Moreover, inhibition of MAPK activation with dominant-negative mutants suppresses IL-3-dependent survival in BaF3 cells. Several targets of MAPK activity in the survival pathway have been proposed, including the family of 90-kDa ribosomal S6 kinases (Rsks); Rsks protect the cells from death through phosphorylation of BAD (so named because it is a member of the Bcl-2 family, and is an agonist causing cell death) at Ser-136 in multiple tissues. In other Ras/MAPK survival studies, evidence points to a role of the anti-apoptotic proteins of the Bcl-2 gene family in mediating survival.

## B. JAK/Stat Signaling Pathway

The Janus family of kinases (JAKs) plays a major role in transducing the signal from cytokine receptors to a family of signal transduction/activation of transcription (Stat) factors. Members of this relatively small family of proteins serve both as transducers of cytoplasmic signals and as nuclear transcription factors, thereby directly converting a stimulus at the cell surface to an alteration in the genetic program. Stats are unique among transcription factors in possessing an SH2 domain that regulates their dimerization and activation via tyrosine phosphorylation. Seven mammalian Stat family members (Stat1–Stat6, with Stat5A and Stat5B encoded in distinct genes) have been molecularly cloned and may represent all Stats in mammalian cells. Aberrant Stat signaling has been suggested to contribute to malignant transformation by promoting cell cycle progression and/or cell survival.

The JAK/Stat pathway is activated by the binding of a specific ligand to its receptor (Fig. 3). Activation of the receptor induces its own dimerization, which is followed by phosphorylation of the receptor and activation of receptor-associated JAK family kinases. The activated JAKs, in turn, phosphorylate the receptor cytoplasmic tails on tyrosine, providing docking sites for the recruitment of monomeric Stats. JAKs then phosphorylate the recruited Stat proteins on tyrosine, inducing their dimerization, nuclear translocation, and DNA binding. In some cases, other tyrosine kinases (TKs), such as Src family kinases, are also involved in Stat activation. There is a very high correlation between the activation of Stat3 by Src and oncogenic transformation. Activation of Stats by other TK families, such as Lck and v-Fps in oncogenic transformation, has also been reported. JAK1 and v-Src cooperate in activation of Stat3 in transformation of NIH 3T3 fibroblasts. Constitutive Stat3 DNA-binding activity induced by Src oncoprotein results in malignant transformation. In contrast, attenuation of Stat3 signaling by the expression its of dominant-negative forms blocks the transforming ability of Src. However, co-expression of dominant-negative Stat3 together with Ras does not activate Stat3 and does not block Ras-induced transformation. The expression of cyclin D1, which associates with cdk4 or cdk6 and controls the progression of cell cycle from $G_1$ to S phase, is elevated in Stat3-expressing cells. MAP kinase 1/2 can modulate the transcriptional activity of Stat3 by phosphorylating Ser-727. Additionally, v-Src-induced activation of Stat3 is also modulated by p38 and the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway. Activation of GPCRs such as angiotensin II and melanocyte-stimulating hormone receptors also activates Stat1, Stat2, and Stat3. The presence of mutant active melanocortin receptors has been demonstrated, raising the possibility that mutant GPCRs expressed in diseases may activate Src and Stat3 to induce morphological changes and neoplastic transformation.

In addition to proliferative and transforming effects, the JAK/Stat pathway also transduces cytokine-mediated survival signals in several cell types. Knockout studies of the Stat family reveal that Stat3 may have a role in cell survival. Stat3 activation is required for GM-CSF-dependent cell survival in BaF-BO3 cell lines, and the cells expressing dominant-negative Stat3 are apoptotic even in the presence of GM-CSF. Downstream effectors of Stat-mediated survival are unclear, but when the survival is apparent, increased Bcl-2 protein expression has been reported. However, there is no evidence to suggest the direct role of Stat activity in Bcl-2 induction. In some models, Stat activation leads to cell cycle arrest and cell death. Stat1 has been reported to trigger cell death through the expression of caspase-1. This apparent contradiction is probably the consequence of at least four JAK isoforms and seven Stat molecules, which may be activated by several cytokines and have distinct DNA-binding properties.

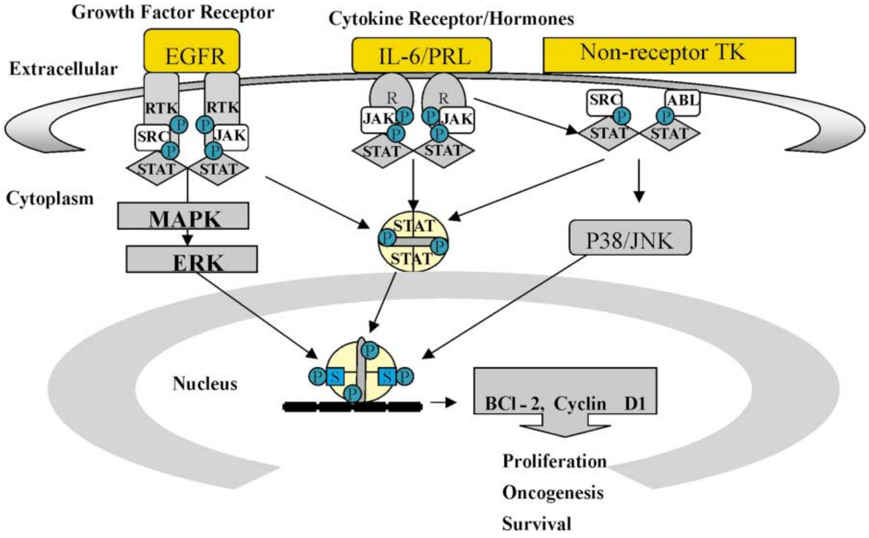

**FIGURE 3** JAK/Stat signaling. Stat signaling is activated by various ligands, including growth factors, hormones, cytokines, or nonreceptor tyrosine kinases (TKs). However, the cellular mechanisms of activation may be different. For example, the binding of interleukin-6 (IL-6) with its receptor causes receptor dimerization and activation of JAKs. JAKs then phosphorylate IL-6 receptors, which enables the IL-6 receptors to recruit and dock monomeric Stats. JAKs then phosphorylate tyrosine residues of Stats, which causes dimerization of Stat proteins. Dimerized Stats are translocated to the nucleus, where they bind to DNA and activate genes associated with cell proliferation and malignant transformation. Receptors for hormones such as prolactin (PRL) or growth hormone also activate this pathway in a similar manner. Epidermal growth factor receptor (EGFR)–receptor tyrosine kinase(RTK)can either directly phosphorylate Stats or can act indirectly through activation of JAKs or Srcs. Nonreceptor TKs such as Src or Abl can also phosphorylate Stats in the absence of ligand-induced receptor activation. ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

## C. PI3-Kinase Signaling Pathway and Cell Proliferation/Survival

Many receptors, including those for growth factors [insulin-like growth factor-I, platelet-derived growth factor (IGF-I, PDGF)], brain-derived neurotrophic factors, neuroendocrine peptides (bradykinin, calcitonin, adrenaline, gastrin), cytokines (IL-3, IL-2), and integrins, transmit survival as well as growth signals through the PI3K pathway. Receptor-induced activation of PI3K activity is biphasic, and only the late phase of activation (3–7 h) is required for cell proliferation.

Eight mammalian PI3Ks have been identified and classified into three main groups on the basis of sequence homology, in *vitro* substrate preference, and method of activation and regulation. Humans express four class I enzymes, which are referred to as Ia and Ib on the basis of their mechanism of activation. The class Ia group consists of classical p110α and two other closely related enzymes, p110β and p110δ. The α and β isoforms are ubiquitous whereas δ isoform expression is more restricted. All class Ia enzymes constitutively associate with the p85 regulatory/adapter subunit to form a heterodimeric complex. At least eight adapter isoforms, encoded by three different genes, have been identified. A majority of the RTK-coupled receptors activate class Ia PI3-kinases through the binding of SH2 domains that are found in all p85 isoforms, although the binding and duration of the resulting signal may vary (Fig. 4). Insulin and PDGF receptors activate PI3-kinase strongly, whereas EGFR has a weak and inconsistent effect. The class Ib group consists of the p110γ catalytic subunit, which associates with the p101 adapter subunit. GPCRs activate class Ib PI3Ks by releasing $G_{\beta,\gamma}$ subunits. It has been suggested that $G_{\beta,\gamma}$ docks on and activates the catalytic subunit of PI3K (Fig. 4).

PI3Ks catalyze the phosphorylation of inositides on the 3 position of the inositol head group. At least four 3-phosphorylated inositol lipids (PIPs) have been identified *in vivo*. These lipids bind to specific binding proteins, which either act as effectors or transmit the PI3K signal onward. PIPs have been described as lipid messengers because they serve as binding sites for proteins that possess a pleckstrin homology (PH) domain. One such protein is c-Akt (also identified as

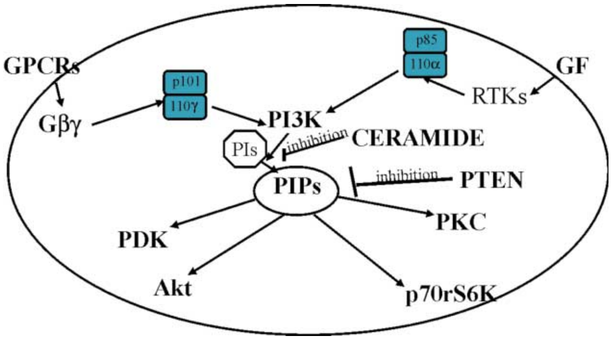

**FIGURE 4** Activation of phosphatidylinositol 3-kinase (PI3K) by G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). GPCRs as well as RTKs activate PI3K, and ceramide can inhibit this process. PI3K catalyzes the phosphorylation of inositides (PIs). Phosphoinositides (PIPs) can activate downstream targets such as phosphoinositide-dependent kinase (PDK), Akt, p70rS6K, and protein kinase C (PKC). The phosphatase/tensin (PTEN) homologue can attenuate PIP action by dephosphorylating the PIPs.

protein kinase B), which is related to protein kinases A and C. Binding of the Akt PH domain to the phospholipids results in Akt translocation to the plasma membrane and phosphorylation at two critical residues, Thr-308 and Ser-473. Phosphorylation at Thr-308 is achieved through additional kinases, such as phosphatidylinositol-dependent kinase 1, which also contains a PH domain and requires PI3K activity for membrane localization. The enzyme that phosphorylates Ser-473 has yet to be identified, but a role for $Ca^{2+}$/calmodulin-dependent protein kinase or cyclic adenosine monophosphate (cAMP)-dependent protein kinase has been suggested. Regulation of Akt function is controlled both by localization to the membrane, which is dependent on available PIPs, and by the level of its phosphorylation. The generation of 3′-phosphoinositols is counterbalanced by lipid phosphatases that dephosphorylate PIPs. The evidence has shown that a phosphatase/tensin (PTEN) homologue that dephosphorylates PIPs and inhibits PI3-kinase activity is inactivated in a majority of prostate tumors and PC cell lines (Fig. 4).

Studies employing relatively specific chemical inhibitors of PI3K (Wortmannin or LY294002) and, more recently, studies using cells transfected with vectors that encode an active or dominant-negative PI3K have led to the identification of several signaling enzymes in addition to Akt, which further amplify the signal initiated by PI3K. These enzymes include the phosphoinositide-dependent kinase (PDK), p70 ribosomal S6 kinase, and possibly protein kinase C. Each of these enzymes are serine/threonine protein kinases that play critical roles in regulating gene expression, cell growth, cell survival, and cell differentiation in response to a number of stimulants. Many of the receptor tyrosine kinases that stimulate PI3K have been localized to microdomains in the plasma membrane that are rich in lipids, including sphingomyelin and cholesterol. More importantly, the microdomains contain ceramide, which, at high levels, may interrupt PI3K signaling.

## D. Akt in Cell Survival Signaling

Inhibition of PI3K activity is sufficient to induce cell death even in the presence of survival factors. However, this death can be overcome by constitutive Akt activity. In some cases, the expression of dominant-negative forms of Akt induces apoptosis. Although Akt is a major, if not the sole, effector of PI3K-induced survival, the precise mechanism by which Akt suppresses death is not yet elucidated. Several Akt identified targets may promote cell survival; however, no one substrate or model has emerged as the clear candidate.

The current understanding of Akt targets can be sorted into essentially two categories: proteins directly involved in signal transduction and enzymes of glucose metabolism (Fig. 5). Among the former is BAD, a pro-apoptotic member of the Bcl-2 family. Akt phosphorylates BAD at Ser-136. When phosphorylated, BAD is sequestered in the cytoplasm by 14-3-3 proteins, unable to heterodimerize with and inactivate the anti-apoptotic protein, Bcl-xL. Overexpression of BAD alone commits a cell to death, but the cell may be rescued by co-expression of activated Akt. However, Akt is incapable of protecting cells expressing BAD with a Ser-136 → Ala mutation. Clearly, Akt can mediate growth factor-dependent survival by reversing the apoptotic activity of BAD. However, this may not be the only mechanism of Akt protection, because Akt-dependent survival can be observed in cells that do not contain BAD. This suggests that Akt-mediated survival is likely to involve other mechanisms in these cells.

In *Caenorhabditis elegans*, a homologous PI3K pathway regulates development and longevity. Mutations in Daf-2 (mammalian insulin receptor homologue) or AGE-1 (mammalian PI3K homologue) lead to developmental arrest at the dauer larval stage. This phenotype can be suppressed by mutations of the Daf-16 allele. Searches for mammalian orthologues of the Forkhead transcription factor Daf-16 have revealed three members to date, AFX,

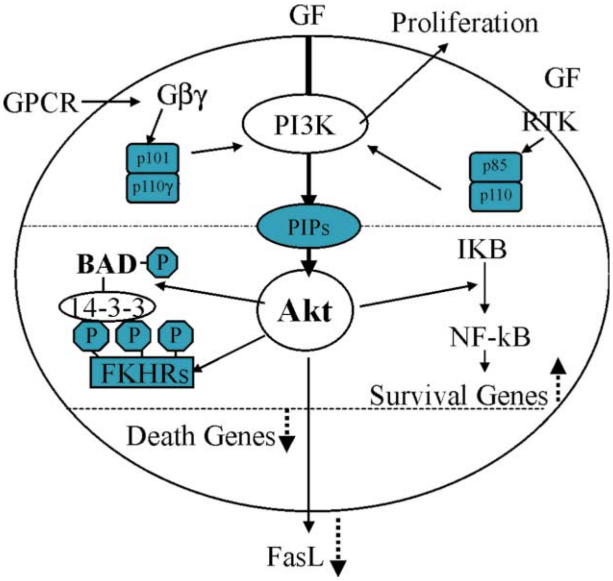

**FIGURE 5** Induction of survival by Akt. Akt regulates survival through phosphorylation of multiple targets involved in the regulation of apoptosis. For example, Akt inhibits apoptosis through regulation of transcription of NF-κB as well as Forkhead family members (FKHRs) by controlling their phosphorylation. Akt also phosphorylates BAD (a Bcl-2 homologue). Phosphorylated forms of BAD and FKHRs are retained by 14-3-3 proteins in the cytoplasm and are thereby prevented from translocating to the nucleus, where they can activate death genes. Akt also down-regulates Fas ligand (FasL). GF, growth factor; IκB, inhibitor κB; PIPs, phosphoinositides; RTK, receptor tyrosine kinase.

FKHR, and FKHRL1. As with BAD, in the presence of survival factors, Forkhead proteins are phosphorylated and retained in the cytosol by 14-3-3 proteins, suggesting a general role of 14-3-3 proteins in Akt-induced inhibition. Nonphosphorylatable mutants of FKHRL1 induce apoptosis in a variety of cell lines, whereas expression of Forkhead mutants with aspartic acid residues to mimic phosphorylation renders cells resistant to death. Transcriptional activity is required for Forkhead function; mutants unable to bind DNA are unable to induce cell death even in the absence of growth factors. When dephosphorylated, Forkhead transcription factors migrate to the nucleus, where they bind insulin response sequences. Among other targets, the Fas ligand (FasL) promoter contains a binding site for FKHRL1. In the absence of survival signals, Forkhead activity leads to FasL expression, which then migrates to the cell surface and activates the Fas-mediated cell death cascade. In fact, FKHRL1-dependent death can be abated in the presence of antibodies that block Fas activation. Consistent with this model, PTEN heterozygous mice develop a lethal polyclonal autoimmune disorder similar to that of Fas-deficient mice. Fas-mediated apoptosis is impaired in PTEN(+/−) mice, but apoptosis can be restored in the presence of PI3K inhibitors. Moreover, PTEN knockout studies reveal high concentrations of PIPs and a concomitant hyperactivation of Akt. Although inactivating mutations of PTEN render cells resistant to apoptosis, overexpression of wild-type PTEN sensitizes the cells to death following their detachment from the extracellular matrix. This potentially explains the frequency of PTEN mutations in late-stage, invasive tumors. Induction of NF-κB by Akt has been reported. Akt survival function in response to platelet-derived growth factor and tumor necrosis factor requires the activation of NF-κB transcriptional activity. Gene targets of the transcription factor NF-κB include a prosurvival member of the Bcl-2 family, A1, and the inhibitors of apoptosis proteins.

## VI. CROSSTALK BETWEEN SIGNALING PATHWAYS

There is considerable evidence for crosstalk between different signaling pathways, and the Src family of kinases may play an important role in this process. c-Src is a member of the Src family of cytoplasmic tyrosine kinases; these kinase are involved in various cellular processes such as proliferation, migration, and differentiation. They are 52- to 62-kDa proteins with several domains, including a catalytic domain, a regulatory domain, and SH2 and SH3 binding domains. The inactive form of c-Src is phosphorylated at Tyr-527 and interacts with its own SH2 domain to mask its catalytic domain. Its activation causes dephosphorylation of Tyr-527, enabling its catalytic domain to interact with other substrates.

Src kinases seem to play an important role in GPCR-induced MAPK activation. Agonist-induced activation of the β-adrenergic receptor (β-AR) causes rapid recruitment of β-arrestin 1 and c-Src. Blocking of β-arrestin 1/c-Src interaction impairs β-AR-induced MAPK activation in 293 cells as well as in other cell systems. A similar role for the β-arrestin 1/c-Src complex in several other systems has been reported. There is also evidence that GPCRs may directly activate c-Src. For example, treatment of normal mesangial cells with endothelin-1 stimulates autophosphorylation of pp60 v-Src. Overexpression of pp60 c-Src in C3H10T1/2 mouse fibroblasts potentiates the intracellular accumulation of cyclic AMP on stimulation of the β-adrenergic receptor.

Recent evidence has shown that pp60 c-Src phosphorylates the tyrosine residues of $G_{\alpha s}$ at positions 37 and 377. Because Tyr-37 is located near the site of $\beta\gamma$ binding, it is likely that phosphorylation of Try-37 would modulate GDP dissociation and GTP activation. Overexpression of c-Src kinase (CSK), which inhibits c-Src activation, impairs $G_{\beta,\gamma}$-mediated tyrosine phosphorylation of EGFR and Shc.

## VII. TUMOR ESCAPE FROM HOST DEFENSE

The immune system is charged with the function of cancer surveillance and has an ability to clear genetically altered cells that have undergone malignant transformation. However, tumor cells develop an ability to avoid this clearance and survive, grow, and metastasize. The mechanisms by which cancer cells escape host immune responses vary among different types of cancers and even within different cancers of a particular type. Generally, tumors accomplish escape by a host of mechanisms, including down-regulation of expression of surface antigens [such as major histocompatibility complex class I (MHC-I) antigens]; increased secretion of immunosuppressive cytokines [such as transforming growth factor-β (TGF-β), IL-10, or vascular endothelial growth factor (VEGF)]; generation of variants of tumor antigens, thereby affecting the ability of immune cells to recognize tumor cells; and overexpression of Fas ligand, which interacts with immune T cells and induces their apoptosis. These actions significantly reduce the ability of immune cells to recognize tumor cells, to associate with them, and to induce cytotoxic response. The tumors that are more efficient in inducing all these responses are more aggressive than those that inefficiently evade the immune response.

## VIII. SUMMARY

Tumor growth occurs because of the increased expression of mitogenic peptides or growth factors. Growth can also be caused by mutations in the receptors for mitogens or growth factors or in the components of signal transduction pathways. Such changes promote increased cell growth and decreased cell attrition. Most of the mitogenic signals generated as a result of these changes are mediated by Ras/Raf/MAPK, JAK/Stat, and/or PI3K/Akt signaling pathways. This is supported by studies showing that a majority of human cancers display persistent activation of one or more of these pathways. Tumor cells can also evade the host immune system by secreting various proteins, which may either confuse or kill the natural killer cells of the immune system. Further understanding of these mechanisms will contribute to a better understanding of the processes associated with tumor growth, and will provide novel approaches to regulate and reverse the growth of cancers.

## Glossary

**G-protein-coupled (heptahelical) receptors** A family of transmembrane receptor proteins that activate G-proteins in response to activation by a specific ligand.

**phosphoinositide 3-kinases** A group of lipid kinases that phosphorylate the 3 position of the inositol ring of inositol phospholipids. They have been shown to interact with growth factors, hormones, and oncogene products to activate diverse signal transduction pathways. They also play a key role in mediating anti-apoptotic, i.e., prosurvival, actions.

**tyrosine kinases** A group of transmembrane proteins with tyrosine-specific phosphorylating activity. The extracellular domain of receptor tyrosine kinases serves as a receptor for agonists, such as growth factors and hormones. The binding of a specific ligand to the extracellular domain of the receptors turns on the kinase activity of their catalytic domain, which is located on the cytoplasmic side of the membrane. Nonreceptor tyrosine kinases lack an extracellular binding domain, and they are activated by phosphorylation of their tyrosine residues.

## See Also the Following Articles

**Adrenocorticosteroids and Cancer • Androgen Receptors and Prostate Cancer • Apoptosis • Crosstalk of Nuclear Receptors with STAT Factors • Epidermal Growth Factor (EGF) Family • Estrogen and Progesterone Receptors in Breast Cancer • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Vascular Endothelial Growth Factor • Vitamin D Effects on Cell Differentiation and Proliferation**

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# CC, C, and CX$_3$C Chemokines[1]

RONALD L. RABIN

*Center for Biogenics Evaluation and Research, U. S. Food and Drug Administration, Maryland*

I. INTRODUCTION
II. CC CHEMOKINES
III. C AND CX$_3$C CHEMOKINES

**The CC chemokines (β-chemokines) constitute a large family of chemoattractant peptides in which the first (N-terminal) two of the four conserved cysteines are adjacent. As in the other major chemokine subfamily, the CXC chemokines, the first pair of cysteines lies within the first 10 amino acids of the mature peptide, and there are disulfide bonds between cysteines 1 and 3 and between cysteines 2 and 4. All non-CXC chemokines are presumed to play a role in adaptive immunity and are involved in the immune response or in development and homeostasis.**

## I. INTRODUCTION

There are currently 28 human CC chemokines, which have been assigned systematic names consisting of "CCL" (CC ligand) followed by the number that is in the designation for the corresponding human gene. CC chemokine family members signal only through chemokine receptors with the prefix "CCR" and do not share receptors with the CXC, C, or CX$_3$C families of chemokines. There are three non-CXC, non-CC chemokines. Two are C (γ) chemokines in which one of the pair of N-terminal region cysteines and one of the downstream cysteines are absent, and one is a CX$_3$C (δ) chemokine in which 3 amino acids separate the first and second cysteines. The receptor for the C chemokines (XCL1 and XCL2) is XCR1 and the receptor for CX$_3$CL1 is CX3CR1. Table 1 shows the systematic and common names, gene alias accession numbers, and receptors for the CC, C, and CX$_3$C chemokines.

All non-CXC chemokines have a presumptive role in adaptive immunity and participate either in the response to immunological challenge and inflammation or in homeostasis. Those in the latter group are important for the organization of secondary lymphoid organs—spleen, lymph nodes, and mucosal-associated lymphoid tissue (MALT).

Three features distinguish the non-CXC chemokines from their CXC counterparts. Although neutrophils migrate toward many of the CXC chemokines, the role of CC family members in neutrophil migration in humans is minimal. Second, although only one CXC chemokine can bind to more than one receptor (CXCL8 binds CXCR1 and CXCR2), seven pro-inflammatory CC chemokines signal through two or three receptors. This promiscuity has impeded attempts to define precise roles for CC chemokines in immune function. Third, although most of the CXC chemokine family members can either enhance or inhibit angiogenesis, CCL2, CCL11, and CX$_3$CL1 are the only non-CXC chemokines shown to be pro-angiogenic, and none have been reported to inhibit angiogenesis.

All chemokine receptors are seven-transmembrane domain proteins that are coupled to G- proteins (7TMDGPCR). Of the four classes of heterotrimeric G-proteins, G$_i$-proteins are those best documented as coupling to chemokine receptors and being essential for chemotaxis. G-protein activation leads to signaling through phospholipase Cβ, phosphatidylinositol-3-kinase-γ, and protein kinase C. The endpoints of these cascades can include chemotaxis, respiratory burst, degranulation, and enhanced cell adhesion. It is important to note that the consequences of signaling can vary according to cellular context. For example, whereas freshly isolated monocytes and lymphocyte subsets express receptors for CCL5 (RANTES) and all receptor-bearing cells can migrate toward CCL5,

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TABLE 1 CC, C, and CX$_3$C Chemokines

| Official names | | | Database accession no. | | | |
|---|---|---|---|---|---|---|
| Protein | Gene chromosome | Common synonyms | Human | Mouse | Subclass | Receptors |
| **CC(β) Chemokines** | | | | | | |
| CCL1 | *SCYA1* 17 | I-309; (mouse) TCA-3 | P22362 | P10146 | 4 cys | CCR8 |
| CCL2 | *SCYA2* 17q11.2–q12 | MCP-1; MCAF:(mouse) JE | P13500 | P10148 | 4 cys | CCR2, DARC |
| CCL3 | *SCYA3* 17q11–q21 | MIP-1α; MIP-1αS; LD78α[e] | P10147 | P10855 | 4 cys | CCR1, CCR5, D6 |
| NA | *SCYA3L1* 17q11–q21 | LD78β, MIP-1αP | P16619 | P10855 | 4 cys | CCR1, CCR5, D6 |
| CCL4 | *SCYA4* 17q11–q21 | MIP-1β | P13236 | P14097 | 4 cys | CCR5, D6 |
| CCL5 | *SCYA5* 17q11.2–q12 | RANTES | P13501 | P30882 | 4 cys | CCR1, CCR3, CCR5, D6, DARC |
| CCL6 (reserved) | *SCYA6* | (Mouse) C10; (mouse) MRP-1 | NA | P27784 | 6 cys | CCR1 |
| CCL7 | *SCYA7* 17q11.2–q12 | MCP-3 | P80098 | Q03366 | 6 cys | CCR1, CCR2, CCR3, D6 |
| CCL8 | *SCYA8* 17q11.2 | MCP-2 | P80075 | AB023418 | 4 cys | CCR1, CCR2, CCR3, CCR5, D6 |
| CCL9 (reserved) | *SCYA9* | (Mouse) MRP-2; (mouse) MIP-1γ | NA | P51670 | 6 cys | CCR1 |
| CCL10 (reserved) | *SCYA10* | | | | 4 cys | |
| CCL11 | *SCYA11* 17q21.1–q21.2 | Eotaxin | P51671 | P48298 | 4 cys | CCR3 |
| CCL12 (reserved) | *SCYA12* | (Mouse)MCP-5 | NA | Q62401 | 4 cys | CCR2 |
| CCL13 | *SCYA13* 17q11.2 | MCP-4 | Q99616 | NA | 4 cys | CCR2, CCR3, D6 |
| CCL14a | *SCYA14* 17q11.2 | CC-1; HCC-1; NCC-2; CCCK-1/CCCK-3; Ckβ1; MCIF | Q16627 | NA | 4 cys | CCR1 |
| CCL14b | 17q11.2 | HCC-3 | | | | Undetermined |
| CCL15 | *SCYA15* 17q11.2 | HCC-2; leukotactin-1 (Lkn-1); MIP-5; CC-2; NCC-3; MIP-1δ | Q16663 | NA | 6 cys | CCR1, 3 |
| CCL16 | *SCYA16* 17q11.2 | HCC-4; LEC; NCC-4; LMC; monotactin-1 (Mtn-1); LCC-1; ILINCK | AB018249 | NA | 4 cys | CCR1, CCR2, CCR5, CCR8 |
| CCL17 | *SCYA17* 16q13 | TARC | Q92583 | AJ242587 | 4 cys | CCR4 |
| CCL18 | *SCYA18* 17q11.2 | DC-CK-1; PARC; MIP-4; AMAC-1; ckβ7 | P55774 | NA | 4 cys | Undetermined |
| CCL19 | *SCYA19* 9p13 | MIP-3β, ELC; exodus-3; ckβ11 | Q99731 | AF059208 | 4 cys | CCR7 |
| CCL20 | *SCYA20* 2q33–q37 | MIP-3α, LARC; exodus-1; (mouse) ST38 | P78556 | AB015136 | 4 cys | CCR6 |
| CCL21 | *SCYA21* 9p13 | 6Ckine; SLC; exodus-2; TCA4; ckβ9 | CAA06653 | AF001980 | 6 cys | CCR7 |
| CCL22 | *SCYA22* 16q13 | MDC; (mouse) dc/b-ck; (mouse) abcd-1 | U83171 | AJ238238 | 4 cys | CCR4 |
| CCL23 | *SCYA23* 17q11.2 | MPIF-1; MIP-3; ckβ8-1 | P55773 | NA | 6 cys | CCR1 |
| CCL24 | *SCYA24* 7q11.23 | MPIF-2; eotaxin-2; ckβ6 | U85768 | NA | 4 cys | CCR3 |
| CCL25 | *SCYA25* | TECK, ckβ15 | O15444 | O35903 | 4 cys | CCR9 |
| CCL26 | *SCYA26* 7q11.23 | Eotaxin-3; MIP-4α | AC005102 | NA | 4 cys | CCR3 |
| CCL27 | *SCYA27* 9p13 | CTACK; ILC; PESKY; ESKINE (mouse) ALP | AJ243542 | AF099931 | 4 cys | CCR10 |
| CCL28 | *SCYA28* | MEC | AF220210 | AF220238 | 6 cys | CCR3, CCR10 |
| **C(γ) Chemokines** | | | | | | |
| XCL1 | *SCYC1* 1q23 | Lymphotactin α; SCM-1α; ATAC | NP_002986 | P47993 | | XCR1 |
| XCL2 | *SCYC2* 1q23–q25 | Lymphotactin β; SCM-1β; ATAC | NP_003166 | P47993 | | XCR1 |
| **CX$_3$C (δ) Chemokines** | | | | | | |
| CX$_3$CL1 | *SCYD1* 16q13 | Fractalkine; (mouse) neurotactin | U91835 | AF071549 | | CX3CR1 |

Adapted from P. M. Murphy, *et al.*, International Union of Pharmacology. XXII. Nomenclature for Chemokine Receptors. *Pharmacol. Rev.* **52**, 145, 2001, with permission from the corresponding author. The systematic names refer only to human chemokines. However, the accession numbers and common names for putative mouse orthologues are included. CCL6, CCL9, and CCL12 lack known human orthologues; the standard name is reserved for a potential human counterpart. In many cases, the same common name applies to human and mouse counterparts, but species-specific names are sometimes used to convey substantially different properties, such as a major difference in sequence (e.g., human I-309 versus mouse TCA-3) or length (e.g., mouse JE versus MCP-1). The number in the systematic name for each chemokine matches that in an alias for the corresponding human gene name, and the roots for gene names correspond to protein roots as follows: SCYA = CCL; SCYB = CXCL; SCYC = XCL; and SCYD = CX3CL, where SCY denotes small cytokine; A, B, C, and D denote the chemokine classes in the gene locus; and L signifies “ligand” in the root of the protein name. Thus, for example, SCYA1 is a gene alias for the human chemokine CCL1. Accession numbers are from the SwissProt database, when available; NA, not available in any database. Also listed are the two nonsignaling chemokine-binding proteins D6 and Duffy antigen receptor (DARC).

only the monocytes respond to CCL5 with a global calcium flux.

The genes for 14 of the 28 non-CXC chemokines are clustered in one of two subregions of chromosome 17 (Table 2). The others are scattered over six chromosomes, although in three instances, pairs of genes for homologous chemokines that share a common receptor are adjacent to each other. These patterns suggest that both dispersion and duplication *in situ* are responsible for generating the gene family.

The chemokines are discussed in groups, first, according to the class of chemokine (CC, C, and then CX$_3$C), second, by their roles in either homeostasis or response to immune challenge, and third, according to similarities in chromosome localization, protein structure, function, and receptor usage, all of which are typically correlated (see Table 2).

**TABLE 2 Chromosomal Organization of CC, C, and CX$_3$C Chemokines**

| Chromosome (subregion) | Chemokine (common name) |
|---|---|
| 2 | CCL20 (MIP-3α) |
| 5 | CCL28 |
| 7 | CCL24 (eotaxin-2) |
| | CCL26 (eotaxin-3) |
| 9 | CCL19 (ELC) |
| | CCL21 (SLC) |
| | CCL27 (CTACK) |
| 16 | CCL17 (TARC) |
| | CCL22 (MDC) |
| 17 (MIP subregion) | CCL4 (MIP-1β) |
| | CCL3 (MIP-1α) |
| | CCL18 (DC-CK1) |
| | CCL23 (MPIF-1) |
| | CCL15 (leukotactin) |
| | CCL14 (HCC-1) |
| | CCL16 (HCC-4) |
| | CCL5 (RANTES) |
| 17 (MCP subregion) | CCL1 (I-309) |
| | CCL13 (MCP-4) |
| | CCL8 (MCP-2) |
| | CCL11 (eotaxin) |
| | CCL7 (MCP-3) |
| | CCL2 (MCP-1) |
| 19 | CCL25 (TECK) |
| 1 | XCL1 (lymphotactin-1) |
| | XCL2 (lymphotactin-2) |
| 16 | CX$_3$CL1 (fractalkine) |

*Note*. The chemokines are listed first according to the chromosomal location of their gene, and from top to bottom, with decreasing distance from the centromere. The most common nonsystematic name is given in parentheses.

## II. CC CHEMOKINES

### A. Structural Features

The secondary and tertiary structures of the CC and CXC chemokines are similar. The N-termini that lead to the first cysteine are elongated, limited in flexibility by the first two cysteines, and except for CCL5, unstructured in solution. After the first two cysteines, there is a loop of approximately 10 residues, the so-called N-loop, followed by a single turn $3_{10}$ helix, three anti-parallel β-strands that form a β-pleated sheet, and an α-helix at the C-terminus. Each of the β-strands and the α-helix is connected by the 30's, 40's, and 50's loops, named for their position in the peptide sequence. The third and fourth cysteines are in the 30's and 50's loops, respectively. Naturally produced chemokines are usually glycosylated, but recombinant proteins that lack glycosylation retain their *in vitro* biological activity. The α-helix and C-terminus contain basic residues that bind to glycosylaminoglycans (GAGs), such as heparin and chondroitin sulfate.

The two disulfide bonds and hydrophobic interactions between the C-terminal helix and the β-sheet are critical for chemokine structure. Several CC chemokines have additional cysteine residues, in one case within the N-loop and in the other cases C-terminal to the fourth conserved cysteine.

Some chemokines multimerize at concentrations necessary for X-ray crystallography and nuclear magnetic resonance (NMR); and quaternary structure is where CXC and CC chemokines differ. The CXC chemokines tend to contain hydrophobic residues along the first β-strand, which is their point of contact. By contrast, CC chemokines have hydrophobic residues surrounding the first two cysteines, and their point of contact is along the extended N-terminus.

The relevance of dimers in chemokine function is controversial. Mutant proteins that cannot dimerize are active *in vitro* and compete well for receptor with wild-type proteins. On the other hand, chemokines are frequently "presented" to circulating leukocytes bound to GAGs on the surface of endothelial cells, which would be expected to lead to oligomerization *in vivo*. Oligomerization of ligands raises the possibility of receptor oligomerization. Dimerization of 7TMDGPCR has been shown in a number of systems including those for chemokine receptors and may result in activation of signaling molecules usually associated with other types of cytokine receptors, such as the Janus kinases. Heterodimerization of CCL3 (MIP-1α) and CCL4 (MIP-1β) has also been reported, suggesting the possibility of receptor heterodimerization. Finally, GAG-mediated multimerization of CCL5 has been reported to lead to activation of *Src* kinase family members independent of 7TMDGPCR signaling.

## B. CC Chemokines with a Role in Homeostasis of the Adaptive Immune System

### *1. CCL19 (ELC) and CCL21 (SLC)—Chromosome 9*

The structure of CCL19 is typical for CC chemokines. However, CCL21 has a 30-amino-acid, highly basic, C-terminal extension that interacts with GAGs on high endothelial venule cells. An additional pair of cysteines in the extension of CCL21 may form a disulfide bond.

CCL19 is expressed at high levels in the thymus medulla including endothelial venules and in lymph nodes by dendritic cells (DCs). Expression is increased by the inflammatory cytokines tumor necrosis factor α (TNFα) and interferon-γ (IFN-γ). CCL21 is also expressed in thymus, but much less than CCL19. CCL21 is expressed in the high endothelial venules that direct migration of cells into lymphoid organs. These two chemokines bind only to CCR7.

By definition, homeostatic migration occurs in the absence of inflammation and allows lymphocytes to survey secondary lymphoid tissue for antigen. This includes migration from the blood across specialized high endothelial cells into lymph nodes and presumably migration within the tissue itself. CCL19 and CCL21 provide this function for naive T cells and a subset of memory T cells that surveys secondary lymphoid tissues for recall antigens. Both of these chemokines trigger rapid integrin-mediated arrest of lymphocytes under flow conditions, emphasizing their importance in migration from the microvasculature. In addition, DCs express CCR7 when they begin to migrate through lymphatics and into the T-cell zones of lymph nodes. Thus, CCL19 and CCL21 are "compartment" chemokines that direct a variety of cell types to T-cell zones of lymphoid organs.

Neutralization of CCL19 in mice results in reduced numbers of T cells in spleens of newborn mice due to defective thymocyte emigration. In the *plt* (paucity of lymph node T cells) mouse, which fails to express both CCL19 and CCL21 in lymphoid organs, naive T cells and DCs do not home into the T-cell areas of lymph nodes, Peyer's patches, and the white pulp of the spleen.

## C. CC Chemokines with Pro-inflammatory Roles in Adaptive Immunity

### *1. CCL20 (MIP-3α)—Chromosome 2*

CCL20 is expressed in thymus, in lymph nodes, and at high levels in epithelium associated with MALT. Inflammatory cytokines and lipopolysaccharide (LPS) induce the expression of CCL20 by peripheral blood mononuclear cells (PBMCs), endothelial and epithelial cells, monocyte and macrophage cell lines, and neutrophils.

CCL20 binds only to CCR6, which is expressed on B cells, a subset of memory T cells, and immature DCs, all of which migrate toward CCL20. Of note, CCL20 is highly active on resting memory T cells, which suggests that this chemokine may be important for their recruitment to inflammatory sites early in an immune response. In addition, of the five CC chemokines that reportedly reversibly arrest hematopoiesis *in vitro*, CCL20 is the only one that does not bind to CCR1.

Although no knockout (KO) mice for CCL20 have been described, CCR6KO mice show a defect in mucosal antibody production. CCL20 expression levels are high in models of allergen-induced pulmonary inflammation, and when CCR6 KO mice are used in these models, pulmonary inflammation is reduced.

In humans, CCL20 is expressed in affected tissues in a variety of autoimmune diseases, such as psoriasis, rheumatoid arthritis, and type I diabetes—all consistent with the role for this chemokine in mediating the migration of memory T cells.

### *2. CCL28—Chromosome 5*

CCL28 is expressed in secondary lymphoid organs and at high levels in mucosal epithelium. CCL28 signals through CCR10, which is also the receptor for CCL27. CCR10 is expressed on skin-derived Langerhans' cells and on T cells infiltrating into skin (see section on CCL27, below).

### *3. CCL11 (Eotaxin)—Chromosome 17, CCL24 (Eotaxin-2), and CCL26 (Eotaxin-3)—Chromosome 7*

CCL11 was discovered as a result of a systematic search for eosinophil chemotactic factors in bronchoalveolar lavage fluid in a guinea pig asthma model. Despite the relatively low level of sequence identity among the "eotaxins" (34–38%), they all bind to the receptor CCR3, which is expressed at high levels on eosinophils and basophils.

A variety of cell types express CCL11, including epithelial and endothelial cells, smooth muscle cells, mast cells, and macrophages. All the eotaxins are expressed in response to interleukin-4 (IL-4) or IL-13, two cytokines that are associated with the type 2 response and that signal through the transcription factor STAT6. However, CCL11 and CCL24 are constitutively expressed in some tissues, and expression of CCL11 is also enhanced by the inflammatory cytokines TNFα or IL-1β. CCL26 expression is limited to vascular endothelium and fibroblasts.

The eotaxins are potent eosinophil and basophil chemoattractants. They also trigger calcium flux, actin polymerization, and eosinophil effector functions including respiratory burst, Leukotriene C4 (LTC4) production, adhesion molecule activation, release of eosinophil granule cationic proteins and preformed IL-4, and internalization of CCR3. CCR3 is a co-receptor for some human immunodeficiency virus-1 (HIV-1) isolates, and CCR3 ligands block CCR3-mediated infection *in vitro*.

CCL11-deficient mice have reduced levels of eosinophils in the intestine compared with wild-type controls, demonstrating that CCL11 is critical for basal trafficking of eosinophils into tissue. Studies using these mice to address the role of CCL11 in allergen-induced eotaxin recruitment into the lung have yielded discrepant results, suggesting that the role of CCL11 in this context is dependent on the strain of mice used. By contrast, CCL11 is critical for allergen-induced eosinophilic gastrointestinal inflammation, since CCL11-deficient mice show no pathology in that model.

CCL11, CCL24, and CCL26 are important either individually or together in diseases that are mediated by eosinophils and are associated with atopy, since CCR3 is the dominant receptor mediating eosinophil recruitment. Human studies have shown an association of type 2 diseases with CCL11, which is elevated in serum in patients with atopic dermatitis and in nasal lavage fluid from patients with allergic rhinitis. Furthermore, asthmatics who are homozygous for a variant of CCL11 (A23T) have lower levels of plasma CCL11 and better lung function than asthmatics who lack the mutation. Since expression of the eotaxins is regulated differently, they may have nonredundant roles *in vivo*, despite sharing a receptor.

### *4. CCL27 (CTACK)—Chromosome 9*

CCL27 is constitutively expressed by keratinocytes, and expression is increased by TNFα and IL-1β and decreased by IL-10. Fibroblasts in inflamed skin also express CCL27. CCL27 binds only to CCR10, which is also the receptor for CCL28. CCR10 is frequently co-expressed with the skin homing molecule cutaneous lymphoid antigen (CLA) on memory T cells, and all T cells invading the skin are CCR10$^+$, suggesting that CCL27 and CCR10 may be important for supporting the inflammatory response in skin and that blockade may attenuate inflammatory skin disease.

### *5. CCL17 (TARC) and CCL22 (MDC)—Chromosome 16*

Both CCL17 and CCL22 are highly expressed in thymus and to a lesser extent in lungs, intestines, skin (by keratinocytes), and secondary lymphoid tissue (by DCs and macrophages). IL-4 and IL-13 enhance and IFN-γ inhibits the expression of CCL22.

Both CCL17 and CCL22 signal only through CCR4, which is expressed on thymocytes, monocytes, activated natural killer (NK) cells, and DCs. CCR4 is also expressed on a subset of memory CD4 T cells, including Th2 cells, on those that express CLA (see section on CCL27, above), and on CD4 regulatory T cells, a newly defined subset that suppresses the immune response.

Expression of CCL22 in the thymus suggests a role in directing the migration of thymocytes during maturation. In murine models, antibody blockade of CCL17 or CCL22 attenuates ovalbumin-induced asthma, consistent with a role for these chemokines in the type 2 response. Concordantly, levels of CCL17 are high in the serum and sputum of asthmatics, and both CCL17 and CCL22 are elevated in patients with atopic dermatitis.

### *6. CCL3 (MIP-1α) and CCL4 (MIP-1β)—Chromosome 17, MIP Subregion*

CCL3 is expressed constitutively in the bone marrow, including by osteoblasts, and is induced during inflammation. Bacterial toxins, viral infections, TNFα, IFN-γ, IL-1β, and IL-6 are some of the stimuli that induce the expression of CCL3 and/or CCL4 *in vitro* from multiple cell types including monocytes/macrophages, DCs, and epithelial cells. In addition, IL-10, IL-4, and transforming growth factor-β attenuate the inflammation-induced expression of these two chemokines.

CCL3 signals through CCR1 and CCR5. CCR5 is the primary receptor for CCL4. Neutrophils, eosinophils, and T cells express CCR1, although CCL3 is not a chemotactic factor for human neutrophils. NK cells and subsets of resting memory T cells, including some but not all Th1 cells, express CCR5. Monocytes express both CCR1 and CCR5.

*In vitro*, CCL3 induces the chemotaxis of CCR1- or CCR5-expressing cells, stimulates adhesion and integrin expression, and may increase IL-2 secretion and proliferation by T cells in response to antigen receptor stimulation. CCL4 is a chemoattractant for CCR5-expressing cells and also activates NK cells to kill tumor cells *in vitro*. Both of these chemokines are expressed in inflamed tissues in mice and humans.

Expression of CCR5 on Th1 cells and induction of CCR1 expression on T cells by IFN-α suggest a role for CCL3 and CCL4 in cell-mediated immunity. Mice treated with monoclonal antibodies against CCL3 or that are CCL3-deficient have an impaired inflammatory response and decreased pathology from viral infections, decreased survival after infection with *Cryptococcus neoformans*, delayed allograft rejection, and attenuated development of experimental autoimmune encephalitis. Mice treated with antibodies to CCL4 have decreased responses to endotoxemia and hapten-induced contact hypersensitivity.

The demonstration that CCL3 and CCL4 (and CCL5) block *in vitro* infection by macrophage-tropic strains of HIV-1 was crucial for the discovery that CCR5 is the major co-receptor used by HIV-1 for fusion and entry. In fact, HIV-1-infected patients with higher levels of circulating CCL3 and CCL4 have an improved prognosis. CCL5 analogues have been used to study the effect of CCR5 blockade on HIV-1 infection, as have "intrakines" containing CCL3 that trap CCR5 in the endoplasmic reticulum to render cells resistant to HIV-1. Clinical studies of CCR5 blockers with the goal of treating HIV-1 infection are under way.

CCL3 reversibly arrests hematopoiesis *in vitro* and was investigated as an agent to protect the bone marrow from toxicity of cancer chemotherapeutics. Unfortunately, to date, the human studies have shown little benefit. CCL15, CCL16, and CCL23 also bind to CCR1 and arrest hematopoiesis.

Inhibition of CCL3 and CCL4 is being explored in animal models of autoinflammatory diseases in which local levels are elevated—rheumatoid arthritis, for example—but has not been tested in humans.

### 7. CCL5 (RANTES)—Chromosome 17, MIP Subregion

CCL5 expression is induced by IFN-γ, TNFα, or IL-1 in T cells, fibroblasts, macrophages, epithelial cells, and endothelial cells. In addition, CCL5 protein is found in eosinophil granules and is released upon exposure to IFN-γ.

CCR1, CCR3, and CCR5 all function as receptors for CCL5. Thus, CCL5 produces signals in monocytes, macrophages, T-cell subsets, DCs, eosinophils, basophils, and microglia. In addition to its chemoattractant activity, CCL5 stimulates eosinophils to secrete eosinophil cationic protein and stimulates basophils to release histamine.

Mononuclear cells in CCL5 KO mice migrate less to sites of cutaneous hypersensitivity and T cells from these mice proliferate less *in vitro* in response to mitogens and specific antigens. The effects of CCL5 overexpression in animal models or challenge in humans vary with the anatomic site and type of challenge. Intratracheal challenge of Sprague–Dawley rats with CCL5-expressing adenovirus showed enhanced monocyte recruitment to the lung by approximately 50-fold, although CCL5 challenge to nasal mucosa of allergic patients induced eosinophil influx. CCL5 is expressed in lungs of patients with chronic eosinophilic pneumonia, suggesting that CCL5 has a role in eosinophil-induced pathology and that CCL5 blockade may be beneficial in treatment of atopic diseases, including asthma.

CCR5 is the major HIV-1 co-receptor, and CCL5 is an effective inhibitor of CCR5-mediated infection *in vitro*. Since increased transcription of the CCL5 gene due to polymorphisms in its promoter is associated with delayed progression of HIV-1 disease, CCR5 blockade with CCL5 or its derivatives may be an effective strategy for prevention of HIV-1 entry into cells. Clinical trials of modified CCL5 proteins to block HIV transmission are under way.

### 8. CCL18 (DC-CK, PARC)—Chromosome 17, MIP Subregion

CCL18 is constitutively expressed by DCs in secondary lymphoid organs and by alveolar macrophages in lung. Expression can be induced by IL-4, IL-10, IL-13, and LPS and inhibited by IFN-γ.

CCL18 attracts naive resting T cells and naive tonsil B cells and thus may participate in primary T-cell and B-cell activation in secondary lymphoid tissue. Since CCL18 is also expressed at high levels in hypersensitivity pneumonitis and by macrophages in atherosclerotic plaques, it may have a role in chronic inflammation as well, presumably by targeting cells other than naive lymphocytes.

### 9. CCL23 (MPIF-1, CKβ8-1) and CCL15 (Leukotactin-1)—Chromosome 17, MIP Subregion

CCL15 and CCL23 are similar in many respects. They share >80% identity, have unusually long amino-termini, 31 and 49 amino acids, respectively, possess an extra pair of (presumably bonded) cysteines, and are transcribed from genes with four exons, one more than the other CC chemokine family members.

There are, however, distinct differences between these two chemokines. CCL23 is the only chemokine with an alternative splice variant in which amino-terminal amino acids are deleted. In addition, although both are expressed in response to IL-4 and LPS, TNFα and IFN-γ also stimulate CCL15 expression, and although both CCL15 and CCL23 signal through CCR1, CCL15 also uses CCR3. Thus, although both chemokines attract monocytes, lymphocyte subsets, and neutrophils, only CCL15 attracts eosinophils.

CCL15 is expressed by leukocytes and is found in the adrenal gland, pancreas, heart, intestine, and liver. CCL15 is also found in atherosclerotic plaques. CCL23 is expressed in pancreas, skeletal muscle, and bone and is chemotactic for osteoclast precursors, suggesting a role in bone remodeling. In addition, the genes for CCL15 and CCL14 (see below) are separated by 14 kb, and bicistronic transcripts are found in colon and liver.

### *10. CCL14 (Hemofiltrate CC Chemokine-1, HCC-1) and CCL16 (HCC-4)—Chromosome 17, MIP Subregion*

CCL14 was purified initially from hemofiltrate collected from patients with chronic renal failure. Two variants lacking the first two and four amino acids of the amino terminus are more potent than the parent protein. In addition to bicistronic transcripts with CCL15 in colon and liver, monocistronic transcripts for CCL14 are found in all tissues examined except brain and placenta. Leukocytes do not express CCL14. The reason for the high levels of CCL14 in normal plasma and for its elevation during chronic renal failure is unknown.

The C-terminus of CCL16 is approximately 20 amino acids longer than most other CC chemokines. CCL16 is expressed in liver and by monocytes, T cells, and NK cells. CCL16 expression is increased by the anti-inflammatory cytokine IL-10.

Both of these chemokines primarily signal through CCR1. CCL16 also binds to CCR2, CCR5, and CCR8. The required concentrations for induction of chemotaxis by CCL16 are 10- to 100-fold higher than those for other chemokines, and CCL16 cannot block CCR5-mediated HIV-1 infection *in vitro*.

In its secreted form, CCL14 binds only to CCR1. However, serine proteases that are found, for example, in tumor cell supernatants, cleave the amino-terminal eight amino acids, resulting in a chemokine that binds to CCR3 and CCR5. Since binding of the cleaved form of CCL14 internalizes CCR5, *in vitro* infection with CCR5-dependent strains of HIV-1 is blocked.

The roles for these chemokines in immunity are unclear. CCL14 protects mice challenged with LPS, and tumors that express this CCL16 are rejected more readily than nonexpressers.

### *11. CCL1 (I-309)—Chromosome 17, MCP Subregion*

CCL1 is expressed by T cells, mast cells, and, in response to LPS or inflammatory cytokines, by monocytes and macrophages. CCL1 signals through CCR8, the only chemokine to do so. CCR8 is expressed on thymocytes, on monocytes, and, similar to CCR4, on Th2 and CD4 regulatory T cells. Thus, like CCR4 and its ligands, CCR8 and CCL1 may have a role in type 2-mediated (allergic) diseases and in preserving the balance between appropriate and overexuberant or misdirected immune responses.

### *12. CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), and CCL13 (MCP-4)—Chromosome 17, MCP Subregion*

These four chemokines are collectively referred to as the "MCPs". Their expression is induced by inflammatory cytokines, such as IL-1, TNFα, IFN-γ, IL-6, and IL-4, in many different cell types including fibroblasts, endothelial cells, smooth muscle cells, monocytes/macrophages, and keratinocytes. Expression of the MCPs is attenuated by glucocorticoids, estrogen, progesterone, and by IL-4, IL-10, IL-13, and TGFβ.

All of the MCPs target CCR2, and all but CCL2 signal through CCR3. In addition, CCL7 and CCL8 signal through CCR1 and CCL8 signals through CCR5, and can inhibit HIV-1 infection *in vitro*. CCR2 is expressed by monocytes, by NK cells, and by subsets of memory CD4 T cells.

The MCPs are best characterized as chemoattractants for monocytes, but they also attract subsets of resting or activated memory CD4 T lymphocytes, NK cells, and DCs. Since CCR3 is expressed on eosinophils and basophils, these cells are targets for all MCPs except CCL2. In addition, the MCPs may modulate the immune response independently of their effects on migration, by increasing type 2 polarization of T cells, stimulating secretion of IL-1 and IL-6 by monocytes, and, in the case of the CCR3 ligands, stimulating basophils to release histamine.

CCL2 has been extensively studied in murine models. Mice transgenic for CCL2 show an accumulation of monocytes in uninflamed tissues. Genetic deletion of CCL2 results in the lack of migration of monocytes after intraperitoneal thioglycollate challenge and decreased ability to mount type 2 responses. CCL2 blockade attenuates murine models of asthma, arthritis, and glomerulonephritis.

In humans, CCL2 is expressed in many pathologic tissues and states, including rheumatoid arthritis synovia, asthma, idiopathic pulmonary fibrosis, psoriasis, multiple sclerosis, and HIV-1 dementia. Plasma levels of CCL2 are elevated in sepsis, congestive heart failure, and acute myocardial infarction. CCL2 is expressed in atherogenic plaques, and atherogenic mice cross-bred to CCR2 KO mice are rescued from atheroma formation. Also interesting are reports that CCL2 expression is decreased by estradiol, perhaps contributing to the differences in the incidence of vascular disease between males and females. Taken together, the animal models and human studies suggest that CCL2 blockade may be an effective therapy for inflammatory diseases and arteriosclerosis.

#### *13. CCL25 (TECK)—Chromosome 19*

CCL25 is unusually long (142 amino acids) due to the unique C-terminus, which is 40 amino acids longer than that of most other chemokines. CCL25 is highly expressed in thymus by DCs and epithelial cells and in small intestinal epithelium. CCR9 is the unique receptor for CCL25 and is highly expressed on CD4$^+$ CD8$^+$ thymocytes. Two observations suggest a role for CCR9 in mucosal immunity. First, CCR9$^+$ T cells co-express the $\alpha4\beta7$ integrin, which mediates homing into mucosal sites. Second, CCL25 attracts cells that secrete IgA, an antibody isotype that confers humoral protection at mucosal sites.

CCR9 KO mice are grossly normal, but competition experiments using CCR9$^{-/-}$ and CCR9$^{+/+}$ chimeric bone marrow suggest a role for migration of T-cell progenitors to or in the thymus. These mice also reveal a role for CCR9 in the generation of intraepithelial $\gamma/\delta$ T cells.

## III. C AND CX$_3$C CHEMOKINES

### A. XCL1 (Lymphotactin $\alpha$) and XCL2 (Lymphotactin $\beta$)—Chromosome 1

XCL1 is an unusual chemokine with only one disulfide bond. The C-terminal extension of XCL1 is conserved across species and contains six hydrophobic and six uncharged polar amino acids (mostly threonine). The NMR structure reveals the conserved chemokine fold of a three-stranded anti-parallel $\beta$-sheet and C-terminal $\alpha$-helix, with disordered N- and C-termini. The C-terminus is necessary for chemokine activity. The XLC1 and XLC2 genes are adjacent on chromosome 1 and the proteins differ by two amino acids.

XCL1 is produced by prothymocytes, activated CD8 T cells, Th1 T cells, $\gamma\delta$ epidermal T cells, and mast cells. XCL1 signals through XCR1 and is a chemoattractant for T cells and NK cells *in vitro* and *in vivo*.

XCL1 can induce anti-tumor immunity in mice synergy with IL-2. There is also evidence that XCL1 may have a role in graft rejection and T-cell-mediated autoimmunity and as a vaccine adjuvant.

### B. CX$_3$CL1 (Fractalkine)—Chromosome 16

CX$_3$CL1 is a 373-amino-acid type 1 transmembrane protein, of which the N-terminal 76 amino acids comprise the chemokine domain and 241 amino acids include a mucin stalk, which is followed by a transmembrane domain and an intracellular domain. A cleaved soluble form can also be produced. CX$_3$CL1 is unique in having 3 amino acids between the first two conserved cysteines. Although the CX$_3$CL1 gene is located between the genes for CCL17 and CCL22, CX$_3$CL1 does not share any more homology with them than with the other CC chemokines.

CX$_3$CL1 is expressed by neurons, macrophages, dendritic cells, and inflamed endothelial cells. It signals through CX3CR1 and is a chemoattractant for T cells, monocytes, and NK cells. The transcription factor p53 is expressed in injured cells, functions as a tumor suppressor, and induces the expression of CX$_3$CL1, thus suggesting a role for this chemokine in recruiting cytotoxic cells and eliminating cells that might undergo malignant transformation. CX$_3$CL1 also signals through CX3CR1 on neurons, but its function in the central nervous system is unknown. CX3CL1 is unique among chemokines in being able to mediate arrest of cells under conditions of flow by direct interaction, without G protein signaling. In addition, CX3CR1 is a co-receptor for HIV-1 and functions as a binding site for the envelope protein of the respiratory syncytial virus. CX$_3$CL1 can block the CX3CR1-mediated entry of these viruses *in vitro*.

CX3CR1 is preferentially expressed on Th1 T cells, and CX3CL1 is found in lesions of psoriasis, a type 1 autoimmune disease. Inhibiting CX$_3$CL1 activity prevented leukocyte infiltration in a rat model of crescentic glomerulonephritis, and CX$_3$CR1 KO mice required less immunosuppression to prevent cardiac allograft rejection.

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## Glossary

**adaptive immunity** A limb of the immune system that responds slowly and that depends on interactions between components of pathogens and antigen-specific T-cell or B-cell receptors. Adaptive immunity improves qualitatively and quantitatively with continued or repeated exposure to pathogen antigens. B and T cells are the primary cells of the adaptive limb.

**antigen** Literally, any molecule that binds antibody. Frequently used to refer to any foreign molecules that stimulate an adaptive immune response.

**dendritic cells** Specialized bone marrow-derived cells that present antigen to T cells and are important for the primary immune response that results in naive to memory T-cell transition.

**innate immunity** A limb of the immune system that responds quickly and that does not depend on interactions between components of pathogens and antigen-specific T-cell or B-cell receptors. The neutrophil is the quintessential innate immune cell.

**integrins** Heterodimeric molecules on the surface of leukocytes that mediate strong adhesion between cells, including adhesion between leukocytes and endothelial cells, which is necessary for proper trafficking. Integrins that are activated have increased affinity for their ligands, which are members of the immunoglobulin superfamily.

**memory B or T cells** B or T cells (or their descendants) that have been activated by their cognate antigen and undergone phenotypic changes resulting in a lower activation threshold and the capability to migrate into peripheral tissues in addition to (or rather than) secondary lymphoid organs.

**naive B or T cells** Mature B or T cells that have never been activated by their cognate antigen. Naive cells can migrate only into secondary lymphoid organs and have a higher threshold for activation than memory cells.

**primary lymphoid organs** Sites of lymphocyte development and maturation. In humans, these are the bone marrow (B cells, NK cells) and thymus (T cells).

**secondary lymphoid organs** Sites of primary stimulation of B and T cells by specific antigen. These include, among others, lymph nodes, spleen, and Peyer's patches.

**Th1** Memory CD4 T cells that secrete IFN-$\gamma$, and not IL-4 or IL-5, and direct an adaptive response to intracellular pathogens such as viruses. Th1 cells guide a "type 1" response, which is important for most host defense and is implicated in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

**Th2** Memory CD4 T cells that secrete IL-4, IL-5, and IL-13, and not IFN-$\gamma$. These Th2 cytokines are important for B cells to divide, mature, and produce antibodies and for response to some parasites, particularly helminths. Th2 cells guide a "type 2" response, which is implicated in allergic diseases and asthma.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • CXC Chemokines • Defensins • Glucocorticoids and Autoimmune Diseases • Pro-Inflammatory Cytokines and Steroids • Tumor Necrosis Factor (TNF)**

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# Chemokines

See *CC, C, and $CX_3C$ Chemokines; CXC Chemokines*

# Cholecystokinin (CCK)

Rodger A. Liddle
*Duke University Medical Center*

I. INTRODUCTION
II. MOLECULAR FORMS
III. DISTRIBUTION
IV. CCK RECEPTORS
V. CHOLECYSTOKININ RELEASE
VI. BIOLOGICAL ACTIONS OF CCK
VII. CLINICAL USES OF CHOLECYSTOKININ

**Cholecystokinin (CCK), a peptide hormone, is produced and secreted by endocrine cells of the upper small intestine following food ingestion. CCK is the major hormone responsible for stimulating pancreatic enzyme secretion and gallbladder contraction. CCK promotes satiety, delays gastric emptying, potentiates insulin secretion, and may regulate bowel motility; CCK may also play a role in learning and memory, anxiety, analgesia, and thermoregulation.**

## I. INTRODUCTION

Cholecystokinin (CCK) was discovered by Ivy and Oldberg in 1928 when they recognized that intestinal extracts could stimulate gallbladder contraction in dogs; the substance responsible for this action was named cholecystokinin [“cholecyst” (gallbladder); “kinin” (to move)]. In 1943, Harper and Raper noted that a similar extract, which they named “pancreozymin,” stimulated pancreatic enzyme secretion. It was not until CCK was purified and its amino acid sequence determined by Mutt in 1968 that it was proved that CCK and pancreozymin were identical, both possessing the ability to stimulate the gallbladder and the pancreas. The original name, cholecystokinin, is the term commonly used today.

Over the past three decades, CCK has been found to have many other biological effects in experimental animals and in human study participants and patients. One of the most noteworthy actions of CCK is its ability to induce satiety and thus reduce food intake. Until the development of reliable assays for measuring blood levels of CCK, the physiological effects of CCK remained controversial. However, it has now been shown in humans that physiological levels of CCK stimulate gallbladder contraction and pancreatic enzyme secretion, inhibit gastric emptying, potentiate insulin secretion, and reduce food intake. Although it is not yet proved physiologically, CCK may regulate bowel motility and, in certain species, may promote pancreatic growth. Less well described but fascinating actions of CCK include effects on learning and memory, anxiety, analgesia, and thermoregulation.

In the small intestine, CCK is produced by discrete endocrine cells within the mucosa. However, CCK is even more abundant in the brain and is found in peripheral nerves innervating the intestine, where it functions as a neurotransmitter.

## II. MOLECULAR FORMS

The original CCK peptide isolated from the intestine was a tritriacontapeptide (CCK-33). Several larger and smaller molecular forms of CCK have since been found in human and animal intestine, brain, and blood. The biologically active region of CCK resides in its carboxyl terminus and all forms of CCK possesses an identical carboxyl five-amino-acid sequence (-Gly-Trp-Asp-Met-Phe-$NH_2$) (Fig. 1). This region is common to gastrin, thus gastrin has some CCK-like activity (albeit weak) and CCK shares some weak gastrin-like activity. The amino acid sequence shared by the two hormones has made it difficult to develop assays for CCK because antibodies directed against the biologically active region of CCK may cross-react with gastrin. This problem is accentuated by the finding that circulating levels of gastrin are 10- to 100-fold greater than those of CCK.

CCK is produced from a single gene that encodes a 115-amino-acid preprohormone. By posttranslational processing, molecular forms of CCK ranging in size from 4 to 83 amino acids have been identified in tissues and blood. However, the major molecular forms of CCK are CCK-8, CCK-33, and CCK-58. In humans, the CCK gene is located in the 3q12–3pter region of chromosome 3. CCK expression is both tissue specific and developmentally regulated. In the intestine, the CCK gene is expressed prenatally; after birth, expression is regulated primarily by ingestion of foods that stimulate CCK secretion. In the central nervous system, stimuli regulating neuronal CCK gene transcription include growth factors, second messengers such as cyclic adenosine monophosphate

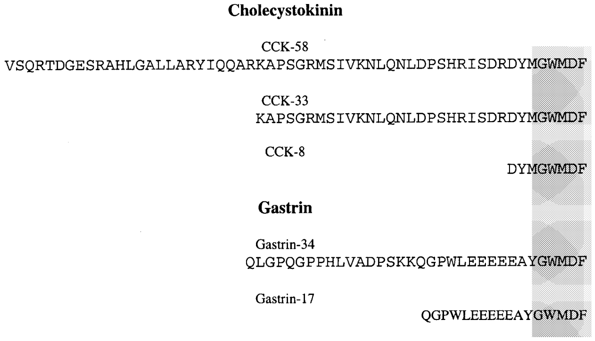
Cholecystokinin
CCK-58
VSQRTDGESRAHLGALLARYIQQARKAPSGRMSIVKNLQNLDPSHRISDRDYMGWMDF
CCK-33
KAPSGRMSIVKNLQNLDPSHRISDRDYMGWMDF
CCK-8
DYMGWMDF
Gastrin
Gastrin-34
QLGPQGPPHLVADPSKKQGPWLEEEEEAYGWMDF
Gastrin-17
QGPWLEEEEEAYGWMDF

**FIGURE 1** Amino acid sequences of the common molecular forms of CCK and gastrin. All biologically active forms of CCK and gastrin share an identical carboxyl-terminal pentapeptide sequence (shaded area).

(cAMP), the neurotransmitter dopamine, and hormones such as estrogen.

## III. DISTRIBUTION

Cholecystokinin cells are flask-shaped cells that are scattered throughout the mucosa of the small intestine. The concentration of CCK cells is greatest in the proximal small intestine and diminishes in a gradient fashion toward the distal jejunum and ileum. CCK cells arise from progenitor cells in the intestinal crypts and, along with enterocytes, migrate up the villus. Residing in the mucosa, the apical surface of CCK cells is open to the lumen of the intestine. Here cells can actually "sample" lumenal contents such as food and releasing factors. These enteroendocrine cells also possess microvilli that increase the exposed surface area, thus allowing greater exposure to potential stimuli.

Like other gastrointestinal hormones, CCK is a "brain–gut" peptide, meaning that the transmitter is found in both the central nervous system and the intestine. In the brain, CCK is highly concentrated in the striatum, hippocampus, and cerebral cortex. The CCK-containing neurons may also synthesize dopamine. Such nerves have been shown to extend to the limbic forebrain and ventromedial hypothalamus, where they may participate in controlling food intake. CCK has been demonstrated to modulate dopamine release, dopamine-mediated reward, and receptor binding and function. These actions have implications for a role for CCK in drug abuse and neurologic and psychiatric disease.

CCK is prevalent in peripheral nerves of the gastrointestinal tract. It is most abundant in nerves innervating the colon, with fewer CCK-containing nerves in the ileum. In these locations CCK is present in myenteric and submucosal plexi, where ganglionic bodies are innervated. CCK is also abundant in the vagus nerve and celiac plexus. In the intestine, CCK stimulates acetylcholine release and causes smooth muscle contraction. Postganglionic CCK-containing neurons also terminate around the islets of Langerhans, where CCK may stimulate islet hormone secretion, e.g., insulin and glucagon release.

## IV. CCK RECEPTORS

CCK exerts its biological actions by binding to specific receptors on its target tissues. Gastrointestinal CCK receptors reside on tissues of the pancreas, gallbladder, stomach, lower esophageal sphincter, ileum, and colon. In the nervous system, CCK receptors are abundant in the brain and on some peripheral nerves. Two types of CCK receptors have been identified. CCK-A (alimentary) receptors are the primary CCK receptor and mediate most of the effects of CCK in the gastrointestinal tract. The CCK-B (brain) receptor is identical to the gastrin receptor and is the major CCK receptor subtype in the nervous system. It is also abundant in the stomach. Both receptors are G-protein-coupled, seven-membrane-spanning proteins, but arise from different genes.

CCK receptor antagonists have been extremely useful in pharmacological and physiological studies to define the physiological role of CCK. The first CCK receptor antagonists useful for *in vitro* studies were cyclic nucleotide analogues (e.g., dibutyryl cGMP). Subsequently, amino acid derivatives (e.g., CR-1409), carboxyl-terminal CCK analogues, and substituted benzodiazepines (e.g., devazepide) were developed and were used *in vivo*. Clinical studies have shown that CCK receptor antagonists inhibit CCK- and meal-stimulated gallbladder contractions, accelerate gastric emptying, and induce hunger (i.e., reverse satiety), indicating that CCK has important physiological roles in each of these processes.

## V. CHOLECYSTOKININ RELEASE

CCK is secreted from specialized endocrine cells of the mucosa (known as I cells) into the extracellular space, where CCK is taken up into the bloodstream. It is by this mechanism that circulating CCK reaches distant target tissues such as the pancreas and gallbladder. Enteric endocrine cells package CCK in secretory granules that are stored along the basolateral surface of the cell, thus allowing CCK to be secreted into the interstitium when the cell is stimulated. *In vivo*, ingested proteins and fat are the major dietary stimulants of CCK release. However, in some species, including humans, and in cell preparations *in vitro*, partially digested nutrients such as amino acids and peptides and fatty acids are potent releasers of CCK, indicating that these components directly interact with the CCK cell.

Circulating blood levels of CCK average approximately 1 p*M* in the fasting state and increase to between 5 and 8 p*M* after eating. Postprandial levels remain elevated for 3–5 h as food empties from the stomach into the upper small intestine. Gastric distention does not influence CCK release. Although fat and protein are the primary stimulants of CCK, carbohydrate has a modest effect on secretion.

It is well recognized that inactivation of protease activity in the lumen of the small intestine of rodents stimulates CCK release and pancreatic exocrine secretion. This phenomenon, known as negative feedback control of CCK release, also applies to other species, including humans. Thus, CCK release is controlled in part by the presence or absence of pancreatic enzymes in the intestine (Fig. 2). This concept indicates that intestinal releasing factors are secreted into the intestine and stimulate CCK secretion. When pancreatic enzymes are present in the intestine, these CCK-releasing factors are inactivated; however, when pancreatic enzymes are inhibited or pancreatic secretion is reduced, these CCK-releasing factors are active and stimulate CCK secretion. Similarly, ingestion of a meal causes temporary binding of trypsin and other digestive enzymes, thus CCK-releasing factors are available to stimulate CCK secretion. To date, a human counterpart of the rodent CCK-specific releasing factor has not been identified.

## VI. BIOLOGICAL ACTIONS OF CCK

CCK is the major hormonal regulator of gallbladder contraction. Coincident with this effect, CCK also relaxes the sphincter of Oddi, which also promotes bile secretion into the intestine. In humans, the predominant CCK receptor type in the pancreas is CCK-B. In most species, CCK-A receptors predominate in the pancreas, and CCK is a potent stimulant of pancreatic secretion. Therefore, in humans, although CCK stimulates pancreatic secretion, its role may be limited.

At physiological blood concentrations that typically occur after a meal, CCK delays gastric emptying; this may be important in the influence CCK has on

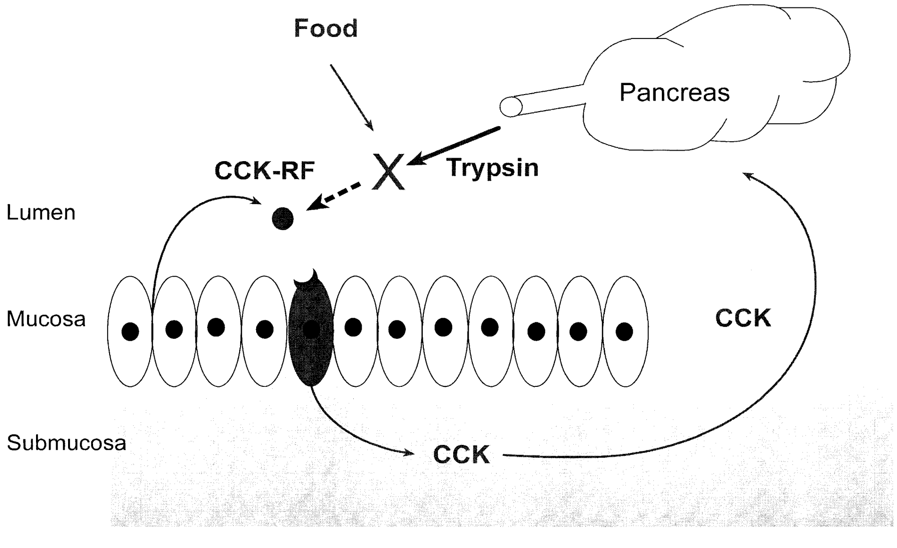

FIGURE 2 Model for regulation of cholecystokinin (CCK) release by an intralumenal CCK-releasing factor (CCK-RF). One or more CCK-RFs stimulate intestinal CCK secretion. CCK-RF is normally secreted in the intestinal lumen, where it is exposed to pancreatic enzymes. Under basal conditions, CCK-RF is inactivated by even small amounts of enzyme; however, following a meal, food competes for enzyme binding, allowing CCK-RF to interact with CCK cells, stimulating hormone secretion. CCK in the circulation stimulates pancreatic secretion, which, following digestion of food, restores CCK-RF and CCK secretion to basal levels. Modified from Liddle, R. A. (1995). Regulation of cholecystokinin secretion by intralumenal releasing factors. *Am. J. Physiol. Gastrointest. Liver. Physiol.* **269**, G319–G327.

reducing food intake and inducing satiety. Due to CCK effects on gallbladder contraction, pancreatic secretion, and gastric emptying, CCK coordinates many digestive processes and plays an important role in the ingestion and digestion of a meal. Although it has been shown that CCK causes relaxation of the lower esophageal sphincter and promotes intestinal motility, it appears that these effects are neural rather than hormonal. CCK receptors have been found on some gastrointestinal and lung cancers, but it is unknown whether CCK plays a role in human cancer growth.

## VII. CLINICAL USES OF CHOLECYSTOKININ

CCK is used along with secretin as a test of pancreatic function. In patients with pancreatic insufficiency, low levels of pancreatic juice are recovered following intravenous injection of these hormones. CCK can also be used clinically to stimulate gallbladder contraction and is helpful in radiographic testing of gallbladder function. For diagnostic purposes, CCK has facilitated the collection of bile and pancreatic juices for cytological examination. Therapeutically, CCK injections have been administered to patients who are unable to eat (e.g., parental alimentation) in order to stimulate gallbladder contraction. This therapy has been effective in reducing gallbladder sludge and in preventing gallstone formation.

Low blood levels of CCK have been reported in patients with celiac disease, in bulimia nervosa, and in conditions that delay gastric emptying. The defect in celiac disease is likely due to reduced CCK secretion from diseased small intestinal mucosa. The cause of abnormal CCK responses in bulimia is unknown but may be related to alterations in gastric emptying, because normal postprandial CCK release is dependent on delivery of food from the stomach to the small intestine. It is not known whether CCK deficiency contributes to any specific pathological consequences. There are no known diseases of cholecystokinin excess.

## Glossary

**gastrin** Gastrointestinal polypeptide hormone produced by G cells of the gastric antrum; stimulates gastric acid secretion.

**G-protein-coupled receptor** A family of cell surface receptor proteins consisting of seven transmembrane regions. The intracellular region interacts with GTP-binding proteins, which, on ligand binding, transduce signals within the cell, leading to a cellular response (e.g., secretion, motility, and growth).

**microvilli** Finger-like extensions along the apical surface of intestinal mucosal cells. On enterocytes, microvilli increase the absorptive surface of the cell; on endocrine cells, microvilli allow potential stimuli greater exposure to their targets (e.g., receptors).

**myenteric plexus** Also known as Auerbach's plexus; a system of nerves and ganglia lying within the longitudinal and circular muscle layers of the intestine. Nerves of the myenteric plexus innervate numerous targets, including the myenteric externa, mucosa, and sympathetic prevertebral ganglia.

**sphincter of Oddi** Muscular region surrounding the distal ends of the common bile duct and pancreatic duct as they enter the duodenum. When constricted, this sphincter prevents flow of bile and pancreatic juice into the duodenum and restricts reflux of duodenal contents back into the bile and pancreatic ducts.

**submucosal plexus** Network of nerves and small ganglia found in the submucosa of the intestine. It is composed of outer and inner layers and transmits secretomotor and vasodilator stimuli to the mucosa. Primary sensory nerves are contained in this plexus, which also communicates with the myenteric plexus.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Eating Disorders • Gastrin • GPCR (G-Protein-Coupled Receptor) Structure • Secretin • Vagal Regulation of Gastric Functions by Brain Neuropeptides**

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# Co-activators and Corepressors for the Nuclear Receptor Superfamily

Brian G. Rowan* and Bert W. O'Malley†
*Medical College of Ohio • †Baylor College of Medicine

I. INTRODUCTION
II. NUCLEAR RECEPTOR CO-ACTIVATORS
III. NUCLEAR RECEPTOR COREPRESSORS
IV. SUMMARY/PERSPECTIVES

**Groups of nuclear-receptor-interacting proteins that regulate the degree of receptor-dependent target gene transcription have been recently identified. These proteins, termed coregulators (both co-activators and corepressors), achieve this effect through multiple mechanisms, including stabilization of nuclear receptors and the basal transcription machinery at the promoter; recruitment of proteins with a variety of enzymatic activities; covalent modification of histones, activators, and other coregulators; and, possibly, turnover of activators and other proteins at the promoter.**

## I. INTRODUCTION

The discovery, structure, and mechanisms of action of the major groups of nuclear receptor coregulators, highlighting both similarities and differences among many of these molecules, are described in this article. Current mechanistic models by which coregulators modulate nuclear receptor action are presented. Tables 1 and 2 summarize some of the major coregulators and their effects. A comprehensive discussion of all nuclear receptor coregulators is beyond the scope of this article, and the reader is referred to recent review articles listed in the bibliography.

### A. The Nuclear Receptor Superfamily

Nuclear receptors constitute a superfamily of ligand-dependent transcription factors that mediate the pleiotropic physiological effects of steroids, retinoids, and other lipophilic ligands through modulating expression of multiple target genes. Nuclear receptors are modular proteins that contain an N-terminal domain (NTD), a centrally located DNA-binding domain (DBD), a dimerization region, a hinge region, a nuclear localization sequence, and a carboxyl-terminal ligand-binding domain (LBD). In addition there are two transcriptional activation functions, activation function-1 (AF-1) and activation function-2 (AF-2), that mediate the gene regulatory functions of the receptor. AF-2 is located in the carboxyl terminus of the protein, is ligand dependent, and contains the major interaction regions for the nuclear receptor coregulators (see later), whereas AF-1 is located in the amino terminus and is ligand independent (Fig. 1).

### B. Type I and Type II Nuclear Receptors

Nuclear receptors are broadly classified as either type I or type II receptors. The steroid receptors include the estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), androgen receptor (AR), and mineralocorticoid receptor (MR). Type II receptors include the thyroid hormone receptor (TR) vitamin D receptor (VDR), all-*trans*-retinoic acid receptor (RAR), and 9-*cis*-retinoic acid receptor (RXR). Grouping as type I or II is based on evolutionary conservation and receptor location in the unliganded state. A third category, the type III

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TABLE 1 Partial Listing of Major Nuclear Receptor Co-activators[a]

| Co-activator | Effect on nuclear receptors |
|---|---|
| SRC family of HAT enzymes | Interact with and co-activates nuclear receptors; possess HAT activity; synergize with CBP in co-activation; SRC-1 and SRC-3 null mice exhibit partial hormone resistance |
| CBP/p300 HAT enzymes | Interact with and co-activate nuclear receptors; possess HAT activity; synergize with SRC family in co-activation |
| P/CAF HAT enzyme | Interacts with nuclear receptors, CBP/p300, and SRC family; possesses HAT activity |
| E3 ubiquitin–protein ligases | E6-AP interacts with and co-activates nuclear receptors independent of ubiquitin ligase activity |
| Kinases | TIF1α interacts with and co-activates nuclear receptors; possesses kinase activity |
| Protein methyltransferases | CARM1 and PMRTs interact with carboxyl terminus of SRC family proteins; synergize with SRC family in co-activation; possess methyltransferase activity; methylate histones and CBP |
| RNA co-activator | SRA is an RNA transcript that interacts with the AF-1 region and co-activates type I receptors; interacts with SRC family proteins |
| TRAPs/DRIPs | Complex of proteins that interact with and co-activate thyroid and vitamin D receptors |

[a] Abbreviations: SRC, steroid receptor co-activator; HAT, histone acetyltransferase; CBP, $Ca^{2+}$/cAMP response element-binding protein; P/CAF, p300/CBP-associated factor; E6-AP, E6-papillomavirus-associated protein; TIF1α, transcription intermediary factor-1α; CARM1, co-activator-associated arginine methyltransferase 1; PMRT, protein arginine N-methyltransferase; SRA, steroid receptor RNA activators; AF-1, activation function-1; TRAP, thyroid hormone receptor-associated protein; DRIP, vitamin D receptor-interacting protein.

receptors, contains the largest number of receptors and includes all of the orphan receptors, i.e., receptors with ligands or modes of regulation that were not known at discovery. In the absence of ligand, type I steroid receptors reside in an inactive complex with heat-shock proteins and immunophilins in either the cytoplasm or the nucleus. Ligand binding results in a receptor conformational change that facilitates dissociation from the inactive complex and association with a dimeric partner. Liganded steroid receptor dimers bind to hormone-responsive element (HRE) sequences in the promoter of steroid receptor target genes. Referred to now as the "activated" form, steroid receptors recruit co-activator proteins (see later) that further facilitate receptor interaction with the promoter and stabilize the basal transcription machinery, resulting in gene transcription (Fig. 2). In contrast to type I receptors, unliganded type II receptors are bound to DNA at HREs and often repress basal transcription of target genes through association with corepressor proteins (see later and Fig. 3). The activation mechanisms for type I and type II receptors are described in detail in later sections. The type III receptors also use similar co-activators/corepressors, but their specific actions are not discussed herein.

TABLE 2 Partial Listing of Major Nuclear Receptor Corepressors[a]

| Corepressor | Effect on nuclear receptors |
|---|---|
| N-CoR/SMRT | Interacts with and corepresses unliganded type II nuclear receptors; represses the partial agonist activity of mixed antagonists for type I nuclear receptors |
| SUN-CoR | Interacts with and corepresses unliganded TR and RevErb; interacts with N-CoR and SMRT |
| TRUP | Inhibits transactivation of TR and RAR |

[a] Abbreviations: N-CoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SUN-CoR, small ubiquitous nuclear coreceptor; TRUP, thyroid hormone receptor uncoupling protein; TR, thyroid hormone receptor; RAR, retinoic acid receptor.

## C. Discovery of Nuclear Receptor Coregulators

Early biochemical studies had identified proteins that interacted with type I steroid receptors in a ligand-dependent manner. These proteins were subsequently cloned using yeast two-hybrid screens from a variety of cDNA libraries and are now referred to as co-activators based on their ability to enhance receptor-dependent gene transcription. Similarly, early biochemical studies identified proteins that interacted with unliganded type II nuclear receptors and presumably facilitated gene silencing. These proteins

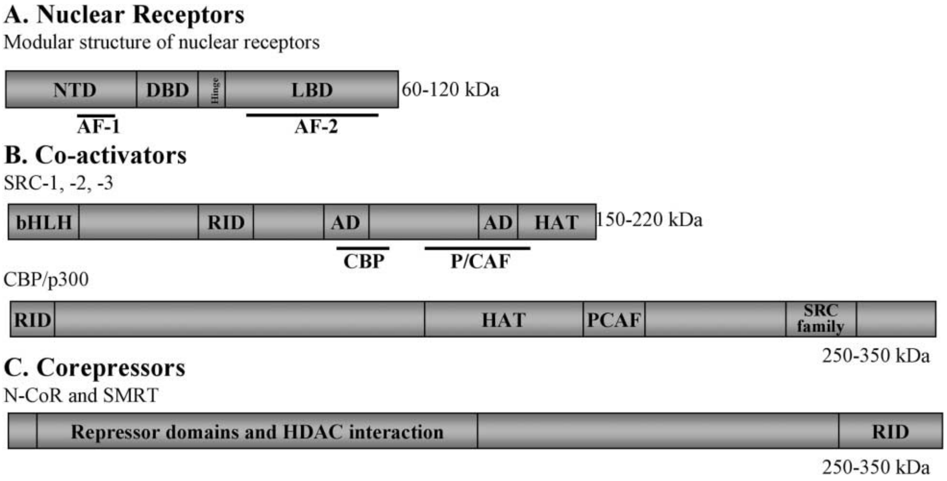

A. Nuclear Receptors
Modular structure of nuclear receptors
NTD
DBD
Hinge
LBD
60-120 kDa
AF-1
AF-2
B. Co-activators
SRC-1, -2, -3
bHLH
RID
AD
AD
HAT
150-220 kDa
CBP
P/CAF
CBP/p300
RID
HAT
PCAF
SRC family
250-350 kDa
C. Corepressors
N-CoR and SMRT
Repressor domains and HDAC interaction
RID
250-350 kDa

**FIGURE 1** Domain structure of receptors, co-activators, and corepressors. (A) Nuclear receptors consist of an N-terminal domain (NTD) containing the ligand-independent activation function-1 (AF-1), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) containing a ligand-dependent activation function-2 (AF-2). (B) The steroid receptor co-activator (SRC) family and CBP/p300 represent major co-activators for the nuclear receptor superfamily. The SRC family members contain a basic helix-loop-helix (bHLH) domain in the amino terminus, the major receptor interaction domain (RID) containing LXXLL motifs in the central region of the protein, activation domains (ADs), histone acetyltransferase (HAT) activity, and domains for interaction with CBP and P/CAF. CBP/p300 contains a RID, HAT activity, and domains for interaction with P/CAF and SRC family members. (C) Nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) are representative of the major nuclear receptor corepressors. These proteins contain repressor domains that interact with histone deacetylases (HDACs) and a RID containing LXXI/HIXXXI/L motifs.

were subsequently cloned using a variety of procedures and are now termed corepressors. The major groups of co-activators and corepressors are described in the following section in a way that illustrates the functional and structural diversity and similarities among many of these molecules.

## II. NUCLEAR RECEPTOR CO-ACTIVATORS

### A. SRC Family of Co-activators

Cloning and characterization of the first authenticated co-activator for the nuclear receptor superfamily were accomplished with the identification of

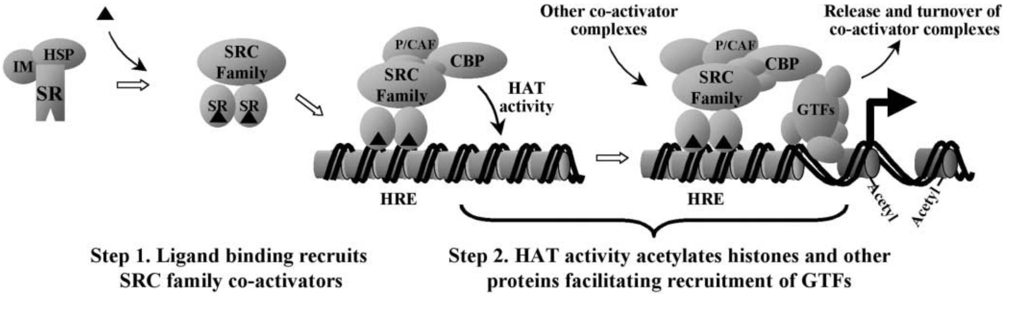
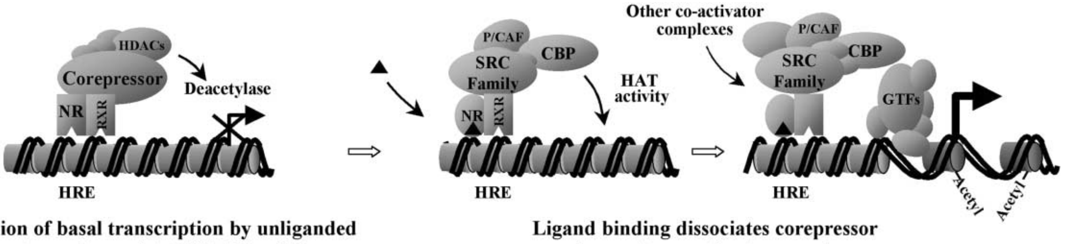

IM
HSP
SR
SRC Family
SR
SR
P/CAF
CBP
SRC Family
HAT activity
HRE
Other co-activator complexes
P/CAF
CBP
SRC Family
GTFs
Release and turnover of co-activator complexes
HRE
Acetyl
Acetyl
Step 1. Ligand binding recruits SRC family co-activators
Step 2. HAT activity acetylates histones and other proteins facilitating recruitment of GTFs

**FIGURE 2** Type I steroid receptor activation of gene transcription: a two-step model (for receptors for estrogen, progesterone, glucocorticoid, androgen, and mineralocorticoid). An unliganded steroid receptor (SR) resides in an inactive complex with heat-shock protein (HSP) and immunophilin (IM). Ligand binding dissociates receptors from HSP and IM; then receptors dimerize and recruit a SR co-activator (SRC) to the promoter (step 1). SRCs possess histone acetyltransferase (HAT) activity and recruit other HATs that acetylate histones, creating a more open chromatin conformation that facilitates binding of general transcription factors (GTFs) (step 2), allowing transcription initiation to proceed. It is hypothesized that other co-activator complexes (thyroid hormone-associated proteins/vitamin D receptor-interacting proteins) are recruited subsequent to the SR co-activators and are released and degraded from the promoter in an as-yet undetermined temporal pattern.



**Repression of basal transcription by unliganded receptor and associated HDAC activity**

**Ligand binding dissociates corepressor and recruits co-activator complexes**

FIGURE 3 Type II nuclear receptors: repression of basal transcription by unliganded receptors and transcriptional activation following ligand binding (for thyroid, retinoic acid, and vitamin D receptors). An unliganded type II nuclear receptor (NR) binds the hormone -responsive element (HRE) predominantly as a heterodimer with retinoid X receptor (RXR) and recruits corepressor proteins with associated histone deacetylases (HDACs). HDACs deacetylate histones, resulting in a closed local chromatin structure, thereby silencing low-level basal gene transcription. Ligand binding to type II receptors changes receptor conformation, resulting in dissociation of corepressors and recruitment of co-activators with associated HAT activity. SRC, Steroid receptor co-activator; CBP, $Ca^{2+}$/cAMP response element-binding protein; P/CAF, p300/CBP-associated factor; GTF, general transcription factor.

steroid receptor co-activator-1 (SRC-1). SRC-1 and other co-activators were discovered using yeast two-hybrid screens of cDNA libraries with the hormone-bound steroid receptor LBD as bait. SRC-1 interacts with nuclear receptors in a ligand-dependent manner and, in general, steroid receptor antagonists that block receptor-dependent gene transcription prevent the interaction of receptor with SRC-1. SRC-1 co-activation was demonstrated in cotransfection assays in which PR-negative cells were transfected with an expression vector for PR, an HRE-containing reporter gene, and cotransfected either with or without an expression vector for SRC-1. Expression of SRC-1 in cells resulted in enhancement of progesterone-dependent reporter gene activity. Although SRC-1 interacts with and co-activates most nuclear receptors, it has a limited ability to co-activate unrelated transcription factors. Subsequent to the discovery of SRC-1, independent laboratories identified two other nuclear receptor co-activators that shared extensive sequence homology to SRC-1 and exhibited similar functional properties with respect to nuclear receptor co-activation. These co-activators, termed TIF2 (GRIP1 and NcoA-2, referred to here as SRC-2) and RAC3 (ACTR, AIB1, P/CIP, and TRAM, referred to here as SRC-3), established an important gene family of co-activators for the nuclear receptor superfamily members that are broadly expressed in mammalian tissues. Because the SRC family and most coregulators lack a DNA-binding function, coregulators must be recruited to the promoter through the interaction with DNA-bound nuclear receptors.

Mutational analysis and biochemical assays have defined several important domains and an enzymatic activity for the SRC family (Fig. 1). The major receptor interaction domain (RID) for all three SRC family members contains several highly conserved LXXLL motifs, where L is leucine and X is any other amino acid. These motifs interact with LBD hydrophobic regions that are exposed on ligand binding and likely adopt an amphipathic α-helical conformation. The LXXLL motif is also conserved in a number of unrelated nuclear receptor coregulators, including $Ca^{2+}$/cAMP response element-binding (CREB)-binding protein (CBP/p300), E6 papillomavirus-associated protein (E6-AP), receptor-interacting protein-140 (RIP140), transcription intermediary factor-1 (TIF1), and components of the TR-associated protein complex. Two activation domains (ADs) that are important for interaction with other activators and/or the basal transcription machinery mediate SRC-1 function. A histone acetyltransferase (HAT) activity is present in the carboxyl terminus of SRC-1, giving the SRC family the potential to modify promoter function through covalent modifications of histones and other locally recruited transacting factors. However, the SRC acetylase activity is likely targeted more to the other proteins in the complex than to histone. In addition, the carboxyl terminus contains domains that interact with two very strong HATs, CBP and p300/CBP-associated factor (P/CAF), bringing additional HAT activity to the promoter for direct histone modification and chromatin remodeling. Indeed, the SRC family has been shown to interact functionally with CBP to enhance steroid receptor gene activation synergistically.

A two-step model for type I steroid receptor induction of gene expression incorporates the discovery of the SRC family in which (1) the liganded receptor recruits SRC co-activators that can (2) open up local chromatin structure through their intrinsic or associated HAT activity and contribute to the stabilization of the preinitiation complex at the promoter (Fig. 2). The *in vivo* significance of co-activators for nuclear receptor action was recently reported for the SRC family. Generation of mice with targeted deletions in SRC-1 and SRC-3 exhibited limited steroid insensitivity. The partial, but not full, hormone resistance phenotype in the SRC knockout models may be explained by functional redundancy among SRC co-activators, as illustrated by a compensatory elevation in the expression of SRC-2 in the SRC-1 null mice.

## B. CBP/p300

CBP and the related orthologue p300 function as co-activators for a large and diverse collection of transcription factors, including members of the nuclear receptor superfamily. CBP possesses HAT enzymatic activity and has domains for interaction with nuclear receptors, all three SRC family members, and P/CAF (Fig. 1). Furthermore, CBP functionally interacts with SRC co-activators to co-activate ER- and PR-dependent gene transcription synergistically in cotransfection assays. However, the physical interaction between SRC co-activators and steroid receptors is by comparison stronger than the interaction between CBP with steroid receptors. Further data indicate there is a sequential recruitment of, initially, a stable complex between the liganded type I steroid receptors and SRC co-activators, followed by recruitment of CBP/p300 and other factors (Fig. 2). Both CBP and p300 null mice exhibit embryonic lethality, possibly indicating the global importance of CBP/p300 to many types of transcription factors.

## C. E3 Ubiquitin-Protein Ligases: E6-AP and RPF-1

Another group of co-activators that bring an entirely different enzymatic function to the promoter are the E3 ubiquitin-protein ligases E6-AP and retina-derived POU domain factor-1 (RPF-1). E6-AP and RPF-1 physically and functionally interact with type I receptors to co-activate target gene transcription, and co-expression of both proteins synergistically enhances transcription. Although both proteins possess ubiquitin ligase activity and are essential components of the ubiquitin proteolysis pathway, the enzymatic activity of E6-AP is dispensable for its co-activation function. The E3 ubiquitin-protein ligases may be involved in ubiquitination and turnover of nuclear receptor complexes at the promoter, providing another potential mechanism of gene regulation by co-activators.

## D. Protein Methyltransferases: CARM1 and PRMTs

The recently discovered co-activator-associated arginine methyltransferase 1 (CARM1) and protein arginine N-methyltransferases (PRMTs) contribute yet another enzymatic activity to the promoter region of nuclear receptor responsive genes. Both CARM1 and PRMTs enhance the transcriptional activity of nuclear receptors, not through a direct physical interaction with receptors but through binding to the carboxyl terminus region of the SRC family of co-activators. Indeed, the presence of an SRC co-activator is prerequisite for CARM1 and PRMTs co-activation of nuclear receptors and for the synergistic enhancement of gene transcription when both proteins are co-expressed. Furthermore, unlike E6-AP enzymatic activity, the methyltransferase enzymatic activity of CARM1 and PRMTs is required for full co-activator function. Co-activator function may be due in part to methylation of histones H3 and H4 by CARM1 and PRMTs, respectively.

## E. SRA

A novel, recently identified co-activator for type I steroid receptors possesses two unique properties: (1) interaction with the amino terminus AF-1 domain of nuclear receptors rather than with the AF-2 domain and (2) function as an RNA transcript rather than as a protein. The steroid receptor RNA activator (SRA) brings a new level of complexity to nuclear receptor-mediated transcription through its novel mechanism and its somewhat restricted pattern of expression. SRA is expressed predominantly in the brain and in tumor tissues. SRA exists in a complex with SRC-1 and may aid in recruiting RNA-binding proteins to steroid receptors at the promoter.

## F. TRAPs/DRIPs

Biochemical studies identified a multiprotein complex that is composed of TR-associated proteins (TRAPs) or vitamin D receptor-interacting proteins (DRIPs); the complex interacts with the liganded LBD of the TR and the VDR. Interestingly, the TRAP/DRIP complex contains no HAT activity and is distinct from SRC-containing complexes. Components of TRAP/DRIP interact with and stabilize components of the general transcription machinery at the promoter, allowing continuous transcription to occur. Similar to the SRCs, one component of the TRAP/DRIP complex, TRAP 220/DRIP 205, contains LXXLL motifs and interacts with the LBD of nuclear receptors in a manner similar to that of SRC co-activators. TRAP 220/DRIP 205 serves as a platform for binding of other components of the TRAP/DRIP complex. The common sites for interaction of TRAP/DRIP and SRC complexes with nuclear receptors suggest a competition between the two complexes for receptor binding. However, emerging data now favor a stepwise recruitment of co-activator complexes to nuclear receptors at the promoter. The current model proposes that the SRC complex is recruited first to remodel chromatin, followed by exchange of the SRC complex with the TRAP/DRIP complex. It is envisioned that the TRAP/DRIP complex stabilizes components of the general transcription factor machinery at the promoter, providing for sustained transcription over time.

## III. NUCLEAR RECEPTOR COREPRESSORS

Selected type II nuclear receptors such as TR and RAR silence basal transcription of target genes in the unliganded state. This was the basis for identification of corepressor proteins that selectively interact with unliganded type II receptors at the promoter of receptor-silenced genes. Relatively fewer examples of nuclear receptor corepressors have been described when compared to the more than thirty nuclear receptor co-activators.

### A. N-CoR and SMRT

Biochemical and cloning strategies led to independent discoveries of two highly homologous proteins that interact with the unliganded TR and RAR. Nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) are corepressors for nuclear receptors as well as for a wide variety of unrelated transcription factors. This unrestricted ability of N-CoR and SMRT to corepress many types of transcription factors may be reflected in the embryonic lethal phenotype of N-CoR (−/−) mice. Similar to co-activators, mutational analysis identified several RIDs in the carboxyl terminus of N-CoR/SMRT that contain an extended hydrophobic motif of the consensus sequence LXXI/HIXXXI/L, where L, I, and H are leucine, isoleucine, and histidine, respectively, and X is any amino acid (Fig. 1). These motifs likely bind to similar regions in the LBDs of type II receptors, as do the co-activators LXXLL motifs. Presumably the extra length of the corepressor hydrophobic motif precludes binding to the liganded receptor yet is permissive for binding to the unliganded receptor. The amino termini of N-CoR and SMRT contain transferable repressor domains that mediate interaction with a variety of histone deacetylases (HDACs). In opposition to the manner in which certain co-activators bring HAT activity to the promoter, N-CoR and SMRT recruit HDACs to the promoter, resulting in deacetylation of histones and other proteins, culminating in a more closed local chromatin conformation that represses basal transcription. On ligand binding, an LBD conformational change promotes dissociation of corepressor proteins and recruitment of HAT-containing co-activator complexes, relieving the repression at the promoter and allowing transcriptional activation to proceed (Fig. 3). Although first identified as proteins that facilitate gene silencing of unliganded TR and RAR, N-CoR and SMRT have also been shown to interact with antagonist-occupied ER and PR and to block the partial agonist activity of mixed antagonists.

### B. Other Corepressors

Although N-CoR and SMRT appear to be the major corepressors for the nuclear receptor superfamily, several other corepressors may also play an important role in gene silencing. Thyroid receptor uncoupling protein (TRUP) appears to have a different mode of action, compared to N-CoR and SMRT. In blocking ligand-dependent activation of gene transcription by TR and RAR, TRUP appears to reduce DNA binding by TR:RXR (retinoid X receptor) and RAR:RXR heterodimers. Small ubiquitous nuclear corepressor (SUN-CoR) is a 16-kDa nuclear protein that represses basal transcription of TR and the orphan nuclear receptor RevErb. Although unrelated to N-CoR, SUN-CoR interacts with N-CoR and SMRT. It is likely that many additional nuclear receptor corepressors will be identified in the coming - years.

## IV. SUMMARY/PERSPECTIVES

The discovery of nuclear receptor coregulators and associated proteins has provided a mechanistic explanation for the long-recognized phenomenon of transcriptional activation and gene silencing exhibited by selected members of the nuclear receptor superfamily. The competing nature of limiting cellular HAT and HDAC activities that are recruited by co-activators and corepressors, respectively, is instrumental in balancing gene repression to gene activation. In addition to HAT and HDAC activities, other coregulators bring a diversity of structural and enzymatic features to receptor-dependent promoters, illustrating the complexity of gene transcription mechanisms. Functional redundancies as well as synergy among coregulators add to this complexity, providing for a fine-tuning of gene expression.

As investigators delve further into the turnover of protein complexes at the promoter, the temporal aspects of co-activator and corepressor complex recruitment, and the tissue-specific, receptor-specific, and ligand-specific coregulators, the model for nuclear receptor gene transcription will necessarily expand. Further challenges will be to describe the role of coregulators at complex promoters and promoters that lack HREs, the detailed tissue- and developmental-specific expression patterns of coregulators, the hormonal regulation of coregulator expression, the promising role of coregulators in directing the action of partial agonists, and the role that membrane signaling pathways play in modulating coregulator activity. The integration of all of these parameters is required to fully understand the complex role of coregulators in nuclear receptor gene transcription.

## Glossary

**co-activator** A biomolecule (predominantly protein) that interacts with transcription factors to enhance transcription factor-dependent target gene transcription.

**corepressor** A biomolecule (predominantly protein) that interacts with transcription factors to repress basal gene expression and to block transcription factor-dependent target gene activation.

**histone acetylase** A protein with enzymatic activity that results in covalent acetylation of histones and other proteins.

**histone deacetylase** A protein with enzymatic activity that results in removal of acetyl groups from histones and other proteins.

**nuclear receptor superfamily** An evolutionarily conserved family of ligand-dependent transcription factors.

## See Also the Following Articles

**Crosstalk of Nuclear Receptors with STAT Factors • Orphan Receptors, New Receptors, and New Hormones • Steroid Hormone Receptor Family: Mechanisms of Action**

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Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999). Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177.

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# Colony-Stimulating Factor-1 (CSF-1)

Xu-Ming Dai and E. Richard Stanley

*Albert Einstein College of Medicine, New York*

I. INTRODUCTION

II. CSF-1 GENOMIC ORGANIZATION, GENE EXPRESSION, BIOSYNTHESIS, AND STRUCTURE

instrumental in balancing gene repression to gene activation. In addition to HAT and HDAC activities, other coregulators bring a diversity of structural and enzymatic features to receptor-dependent promoters, illustrating the complexity of gene transcription mechanisms. Functional redundancies as well as synergy among coregulators add to this complexity, providing for a fine-tuning of gene expression.

As investigators delve further into the turnover of protein complexes at the promoter, the temporal aspects of co-activator and corepressor complex recruitment, and the tissue-specific, receptor-specific, and ligand-specific coregulators, the model for nuclear receptor gene transcription will necessarily expand. Further challenges will be to describe the role of coregulators at complex promoters and promoters that lack HREs, the detailed tissue- and developmental-specific expression patterns of coregulators, the hormonal regulation of coregulator expression, the promising role of coregulators in directing the action of partial agonists, and the role that membrane signaling pathways play in modulating coregulator activity. The integration of all of these parameters is required to fully understand the complex role of coregulators in nuclear receptor gene transcription.

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# Colony-Stimulating Factor-1 (CSF-1)

XU-MING DAI AND E. RICHARD STANLEY
*Albert Einstein College of Medicine, New York*

I. INTRODUCTION
II. CSF-1 GENOMIC ORGANIZATION, GENE EXPRESSION, BIOSYNTHESIS, AND STRUCTURE

III. CSF-1 RECEPTOR, SIGNAL TRANSDUCTION, AND CELLULAR RESPONSE
IV. CSF-1 BIOLOGY
V. CSF-1 AND THE CSF-1R IN DISEASES
VI. SUMMARY

**Colony-stimulating factor-1 (CSF-1) is the primary regulator of mononuclear phagocytic lineage cells, including monocytes, tissue macrophages and osteoclasts. As one of a group of four growth factors defined by their ability to stimulate hematopoietic progenitor cells to form colonies of mature progeny cells in semisolid culture media, colony-stimulating factor-1, also known as macrophage CSF, may play a role in atherosclerosis, glomerulonephritis, Alzheimer's disease, and in some myeloproliferative diseases and neoplasms.**

## I. INTRODUCTION

The primary regulator of cells of mononuclear phagocytic lineage, colony-stimulating factor-1, promotes differentiation of the committed but undifferentiated monoblast precursor to the mature, terminally differentiated cell; CSF-1 supports the survival, proliferation, and function of all cells of the lineage. It also regulates the function of cells of the female reproductive tract. CSF-1 is synthesized by a variety of different cell types, including fibroblasts, endothelial cells, bone marrow stromal cells, osteoblasts, keratinocytes, astrocytes, myoblasts, thyrocytes, adipocytes, liver parenchymal cells, mesothelial cells, and uterine epithelial cells. Ovarian granulosa cells, oviduct epithelium, and uterine epithelial cells synthesize large amounts of CSF-1 during pregnancy under the control of a variety of female steroid hormones. The effects of CSF-1 are mediated by the CSF-1 receptor (CSF-1R) that is expressed on osteoclasts and tissue macrophages and their progenitors, as well as on embryonic cells, decidual cells, and trophoblastic cells. The reported CSF-1-producing cells and CSF-1R-bearing cell types are summarized in Table 1. CSF-1 appears to be involved in several disease states, including atherosclerosis, glomerulonephritis, and Alzheimer's disease, in some pathological pregnancy states, and, via autocrine or paracrine regulation, in the development or progression of myeloproliferative diseases, leukemias, and neoplasms of the ovary, uterus, and breast, for which elevated serum levels of CSF-1 are a tumor marker.

TABLE 1 CSF-1-Producing and CSF-1R-Bearing Cells

| CSF-1-producing cells | CSF-1R-bearing cells |
|---|---|
| Endothelial cells | Monocytes |
| Fibroblasts | Tissue macrophages |
| Monocytes | Kupffer cells |
| T cells | Langerhans cells |
| B cells | Neurons |
| Osteoblasts | Microglia |
| Bone marrow stromal cells | Peripheral B cells |
| Mesothelial cells | Osteoclasts |
| Liver parenchymal cells | Trophoblastic cells |
| Keratinocytes | |
| Myoblasts | |
| Astrocytes | |
| Adipocytes | |
| Ovarian granulosa cells | |
| Grandular cells of uterus | |
| Oviduct epithelium | |
| Uterine epithelial cells | |
| Other epithelial cells | |

## II. CSF-1 GENOMIC ORGANIZATION, GENE EXPRESSION, BIOSYNTHESIS, AND STRUCTURE

CSF-1 cDNAs have been cloned from several species, including humans, mice, rabbits, rats, cows, and cats. Sequence comparison indicates that the CSF-1 gene is highly conserved. The human CSF-1 gene, located on chromosome 1p13–p21, spans approximately 21 kb and is composed of 10 exons. The genomic organization of the human CSF-1 gene, the posttranscriptional differential splicing of CSF-1 messenger RNA, and the biosynthesis of various forms of CSF-1 protein are summarized in Fig. 1.

Through differential mRNA splicing and posttranslational proteolytic processing and modification, mouse and human CSF-1s are each expressed in three forms: a secreted glycoprotein (80–100 kDa), a secreted chondroitin sulfate-containing proteoglycan (130–160 kDa), and a biologically active membrane-spanning cell surface glycoprotein form (68–86 kDa). All of these forms are homodimeric and highly glycosylated with N- and O-linked oligosaccharides. The cell surface form is involved in local regulation and the proteoglycan form may be sequestered to particular tissue matrices to function locally. The specific functions and biological significance of these various forms of CSF-1 are currently under intensive investigation both *in vivo* and *in vitro*.

At least four types of mature CSF-1 mRNAs have been isolated. Differential splicing at exon 6 in the coding region gives rise to mRNAs encoding either

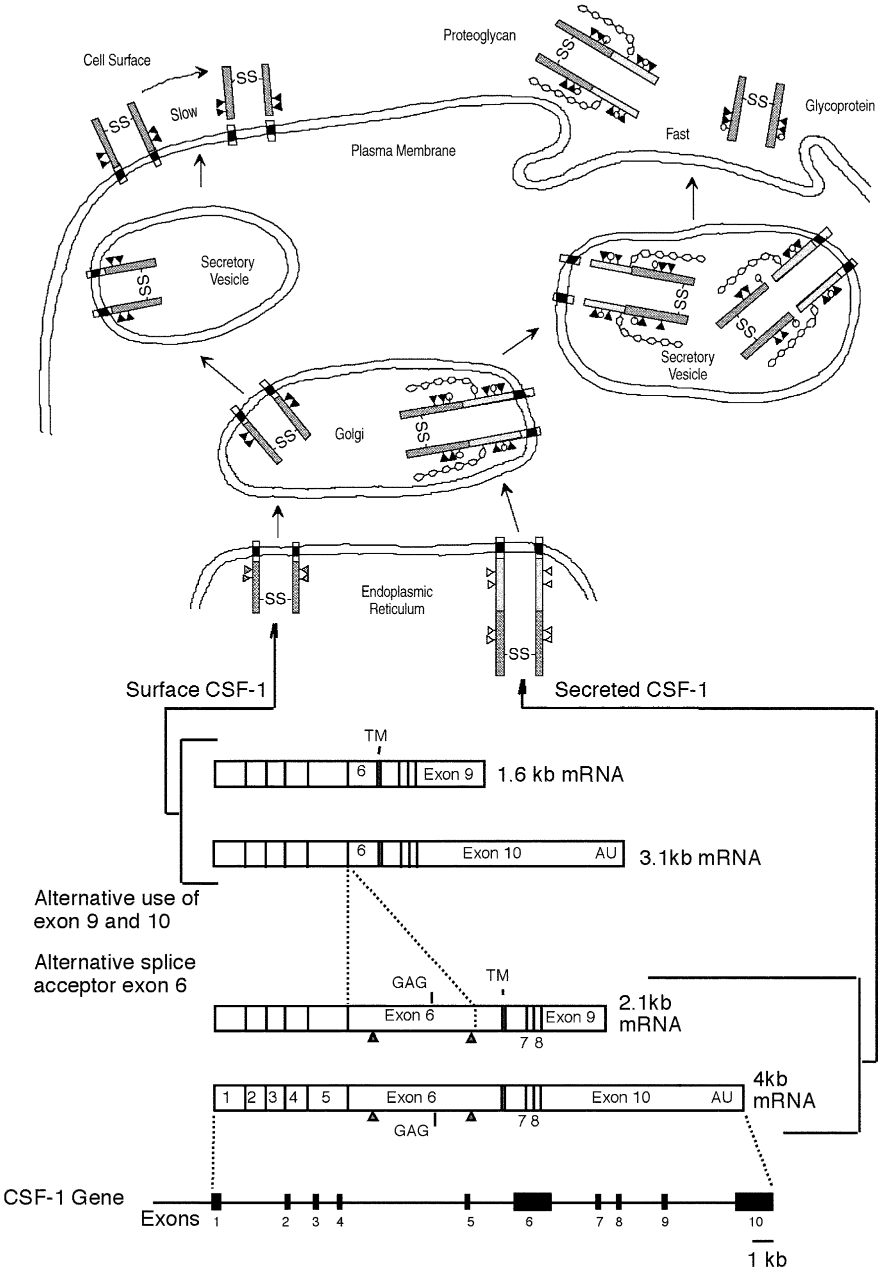
Cell Surface
Slow
SS
Proteoglycan
Plasma Membrane
Fast
Glycoprotein
Secretory Vesicle
Golgi
Endoplasmic Reticulum
Surface CSF-1
Secreted CSF-1
TM
6
Exon 9
1.6 kb mRNA
Exon 10
AU
3.1kb mRNA
Alternative use of exon 9 and 10
Alternative splice acceptor exon 6
GAG
Exon 6
7 8
2.1kb mRNA
1 2 3 4 5
4kb mRNA
CSF-1 Gene
Exons 1 2 3 4 5 6 7 8 9 10
1 kb

**FIGURE 1** CSF-1 genomic organization, expression, and structure. The CSF-1 gene is localized to human chromosome 1p13–p21 and mouse chromosome 3. The bottom half of the diagram shows the intron–exon structure and four representative human cDNA clones that have been sequenced. Similar mouse clones have been isolated. Exons 1–10 and the transmembrane (TM) domain are indicated. The 4- and 2.1-kb mRNAs arise from alternative usage of untranslated regions (AU) encoded by exons 9 and 10, the latter exon encoding putative mRNA instability sequences. The 1.6- and 3.1-kb mRNAs are products of a splicing reaction that results in the use of a short form of exon 6. The approximate intracellular proteolytic cleavage sites (arrowheads) and the chondroitin sulfate glycosaminoglycan addition site (GAG) are also shown. The top half of the diagram shows the processing of CSF-1 homodimers encoded by both the short and long coding regions. Hatched regions represent those present in the mature secreted or released glycoprotein forms whereas both hatched and filled regions are present in the major secreted proteoglycan form. N-Linked (filled arrowheads) and O-linked (open circles) glycosylation sites, the chondroitin sulfate chain (linked, open hexagons), and the transmembrane domain (filled region) are shown. Reproduced from Stanley (1998), with permission.

secreted or cell surface CSF-1 and there is alternative use of exon 9 and 10 in the 3′ untranslated region. These exons may confer differential stability to the CSF-1 mRNA. The CSF-1 gene is expressed either constitutively (usually at low levels) or is induced in response to a variety of factors during development or in pathophysiological states. Interferon γ (IFNγ), tumor necrosis factors (TNFα and TNFβ), interleukin-1 (IL-1), IL-3, IL-4, granulocyte/macrophage colony-stimulating factor (GM-CSF), and bacterial lipopolysaccharide (LPS) induce CSF-1 production by monocytes, endothelial cells, fibroblasts, T cells, and polymorphonuclear leukocytes.

The N-terminal 149 amino acids of the full-length CSF-1 precursor, which is highly conserved in all biologically active forms of CSF-1, are necessary and sufficient for *in vitro* biological activity. The crystal structure of this region reveals that the CSF-1 monomer has an antiparallel four-α-helical bundle/antiparallel β-ribbon structure, similar to the structure of other cytokines, e.g., GM-CSF. The CSF-1 dimer is formed by the disulfide bonding of two monomers end to end, yielding a very flat, elongated structure.

## III. CSF-1 RECEPTOR, SIGNAL TRANSDUCTION, AND CELLULAR RESPONSE

The unique, high-affinity CSF-1R is encoded by the c-*fms* protooncogene. The CSF-1R gene is localized to human chromosome 5q33.3 and to mouse chromosome 18. The human CSF-1R gene is 58 kb long and is composed of 21 introns and 22 exons. Exon 1 encodes untranslated sequences and is 26 kb upstream of exon 2, which encodes the signal peptide. Gene transcription occurs in a tissue-specific fashion from two distinct promoters. In human placental trophoblast, transcription is initiated upstream of exon 1. In macrophages, it is initiated immediately upstream of exon 2. The receptor possesses a highly glycosylated extracellular ligand-binding domain (493 amino acids), a hydrophobic transmembrane domain (25 amino acids), an intracellular tyrosine kinase domain (436 amino acids) interrupted by a kinase insert domain (73 amino acids), and a C-terminal tail (Fig. 2). The extracellular domain of the CSF-1R is characteristic of members of the immunoglobulin gene superfamily.

Signaling by the CSF-1R is initiated by the binding of the divalent CSF-1 dimer ($K_d = 0.1\ nM$) to the CSF-1R, which stabilizes a dimerized receptor–ligand complex; this results in the activation of the kinase domain and autophosphorylation of the receptor on the multiple tyrosine residues that serve as binding sites for SH2 and protein tyrosine-binding domain-containing downstream signaling proteins, including Src, p85/phosphatidylinositol 3-kinase (PI3K), and Cbl. These and other, primarily cytosolic, proteins become tyrosine phosphorylated. These events are followed by CSF-1R extracellular domain disulfide bonding and further tyrosine phosphorylation, serine phosphorylation, and Cbl-regulated multiubiquitination of the receptor, all of which precede internalization and intralysosomal destruction of the receptor–ligand complex. The juxtamembrane region of the CSF-1R has been implicated in internalization. The C-terminal tail of the CSF-1R, presumably via the binding of Cbl to Tyr-974, has been shown to regulate signal transduction negatively. Its truncation, in conjunction with point mutations in the extracellular domain of the v-*fms* product (encoded by the Susan McDonough strain of feline sarcoma virus), leads to ligand-independent signaling and transforming activity. The major CSF-1R signaling pathways, the signaling molecules involved, and the cellular response to each signaling pathway in myeloid cells are summarized in Fig. 2.

*In vitro* experiments with cultured primary bone marrow-derived macrophage (BMM) and CSF-1-responsive macrophage cell lines, such as BAC1.2F5 and RAW246, have shown multiple cellular effects of CSF-1. These include the stimulation of cell proliferation, survival, differentiation, and protein synthesis, and inhibition of protein degradation. Macrophages are also induced to undergo morphological changes, including spreading and extension of lamellipodia and formation of ruffles on the cell surface, followed by cell polarization and increased motility. The small G-proteins and protein tyrosine phosphatase-ϕ are involved in mediating these effects. In addition, CSF-1-enhanced invasiveness of CSF-1R-expressing macrophage and carcinoma cell lines has been shown in a human amniotic basement membrane invasion assay. This response may be correlated with the enhancement of breast cancer metastasis by CSF-1 and macrophages in animal model systems. CSF-1 has also been shown to prime macrophages to respond to other stimuli, such as LPS, by releasing cytokines (TNFα, IL-1, and IL-6), and to stimulate the synthesis and surface expression of the macrophage scavenger receptor and to maintain the expression of CD36, an oxidized low-density lipoprotein (LDL) scavenger receptor. In addition, trophoblasts, decidual cells, microglia, and CD5 + B lymphocytes are all responsive to CSF-1 stimulation *in vitro*. Multiple signaling pathways activated by CSF-1 stimulation induce the expression of the cyclin

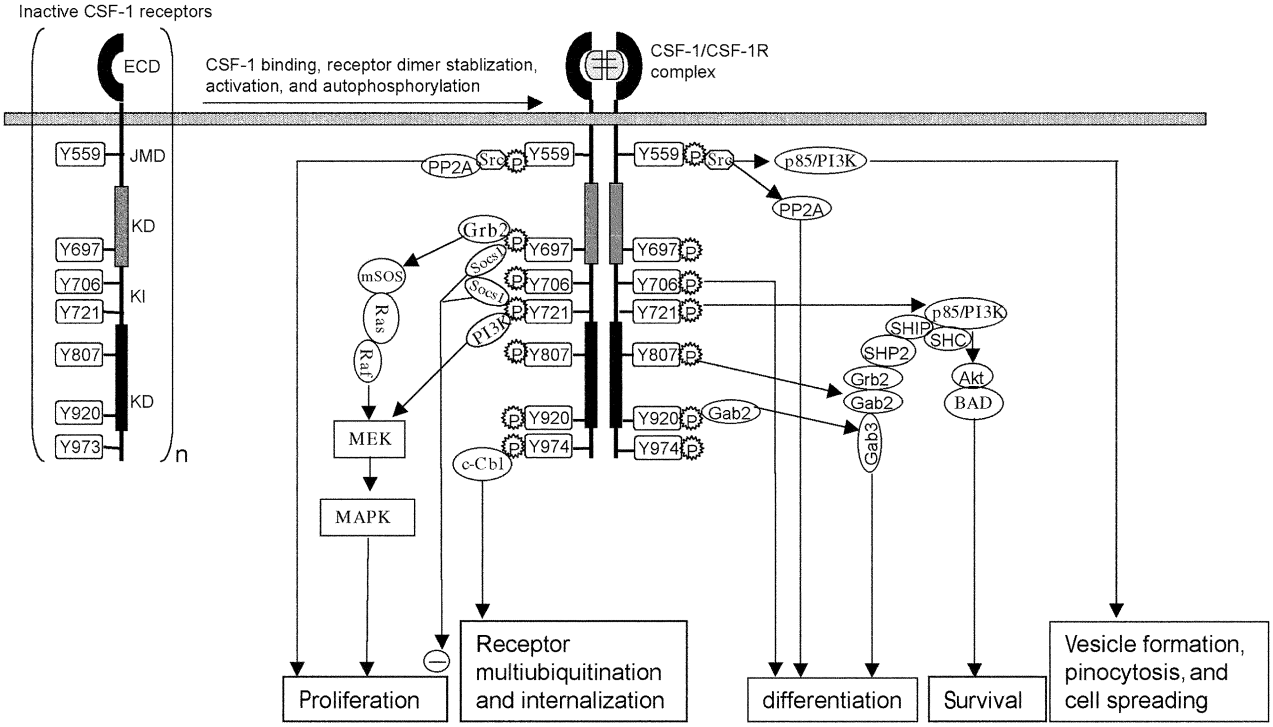
Inactive CSF-1 receptors
ECD
CSF-1 binding, receptor dimer stablization, activation, and autophosphorylation
CSF-1/CSF-1R complex
Y559
JMD
KD
Y697
Y706
KI
Y721
Y807
KD
Y920
Y973
n
PP2A
Src
P
Y559
Y559
P
Src
p85/PI3K
PP2A
Grb2
P
Y697
Y697
P
mSOS
Socs1
Socs1
P
Y706
Y706
P
Y721
Y721
P
Ras
PI3K
P
Y807
Y807
P
p85/PI3K
SHIP
SHC
SHP2
Raf
Grb2
Akt
Gab2
BAD
MEK
P
Y920
Y920
P
Gab2
P
Y974
Y974
P
Gab3
c-Cbl
MAPK
Proliferation
Receptor multiubiquitination and internalization
differentiation
Survival
Vesicle formation, pinocytosis, and cell spreading

**FIGURE 2** CSF-1/CSF-1 receptor signaling pathways and cellular responses to CSF-1. The CSF-1 homodimer binds to the extracellular domain (ECD) of the CSF-1R to form a CSF-1/CSF-1R complex, leading to activation of the kinase domain (KD) and phosphorylation of the receptor in specific tyrosine residues. These phosphorylated tyrosine residues create binding sites for Src homology region 2 (SH2) domain and protein tyrosine-binding domain-containing signaling molecules that may also become tyrosine phosphorylated and that regulate cell proliferation, differentiation, survival, and spreading, as well as the CSF-1R multiubiquitination, internalization, and lysosomal degradation. JMD, Juxtamembrane domain; KI, kinase insert domain.

D1 and D2 genes in the macrophage proliferative response, and CSF-1-induced protein phosphatase 2A gene expression is similarly required for cell cycle progression. CSF-1 induces myeloid lineage differentiation by activating differentiated cell-specific gene expression [e.g., the urokinase plasminogen activator (uPA) gene] through the action of transcription factors such as PU.1 and Ets/activator protein 1(AP-1) elements. During the course of differentiation, CSF-1R gene expression is also up-regulated by these transcription factors.

## IV. CSF-1 BIOLOGY

Circulating CSF-1 is believed to be synthesized by endothelial cells lining the small blood vessels. Of the circulating CSF-1 (half-life 10 min), 94% is cleared by CSF-1R-mediated endocytosis and by intracellular degradation by liver Kupffer cells and splenic macrophages, only 5% being filtered through the kidney. This clearance mechanism represents a coarse negative feedback control on macrophage production, whereby the number of sinusoidally located end cells determines the steady-state concentration of circulating growth factor. In addition, many different cell types synthesize CSF-1 locally, including osteoblasts and epithelial cells of the female reproductive tract. By virtue of local synthesis of the long-lived cell surface CSF-1 and of localized sequestration of the secreted proteoglycan CSF-1, both of these forms may participate in short-range regulation of target cells.

The phenotypes of mice homozygous for the inactivating mutation in the CSF-1 gene, *osteopetrotic* (*op*, $Csf1^{op}$), and the phenotypes of gene targeted $Csf1r^{-}/Csf1r^{-}$ mice are similar (Table 2). These mice are osteopetrotic and toothless due to a deficiency in osteoclasts. Mouse $Csf1^{op}/Csf1^{op}$ phenotypes were all readily corrected by transgenic expression of full-length CSF-1. Though many of the overt phenotypes of these mice are significantly corrected by restoration of circulating CSF-1, failure to rescue several phenotypes by daily injection with human recombinant CSF-1 (Table 3) indicates that CSF-1 also acts

**TABLE 2 Shared Phenotypes of $Csf1^{op}/Csf1^{op}$ and $Csf1r^{-}/Csf1r^{-}$ Mice**

Toothlessness
Reduced weight
Reduced postnatal viability[a]
Osteopetrosis[a]
Osteoclast deficiency[a]
Reduced bone marrow cellularity
Reduced numbers of tissue macrophages[a]
Reduced dermal thickness
Reduced auditory and visual processing
Spontaneous emphysema[b]

Female reproductive defects
- Extended estrus cycle
- Reduced pregnancy rate
- Reduced post-implantation survival
- Abnormal mammary gland development[b]
- Failure of lactating mammary gland development

Male reproductive defects
- Reduced testosterone production
- Reduced mating capability
- Reduced numbers of viable sperm

[a] Phenotype more severe in $Csf1r^{-}/Csf1r^{-}$ than in $Csf1^{op}/Csf1^{op}$.
[b] Phenotype not studied in $Csf1r^{-}/Csf1r^{-}$ mice.

locally. Studies in $Csf1^{op}/Csf1^{op}$ mice have established the central role of CSF-1 in the regulation of osteoclast progenitor cell differentiation, tissue macrophage production, and various biological functions. $Csf1r^{-}/Csf1r^{-}$ mice have some more severe phenotypes than do $Csf1^{op}/Csf1^{op}$ mice (Table 2). These data indicate that all CSF-1 functions are mediated by CSF-1 receptor and there are some CSF-1-independent effects of the CSF-1R.

Initially CSF-1 alone was shown to stimulate cultured murine bone marrow progenitor cells to form colonies of macrophages. Subsequent studies conclusively proved the central role of CSF-1 in the supporting mononuclear phagocyte proliferation, differentiation, and survival. CSF-1 also synergizes with cytokines (stem cell factor, IL-1, IL-3, and IL-6) that act on multipotent cells to stimulate the differentiation of multipotent hematopoietic cells into cells

**TABLE 3 CSF-1 Requirements for Tissue Macrophage Development and the Effects of Postnatal Circulating CSF-1 and the Full-Length CSF-1 Transgene on Restoration of Macrophages in $Csf1^{op}/Csf^{op}$ Mice[a]**

| Tissue | Criterion for detection | CSF-1 requirement | Effect of postnatal circulating CSF-1 | Response to full-length CSF-1 transgene |
|---|---|---|---|---|
| Muscle | F4/80 | Complete | None | Complete |
| Tendon | F4/80 | Complete | None | Complete |
| Dermis | F4/80 | Complete | Partial/none[b] | Complete |
| Periosteum | F4/80 | Complete | None | Complete |
| Synovium | F4/80 | Complete | None | Complete |
| Kidney | F4/80 | Complete | Complete | Complete |
| Retina | F4/80 | Complete | Complete | ND |
| Peritoneal cavity | NSE | Complete | None | ND |
| Pleural cavity | NSE | Complete | None | ND |
| Adrenal gland | F4/80 | Partial | None | Complete |
| Bladder | F4/80 | Partial | Partial/none[b] | Partial |
| Salivary gland | F4/80 | Partial | Partial | Complete |
| Liver | F4/80 | Partial | Complete | Complete |
| Stomach | F4/80 | Partial[c] | Partial/none[b] | Complete |
| Gut | F4/80 | Partial[c] | Partial/none[b] | Complete |
| Spleen | F4/80 | Partial | Complete | ND |
| Langerhans cells | F4/80 | Independent | None | ND |
| Thymus | F4/80 | Independent | None | Complete |
| Lymph node | F4/80 | Independent | None | None |
| Bone marrow macrophage | F4/80 | Partial | Partial | ND |
| Bone marrow monocytes | F4/80 | Independent | None | None |
| Spleen | MOMA-1 | Complete | Complete | Complete |
| Osteoclasts | TRAP | Complete | Complete | Complete |

[a] Abbreviations: NSE, nonspecific esterase; TRAP, tartrate-resistant acid phosphatase staining; ND, not determined.
[b] Localized subpopulations exhibited different responses to restoration of circulating CSF-1.
[c] Localized subpopulations within these tissues are completely dependent.

committed to the mononuclear phagocytic lineage. With osteoprotegerin ligand, CSF-1 is required for *in vitro* generation of osteoclasts from their monocytic precursors. CSF-1 also appears to play a role in regulating osteoclast function by increasing survival, cell size, and multinucleation and by stimulating motility and spreading. Recently, CSF-1 was identified as a growth factor for mouse primary stromal initiating cells, which are defined by their ability to support the proliferation of B and myeloid lineage cell lines and which are not hematopoietic. This suggests that CSF-1, together with other necessary cytokines, could participate in the establishment of a functional hematopoietic microenvironment.

Local CSF-1 expression in the mouse female reproductive tract coincides with CSF-1R expression. CSF-1 regulates the estrus cycle, pregnancy rate, postimplantation embryonic viability, and branching morphogenesis of the developing and lactating mammary gland. Although CSF-1 is not absolutely required at any stage during pregnancy, it affects many stages and, if assessed by their ability to produce viable offspring beyond weaning, *op*/*op* females are almost completely infertile. CSF-1 regulates the development of testicular macrophages that normally contact the testosterone-producing Leydig cells. Associated with an absence of these testicular macrophages, male *Csf1*$^{op}$/*Csf1*$^{op}$ mice have a low libido, abnormal Leydig cell morphology, and reduced testosterone levels. Recently, CSF-1 and its major target cells in the central nervous system, microglia, have been shown to play a role in establishing the feedback sensitivity to circulating steroid hormones in the hypothalamus. This may partly explain the reproductive defects in *Csf1*$^{op}$/*Csf1*$^{op}$ mice.

The CSF-1R is predominantly expressed in mononuclear phagocytes and their progenitors and cells of the female reproductive tract. Thus, the pleiotropic nature of the *Csf1*$^{op}$/*Csf1*$^{op}$ phenotype suggests that one group of broadly distributed target cells, the macrophages, plays important regulatory roles in tissues (Fig. 3). Tissue macrophages that require CSF-1 for their development are found primarily in tissues undergoing rapid morphogenesis or tissue remodeling. A current hypothesis to explain the broad biological effects of CSF-1 is that CSF-1

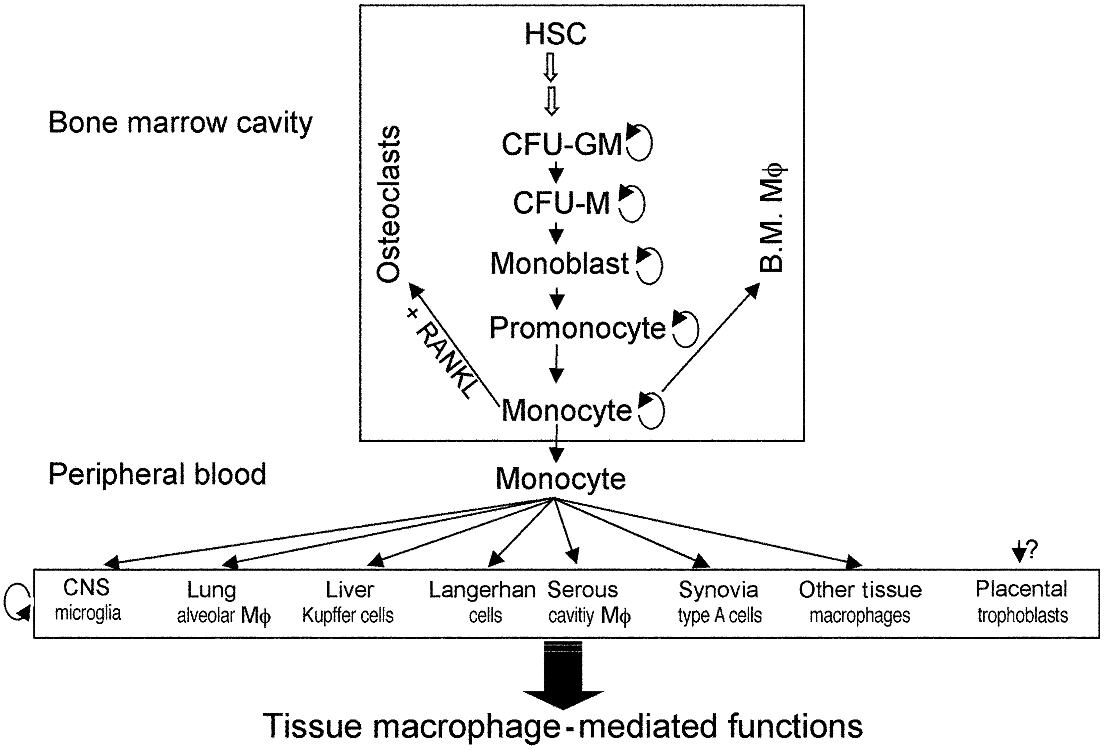

**FIGURE 3** Regulation of mononuclear phagocyte production by CSF-1. CSF-1 is the primary regulator of mononuclear phagocytes. In combination with other hematopoietic growth factors, CSF-1 supports multipotent progenitor differentiation to CSF-1-dependent bone marrow colony-forming cells *in vitro*; in combination with RANKL, CSF-1 is able to support osteoclast differentiation both *in vivo* and *in vitro*. HSC, Hematopoietic stem cell; CFU-GM, colony-forming unit (granulocyte/macrophage); CFU-M, colony-forming unit (macrophage); Mø, macrophage.

regulates the development of cells (macrophages and osteoclasts) that have important trophic and/or scavenger roles for the development and/or function of the tissue in which they reside (Fig. 4).

## V. CSF-1 AND THE CSF-1R IN DISEASES

In normal adults, the reported average serum level of CSF-1 is about 4.46 ± 1.33 ng/ml and the range is from 1.7 to 8.4 ng/ml. Serum CSF-1 levels are significantly elevated during pregnancy. Compared with adults, CSF-1 levels are higher in the newborn infant. Increased circulating CSF-1 has been associated with many human diseases and local CSF-1 expression is also believed to be either pathological or protective in various states.

### A. Neoplasms

Viral oncogenic forms of *fms* cause feline fibrosarcomas. The 5′ end of the CSF-1R is used approximately 20% of the time as an integration site for Friend murine leukemia virus-induced mouse myeloid leukemias. Autocrine regulation by CSF-1 is a characteristic of all irradiation-induced leukemias in SJL/J mice and of some *myc* retrovirus-induced mouse tumors. Transfection of ovarian cancer cells with CSF-1 cDNA enhances their tumorigenicity in nude mice, with the formation of invasive metastases. In polyoma middle T-antigen-induced mammary tumors in mice, CSF-1, via its action on tumor-associated macrophages, promotes mammary carcinoma progression and metastasis. In addition, mouse CSF-1 antisense treatment suppresses the growth of metastatic human tumor xenografts in mice.

Circulating levels of CSF-1 are elevated in patients with myeloid and lymphoid malignancies and carcinomas of the ovary, endometrium, breast, head, and neck and in prostatic cancer with bone metastasis. In the metastases of epithelial ovarian tumors, overexpression of the CSF-1 receptor in addition to epithelial cell-derived CSF-1 is associated with poor prognosis and decreased survival. High levels of CSF-1 in the ascites of these patients correlate with advanced presentation and poor outcome. Furthermore, tyrosine phosphorylated forms of the CSF-1R (pTyr-809 and pTyr-723) are detectable in a significant proportion of ovarian tumors. pTyr-809 is associated with enhanced local invasiveness and pTyr-723 is associated with enhanced metastatic potential. Their expression is correlated with a highly significant decrease in survival and increased risk of recurrence. The activated CSF-1R may be used as a predictor of poor outcome in advanced epithelial ovarian carcinoma. The more invasive human breast cancer cell lines express elevated CSF-1, CSF-1 receptor, and uPA. The CSF-1R is also expressed in prostatic carcinoma cells. Assignment of the GM-CSF, CSF-1, and CSF-1R genes to a specific region of human chromosome 5 provides evidence for linkage of a family of genes regulating hematopoiesis and for their involvement in the deletion (5q) in myeloid disorders. In some cases of human myeloid leukemias, the CSF-1 and CSF-1R genes are simultaneously and stably expressed.

These studies suggest that there is not only autocrine/paracrine growth control by CSF-1 in selected human neoplasias but also an indirect enhancing effect of CSF-1 stimulation of tumor-associated macrophages in tumor progression and metastasis.

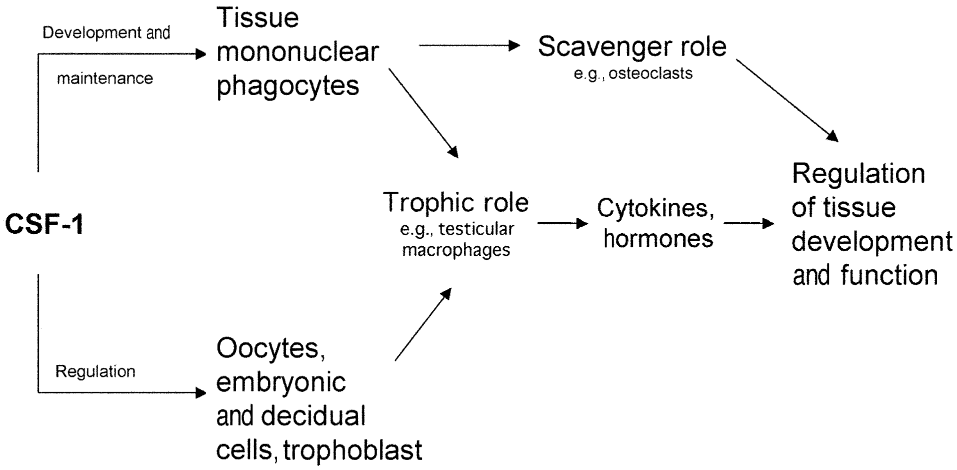
Development and maintenance
Tissue mononuclear phagocytes
Scavenger role
e.g., osteoclasts
CSF-1
Trophic role
e.g., testicular macrophages
Cytokines, hormones
Regulation of tissue development and function
Regulation
Oocytes, embryonic and decidual cells, trophoblast

**FIGURE 4** Regulation by CSF-1 of target cells that have trophic and/or scavenger roles in tissues. Reproduced from Stanley (1998), with permission.

## B. Atherosclerosis

Monocyte-derived macrophage accumulation and their transformation into lipid-laden foam cells after taking up modified lipoproteins in the arterial intima represent the initial atherosclerotic cellular lesion. In low-density lipoprotein receptor-null or apolipoprotein E-null mice, the Csf1$^{op}$ mutation has protective effects on the development of atherosclerosis, despite the inhibitory effect of CSF-1 on cholesterol clearance. CSF-1 also enhances vascular plaque rupture by triggering apoptosis of vascular smooth muscle cells. A reduction of the CSF-1 expression is a potential mechanism underlying the reduction of neointimal proliferation by intracoronary brachytherapy. Increased CSF-1 levels are correlated with the increased aortic calcification index and the elevated atherosclerosis in the hemodialysis patient.

## C. Glomerulonephritis

Glomerular and interstitial macrophage accumulation is one of the major inflammatory aspects of most types of human glomerulonephritis. The increase of both locally produced and circulating CSF-1 has been shown to be correlated with enhanced monocyte infiltration, the local proliferation of macrophages and their activation, and glomerular injury and proteinuria. In particular, the renal tubular epithelium is a major site of production of CSF-1 during experimental renal injury. The ectopic expression of CSF-1 in the kidneys of autoimmune MRL-Fas$^{lpr}$ mice causes local tissue injury accompanied by extensive macrophage infiltration. A proinflammatory role of CSF-1 has been assumed. In addition, based on the experiments carried out with a drug-induced hypercholesterolemia rat model, a preventive role of CSF-1 on the progression of lipid-induced nephrotoxicity in diabetic nephropathy has been proposed.

## D. Alzheimer's Disease

In Alzheimer's disease (AD), inflammation mediated by microglia plays an important role in neuron injury and cognitive decline. CSF-1 and its receptor are involved in the inflammatory process. There is increased expression of the CSF-1R and CSF-1 in both human AD brain and transgenic mouse AD models. Both *in vitro* and *in vivo* studies have proved the powerful proliferative and proinflammatory effects of CSF-1 on microglia. CSF-1 also augments β-amyloidal-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells.

## E. Other Diseases

Elevated serum CSF-1 levels have been found in following chemotherapy treatment and in several other human pathological states, such as preeclampsia in pregnant patients, infection, hepatic injury, hemophagocytic syndrome, thalassemia, and ischemic heart disease, indicating a possible role of CSF-1 in these diseases. In addition, low maternal serum CSF-1 levels in the first trimester have been associated with unexplained recurrent abortion. Also, low levels of CSF-1, or lack of sufficient increase of serum CSF-1 after the first trimester, have been strongly associated with adverse pregnancy outcome, including low body weight or preterm delivery, severe gestational hypertension, and preeclampsia. In contrast, other studies have shown that placental and blood levels of CSF-1 are higher in preeclampsia than in normal pregnancies. The specific roles of CSF-1 in pregnancy need to be further defined.

## F. Potential Therapeutic Applications

Preclinical studies have shown encouraging potential therapeutic applications of CSF-1, including its use in the treatment of infections and malignancies, to accelerate hematopoietic recovery following chemotherapy or bone marrow transplantation, to reduce the risk of atherosclerosis, and to improve fertility.

Administration of pharmacological doses of recombinant human CSF-1 in mice and nonhuman primates has been shown to result in (1) an increase of up to 10-fold in the circulating monocyte concentration, (2) an increase in macrophage numbers in certain areas of the periphery, (3) a stimulation of the cycling of bone marrow progenitors for granulocytes, macrophages, megakaryocytes, and erythrocytes, and (4) stimulation of natural killer (NK) cell activity and macrophage/monocyte antibody-dependent cellular cytotoxicity to protect against lethal *Escherichia coli* and *Candida* infections and to reduce tumor metastases and increase survival of mice bearing melanomas. CSF-1 administration to hyperlipidemic rabbits or cynomolgus monkeys rapidly lowers blood cholesterol levels by enhancing the clearance of lipoproteins containing apolipoprotein B-100 via both LDL receptor-dependent and -independent pathways. CSF-1 increases the uptake of acetylated LDL into macrophages by up-regulating scavenger receptors and stimulates the efflux of free LDL from macrophages by up-regulating both the LDL receptor and cholesterol ester hydrolase. Exposure-based safety evaluations show that recombinant human CSF-1 is free of toxicity at all tested dosages, except for a transient decrease of platelet counts (thrombocytopenia). Thrombocytopenia is the major dose-related adverse effect of CSF-1 administration. It has been reported to happen to all the species studied. No effect of CSF-1 administration has been found on thrombocytopoiesis. The thrombocytopenia is thought to be due to the increased monocyte/macrophage activity causing shortened platelet survival.

The innate immune system consists largely of professional phagocytes of the granulocyte and monocyte lineages [polymorphonuclear leukocytes (PMLs), circulating monocytes, and tissue-based macrophages], which play a critical role in host recognition of, and responses to, bacterial, fungal, and parasitic pathogens. CSF-1 enhances the cytotoxicity, superoxide production, phagocytosis, chemotaxis, and secondary cytokine production by macrophages. It has been shown to augment the *in vitro* antifungal activity of monocytes/macrophages against both conidia and hyphae of *Aspergillus fumigatus*, partly via enhancement of oxidation-dependent mechanisms. Therapeutic effects of CSF-1 in combination with a systemic antifungal agent (amphotericin B; fluconazole) have been shown in both mouse and rat systemic fungal infection models. The effects of CSF-1 administration have also been reported to suppress the acute virus-induced myocarditis and the infiltration of glomerular macrophages in lipid-induced nephrotoxicity.

Clinical trials with CSF-1 have mainly been based on its specificity for mononuclear phagocytic cells. In the reported phase I and phase II trials, CSF-1 has been used in the adjunctive treatment of infectious complications during neutropenia, for example, the treatment of patients with invasive fungal infections after bone marrow transplantation.

Because elevated serum CSF-1 levels or local production are involved in some chronic pathological processes, the development of effective CSF-1/CSF-1R antagonists is another potential area of application. For example, *in vitro* experiments showed that CSF-1 increased the frequency of human immunodeficiency virus (HIV) infection in monocyte-derived macrophages, the amount of HIV mRNA expression per infected cell, and the level of proviral DNA expressed per infected cell. An autocrine/paracrine mechanism in sustaining HIV replication in macrophages was proposed. In addition, CSF-1 is involved in the undesired recruitment of macrophages to the site of transplanted kidney allografts and plays a pathogenic role in acute rejection. CSF-1 could be a potential target for immunosuppression in kidney transplantation patients. Potential therapeutic approaches could involve the use of anti-CSF-1R antibodies, CSF-1R extracellular domain or anti-sense RNA to CSF-1, and the CSF-1R. Indeed, as indicated earlier, blocking host synthesis of CSF-1 with anti-CSF-1 antisense in mice bearing metastatic human embryonic and colon carcinoma xenografts has been shown to block tumor progression and improve survival.

## VI. SUMMARY

CSF-1 is the primary regulator of the differentiation, proliferation, and functioning of mononuclear phagocytic lineage cells, including monocytes, tissue macrophages such as skin Langerhans cells, liver Kupffer cells, bone osteoclasts and neuronal microglia. All CSF-1 function is mediated by a high-affinity receptor (CSF-1R) encoded by the c-*fms* protooncogene. Abnormal CSF-1/CSF-1R regulation is involved in a variety of disease states. CSF-1 is of potential diagnostic and therapeutic significance in disease management.

## Acknowledgments

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## Glossary

**colony-stimulating factors** Group of four growth factors defined by their ability to stimulate hematopoietic progenitor cells to form colonies of mature progeny cells in semisolid culture media. CSF-1 supports formation of macrophage colonies, granulocyte CSF supports formation of granulocyte colonies, granulocyte/macrophage CSF supports formation of colonies of granulocytes and macrophages, and interleukin-3, also known as multilineage CSF, supports formation of colonies containing cells derived from multiple lineages.

**mononuclear phagocytic lineage** Term used to describe the lineage of the colony-stimulating factor-1-responsive hematopoietic precursor of macrophage → monoblast → promonocyte → monocyte → macrophage. Mononuclear phagocytes generally include tissue macrophages, microglia, Kupffer cells, Langerhans cells, synovial type A cells, and osteoclasts. These cells are derived from common hematopoietic precursors and their differentiation is regulated by CSF-1.

**osteopetrosis** bone imbalance disorder characterized by accumulation of excessive bone mass resulting from a reduction in bone resorption relative to bone formation.

## See Also the Following Articles

**Flt3 Ligand • Granulocyte Colony-Stimulating Factor (G-CSF) • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Interferons: α, β, Ω, and τ • Osteogenic Proteins**

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# Contractility

See *Uterine Contractility*

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# Corpus Luteum: Regression and Rescue

ANTHONY J. ZELEZNIK
*University of Pittsburgh School of Medicine*

I. OVERVIEW OF THE LUTEAL PHASE
II. THE ROLE OF LH IN LUTEAL FUNCTION
III. LH SECRETION AND THE REGRESSION OF THE CORPUS LUTEUM
IV. LUTEAL RESPONSIVENESS TO LH
V. RESCUE OF THE CORPUS LUTEUM DURING EARLY PREGNANCY
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luteal cell proliferation, survival, and steroid production. The steroidogenic cells of the corpus luteum cease to proliferate and this is associated with the decline in the expression of proliferating cell nuclear antigen (PCNA). Rescue of the corpus luteum by hCG is not associated with either the resumption of steroidogenic cell proliferation or the expression of PCNA. Likewise, hCG does not appear to dramatically increase mRNA levels for the enzymes involved in progesterone production. Rather, it seems that the rescue of the corpus luteum during early pregnancy is the result of the extremely high concentrations of hCG in blood that serve to maximally stimulate progesterone production by the aging corpus luteum until the placenta assumes the principal burden of progesterone production for the remainder of pregnancy.

## Glossary

**human chorionic gonadotropin** A glycoprotein hormone similar to LH that is produced by the trophoblast cells of the early embryo and placenta.

**luteinizing hormone** A glycoprotein hormone produced by the anterior pituitary gland.

**luteolysis** The cessation of progesterone production at the end of a nonfertile menstrual cycle.

**luteotropin** Any substance that stimulates the function of the corpus luteum.

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Folliculogenesis • Gonadotropin-Releasing Hormone (GnRH) • Gonadotropin-Releasing Hormone and Puberty • Luteinizing Hormone (LH) • Ovulation • Progesterone Action in the Female Reproductive Tract • Vascular Endothelial Growth Factor**

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# Corpus Luteum in Primates

Richard L. Stouffer
*Oregon National Primate Research Center, Oregon Health & Science University*

I. INTRODUCTION
II. DEVELOPMENT OF THE CORPUS LUTEUM
III. FUNCTION OF THE CORPUS LUTEUM
IV. REGRESSION OF THE CORPUS LUTEUM
V. RESCUE OF THE CORPUS LUTEUM
VI. SUMMARY AND CLINICAL RELEVANCE

**The corpus luteum is the endocrine tissue in the ovary of mammals that differentiates from the mature follicle after ovulation has occurred. The corpus luteum plays two key roles during the reproductive life of primates, including women. First, with its limited functional tenure during the ovarian/menstrual cycle, reproductive potential and cyclicity are maintained. Second, with its timely rescue during the fertile cycle, early pregnancy is sustained until sufficient placental function is acquired.**

## I. INTRODUCTION

A key feature of ovarian function in mammalian species is the development of the corpus luteum from the ovulatory follicle and its subsequent role in producing hormonal factors, notably progesterone, that are essential for the initiation and maintenance of pregnancy. Considering the importance of this process, the species differences in the structure, function, lifespan, and regulation of the corpus luteum are remarkable. Important differences between the corpus luteum of typical laboratory animals, such as rats, mice, and rabbits, and that of domestic animals limit our understanding of the control of the primate corpus luteum but certainly provide a strong rationale for further studies.

Primates are among the species that have ovarian cycles with "long luteal phases," i.e., the corpus luteum differentiates after ovulation and functions for a sufficient period of time to permit movement of the early embryo through the oviduct, preparation of the uterus for implantation, and embryo attachment plus trophoblast invasion into the endometrium. If the oocyte released after ovulation is not fertilized and/or implantation does not occur, the functional lifespan of the corpus luteum is limited to approximately 2 weeks, corresponding to the luteal phase of the menstrual cycle. Luteolysis near the end of the menstrual cycle typically occurs before the developing placenta can initiate local processes (e.g., progesterone production) to keep the uterus in a quiescent, supportive state throughout pregnancy. Thus, a critical interval in early pregnancy is when the conceptus signals to the mother (i.e., maternal recognition of pregnancy) that intrauterine pregnancy is occurring, the primate corpus luteum is "rescued" from impending regression, and luteal function is extended until essential activities are replaced by placental function (i.e., the luteal–placental shift).

The following sections address our current understanding, or lack thereof, of the processes controlling the development, function, and lifespan of the primate corpus luteum.

## II. DEVELOPMENT OF THE CORPUS LUTEUM

Differentiation of the corpus luteum from the wall of the ovulatory follicle (Fig. 1) involves two pivotal events. One is the conversion of granulosa cells lining the inside of the wall into luteal cells (sometimes referred to as granulosa-luteal cells). In the preovulatory follicle, the granulosa cells are small and display limited steroidogenic activity, primarily converting androgen to estrogen by aromatization. During luteinization, the granulosa cells hypertrophy and acquire increased quantities of lipid droplets and cellular organelles (e.g., smooth endoplasmic reticulum and mitochondria with lamellar cristae) associated with steroidogenesis. The second event is the disruption of the basement membrane between the avascular granulosa and vascular theca cell compartments, followed by proliferation and migration of vascular elements into the luteinizing granulosa layer. Luteal angiogenesis is so extensive that approximately half of all cells comprising the corpus luteum are endothelial or perivascular cells in sinusoidal capillaries adjacent to steroidogenic luteal cells. The fate of follicular theca cells during development of the primate corpus luteum is controversial. Some follicular theca cells may degenerate or be absorbed into the surrounding stroma, but there is little evidence that they disperse into the luteal parenchyma as theca luteal cells, which is typical of many nonprimate species. Others remain as paraluteal cells (Fig. 1)

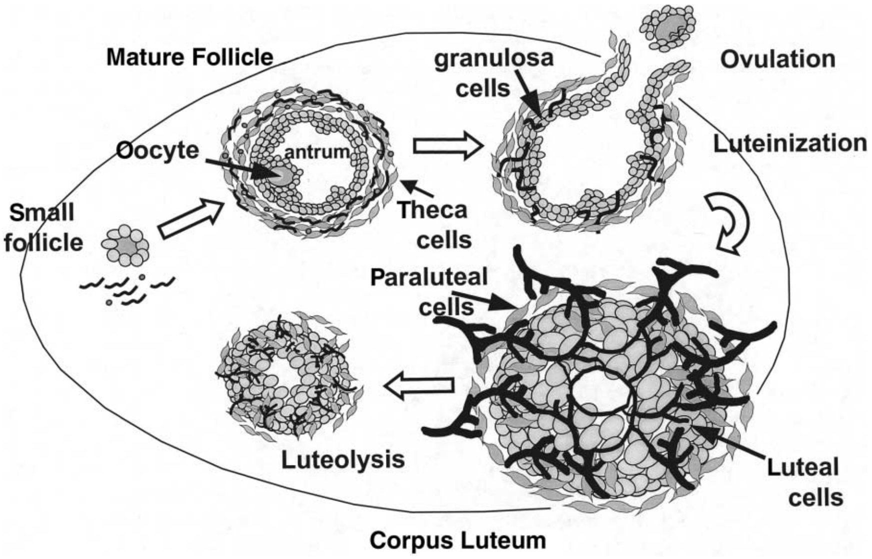

FIGURE 1 Tissue remodeling and angiogenesis associated with conversion of the ovulatory follicle into the corpus luteum in primates. In the mature follicle, vascularization (black blood vessels) is limited to the theca cell layers. A basement membrane separates the theca from the avascular granulosa cell layer, which encloses the oocyte and a fluid-filled antrum. Near the time of ovulation, the basement membrane breaks down and angiogenesis occurs in the granulosa layer. Granulosa-derived luteal cells hypertrophy but remain generally segregated from theca-derived paraluteal cells found around the outside of and along infoldings in the corpus luteum. At luteolysis, both the luteal cells and the microvasculature degenerate. Adapted from Hazzard and Stouffer (2000), with permission.

clustered along the periphery and within infoldings of the luteal gland. By day 6–7 after ovulation, the corpus luteum is well developed, with the antrum of the antecedent follicle replete with luteinized steroidogenic cells and the vascular elements connected to venules running from the center back through the luteal tissue to veins outside the gland.

It is generally accepted that the midcycle surge of gonadotropins, particularly luteinizing hormone (LH), secreted by the anterior pituitary is the stimulus for periovulatory events in the primate follicle. The duration of the LH surge in primates (~48 h) is considerably longer than that in nonprimates (e.g., 4–8 h in rodents and rabbits). Recent evidence suggests that surge requirements vary for periovulatory events, with resumption of oocyte maturation requiring less LH exposure than that for optimal luteal development. Why primate follicles appear to require such a prolonged LH surge, particularly for luteinization, remains unknown. It could be related to their predominant reliance on LH as a luteotropic hormone, whereas other species rely on other pituitary hormones (e.g., prolactin in rodents) or local factors (e.g., estrogen in rabbits) as primary luteotropins.

Both granulosa and theca cells of the preovulatory follicle contain LH receptors, and it is generally believed that the surge levels of LH act via receptor–adenylate cyclase-coupled, cyclic AMP (cAMP)-mediated intracellular pathways to modulate gene transcription and consequent changes in cellular levels of enzymes or proteins. Such events produce transient changes related to ovulation and luteinization or stable changes related to luteal cell phenotype. Recent studies using controlled ovarian cycles, where events can be examined at precise time intervals after exposure to an ovulatory gonadotropin bolus, are providing insight into the kinetics and array of changes occurring in the luteinizing follicle. For example, granulosa cell proliferation is inhibited early (within 12 h) during the gonadotropin surge, in association with suppressed expression of mRNAs for key promoters of cell cycle progression (e.g., cyclin B1) and increased expression of cell cycle inhibitors (e.g., the cyclin-dependent kinase inhibitors $P21^{Cip1}$ and $P27^{Kip1}$). The ability of luteinizing granulosa cells to bind low-density lipoprotein (LDL) and to convert LDL-derived cholesterol to progesterone increases markedly after the gonadotropin surge and is associated with increased expression and/or activity of LDL receptor, StAR, P450 side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD). A major action of the LH surge is to promote further differentiation of granulosa cells into the progesterone-producing luteal cells.

Another action of the midcycle gonadotropin surge is to modulate the production of paracrine or autocrine factors and follicular/luteal sensitivity to hormones or local factors. In primates, as well as nonprimates, the LH surge induces the expression of one isoform of cyclooxygenase (COX-2) with a resulting increase in prostaglandin (PG) levels in follicular fluid of ovulatory follicles. Since elevated PG levels occur in various species ~10 h before follicle rupture, there is speculation that this event controls the timing of ovulation in mammals. Whether COX-2 expression is important for luteinization is unclear. Another important action of the LH surge is to promote the production of angiogenic factors, such as vascular endothelial growth factor (VEGF; notably VEGF-A) and angiopoietin (Ang; particularly Ang-1), by luteinizing granulosa cells. Systemic or intrafollicular injection of VEGF-neutralizing agents into monkeys around the time of ovulation impairs angiogenesis/vascular function and progesterone secretion by the developing corpus luteum. The data are consistent with the concept that angiogenic factors produced by luteinizing granulosa cells, in direct response to LH, act in a paracrine manner to promote the development and maturation of new blood vessels in the corpus luteum. The LH surge also markedly alters the expression of steroid hormone receptors, and presumably local steroid action, in the primate follicle. Whereas estrogen receptors (ERβ) are suppressed, progesterone receptors (PRs) are induced in luteinizing granulosa cells concomitant with their increased ability to produce progesterone. Progesterone produced in the early response to the LH surge is essential for ovulation and luteinization to occur in primate follicles (Fig. 2). The cellular mechanisms of progesterone action are under investigation, but it is likely that this steroid promotes the expression/activity of specific proteases (e.g., matrix metalloproteinases, MMP-1) and their endogenous inhibitors (e.g., TIMP-1) to regulate the tissue remodeling that is critical for ovulation or luteinization. In addition, progesterone may act as an anti-atretic, pro-differentiative factor in the ovulatory follicle. The LH surge also modulates the production, sensitivity, or action of growth factors, such as the insulin-like growth factor-binding proteinsystem, in the developing corpus luteum.

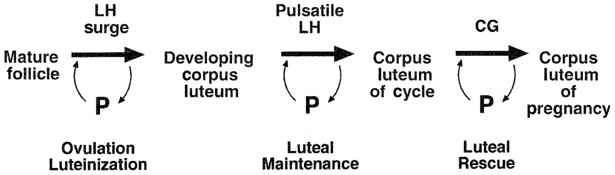

FIGURE 2 Schematic of the hypothesis that the luteotropic actions of LH during the menstrual cycle and CG in early pregnancy involve at least two mechanisms: (1) a direct action on luteinizing follicle and luteal cells and (2) an indirect action via stimulation of luteal progesterone (P) and P receptor expression. Thus, both endocrine (LH and CG) and local (P) factors are essential for the development and maintenance of the primate corpus luteum.

## III. FUNCTION OF THE CORPUS LUTEUM

The primate corpus luteum synthesizes and secretes remarkable amounts of progesterone, the steroid hormone that is essential for the initiation and maintenance of pregnancy. Localization of P450-SCC and 3β-HSD protein and enzyme activity to granulosa luteal cells and paraluteal cells indicates that both can contribute this hormone, although the former is a much more numerous cell population. However, the primate corpus luteum produces at least three other substances that may have endocrine functions during the menstrual cycle or early pregnancy. Moreover, they appear to originate from specific luteal cell types.

Unlike in many nonprimate species, the luteal tissue of monkeys, apes, and humans produces significant amounts of estrogen. Immunohistochemical studies indicate that P450-C17, the enzyme catalyzing androgen synthesis, is confined to the theca layer of preovulatory follicles and the paraluteal cells in the corpus luteum. In contrast, P450-aromatase, the enzyme converting androgen to estrogen, is found in the granulosa layer of the follicle and luteal cells in the central parenchyma of the corpus luteum. These findings suggest that a two-cell model for estrogen synthesis, similar to that proposed for the follicle, is retained in the primate corpus luteum. Paraluteal cells may synthesize androgens that diffuse or are carried by the local microvessels to granulosa luteal cells where they serve as a precursor for estrogen production.

Compartmentalization of luteal cell activities is not limited to steroidogenesis; it involves two peptide or protein hormones, inhibin and relaxin. Whereas inhibin (primarily inhibin B) production typically ends following the LH surge in many species, the primate corpus luteum synthesizes and secretes significant amounts of inhibin A. Investigators report immunostaining for inhibin subunits primarily in granulosa luteal but not paraluteal cells; these cells likely produce other members of the inhibin receptor family, including the binding proteins follistatin and follistatin-related gene product, that neutralize inhibin/activin activity. Relaxin is also produced by the primate corpus luteum, with levels becoming detectable by the late luteal phase of the menstrual cycle, concomitant with relaxin immunostaining in granulosa luteal cells. Although their presence in the circulation has led to speculation regarding endocrine actions [e.g., inhibin control of pituitary follicle-stimulating hormone (FSH) secretion or relaxin stimulation of uterine growth or pituitary GH/prolactin secretion], such roles await verification. Nevertheless, it is likely that they locally modulate follicular or luteal function, e.g., inhibin suppression of FSH or LH actions in the ovary.

It is generally accepted that the low levels of LH circulating during the luteal phase of the menstrual cycle are required for normal function of the primate corpus luteum. The function and lifespan of the corpus luteum end abruptly at any stage of the luteal phase if LH support is compromised. To date, LH remains the only luteotropic hormone identified during the menstrual cycle. A possible regulatory role for prolactin, a luteotropic hormone in some species, remains unsubstantiated in primates despite considerable investigation. Participatory roles for other hormones, notably those regulating cell metabolism (e.g., GH or insulin), require further investigation.

Although pituitary LH appears to be the essential luteotropin, its precise actions in maintaining the structure and function of the primate corpus luteum are ill defined. Primate luteal tissue contains specific LH–chorionic gonadotropin (CG)-binding sites that belong to the G-protein-coupled family of membrane receptors that contain seven-transmembrane domains and activate primarily the adenylate cyclase system. cAMP generated by this signal transduction system acts as an intracellular "second messenger" to elicit LH's biologic effects. LH and cAMP stimulate the production of progesterone and estrogen by primate luteal cell preparations. However, the population(s) of luteal cells that are targets for LH action is controversial. Initial evidence from domestic animals suggested that the "small" luteal cells of putative theca origin respond acutely to LH with enhanced progesterone secretion, whereas "large" luteal cells of granulosa cell origin did not respond to LH while producing large amounts of progesterone. However, since small and large cells from corpora lutea of the natural cycle contain equal numbers of LH-binding sites, a role for LH in large luteal cells of nonprimates, including rodents, cannot be ruled out. Investigations in nonhuman primates suggest that large—not small—cells respond to LH–CG and cAMP with enhanced progesterone production in a stage-dependent manner. Comparable studies on human luteal cells are difficult to interpret due to the need to use proteases for tissue dispersion. The data generally support the concept that large luteal cells produce greater amounts of progesterone than small luteal cells, but their origin (granulosa vs theca) and LH responsiveness may be species-dependent and vary during the luteal lifespan.

It is unclear how acute actions of LH to stimulate progesterone production relate to chronic actions to sustain the structure and function of the corpus luteum. It is likely that other substances produced by the corpus luteum act to modulate, and perhaps mediate, the actions of LH in controlling luteal development and function. Examples include lipid derivatives (prostaglandins and leukotrienes; see following section), peptides/proteins, and the steroid hormones themselves. The corpus luteum of certain species, notably, nonhuman primates and humans, express PRs throughout the luteal phase of the reproductive cycle and into early pregnancy. This contrasts with other species, such as rodents and rabbits, in which luteinizing granulosa cells express PR for only a short interval (12–72 h) around ovulation. Recent reports suggest that the local actions of progesterone include promotion of luteal cell differentiation while preventing cell degeneration (via apoptosis), perhaps by sustaining luteal sensitivity to, or production of, luteotropic factors (e.g., via LH receptor synthesis or enhanced ratio of luteotropic:luteolytic PG production) and preventing luteal sensitivity to, or production of, luteolytic factors (e.g., via inhibition of estrogen receptor expression or sequestration and action of immune cells). Since luteal PR expression is sustained in species in which LH is the primary luteotropin, it appears that the effects of LH–CG involve at least two mechanisms: (1) a direct, acute action on luteal cells to promote progesterone production and (2) an indirect, chronic action via the local effects of progesterone to promote luteal structure and function (Fig. 2).

## IV. REGRESSION OF THE CORPUS LUTEUM

The factors responsible for the regression of the primate corpus luteum near the end of the menstrual cycle remain an enigma. In many nonprimate species, luteolysis is controlled by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted by the uterus near the end of the ovarian cycle; prevention or neutralization of this luteolytic signal, e.g., via hysterectomy (removal of the uterus), prolongs the luteal lifespan. However, in primates, the demise of the corpus luteum is not the result of a luteolytic signal from the uterus, since hysterectomy does not alter cyclic ovarian function in monkeys or women.

Research has focused on mechanisms involving three substances: one endocrine (LH) and two local (estrogen and $PGF_{2\alpha}$) factors. LH is secreted in a pulsatile manner by the pituitary, and since the frequency of LH pulses declines by the midluteal phase, investigators proposed that this apparent reduction in luteotropic support causes luteolysis. However, subsequent studies demonstrated that the length of the luteal lifespan was *not* altered if the frequency of LH pulses was not allowed to decline [by giving exogenous gonadotropin-releasing hormone (GnRH) pulses at frequent intervals] or if the frequency of LH pulses was prematurely reduced in the early luteal phase. While this hypothesis appears untrue, considerable evidence supports the concept that the corpus luteum becomes less sensitive to LH as its lifespan progresses during the menstrual cycle. The causes for the apparent reduction in luteal sensitivity to LH and its role in controlling luteal lifespan remain unclear.

The lack of a uterine or pituitary role in controlling luteal regression led investigators to propose "self-destruct" mechanisms whereby local factors synthesized in the ovary or corpus luteum initiate luteolysis near the end of the menstrual cycle. Based on the unique ability of primate luteal tissue to synthesize estrogen, plus reports that systemic or local delivery of estradiol into the monkey and human corpus luteum induces premature luteolysis, it was proposed that estrogen was the local luteolytic factor. However, subsequent studies led to the perception that exogenous estrogen did not directly induce luteolysis, but suppressed luteal function via its potent negative feedback inhibition of pituitary LH secretion. Unsuccessful efforts to detect appreciable levels of the classical genomic estrogen receptor, ER-$\alpha$, further reduced enthusiasm for a local luteolytic action of estrogen. However, the recent discovery that the primate corpus luteum expresses the $\beta$ isoform of ER, plus evidence from the monkey model that ER-$\beta$ expression is highest at the time of luteal regression, indicates that the issue of local estrogen action in primate luteal tissue warrants reevaluation.

Because the primate corpus luteum synthesizes PGs, including the PG that is the luteolysin in nonprimates, a self-destruct scenario similar to that described for estrogen was envisioned for $PGF_{2\alpha}$. Early *in vivo* studies with exogenous $PGF_{2\alpha}$ or its analogues yielded variable results, in part due to rapid metabolism, stage-specific sensitivity of the corpus luteum, or high levels of endogenous PGs in luteal tissue. However, intraluteal administration of $PGF_{2\alpha}$ induces premature luteolysis in monkeys and women. Evidence for anti-gonadotropic effects of $PGF_{2\alpha}$ on primate luteal cells *in vitro*, plus the presence of PG receptors in luteal tissue, supports a direct action of $PGF_{2\alpha}$ in the corpus luteum of the menstrual cycle. Nevertheless, it has been difficult to substantiate a physiologic role for endogenous $PGF_{2\alpha}$ in the initiation of luteolysis. Administration of general inhibitors of PG synthesis (cyclooxygenase blockers) either orally or intraluteally to monkeys did not extend the luteal lifespan as one would expect if it prevented the synthesis and action of a luteolytic PG. In fact, intraluteal infusion caused premature luteolysis; these results support the *in vitro* evidence that other PGs of the E, I, and D series have stimulatory or steroidogenic actions in the corpus luteum. Thus, it is possible that the functional lifespan of the primate corpus luteum is influenced by a balance between luteotropic and luteolytic PGs, with PGE or PGI promoting luteal function and, as the tissue becomes responsive to PGF, the latter inhibiting luteal function. It is also possible that other arachidonate products, such as the leukotrienes or epoxide derivatives, influence luteal function.

Although the luteolytic signal(s) remains elusive, cellular and molecular approaches are providing new insight into the processes controlling luteal structure and function. The corpus luteum consists of dynamic populations of luteal and nonluteal cells whose numbers and characteristics change as the luteal lifespan progresses. It appears that there is a loss of large steroidogenic luteal cells as the primate corpus luteum ages. Since it was suggested that small luteal cells differentiate into large cells in response to LH, the decline could be related to a reduction in luteal differentiation or alternatively the onset of luteal degeneration. Likewise, there is a decline in small cell, and then large cell, responsiveness to $PGE_2$ and LH–CG as the monkey corpus luteum ages. Thus, the declining steroidogenic output is due, at least in part, to changes in cell populations and their responsiveness to luteotropic factors. Changes in luteal vasculature may also be critical during luteolysis (Fig. 1); the volume density of blood vessels declines during luteal regression in many species, including humans. Large portions of luteal capillary beds degenerate during luteolysis, although larger vessels are maintained perhaps to assist in resorption of the luteal mass. Although the mechanisms remain unclear, it is hypothesized that the balance between angiogenic factors that stabilize vessels (VEGF, angiopoietin-1) and those that destabilize vessels (angiopoietin-2) shifts in the corpus luteum at luteolysis. Finally, local actions of resident or migrating immune cells, including macrophages and T-lymphocyte subsets, may contribute to endothelial and luteal cell destruction during luteolysis via humoral or nonhumoral mechanisms. Whether molecular pathways such as the generation of highly toxic reactive oxygen species or initiation of apoptosis play key roles in luteolysis in primates, as proposed in other species, awaits further investigation.

## V. RESCUE OF THE CORPUS LUTEUM

It is widely accepted that CG secreted by the implanting embryo and syncytiotrophoblast of the placenta is responsible for rescuing the primate corpus luteum from its impending demise at the end of the menstrual cycle and extending luteal function in early pregnancy (Fig. 3). Passive or active immunization of nonhuman primates and women against CG produces infertility despite normal ovulatory menstrual cycles. Moreover, administration of human chorionic gonadotropin (hCG) to mimic the patterns and levels of endogenous CG in early pregnancy elicits changes in the corpus luteum that are characteristic of maternal recognition of pregnancy: (1) the lifespan of the corpus luteum is extended and timely menstruation does not occur, (2) circulating levels of progesterone increase transiently and then decline as the time of the luteal-placental shift approaches, and (3) nonsteroidogenic activities, including relaxin and inhibin A production, also increase. Thus, CG in the absence of any other embryonic factors is capable of rescuing the corpus luteum of early pregnancy.

Although CG can be first detected, initially in utero-ovarian blood and then in the general circulation, around the time of implantation, the patterns and duration of CG and luteal progesterone secretion vary among primate species. In humans and apes, CG levels peak in the first trimester of pregnancy and then decline, but remain at substantial levels throughout gestation. However, in baboons and Old World monkeys, CG levels decline to undetectable levels by midpregnancy. In New World monkeys, CG levels

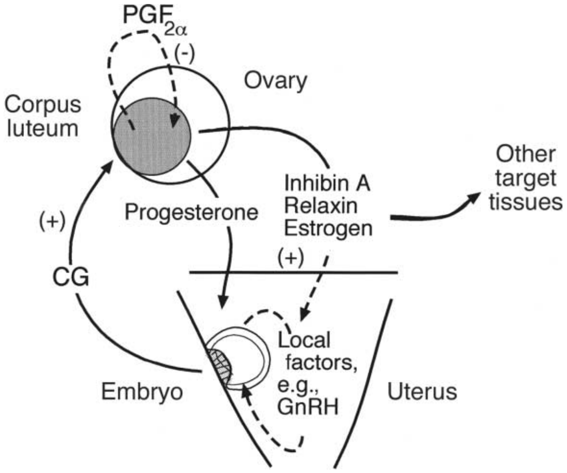

**FIGURE 3** Endocrine and local mechanisms during rescue of the primate corpus luteum at pregnancy initiation. Solid arrows indicate established pathways, whereas dotted arrows indicate proposed pathways. Embryo-derived CG, likely produced in response to local GnRH or other factors, prevents the timely luteal regression at the end of the menstrual cycle. CG may prevent the action of local luteolytic factors, e.g., $PGF_{2\alpha}$. Thus, the corpus luteum continues to produce progesterone (and other putative hormones), which sustains intrauterine pregnancy until the luteal–placental shift. Reprinted from Stouffer and Hearn (1998), with permission.

do not peak until midpregnancy and decline just before parturition. In humans, apes, and Old World monkeys, CG increases serum progesterone levels above those observed during the menstrual cycle. In women, luteal progesterone production remains elevated for 2–3 weeks and the luteal-placental shift does not occur until the sixth week of pregnancy. In contrast, luteal progesterone production in Old World monkeys is enhanced for only a short interval (~4 days) before declining, despite continued increases in circulating CG. In these monkeys, the luteal-placental shift occurs by the third week of pregnancy. In New World monkeys, circulating progesterone levels at pregnancy initiation are simply maintained at concentrations observed during the ovarian cycle. The time of the luteal–placental shift is not well defined in these animals.

CG not only extends the progestational function of the corpus luteum, it promotes other activities even when progesterone production may be declining. These include the synthesis of the estrogens estradiol and estrone, which remain at elevated levels in monkeys even when progesterone levels begin to decline. They also include relaxin and inhibin A. That the corpus luteum is the primary source of inhibin in early pregnancy is confirmed by evidence from women lacking endogenous luteal function, in which circulating inhibin levels failed to rise through 4–6 weeks of pregnancy (maintained by exogenous progestin therapy) compared to normal gestation. Thus, early pregnancy can be considered the final interval of luteal differentiation in primates—a process that starts with the LH surge at ovulation and ends with CG secretion postimplantation. Although progesterone of luteal origin is essential for the maintenance of early pregnancy in primates, the roles of other luteal products, including estrogen, relaxin, and inhibin A, await clarification.

It remains unclear how production of another LH-like hormone, i.e., CG, rescues the primate corpus luteum of early pregnancy. CG is a glycoprotein hormone that is structurally similar to LH; the α-subunits are identical and there is remarkable similarity (up to 85% homology) in the first 114 amino acids of the LHβ- and CGβ-subunits. However, CG is unique due to the presence of a 24- to 31-amino-acid extension to the C-terminus of CGβ and different carbohydrate moieties attached to both subunits. These differences markedly increase CG's circulating half-life and, hence, bioactivity. Nevertheless, research in nonhuman primates and humans supports the concept that both pituitary LH and placental CG share a common receptor and activate similar cAMP-mediated pathways in gonadal cells. Indeed, the effects of administered CG so closely resemble those of pituitary LH that, because of its availability, hCG is used clinically in place of LH to promote a number of ovarian events, including ovulation and corpus luteum function. Very recently, Zeleznik reported that exogenous LH is capable of mimicking CG's action in early pregnancy. Continuous administration of increasing amounts of recombinant human LH or hCG beginning around the expected time of implantation increased progesterone levels and prevented timely luteolysis during the menstrual cycle in rhesus monkeys. These data, plus those from *in vitro* studies on primate ovarian cells, indicate that the actions of LH and CG are comparable; i.e., CG's ability to rescue the primate corpus luteum in the presence of circulating LH support is not due to a unique activity intrinsic to the CG molecule.

It is possible that CG provides an incremental increase in luteotropic support that prevents the luteolytic process. Because of differences in secretion patterns and circulating half-life between LH and CG, there are qualitative and quantitative differences in the gonadotropin milieu at pregnancy initiation. The intermittent pulses of LH secretion translate into intervals of gonadotropin support and withdrawal during the mid- to late luteal phase that correspond positively with luteal steroidogenic activity. Since CG has a much longer half-life than LH and is produced in exponentially increasing amounts, CG circulates continuously and ultimately rises to levels that obscure any LH pulse. Thus, the conceptus offers uninterrupted (as opposed to episodic) and increasing levels of luteotropic support for the corpus luteum. Whether this change in luteotropic support prevents a luteolytic signal from occurring or bypasses the signal to prevent luteal regression is unknown.

For ethical and practical reasons, there has been little research on the cellular and molecular changes during CG rescue of the corpus luteum in early pregnancy. Models simulating early pregnancy via exogenous CG exposure provide some insight. Morphologic studies identified changes in granulosa luteal cells suggestive of the rapid use of steroid precursors and/or reduced storage of precursors (fewer lipid droplets) for steroid synthesis and increased production of secretory proteins (more membrane-bound granules). In contrast, paraluteal cells displayed changes suggestive of increased steroid precursor accumulation (more lipid droplets) and steroidogenesis (agranular endoplasmic reticulum). The increased luteal mass and steroidogenic activity are not, however, accompanied by a renewal of angiogenesis in the corpus luteum. The marked decline in endothelial cell proliferation that occurs around luteolysis is not prevented by CG. However, this does not rule out the possibility that CG has other effects on the vasculature, either directly or via vascular-specific factors, to influence vessel function or blood flow.

CG exposure results in divergent changes in luteal expression of mRNAs and proteins involved in steroid and peptide hormone production. CG may transiently stimulate progesterone production in the monkey corpus luteum by enhancing the uptake and availability of cholesterol stores (e.g., LDL) for existing steroidogenic machinery, not by stimulating key enzymatic steps (e.g., P450-scc or 3β-HSD). However, early time points (1–3 days of CG exposure) are needed to evaluate this issue. In contrast, CG markedly increases the expression and/or enzyme activity for androgen (P450-c17) and estrogen (P450-aromatase) production in the corpus luteum. With the discovery of two relaxin genes, H1 and H2, in human, it was noted that only the H2 gene is expressed by the corpus luteum, especially in pregnancy. Although transcriptional and/or posttranscriptional control of relaxin expression likely occurs in the corpus luteum, whether CG directly regulates these processes is unknown. There are no reports of acute stimulation of relaxin mRNA or protein production by CG in primate luteal tissue or cells *in vitro*.

Consideration of direct versus indirect effects of CG action must take into account the dynamics of LH–CG receptor–effector systems in primate luteal cells. Unlike in rodent systems where exposure to a large bolus of LH or CG results in the loss or "down-regulation" of gonadotropin receptors, the scenario in primate luteal tissue during exposure to high, rising levels of CG appears somewhat different. Although many of the LH–CG receptors appear occupied, there is a remarkable constancy in the total receptor population—the number of unoccupied sites declines and the number of occupied sites increases with the duration and level of CG exposure. Nevertheless, homologous desensitization of the adenylate cyclase system becomes apparent, as cAMP production in response to LH or CG, but not PGE, PGI, or forskolin, becomes severely impaired. It is possible that the uncoupling of LH–CG receptors from cAMP production is an important step in the transient progestational response of the corpus luteum to CG in early pregnancy. However, the continued response to paracrine factors, including luteotropic PGs or progesterone itself, may promote luteal structure and function. As such, it is noteworthy that a blockade of progesterone production causes structural regression of the corpus luteum despite continued exposure to rising levels of CG during simulated early pregnancy in rhesus monkeys.

## VI. SUMMARY AND CLINICAL RELEVANCE

Although our knowledge of the function and control of the primate corpus luteum has increased significantly in the past decade, further studies are needed. A better understanding of the interactions between gonadotropins and local factors, between cell types, and between molecular pathways in luteal cells is required to elucidate the processes controlling the development, function, and lifespan of the corpus luteum during the menstrual cycle and early pregnancy (Fig. 4). Further progress is essential for evaluating and treating the pathophysiology leading to luteal dysfunction, including recognized disorders such as luteinized unruptured follicle syndrome, short or insufficient luteal phases, or the consequences of ovarian hyperstimulation syndrome occurring during the luteal phase and early pregnancy in assisted reproductive technology protocols. A better understanding of the endocrine, cellular, and molecular defects that lead to abnormal luteal function carries

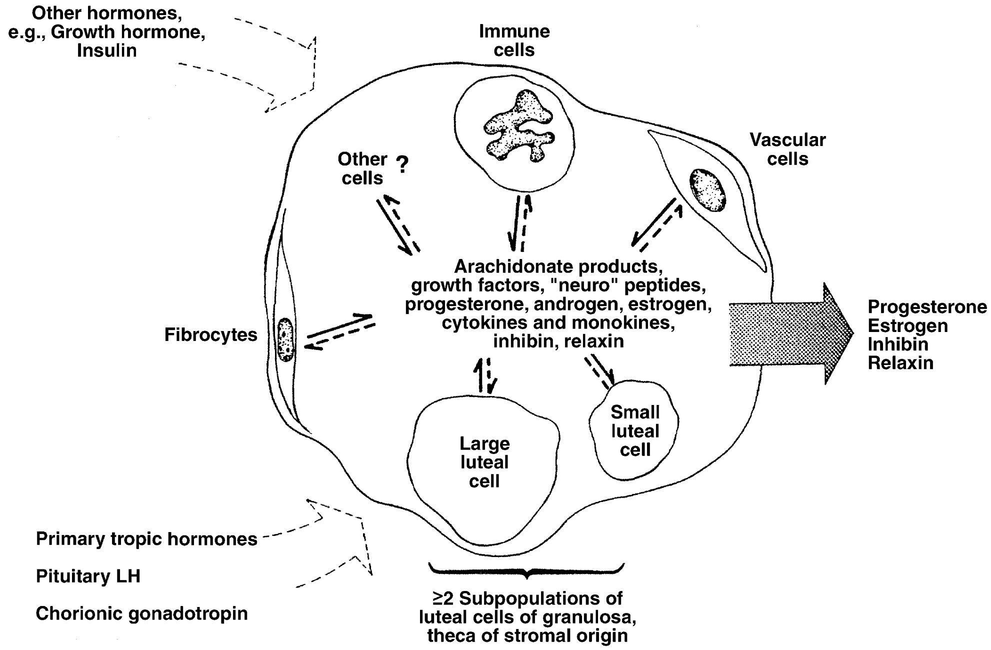
Other hormones, e.g., Growth hormone, Insulin
Immune cells
Vascular cells
Other cells ?
Arachidonate products, growth factors, "neuro" peptides, progesterone, androgen, estrogen, cytokines and monokines, inhibin, relaxin
Fibrocytes
Progesterone
Estrogen
Inhibin
Relaxin
Large luteal cell
Small luteal cell
Primary tropic hormones
Pituitary LH
Chorionic gonadotropin
≥2 Subpopulations of luteal cells of granulosa, theca of stromal origin

FIGURE 4 Conceptual summary of the multiple cell types in the primate corpus luteum and their many products that may serve as autocrine and paracrine regulators of luteal function or as classical hormones. Solid arrows denote secretion, whereas dotted arrows denote local action on target cells. Hormones from the pituitary, placenta, and other endocrine glands that act on the corpus luteum to regulate function or lifespan are also represented. Adapted from Stouffer (1996), with permission.

with it the hope of ameliorating or preventing the infertility, recurrent miscarriage, or serious side effects (e.g., fluid imbalance) inherent in these disorders. Likewise, the ephemeral nature of the corpus luteum offers unique opportunities for studying processes such as tissue differentiation and degeneration, angiogenesis, and inflammatory/immune response in the healthy adult, which can complement concepts arising from research during embryogenesis or pathologic states.

## Glossary

**corpus luteum** The endocrine gland in the ovary of primarily mammals that differentiates from the mature follicle after the ovulatory surge of pituitary gonadotropic hormones.

**luteal peptide/protein production** The synthesis and secretion of nonsteroid hormones, notably inhibin A and relaxin, by the primate corpus luteum.

**luteal–placental shift** The transfer of critical hormonal functions from the corpus luteum to the placenta during gestation; thereafter, the corpus luteum is not essential for maintaining intrauterine pregnancy.

**luteal steroidogenesis** The synthesis and secretion of steroid hormones, notably progesterone and estrogens, by the primate corpus luteum.

**luteinization** The processes whereby the corpus luteum differentiates from the somatic cells of the follicle wall, including conversion of granulosa cells to luteal cells and neovascularization of the luteinizing granulosa layer.

**luteolysis** The processes whereby the corpus luteum regresses near the end of the nonfertile menstrual cycle or, presumably, after the luteal–placental shift in early pregnancy.

**luteolytic factors** Putative local substances, such as prostaglandin $F_{2\alpha}$, that cause regression of the corpus luteum.

**luteotropic hormones** Blood-borne factors, particularly luteinizing hormone from the pituitary gland and chorionic gonadotropin from the placenta, that promote the structure and function of the primate corpus luteum.

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Folliculogenesis, Early • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Ovulation • Progesterone Action in the Female Reproductive Tract**

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# Corticotropin-Releasing Hormone (CRH)

David T. Breault and Joseph A. Majzoub
*Harvard Medical School*

I. INTRODUCTION
II. ANATOMIC DISTRIBUTION
III. GENE AND PROTEIN STRUCTURE
IV. REGULATION OF EXPRESSION
V. REGULATION OF CRH SECRETION
VI. RECEPTORS
VII. SIGNAL TRANSDUCTION
VIII. CRH-BINDING PROTEIN
IX. ROLE IN PHYSIOLOGY
X. PATHOPHYSIOLOGY
XI. CRH-RELATED PEPTIDES

**Corticotropin-releasing hormone (CRH) is the major regulator of the hypothalamic-pituitary-adrenal axis and plays a role in the normal development of the adrenal, brain, and lung. In addition, CRH, and the family of CRH-related peptides, may have regulatory roles in a number of other systems, including the gastrointestinal system, the immune system, the autonomic nervous system, the reproductive system, and behavior.**

## I. INTRODUCTION

CRH derives its name from its role in the anterior pituitary, where it mediates the release of corticotropin (ACTH) leading to the release of adrenocortical steroids. In addition, CRH controls the secretion of β-endorphin and other proopiomelanocortin (POMC)-derived peptides from the anterior pituitary. CRH is the major hypothalamic activator of the hypothalamic–pituitary–adrenal (HPA) axis. In addition, CRH is an important mediator of the stress response, which integrates endocrine, autonomic, immunologic, and behavioral reflexes. Beyond its role as an endocrine hormone, increasing evidence suggests a role for CRH in the regulation of gastrointestinal and reproductive function.

Recently, a family of CRH-related peptides (CRH-RPs) has been identified. These factors likely play a role in integrating multiple aspects of the stress response. Both CRH and CRH-related peptides interact with two different transmembrane, G-protein-coupled cell surface receptors, CRH-R1 and CRH-R2, which differ in their patterns of tissue distribution. In addition, the binding affinities for CRH and the various CRH-RPs to the two receptors differ considerably and likely contribute to the different actions of these peptides.

## II. ANATOMIC DISTRIBUTION

CRH has been localized to the nerve cell bodies in and near the dorsomedial parvicellular division of macrophages, microglia, Kupffer cells, Langerhans cells, synovial type A cells, and osteoclasts. These cells are derived from common hematopoietic precursors and their differentiation is regulated by CSF-1.

**osteopetrosis** bone imbalance disorder characterized by accumulation of excessive bone mass resulting from a reduction in bone resorption relative to bone formation.

## See Also the Following Articles

**Flt3 Ligand • Granulocyte Colony-Stimulating Factor (G-CSF) • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Interferons: α, β, Ω, and τ • Osteogenic Proteins**

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# Contractility

See *Uterine Contractility*

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# Corpus Luteum: Regression and Rescue

ANTHONY J. ZELEZNIK
*University of Pittsburgh School of Medicine*

I. OVERVIEW OF THE LUTEAL PHASE
II. THE ROLE OF LH IN LUTEAL FUNCTION
III. LH SECRETION AND THE REGRESSION OF THE CORPUS LUTEUM
IV. LUTEAL RESPONSIVENESS TO LH
V. RESCUE OF THE CORPUS LUTEUM DURING EARLY PREGNANCY
VI. CELLULAR MECHANISMS INVOLVED IN THE REGRESSION AND RESCUE OF THE PRIMATE CORPUS LUTEUM

**In primate menstrual cycles, the luteal phase begins with the ovulation of the graafian follicle and the transformation of the ruptured follicle into a corpus luteum. In menstrual cycles in which pregnancy does not occur, the corpus luteum must regress in order for a new cycle to be initiated; however, if fertilization and implantation do occur, the lifespan of the corpus luteum must be extended until the placenta becomes the principal source of progesterone needed to sustain the pregnancy. The extant knowledge regarding the physiological and cellular mechanisms responsible for the regression of the corpus luteum at the end of nonfertile menstrual cycles and its rescue during early pregnancy is examined in this article.**

## I. OVERVIEW OF THE LUTEAL PHASE

The primate menstrual cycle consists of a follicular phase during which an ovarian follicle and its enclosed oocyte mature in preparation for ovulation and fertilization. The luteal phase commences upon the ovulation of the graafian follicle and the transformation of the ruptured follicle into a corpus luteum. The major steroid produced by the ovary during the follicular phase is estrogen, which is responsible for the proliferation of the uterine endometrium. The major steroid produced during the luteal phase is progesterone, which is responsible for the transformation of the proliferative endometrium into the secretory endometrium, a change that is absolutely required for the implantation of the fertilized oocyte and the successful initiation and maintenance of pregnancy.

In menstrual cycles in which pregnancy does not occur, the luteal phase lasts 14–16 days, following which the production of progesterone by the corpus luteum ceases and menstruation occurs. The regression of the corpus luteum at the termination of nonfertile menstrual cycles is essential for the initiation of a new cycle because the secretory products of the corpus luteum (estrogen, progesterone, and inhibin) act to suppress follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion, which, in turn, inhibits the development of a preovulatory follicle. By contrast, in menstrual cycles in which successful fertilization and implantation occur, the lifespan of the corpus luteum must be prolonged until the placenta assumes the major burden of producing progesterone and sustaining pregnancy. For successful reproduction and preservation of the species, the primate corpus luteum must have the ability to determine when to die and when to live beyond its typical lifespan.

Fig. 1 illustrates the patterns of LH secretion, luteal cell steroidogenic capacity, and serum progesterone levels during a typical luteal phase. Fig. 1A shows that LH secretion by the pituitary gland is pulsatile in nature, owing to the intermittent secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus. During the early luteal phase of the menstrual cycle, LH pulses occur at a frequency of approximately one pulse every hour. However, as the luteal phase continues, the frequency of LH pulses diminishes to one pulse every 4 to 8 h during the mid to late luteal phase. This decline in LH pulse frequency is the result of a direct action of progesterone on the hypothalamus to reduce the frequency of GnRH secretory episodes.

Fig. 1B summarizes the results of *in vitro* studies designed to investigate the secretory capacity of the corpus luteum. The secretion of progesterone on a per cell basis appears to be highest during the early luteal phase and progressively declines as the corpus luteum ages. Likewise, the expression of mRNAs for the major enzymes involved in progesterone production also appears to be highest in the newly formed corpus luteum and declines thereafter. As illustrated in

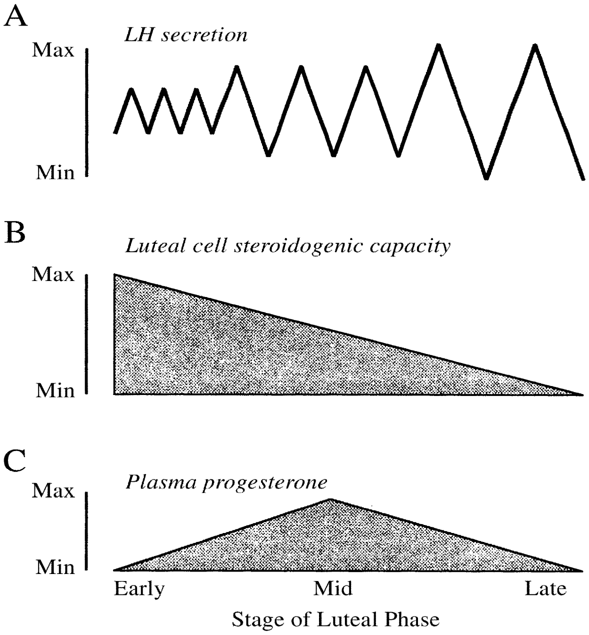

**FIGURE 1** Pattern of LH secretion (A), luteal cell steroid production (B), and serum progesterone levels (C) throughout the luteal phase of the nonfertile menstrual cycle. Details are presented in the text.

Fig. 1C, the secretory activity of the corpus luteum, as reflected by serum progesterone levels, is low during the early luteal phase, rises and peaks during the midluteal phase, and declines thereafter. The discrepancy between the absolute steroidogenic capacity and serum progesterone levels during the early luteal phase is likely due to the fact that the vascular supply to luteal cells in the early luteal phase is not completely formed, hence the delivery of cholesterol via low-density lipoproteins to the luteal cells is limiting.

## II. THE ROLE OF LH IN LUTEAL FUNCTION

During the final stages of follicular development, the granulosa cells of the maturing follicle acquire LH receptors on their cell surface and the expression of the LH receptor is carried over to luteal cells following ovulation. The presence of circulating LH is obligatory for the functioning of the corpus luteum. As depicted in Fig. 2, when macaque monkeys are treated with a GnRH antagonist (which blocks pituitary LH secretion), there is a rapid and sustained fall in serum progesterone production by the corpus luteum and premature menstruation ensues. These results as well as those of many other similar studies have convincingly demonstrated that LH is the major luteotropin of the primate menstrual cycle.

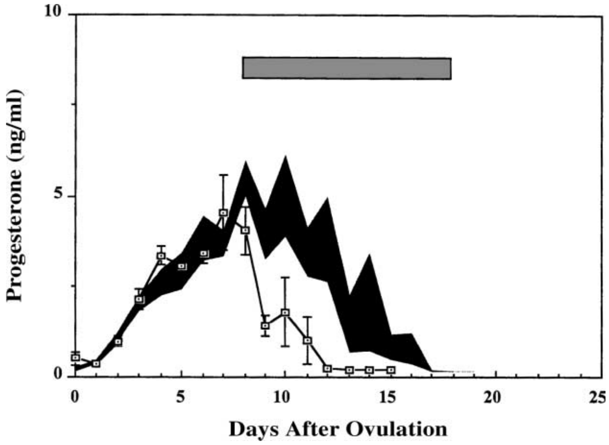

**FIGURE 2** Effect of a GnRH antagonist on progesterone production by the corpus luteum. Macaque monkeys were treated with a GnRH antagonist during the midluteal phase of the menstrual cycle as shown by the shaded rectangle at the top of the graph. Serum progesterone concentrations declined to undetectable levels over a period of 3 days following the beginning of the antagonist treatment, and premature menstruation occurred.

## III. LH SECRETION AND THE REGRESSION OF THE CORPUS LUTEUM

Because LH is absolutely required for the production of progesterone by the corpus luteum, it would be reasonable to think that the regression of the corpus luteum could be caused by the reduction in LH pulse frequency that occurs during the mid through late luteal phase of the menstrual cycle, as depicted in Fig. 1. However, this is not the case. Women who lack the ability to produce GnRH and monkeys whose endogenous GnRH secretion is terminated by lesions in the hypothalamus fail to undergo menstrual cycles due to the absence of FSH and LH secretion. However, menstrual cycles can be restored by the administration of exogenous GnRH at a frequency of one pulse per hour. In these subjects, the frequency of LH pulses during the luteal phase is maintained at one pulse per hour (i.e., there is no decline in LH pulse frequency during the late luteal phase), yet corpus luteum regression occurs at the expected time without any decline in LH pulse frequency. Similarly, a premature reduction in LH pulse frequency to one pulse every 8 h during the early luteal phase does not result in premature luteal regression.

## IV. LUTEAL RESPONSIVENESS TO LH

Given the aforementioned finding that luteal regression during nonfertile menstrual cycles occurs in the presence of an apparently unchanging LH stimulus, the next likely explanation for luteal regression is that as the corpus luteum ages, it becomes increasingly unresponsive to the ambient circulating levels of LH. This possibility was tested by infusing LH into macaque monkeys whose endogenous LH secretion was blocked with a GnRH antagonist. As shown in Fig. 2, treatment with a GnRH antagonist results in premature luteal regression. As shown in Fig. 3, when exogenous LH was infused into GnRH antagonist-treated animals, the luteolytic effects of the GnRH antagonist were overcome. However, the functional lifespan of the corpus luteum was not prolonged beyond its normal length, despite the fact that circulating LH levels in these LH-infused animals were approximately 10 times greater than those normally encountered during the spontaneous luteal phase. The finding that the lifespan of the corpus luteum is not prolonged in the presence of markedly elevated LH levels provides strong evidence that the regression of the corpus luteum of nonfertile menstrual cycles is due to an age-related reduction in LH responsiveness of the aging

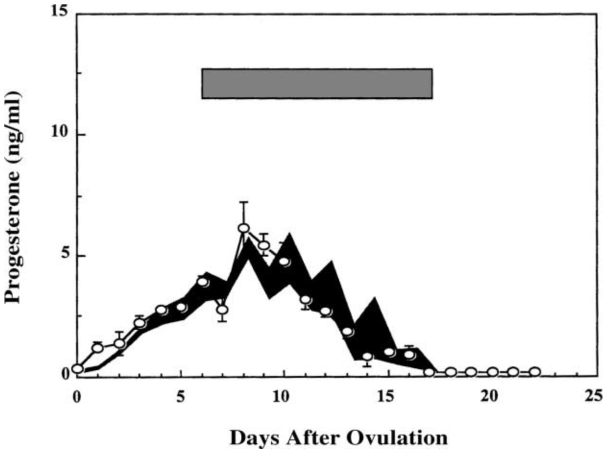

FIGURE 3 Effect of a continuous infusion of luteinizing hormone on the corpus luteum. Macaque monkeys were treated with a GnRH antagonist exactly as shown in Fig. 2. In addition, they received a continuous infusion of LH on the days indicated by the shaded rectangle at the top of the graph. The LH treatment overrode the luteolytic effect of the GnRH antagonist but did not prolong the functional lifespan of the corpus luteum.

corpus luteum such that it can no longer respond adequately to the ambient concentrations of LH in the blood.

## V. RESCUE OF THE CORPUS LUTEUM DURING EARLY PREGNANCY

In menstrual cycles during which successful fertilization and implantation occur, the lifespan of the corpus luteum and its progesterone-secreting ability must be prolonged until the placenta becomes the principal source of progesterone. In humans, this amounts to a period of approximately 4 weeks beyond the normal lifespan of the corpus luteum. The lifespan of the corpus luteum is prolonged by the production of human chorionic gonadotropin (hCG) by the trophoblast cells of the embryo. hCG is a glycoprotein hormone that shares extensive structural homology with LH. Functionally, both hCG and LH bind to the same cell surface receptor and both stimulate cyclic AMP (cAMP) and progesterone production by luteal cells. The principal difference between LH and hCG is that during early pregnancy, circulating hCG concentrations are at least 100 times greater than those of LH. These high hCG concentrations are able to overcome the diminished responsiveness of the corpus luteum to LH and thereby extend the functional lifespan of the corpus luteum.

## VI. CELLULAR MECHANISMS INVOLVED IN THE REGRESSION AND RESCUE OF THE PRIMATE CORPUS LUTEUM

The fact that the corpus luteum of the menstrual cycle has a highly reproducible 14- to 16-day lifespan among both humans and subhuman primates suggests that the timely regression of this tissue is internally programmed. Furthermore, programmed cell death (apoptosis) is well documented in the ovary as atresia of follicles manifests all the hallmarks of apoptosis. Despite the attractiveness of apoptosis as a cellular mechanism for luteal regression in primates, the extant data do not indicate widespread apoptosis, as reflected by histological evidence of DNA fragmentation of luteal cells during the process of luteal regression. Although some cells within the corpus luteum exhibit DNA fragmentation, they constitute only a small minority of the regressing tissue. Furthermore, although the anti-apoptotic protein BCL-2 is expressed in the corpus luteum, its level of expression does not seem to change markedly either during the regression of the corpus luteum or during its rescue by hCG.

An interesting possibility is that the microvascular vascular support of the corpus luteum may decline as the corpus luteum ages, which could contribute to the decline in function and subsequent death of the luteal parenchymal cells by depriving them of blood-borne nutrients and oxygen. Evidence continues to accumulate that vascular endothelial growth factor (VEGF) plays an important role in both the development of blood vessels that occurs during the formation of the corpus luteum and the stabilization of the luteal microvasculature thereafter. Moreover, VEGF production appears to decline during the late luteal phase, and treatment of women with hCG increases both VEGF and the endothelial cell mass of the corpus luteum. Thus, VEGF and its effects on the luteal vasculature could be involved in both luteal regression and luteal rescue.

In addition to changes in the luteal vasculature, it appears that during the process of luteinization (formation of the corpus luteum), luteal cells undergo terminal differentiation, which predestines them to regress. In addition, the process of luteinization is associated with loss of the cAMP-dependent nuclear transcription factor CREB (cAMP response element-binding protein), which controls the expression of some cAMP-regulated genes. Because the corpus luteum is absolutely dependent on LH (and its second messenger cAMP), the loss of CREB expression could remove a subset of luteal cell genes that participate in luteal cell proliferation, survival, and steroid production. The steroidogenic cells of the corpus luteum cease to proliferate and this is associated with the decline in the expression of proliferating cell nuclear antigen (PCNA). Rescue of the corpus luteum by hCG is not associated with either the resumption of steroidogenic cell proliferation or the expression of PCNA. Likewise, hCG does not appear to dramatically increase mRNA levels for the enzymes involved in progesterone production. Rather, it seems that the rescue of the corpus luteum during early pregnancy is the result of the extremely high concentrations of hCG in blood that serve to maximally stimulate progesterone production by the aging corpus luteum until the placenta assumes the principal burden of progesterone production for the remainder of pregnancy.

## Glossary

**human chorionic gonadotropin** A glycoprotein hormone similar to LH that is produced by the trophoblast cells of the early embryo and placenta.

**luteinizing hormone** A glycoprotein hormone produced by the anterior pituitary gland.

**luteolysis** The cessation of progesterone production at the end of a nonfertile menstrual cycle.

**luteotropin** Any substance that stimulates the function of the corpus luteum.

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Folliculogenesis • Gonadotropin-Releasing Hormone (GnRH) • Gonadotropin-Releasing Hormone and Puberty • Luteinizing Hormone (LH) • Ovulation • Progesterone Action in the Female Reproductive Tract • Vascular Endothelial Growth Factor**

## Further Reading

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# Corpus Luteum in Primates

Richard L. Stouffer
*Oregon National Primate Research Center, Oregon Health & Science University*

I. INTRODUCTION
II. DEVELOPMENT OF THE CORPUS LUTEUM
III. FUNCTION OF THE CORPUS LUTEUM
IV. REGRESSION OF THE CORPUS LUTEUM
V. RESCUE OF THE CORPUS LUTEUM
VI. SUMMARY AND CLINICAL RELEVANCE

**The corpus luteum is the endocrine tissue in the ovary of mammals that differentiates from the mature follicle after ovulation has occurred. The corpus luteum plays two key roles during the reproductive life of primates, including women. First, with its limited functional tenure during the ovarian/menstrual cycle, reproductive potential and cyclicity are maintained. Second, with its timely rescue during the fertile cycle, early pregnancy is sustained until sufficient placental function is acquired.**

## I. INTRODUCTION

A key feature of ovarian function in mammalian species is the development of the corpus luteum from the ovulatory follicle and its subsequent role in producing hormonal factors, notably progesterone, that are essential for the initiation and maintenance of pregnancy. Considering the importance of this process, the species differences in the structure, function, lifespan, and regulation of the corpus luteum are remarkable. Important differences between the corpus luteum of typical laboratory animals, such as rats, mice, and rabbits, and that of domestic animals limit our understanding of the control of the primate corpus luteum but certainly provide a strong rationale for further studies.

Primates are among the species that have ovarian cycles with "long luteal phases," i.e., the corpus luteum differentiates after ovulation and functions for a sufficient period of time to permit movement of the early embryo through the oviduct, preparation of the

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# Corticotropin-Releasing Hormone (CRH)

David T. Breault and Joseph A. Majzoub
*Harvard Medical School*

I. INTRODUCTION
II. ANATOMIC DISTRIBUTION
III. GENE AND PROTEIN STRUCTURE
IV. REGULATION OF EXPRESSION
V. REGULATION OF CRH SECRETION
VI. RECEPTORS
VII. SIGNAL TRANSDUCTION
VIII. CRH-BINDING PROTEIN
IX. ROLE IN PHYSIOLOGY
X. PATHOPHYSIOLOGY
XI. CRH-RELATED PEPTIDES

**Corticotropin-releasing hormone (CRH) is the major regulator of the hypothalamic-pituitary-adrenal axis and plays a role in the normal development of the adrenal, brain, and lung. In addition, CRH, and the family of CRH-related peptides, may have regulatory roles in a number of other systems, including the gastrointestinal system, the immune system, the autonomic nervous system, the reproductive system, and behavior.**

## I. INTRODUCTION

CRH derives its name from its role in the anterior pituitary, where it mediates the release of corticotropin (ACTH) leading to the release of adrenocortical steroids. In addition, CRH controls the secretion of β-endorphin and other proopiomelanocortin (POMC)-derived peptides from the anterior pituitary. CRH is the major hypothalamic activator of the hypothalamic–pituitary–adrenal (HPA) axis. In addition, CRH is an important mediator of the stress response, which integrates endocrine, autonomic, immunologic, and behavioral reflexes. Beyond its role as an endocrine hormone, increasing evidence suggests a role for CRH in the regulation of gastrointestinal and reproductive function.

Recently, a family of CRH-related peptides (CRH-RPs) has been identified. These factors likely play a role in integrating multiple aspects of the stress response. Both CRH and CRH-related peptides interact with two different transmembrane, G-protein-coupled cell surface receptors, CRH-R1 and CRH-R2, which differ in their patterns of tissue distribution. In addition, the binding affinities for CRH and the various CRH-RPs to the two receptors differ considerably and likely contribute to the different actions of these peptides.

## II. ANATOMIC DISTRIBUTION

CRH has been localized to the nerve cell bodies in and near the dorsomedial parvicellular division of the paraventricular nucleus (PVN) of the hypothalamus. These neurons send a rich fiber plexus to the median eminence and other hypothalamic and midbrain targets. These neurons are largely controlled by serotoninergic input from the amygdala and hippocampus of the limbic system and brainstem regions involved in autonomic functions. CRH expression has also been localized in many other parts of the central nervous system including the cerebral cortex, limbic system, cerebellum, brainstem, and spinal cord. It is also present in the adrenal medulla and sympathetic ganglia of the autonomic nervous system (Fig. 1).

## III. GENE AND PROTEIN STRUCTURE

The CRH gene is composed of two exons and an 800 bp intron and is highly conserved among species. The entire protein-coding region is contained in the second exon. Human CRH is processed from a prepro-CRH molecule 196 amino acids in length, which contains a hydrophobic signal sequence, required for secretion, at its amino-terminal end. The middle of the molecule contains 124 amino acids of unknown function. The carboxyl-terminal end of prepro-CRH contains the 41-amino-acid sequence of the mature peptide hormone, separated from the amino-terminus by a protease-sensitive basic residue. Amidation of the carboxyl-terminus of CRH is required for biological activity. Human CRH, rat CRH, and mouse CRH have amino acid sequence identity, and ovine CRH differs by only 7 amino acid residues.

The promoter region of the CRH gene contains several putative *cis*-regulatory elements including a cyclic AMP (cAMP)-response element (CRE), activator protein-1 sequences, and glucocorticoid-response elements. The intron contains a restrictive element-1 sequence that binds restrictive element silencing transcription factor and that functions to restrict CRH expression to neuronal cells (Fig. 2).

## IV. REGULATION OF EXPRESSION

The stimulation and inhibition of CRH expression by cAMP-dependent protein kinase A and glucocorticoids, respectively, are mediated by changes in CRH gene transcription. Stimulation occurs following the cAMP-induced phosphorylation of cAMP regulatory element-binding (CREB) protein, which binds to the CRE located in the CRH promoter and recruits the transcriptional activator, CREB-binding protein to the transcriptional apparatus. Experimental evidence suggests that glucocorticoid-mediated repression of CRH transcription results from a direct interaction between the activated cAMP-dependent transcription factor CREB and the activated glucocorticoid receptor. Transcription factor-mediated changes in chromatin structure, likely working through tissue-specific regulatory elements in the CRH gene, contribute to tissue-specific regulation. The brain serves as an example of tissue-specific glucocorticoid responsiveness. Glucocorticoids have a negative

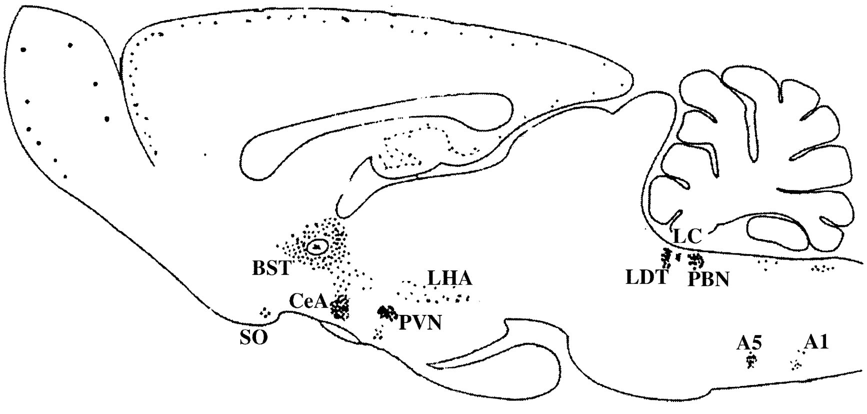


**FIGURE 1** Distribution of CRH in brain. Immunoreactive CRH is identified in rat brain. A1, A1 noradrenaline cells; A5, A5 noradrenaline cells; BST, bed nucleus of the stria terminalis; CeA, central amygdaloid nucleus; LDT, lateral-dorsal tegmental nucleus; LHA, lateral hypothalamic area; PBN, parabrachial nucleus; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus of the hypothalamus. Reprinted from *Peptides* **22**, Smagin, G. N., Heinrichs, S. C., Dunn, A. J. The role of CRH in behavioral responses to stress, pp. 713–724. Copyright (2001), with permission from Elsevier Science.

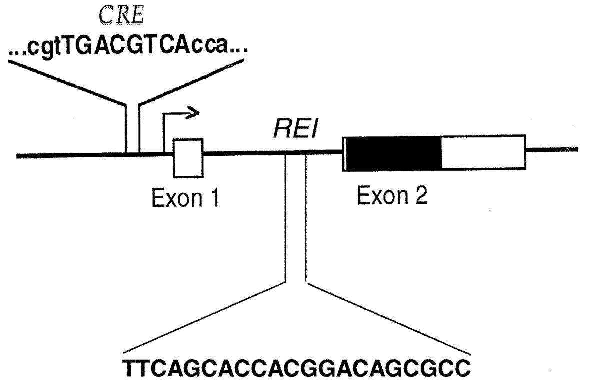


FIGURE 2 Structure of the CRH gene. Depicted are the two exons (rectangles) with the single intervening intron. The intron contains a restrictive element-1 (RE1) sequence at its 3′-end. The promoter contains a canonical cAMP-response element (CRE). The bent arrow depicts the transcription initiation site. The black area within exon 2 denotes the portion of the gene that encodes prepro-CRH.

regulatory effect on CRH gene transcription in the PVN where negative feedback regulation on the HPA axis occurs. However, in the amygdala, a brain region involved in the behavioral stress response, glucocorticoids increase CRH expression.

## V. REGULATION OF CRH SECRETION

A number of neurotransmitters have been implicated in both the positive and the negative regulation of CRH release. Acetylcholine, norephinephrine, histamine, and serotonin increase hypothalamic CRH release, and γ-aminobutyric acid inhibits it. Additional factors that have been implicated in the regulation of CRH release include angiotensin, vasopressin, neuropeptide Y, substance P, atrial natriuretic peptide, activin, melanin-concentrating hormone, β-endorphin, and possibly CRH itself. In addition, cytokines such as interleukin-1β (IL-1β), tumor necrosis factor, eicosanoids, and platelet-activating factor have all been shown to activate the HPA axis by increasing hypothalamic CRH expression (see below).

## VI. RECEPTORS

CRH binds to sites in the anterior lobe of the human pituitary, which correlate with the distribution of corticotrophs. CRH receptors in the anterior pituitary gland are low-capacity, high-affinity receptors, with a $K_d$ for CRH binding of approximately 1 nM. To date, two CRH receptor genes have been identified in humans and other mammals, with a third gene described in the catfish. Both receptors consist of seven-transmembrane regions and are coupled to adenylate cyclase via $G_s$. Both receptors have at least one additional isoform resulting from an alternate splicing event.

The tissue distribution of the type 1 and type 2 receptors varies considerably. CRH-R1 is expressed throughout the brain, being concentrated in anterior pituitary corticotrophs. The distribution of the CRH-R2 splice variants, in contrast, is quite distinct. CRH-R2 α is expressed exclusively in the brain, being more widely distributed than CRH-R1. CRH-R2 β is expressed primarily in the periphery, with the highest levels found in heart and skeletal muscle. The distinct localization of these receptor types suggests they may have functionally different roles.

CRH-R1 binds and is activated by both CRH and urocortin (see below). This receptor mediates the actions of CRH at the corticotroph as well as some aspects of the behavioral stress response, including fear and anxiety. CRH-R2 binds urocortin with over 20-fold higher affinity, compared with CRH (see below). Experimental evidence suggests that this receptor may be involved in blood pressure control, consistent with its anatomic localization.

Mice deficient in CRH-R1 and CRH-R2 have provided important clues to help define their functional roles, with CRH-R1 mediating and CRH-R2 attenuating behavioral stress responses. Studies employing specific agonists and antagonists further suggest that CRH-R2 likely mediates pro-anxiety as well as anxiolytic behaviors in a brain region-specific manner. CRH-R2 has been shown to reduce feeding behavior and decrease gastrointestinal motility.

## VII. SIGNAL TRANSDUCTION

CRH, acting via the type 1 CRH receptor, stimulates adenylate cyclase activity, increasing cAMP levels in anterior pituitary corticotrophs. CRH stimulates ACTH release via the cAMP-protein kinase A pathway, which is responsible for both the increase in POMC transcription and peptide synthesis and the rise in intracellular calcium resulting in ACTH secretion. Forskolin, a direct stimulator of adenylate cyclase activity, and 8-bromo-cAMP, a cAMP analogue, both markedly stimulate ACTH release and increase CRH-stimulated ACTH release. CRH mediates its stimulation of POMC transcription via the POMC CRH-responsive element.

## VIII. CRH-BINDING PROTEIN

The CRH-binding protein (CRH-BP) was first inferred from studies in pregnant humans in whom a high level of immunoreactive CRH was detected in peripheral blood. Levels as high as 10 ng/ml have been documented during pregnancy, which is 10 to 100 times higher than CRH levels in the hypophyseal portal system. The presence of CRH-BP is thought to block the effects of CRH in the pregnant mother, thus preventing or attenuating the activation of her HPA axis. The role of CRH-BP is not fully understood, though its intracellular distribution in corticotrophs and its association with neurons both synthesizing CRH and regulating its release suggest that it may serve to modulate the effects of CRH. In addition to humans, it is expressed in great apes, who also have high levels of CRH during pregnancy, and in the rat. In humans, it is synthesized in liver, placenta, and brain, but in rat, its expression is restricted to the brain.

## IX. ROLE IN PHYSIOLOGY

The HPA axis has a role in mediating the actions of many physiologic systems. Major actions include the following: (1) influencing the fetal development of major organ systems, including lung, liver, and gut; (2) metabolic functions, including the maintenance of normal blood glucose levels during the fasting state via glycogenolysis and gluconeogenesis; (3) modulation of immune function; and (4) maintenance of cardiovascular tone. In response to all forms of stressful stimuli, the HPA axis is called upon to exert the "fight or flight" response, which is mediated through each of the above actions. For example, hypoglycemia stimulates a counterregulatory response involving the HPA axis, which results in gluconeogenesis and lipolysis. Additional examples include hypovolemia and hemorrhage, both of which result in direct stimulation of the HPA axis. In addition, CRH, acting both directly and via the HPA axis, has a role in regulating a number of neuroendocrine functions including (1) behavioral activity; (2) food intake; (3) reproduction; (4) growth regulation; and (5) autonomic functions (Fig. 3).

### A. Endocrine System

CRH is the key factor in the HPA axis leading to the release of ACTH, which acts on the adrenal cortex to release glucocorticoids and other steroid hormones, including androgens and to a lesser extent aldosterone. CRH can also directly stimulate glucocorticoid release from the adrenal gland. Vasopressin, co-synthesized along with CRH in hypothalamic PVN neurons, acts in concert with CRH to stimulate ACTH secretion.

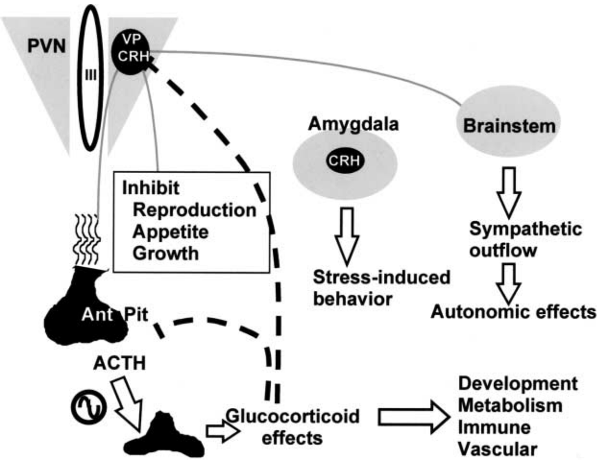


**FIGURE 3** Actions of CRH and the HPA axis. CRH in the PVN, acting as a hormone, is released at the median eminence, and transported by the hypophyseal portal system to the anterior pituitary, where, in conjunction with vasopressin (VP), it stimulates the synthesis and secretion of ACTH in a circadian manner. ACTH enters the systemic circulation, where it acts in the adrenal to stimulate steroidogenic enzymes to synthesize cortisol. Cortisol has multiple actions, including those in development, metabolism, immune function, and vascular regulation. Hypothalamic CRH may also have direct actions in inhibiting reproduction, appetite, and growth. CRH (or other CRH-RPs) in the amygdala and brainstem may participate in the stress response by stimulating stress-related behaviors and activation of the autonomic nervous system, respectively.

As with other endocrine systems, this axis is organized and regulated through a series of negative feedback loops. The negative regulation of glucocorticoids on CRH and ACTH release represents long and short feedback loops, respectively. The ability of ACTH from the pituitary to inhibit CRH release is another example of a short feedback loop.

The circadian rhythm is generated in the suprachiasmatic nucleus. Signals are sent via efferent inputs to the PVN, which modulates CRH release. In response, ACTH is secreted in a pulsatile fashion, which gives rise to a corresponding rise in glucocorticoid levels. In humans, peak hormonal levels are seen in the early morning hours. In rodents and other nocturnal animals, the peak occurs in the evening. CRH is required for the presence of this rhythm, as CRH-deficient mice have a very low or absent rhythm in cortisol secretion. However, the rhythm is restored by infusion of constant amounts of CRH into these animals, indicating that variation in CRH is not required for rhythm generation.

The precise role for the circadian rhythm has not been fully established. Some human data suggest that the rhythm in some way modulates the early morning rise in plasma glucose. It has also been postulated that the daily rise in CRH and ACTH helps prevent adrenocortical apoptotic cell death, which would result without the tropic input of ACTH. It is possible that this daily rise is sufficient to allow the adrenal cortex to maintain an adequate functional reserve, which may be required to respond to a stressful stimulus, while not allowing the excessive ACTH stimulation that would result in Cushing's syndrome (see below).

### B. Gastrointestinal System

The stimulation of colonic motility is a common response to a variety of stressors. This gastrointestinal response to stress, as measured by defecation in rodent studies, can be mimicked by CRH infusion into either the brain or the periphery and is blocked by CRH-R1 antagonists (see below). Its administration induces bowel emptying by increasing colonic motility and it inhibits gastric acid secretion and gastric emptying. Indirectly, as part of the neuroendocrine stress response, CRH also inhibits feeding behavior even in food-deprived experimental animals. It is possible that one of the CRH-RPs, such as urocortin or urocortin III, rather than CRH, is the endogenous peptide that mediates these gastrointestinal actions.

### C. Immune System

There is increasing evidence that the neuroendocrine system and the immune system work together in the regulation of both the stress response and the immune response. CRH is thought to have both direct and indirect effects on the immune system. Indirectly, CRH may down-regulate inflammation through the release of glucocorticoid, whereas a direct, pro-inflammatory action of CRH may occur in peripheral tissues. These direct actions are not fully understood but may include mast cell degranulation, nitric oxide-dependent vasodilation, enhanced vascular permeability, leukocyte proliferation, and cytokine release by activated leukocytes. Further support for a direct effect comes from experimental animal systems in which subcutaneous inflammation gives rise to immunoreactive CRH within leukocytes, monocytes, macrophages, fibroblasts, endothelial cells, epidermal cells, and synovial membrane-lining cells. These studies have been extended to show that CRH antiserum decreases the inflammatory response.

The integration of the neuroendocrine and immune responses to stress relies on the balancing effects of multiple factors. Several such factors, including CRH and IL-1, have been shown to manifest both stimulatory and inhibitory potential. IL-1, produced by stimulated macrophages and monocytes, serves as a potent pro-inflammatory compound. In contrast, IL-1 has also been shown to act centrally, by directly stimulating the HPA axis, to increase the secretion of CRH and ACTH, leading to increased levels of anti-inflammatory glucocorticoid. These dual actions of IL-1 and CRH to mediate both pro- and anti-inflammatory responses may illustrate a fundamental regulatory mechanism integrating the immune system with the HPA axis.

### D. Autonomic Nervous System

As part of the fight or flight response to stress, CRH has direct effects on the autonomic nervous system. Experimental evidence suggests that CRH activates, via the locus ceruleus within the brainstem, the sympathetic nervous system. Central administration of CRH results in rapid increases in the plasma concentration of norepinephrine and epinephrine. These increases are followed by an increase of plasma glucose and glucagon and are associated with an increase in heart rate, respiratory activity, and oxygen consumption. In addition, CRH, via stimulation of ACTH and cortisol production, results in the conversion of norepinephrine to epinephrine in the adrenal medulla. This is likely mediated by the stimulation within the medulla of phenylethanolamine *N*-methyltransferase.

### E. Behavioral Effects

As an important integrator of the stress response, CRH and CRH-RPs (see below) have been implicated as mediators of behavioral responses to stress. CRH and urocortin III are present within the amygdala, an area of the brain that mediates behaviors associated with fear and anxiety, such as decreased exploration and appetite. Consistent with this idea, CRH-R1-deficient mice exhibit decreased anxiety-related behaviors. However, CRH-deficient mice have normal behavioral stress responses that are blocked by a CRH-R1-specific antagonist, suggesting that another CRH-RP, acting through the CRH-R1 receptor, is anxiogenic. Linear growth is also decreased in response to chronic stress. Whether this is due to the decrease in food intake observed with stress or to an independent mechanism, possibly involving CRH or a CRH-RP, is not known.

In humans, dysregulation of CRH may be involved in mental illnesses such as major depression, posttraumatic stress disorder, anorexia nervosa, and panic disorder. For this purpose, CRH-R1 receptor antagonists are currently being tested in clinical trials.

## F. Reproductive System

There is considerable evidence that stress has a major negative effect on reproduction. As part of the neuroendocrine response to stress, CRH has been implicated as a major inhibitor of reproductive function in both sexes. Intense or prolonged stress, for example, has been shown to inhibit gonadotropin secretion. Additional central effects include attenuation of sexual behavior. However, CRH-deficient mice have normal stressor-induced inhibition of reproductive function, indicating that CRH is not absolutely required for this function.

Placental CRH production is found only among primates, being derived from the synciotrophoblasts. It is secreted into both the maternal and the fetal circulations (Fig. 4). During the second half of gestation, both humans and chimpanzees show an exponential rise in maternal CRH blood levels. The fetal concentration of CRH is approximately 1/10 that found in the maternal circulation. The role of placental CRH is not clear but it may function in the initiation of parturition and/or in the regulation of fetal development. Unlike hypothalamic CRH, which is inhibited by cortisol (as part of a negative feedback loop), rising levels of cortisol secreted by the fetal adrenal appear to stimulate placental CRH. Placental CRH, via the umbilical vein, enters the fetal circulation and may stimulate fetal ACTH release to cause a further increase in fetal cortisol release, thus completing a positive feedback loop. This may explain in part the marked, 10- to 100-fold rise in maternal CRH levels that occur in the last part of human pregnancy. The rise in fetal cortisol is also required for the proper maturation of lung, liver, bowel, and other tissues. The stimulation of fetal ACTH by placental CRH would also promote the synthesis and secretion of fetal dehydroepiandrostenedione sulfate, which is the obligate precursor for estradiol produced by the placenta. Estradiol is necessary for the production of placental oxytocin and prostaglandins, which are the major effectors of uterine contraction during labor and delivery. The concomitant stimulation of both fetal cortisol and dehydroepiandrosterone would thus couple the glucocorticoid effects on fetal organ maturation with the timing of parturition, of obvious benefit for postnatal survival.

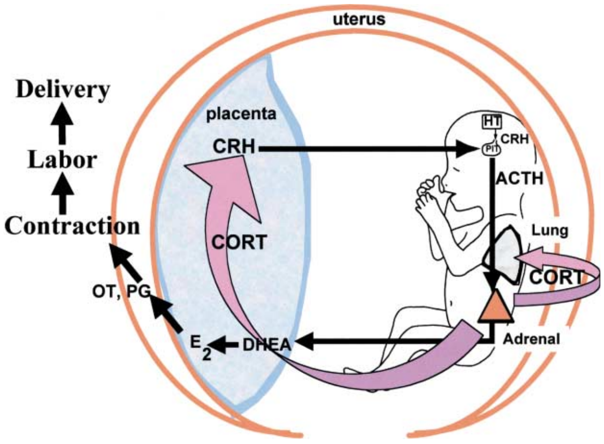


FIGURE 4 CRH and the placental-fetal unit. Placental CRH travels via the umbilical vein to the fetus, where it stimulates the pituitary to release ACTH. ACTH travels to the fetal adrenal, where it stimulates the production of cortisol (CORT) and dehydroepiadrosterone (DHEA) sulfate. Cortisol promotes the maturation of fetal lung and other organs and also feeds back, via the umbilical artery, to the placenta, where it stimulates a further rise in CRH. In the placenta, DHEA is converted to estradiol ($E_2$). Estradiol stimulates oxytocin (OT) and prostaglandin (PG) activity, leading to uterine contraction, labor, and delivery.

## G. Fetal Development

The HPA axis is the first endocrine system to develop. In humans, the axis is active by the eighth week of gestation and is under negative feedback control by glucocorticoids at that time (as evidenced by fetuses with genetic enzymatic blockade of adrenal steroidogenesis). A number of developing organ systems, including the central nervous system, lung, and gut, appear to require the early presence of the HPA axis for normal development.

Hypothalamic CRH may not be responsible for the initial activation of the HPA axis, as corticotroph development occurs normally in the complete absence of CRH. It is intriguing that extrahypothalamic sources of CRH are present at time periods preceding hypothalamic CRH expression. For example, fetal lung in the mouse produces CRH immediately preceding the surge in fetal glucocorticoid levels. This rise in glucocorticoid levels is required for pulmonary differentiation and postnatal survival. It is unclear whether lung CRH plays a significant paracrine or endocrine role in this process.

## X. PATHOPHYSIOLOGY

### A. CRH Excess

Ectopic CRH secretion from a variety of different human tumors results in Cushing's syndrome due to the overproduction of ACTH and glucocorticoid. Clinical characteristics include central obesity, muscle wasting and weakness, facial plethora, glucose intolerance, thinning of skin with purple striae, osteopenia, easy bruising, excessive hair growth, and hypertension. In children, glucocorticoid excess also results in growth failure. CRH overproduction is probably not the cause of the most common form of Cushing's syndrome, termed Cushing's disease, which is due to a primary neoplasm of corticotroph cells in the anterior pituitary.

An experimental model of CRH excess in mice exhibits clinical features similar to those of Cushing' syndrome, including truncal obesity, hair loss, thin skin, and brittle bones, and in addition manifests altered leukoctye counts and increased anxiety-like behaviors.

### B. CRH Deficiency

CRH deficiency, also referred to as secondary adrenal insufficiency, results in adrenal atrophy with impaired glucocorticoid production. A mouse model of complete CRH deficiency results in mice with small adrenal glands and a severely deficient hormonal response to stress. The adrenal insufficiency resulting from long-term glucocorticoid therapy may be due in part to continued suppression of hypothalamic CRH secretion as well as pituitary ACTH.

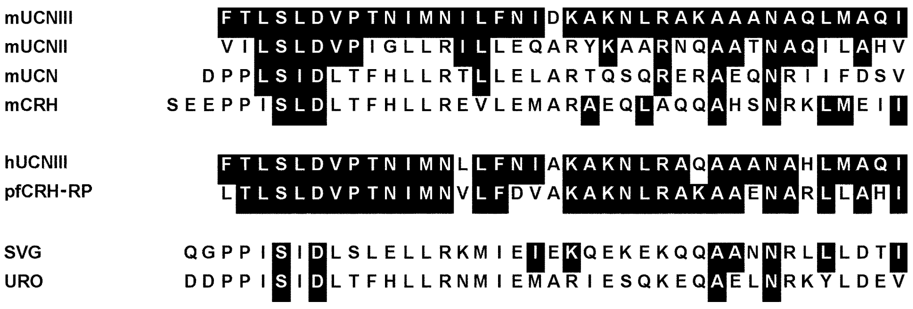
In general, the clinical features associated with glucocorticoid insufficiency are dependent on whether the CRH levels are low or high. When CRH levels are high, as in primary adrenal failure, characteristic findings include anorexia, weakness, excessive fatigue, weight loss, decreased fertility, and hyperpigmentation. Secondary adrenal insufficiency, as with isolated CRH deficiency, typically does not show these findings. This observation is further underscored by the phenotype of CRH-deficient mice; despite low basal glucocorticoid levels and an impaired stress response, these mice have normal longevity, fertility, food consumption, body composition, and activity level.

## XI. CRH-RELATED PEPTIDES

Since its discovery in 1981, CRH has been postulated to mediate both the hormonal and the behavioral responses to stressful stimuli. Several lines of evidence have suggested that CRH is not the sole mediator of the stress response and have led to the identification of CRH-like molecules in rodents and humans (Fig. 5). Before the discovery of CRH, two previously discovered CRH-RPs, urotensin in the fish and sauvagine in the frog, hinted at the possibility of finding other CRH-RPs in mammals.

### A. Urocortin

When CRH was initially identified, it was considered to be homologous to fish urotensin and amphibian sauvagine. The later identification of factors more closely related to CRH in these species prompted the search for the mammalian homologue to urotensin. These efforts led to the discovery of urocortin, a 122-amino-acid propeptide that undergoes a cleavage event to form the mature 40-amino-acid peptide.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mUCNII V I L S L D V P I G L L R I L L E Q A R Y K A A R N Q A A T N A Q I L A H V
mUCN D P P L S I D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V
mCRH S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I I
hUCNIII F T L S L D V P T N I M N L L F N I A K A K N L R A Q A A A N A H L M A Q I
pfCRH-RP L T L S L D V P T N I M N V L F D V A K A K N L R A K A A E N A R L L A H I
SVG Q G P P I S I D L S L E L L R K M I E I E K Q E K E K Q Q A A N N R L L L D T I
URO D D P P I S I D L T F H L L R N M I E M A R I E S Q K E Q A E L N R K Y L D E V

FIGURE 5 CRH and related peptides. Alignment of the peptide sequences of mouse CRH (mCRH), mouse urocortin (mUCN), mouse urocortin II (mUCNII), mouse urocortin III (mUCNIII), human urocortin III (hUCNIII), pufferfish CRH-related peptide (pfCRH-RP), frog sauvagine (SVG), and fish urotensin (URO) is shown. Identity is indicated by a black background. Urocortins II and III are closely related to pufferfish CRH-related peptides, but are not closely related to CRH or urocortin.

Urocortin was first cloned in rat and shares 45% sequence identity with rat CRH. Rat urocortin and human urocortin are 95% identical within the mature peptide region.

Urocortin's primary site of expression is the Edinger-Westphal nucleus, although it is found at scattered sites throughout the brain, including the hypothalamus and pituitary. In addition, it is found in human placenta and fetal membranes. Like CRH, it binds to CRH-BP with high affinity, suggesting that its functions may be modulated by the binding protein. Urocortin binds to all CRH receptors with a higher affinity than does CRH and binds to CRH-R2 β receptor with 40-fold higher affinity. Urocortin serves as a potent ACTH secretagogue (although due to its restricted anatomic distribution it is unlikely to be an important regulator of ACTH release) and appears to mediate some stress-induced behaviors including increased anxiety and possibly anorexia. It may also be involved in sodium and water balance.

The identification of urocortin established that CRH was not the sole mediator of the stress response. It further raised the possibility that urocortin may mediate some of the behaviors previously attributed to CRH. The search for additional CRH-RPs that might be involved in regulating the stress response has led to the identification of two additional factors.

## B. Urocortin II

Based on the above observations, the existence of additional CRH-like molecules was proposed and subsequently identified based on sequence homology to CRH, urocortin, and CRH-like factors identified in lower species. Searching the public DNA database, urocortin II (also termed stresscopin-related peptide) was recently identified based on its similarity to consensus amino acid sequences of known CRH-RPs. It encodes a 38-amino-acid peptide that is a selective CRH-R2 agonist with no affinity for CRH-R1. It is expressed in the hypothalamic magnocellular neurons of the paraventricular and supraoptic nuclei. Based on experimental data, it may be involved in appetite suppression, without a role in more generalized behavioral responses.

A search of the human DNA database (see below) led to the discovery of the human orthologue of urocortin II, stresscopin-related peptide. In addition to the functions described for urocortin II, the expression profile of stresscopin-related peptide was extended to include peripheral tissues such as the heart and gut.

## C. Urocortin III

Using a two-part search strategy of the human DNA database, a third CRH-like peptide was identified and named stresscopin (also termed urocortin III). Human urocortin III encodes a 161-amino-acid prepropeptide with a mature peptide product of 40 amino acids. Receptor studies show selective CRH-R2 binding. Behavioral studies in mice demonstrate that urocortin III, as with urocortin II, results in suppression of appetite, decreased gastric emptying, and decreased heat-induced edema. Consistent with the lack of binding to CRH-R1, urocortin II and III have essentially no role in mediating ACTH release and elevation of glucocorticoid levels. A broad tissue survey demonstrates urocortin III expression in brain, gastrointestinal tract, adrenal, pancreas, thyroid, skeletal muscle, cardiac muscle, and spleen.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Corticotropin-Releasing Hormone Pharmacology • Corticotropin-Releasing Hormone, Stress, and the Immune System • Stress**

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# Corticotropin-Releasing Hormone Pharmacology

Robert F. Quintos, Sarah C. Coste, and
Mary P. Stenzel-Poore
*Oregon Health and Science University, Portland*

I. INTRODUCTION
II. CRH STRUCTURE AND GENE EXPRESSION
III. CRH RECEPTORS 1 AND 2
IV. CRH-BINDING PROTEIN
V. UROCORTIN, UROCORTIN II, AND UROCORTIN III
VI. CONCLUSION

**Corticotropin-releasing hormone (CRH) produced in the hypothalamus is the primary activator of the hypothalamic–pituitary–adrenal axis and the central mediator of the neuroendocrine response to stress. CRH and a family of receptors and other peptides represent a central and peripheral nervous system regulatory network of components that act in concert to respond to stressors. Within this network, each regulatory component has a unique distribution and pharmacology.**

## I. INTRODUCTION

Harris first proposed in 1950 the theory that pituitary action on the adrenal cortex was regulated by the hypothalamus. He had demonstrated that electrical stimulation of the hypothalamus activated the adrenal cortex, and based on this finding hypothesized that on stimulation, the hypothalamus activated the pituitary via factors released into the hypophyseal circulation. Five years later, the presence of these hypothalamic factors was confirmed by demonstrating increased corticotropin secretion by the pituitary *in vitro*. Over the next several years, a number of purified hypothalamic extracts and natural substances, such as vasopressin, norepinephrine, and angiotensin II, were shown to release adrenocorticotropic hormone (ACTH), but none of these mediators demonstrated characteristics that were consistent with being the primary corticotropin-releasing factor. Corticotropin-releasing hormone (CRH) was isolated and sequenced 25 years later.

## II. CRH STRUCTURE AND GENE EXPRESSION

In 1981, Vale and colleagues reported the isolation and amino acid sequence of ovine CRH (Fig. 1). In addition, they demonstrated the potency of CRH stimulation of ACTH release from anterior pituitary cells.

### A. Localization

CRH is widely distributed throughout the brain and in peripheral body organs and tissues (Table 1). CRH-specific immunoreactivity reveals a wide distribution throughout the central nervous system, with high densities of CRH-positive neurons within the parvocellular region of the paraventricular nucleus, lateral hypothalamus, and locus coeruleus. Additionally, dense CRH fiber networks have been detected in the lateral septum, central nucleus of the amygdala, median eminence, stria terminalis, pituitary, and medial vestibular nucleus. This localization is consistent with the physiological role that CRH plays in regulating both the autonomic and the neuroendocrine response to stress. In the periphery, CRH mRNA is expressed in a wide variety of tissues as well, including the lung, heart, gut, kidney, adrenal gland, testis, ovary, pituitary, and spleen.

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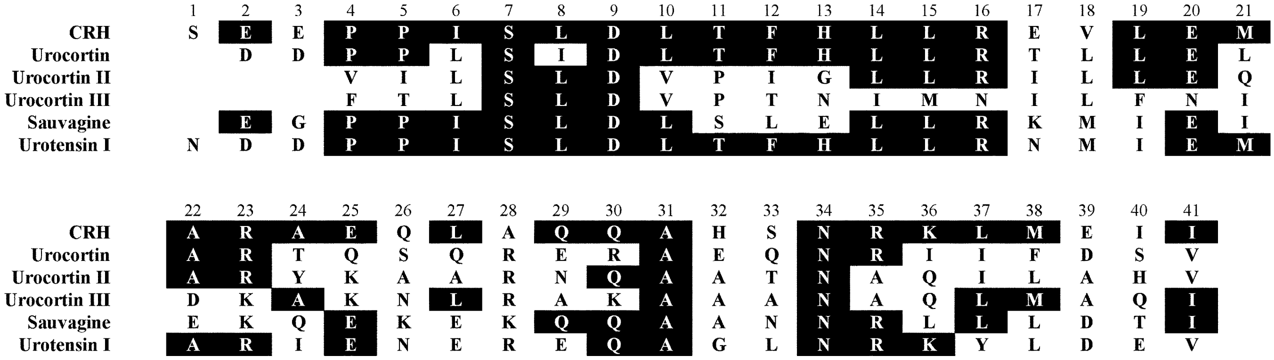

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRH | S | E | E | P | P | I | S | L | D | L | T | F | H | L | L | R | E | V | L | E | M |
| Urocortin | | D | D | P | P | L | S | I | D | L | T | F | H | L | L | R | T | L | L | E | L |
| Urocortin II | | | | V | I | L | S | L | D | V | P | I | G | L | L | R | I | L | L | E | Q |
| Urocortin III | | | | F | T | L | S | L | D | V | P | T | N | I | M | N | I | L | F | N | I |
| Sauvagine | | E | G | P | P | I | S | L | D | L | S | L | E | L | L | R | K | M | I | E | I |
| Urotensin I | N | D | D | P | P | I | S | L | D | L | T | F | H | L | L | R | N | M | I | E | M |

| | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRH | A | R | A | E | Q | L | A | Q | Q | A | H | S | N | R | K | L | M | E | I | I |
| Urocortin | A | R | T | Q | S | Q | R | E | R | A | E | Q | N | R | I | I | F | D | S | V |
| Urocortin II | A | R | Y | K | A | A | R | N | Q | A | A | T | N | A | Q | I | L | A | H | V |
| Urocortin III | D | K | A | K | N | L | R | A | K | A | A | A | N | A | Q | L | M | A | Q | I |
| Sauvagine | E | K | Q | E | K | E | K | Q | Q | A | A | N | N | R | L | L | L | D | T | I |
| Urotensin I | A | R | I | E | N | E | R | E | Q | A | G | L | N | R | K | Y | L | D | E | V |

FIGURE 1 Alignment of amino acid sequences of corticotropin-releasing hormone (CRH)-related peptides. Mammalian (mouse) sequences are shown for CRH and the urocortins. Sequences of amphibian sauvagine and teleost urotensin are shown for comparison. Homologies to CRH are highlighted in black.

## B. Structure and Peptide Processing

The structure and nucleotide sequence of CRH are conserved across species. The CRH gene is located on chromosome 8 (8q13) in the human genome and on chromosome 3 in the mouse and is organized as two exons separated by a single intron. The first exon encodes the majority of the 5′-untranslated region of the mRNA and the second exon contains the entire prohormone sequence.

CRH is produced in an inactive form as a 21-kDa, 196-amino-acid precursor, preproCRH, which undergoes several processing steps to yield the mature active protein (Fig. 2). Within the parvocellular neurons of the hypothalamic paraventricular nucleus, this large precursor undergoes cleavage at

TABLE 1 Tissue Distribution of CRH and Related Peptides

| | Central nervous system | | | |
|---|---|---|---|---|
| Peptide | Cortical | Subcortical | Brain stem | Periphery |
| CRH | Wide distribution, greatest in piriform cortex | Parvocellular region of the paraventricular nucleus, lateral hypothalamus, lateral septum, central nucleus of the amygdala, median eminence, stria terminalis, medial vestibular nucleus, bed nucleus of stria terminalis, ventromedial nucleus | Locus coeruleus, central gray, lateral parabrachial nucleus, lateral reticular nucleus, all layers of cerebellum | Pituitary, adrenals, testis, ovary, gut, heart, lung, and spleen |
| Ucn | External cortical layer, dentate gyrus of hippocampus | Lateral septum, lateral superior olivary nucleus, supraoptic nucleus, and lateral hypothalamus | Edinger–Westphal nucleus, interpeduncular nucleus, sphenoid nucleus, periaqueductal gray, medial vestibular nucleus, autonomic and motor nuclei of the brain stem | Thymus, spleen, stomach, gut, kidney, testis, liver, and heart |
| Ucn II | Meninges | Paraventricular, supraoptic and arcuate nucleus of the hypothalamus, focus coeruleus | Motor nuclei of the brain stem and spinal ventral horn | Heart, adrenals, and peripheral blood |
| Ucn III | Meninges | Median preoptic nucleus, bed nucleus of the stria terminalis, anterior and lateral hypothalamus, anterior periventricular nucleus, medial amygdaloid nucleus | Superior paraolivary nucleus | Skin and small intestine |

the C-terminal dibasic site, Lys$^{153}$-Arg$^{154}$, to yield a 43-residue peptide. Current opinion holds that prohormone convertases (PCs) are responsible for this initial processing step, and a number of PCs have been implicated. Following cleavage, the C-terminal lysine residue is removed by the enzyme carboxypeptidase H, thereby exposing the carboxyl group of a glycine residue. The glycine residue is subsequently converted into an amide by peptidylglycine α-amidating monooxygenase, rendering the mature peptide. The fully processed active CRH(1–41) is then secreted into the portal circulation.

CRH is a distinct 41-residue peptide, but contains regions homologous to other known peptides, e.g., angiotensinogen in mammals and the nonmammalian peptides sauvagine and urotensin I. Moreover, like its nonmammalian counterparts, the secondary structure of CRH consists of a long internal helix spanning 25 amino acids, which then joins a seven-peptide α-helical C-terminal fragment. Though relatively small, the length of this terminal α-helix is essential for maintaining the secondary structure (Fig. 3).

## C. Determinants of Affinity

The entire COOH-terminal region in CRH is required for full potency, as evidenced by a 100-fold decrease in potency following deletion of the COOH-terminal free acid. However, as alluded to previously, the primary determinant of receptor affinity in this region appears to be the α-helical secondary structure rather than the exact chemical identities of the residues. Experimental alterations in various residues in this region have little effect on affinity, as long as the α-helix is preserved. In contrast, modifications in the N-terminal portion beyond the first two or three residues alter the potency of the peptide in ACTH release but maintain its receptor affinity.

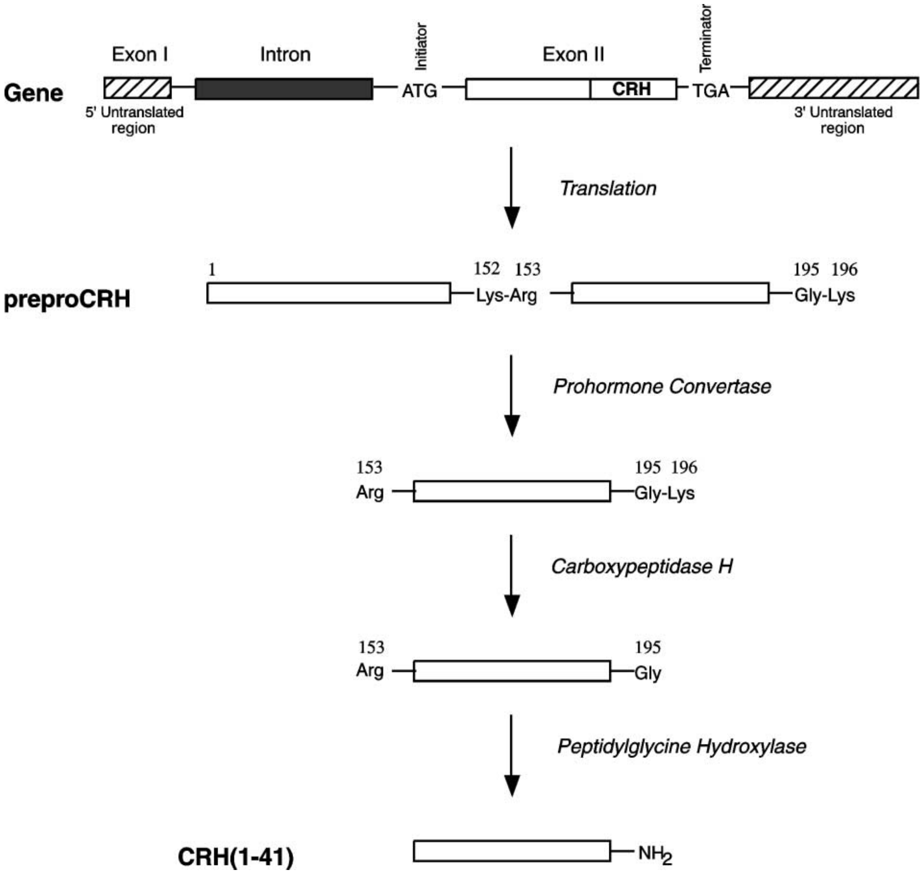
Gene
Exon I
Intron
Initiator
Exon II
Terminator
ATG
CRH
TGA
5' Untranslated region
3' Untranslated region
Translation
preproCRH
1
152 153
Lys-Arg
195 196
Gly-Lys
Prohormone Convertase
153
Arg
195 196
Gly-Lys
Carboxypeptidase H
153
Arg
195
Gly
Peptidylglycine Hydroxylase
CRH(1-41)
NH2

**FIGURE 2** Translation and processing of corticotropin-releasing hormone (CRH). PreproCRH undergoes two major posttranslational processing events to yield a mature, bioactive peptide. First, prohormone convertases cleave the precursor at a dibasic moiety (Lys-Arg) that precedes the beginning of the mature peptide. Second, a C-terminal amino acid (Lys) is cleaved by carboxypeptidase H, and the exposed Gly residue is modified by a peptidylglycine α-amidating monooxygenase, resulting in a C-terminal carboxamide group and the mature peptide.

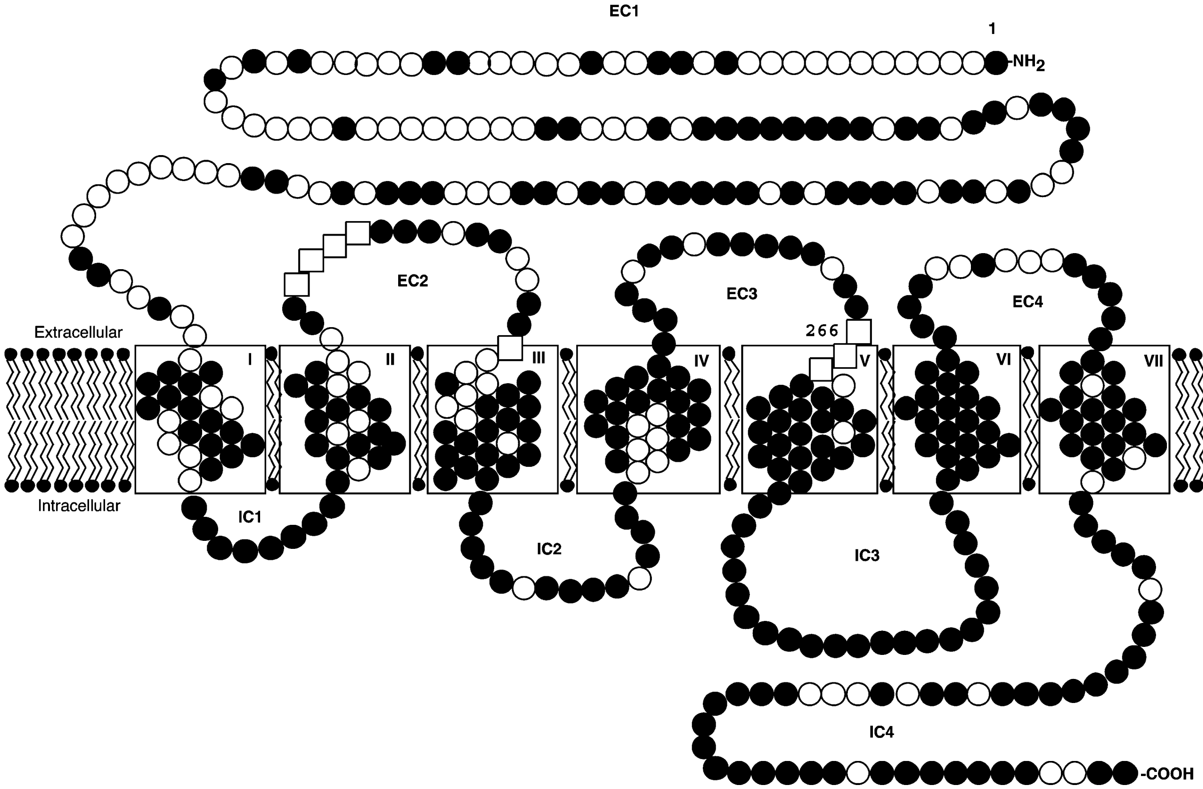

**FIGURE 3** Schematic model of the human CRH-R1 and CRH-R2 receptors. Solid symbols represent conserved amino acids, open symbols represent divergent residues. Amino acids important for high-affinity CRH binding are represented by squares. Residue numbering is based on the sequence of human CRH-R1. EC, Extracellular; IC, intracellular. Transmembrane regions are indicated by Roman numerals.

Specifically, amino acids 4–8 are essential for receptor activation, because their deletion renders the peptide essentially inactive. Additionally, cleavage of these first few residues significantly affects CRH receptor binding. This detrimental effect on CRH affinity is most likely related to disruption of its secondary structure. Modifications that stabilize the α-helical configuration in these nonbinding truncated forms confer binding ability to these peptides and have led to the development of potent CRH antagonists, such as ovine α-helical CRH(10–41) and rat/human α-helical CRH(9–41), both of which are truncated α-helical structures.

## III. CRH RECEPTORS 1 AND 2

### A. Genetics

Currently, two major types of CRH receptors have been cloned and characterized. These receptors, CRH-R1 and CRH-R2, belong to the family of G-protein-coupled seven-transmembrane receptors that are positively coupled to adenylate cyclase. The two receptors, which share approximately 70% homology, are products of two distinct genes. CRH-R1 exists generally as a single functional form (CRH-R1α) and CRH-R2 is expressed as three distinct functional subtypes (CRH-R2α, CRH-R2β, and CRH-R2γ).

### B. Expression

There is little overlap in tissue distribution of the two major CRH receptors (Table 2). In general, CRH-R1 is highly expressed in neocortical, cerebellar, and sensory relay structures, whereas CRH-R2 is mainly limited to subcortical structures. CRH-R1 is also expressed highly in the hippocampus, the hypothalamus, the ACTH-secreting cells in the pituitary, and the brain stem. The CRH-R2 isoforms have distinct patterns of expression, with little overlap among themselves. The greatest distinction is seen between the α- and β-subtypes. CRH-R2α is expressed primarily in the brain whereas CRH-R2β has significant peripheral expression. Within the central nervous system (CNS), both receptors localize to

TABLE 2 CRH Receptors: Pharmacological Profile and Anatomic Regions of Predominant Expression

| Parameter | CRH-R1 | CRH-R2α | CRH-R2β | CRH-R2γ |
|---|---|---|---|---|
| Relative potency | CRH ≥ Ucn >>> Ucn II ≥ Ucn III | Ucn ≈ Ucn II ≈ Ucn III >>> CRH | Ucn ≈ Ucn II ≈ Ucn III >>> CRH | Ucn >> CRH |
| Localization | Central nervous system—pituitary corticotropes, cerebellum, amygdala, hippocampus, brain stem | Central nervous system—hypothalamus, lateral septum, olfactory bulb, cortex | Central nervous system—choroid plexus, cerebral arterioles; periphery—cardiac atria and ventricles (myocardium, epicardium, and arterioles), skeletal muscle, arterioles, lungs, intestine | Central nervous system—lateral septum, hippocampus, amygdala, nucleus accumbens, midbrain, frontal cortex |

subcortical structures; CRH-R2α serves as the neuronal receptor and CRH-R2β is expressed in nonneuronal structures of the choroid plexus and cerebral arterioles. In addition, CRH-R2β has been established as the peripheral CRH receptor, with high levels of expression in the heart and skeletal muscle and lower levels in lung and intestine. CRH-R2γ is unique in that it has been found only in humans thus far. It is expressed only in the CNS, with highest concentrations in the septum and hippocampus.

## C. Structure

CRH-R1, which was the first receptor identified, consists of 415 amino acids and is 98% homologous across species. Characteristic of the G-protein family, CRH-R1 has five putative N-linked glycosylation sites in the N-terminal extracellular domain as well as several potential targets for protein kinase C phosphorylation in the first and second intracellular loops. As previously mentioned, CRH-R1 exists as one functional isoform, although a truncated variant and forms with insertions within the first intracellular loop have been cloned.

In contrast, CRH-R2 exists in three distinct functional isoforms, varying slightly in residue length and differing in their N-termini due to alternative splicing. The α-, β-, and γ-subtypes are 411, 431, and 397 amino acids in length, respectively, each with five predicted N-glycosylation sites, analogous to CRH-R1. The regions of highest identity with CRH-R1 are seen within the fifth and sixth transmembrane domain, the primary site of G-protein coupling (Fig. 3).

## D. Binding Affinity

CRH-R1 and CRH-R2 differ considerably in their pharmacological profiles (Table 2). CRH-R1 demonstrates reversible, high-affinity binding to CRH as well as to urocortin (Ucn), a recently identified CRH-related peptide (discussed later). CRH-R1 exhibits greater affinity for CRH than does CRH-R2. Compared to CRH-R2α, CRH-R1 shows ~13-fold greater affinity for CRH in rats and a >50-fold greater affinity in humans. Comparison to CRH-2β yields a similar range, 4-fold greater affinity in mice and >50-fold in rats and human. Data from humans reveals a 25-fold greater affinity compared with CRH-R2γ.

CRH-R1 as well as all CRH-R2 subtypes bind Ucn with uniform high affinity. Whereas CRH-R1 binds both CRH and Ucn with similar affinity across species, the CRH-R2 receptors consistently favor Ucn. This peptide as well as the recently identified Ucn II and Ucn III are discussed in Section V.

## E. Determinants of Affinity

The N-terminal extracellular domain is the primary determinant of ligand selectivity in CRH-R1. The ligand selectivity of CRH-R1 resides in five N-terminal amino acids, Gly-76, Gly-81, Val-83, His-89, and Leu-90. In particular, at least two of these residues, Gly-76 and Gly-81, are instrumental in the formation of the binding pocket of CRH-R1. Mutation in either one or both of these amino acids abolishes the affinity of the receptor for CRH. In addition, two regions within the second extracellular domain, amino acids 175–178 and His-189, have been found important for CRH binding (Fig. 3).

In contrast, the N-terminal domain plays little if any role in determining affinity in CRH-R2. Instead, the major determinants of ligand selectivity in CRH-R2 have been localized to the border region between the third extracellular domain and the fifth transmembrane domain. Substitution of Val-266, Tyr-267 reduces the affinity for CRH 10-fold without affecting affinity for Ucn.

## IV. CRH-BINDING PROTEIN

### A. Discovery

The existence of CRH-binding protein (CRH-BP) was first postulated shortly after the placenta was identified as a major source of CRH production. The observations were made that the normally negligible circulating levels of CRH dramatically increased during the third trimester of pregnancy to levels comparable to that of the hypophyseal portal circulation. Despite these extraordinary levels of CRH in the systemic circulation, no appreciable increase in ACTH secretion could be demonstrated. These observations have since led to the cloning and sequencing of the CRH-binding protein.

### B. Localization

The tissue distribution of CRH-BP is distinct among species. In humans, it is expressed in the brain, liver, and the placenta, whereas in rats and mice it has been identified only in the brain and pituitary. Additionally, CRH-BP has been demonstrated in human plasma in both males and nonpregnant females. In the brain, CRH-BP is distributed diffusely throughout the cerebral cortex, as well as in a number of subcortical structures. The protein is notably absent in the paraventricular nucleus of the hypothalamus, the acknowledged primary source of CRH in the hypophyseal portal circulation. Meanwhile, CRH-BP shows widespread and preferential expression in pituitary corticotrophs, the major target cell for CRH. The physiologic role of this protein has yet to be elucidated. Given its inhibitory action on both CRH and Ucn and its tissue distribution, CRH-BP most likely functions to maintain homeostasis during the central and peripheral stress response as well as during pregnancy.

### C. Genetics

The gene encoding CRH-BP is located on chromosome 13 in mice and on the long arm of chromosome 5 (locus 5q) in humans. The gene is an 18-kb structure composed of seven exons and six introns. The nucleotide sequences of the protein-encoding exons are highly homologous in the human and rat, with all exon/intron boundaries conserved. In addition, transcriptional elements have been conserved: there is $>85\%$ homology of the first 215 bp of the $5'$ flanking DNA between rat and human genes. However, because there are multiple initiation sites in the rat brain but only one site in the human liver, transcription initiation may be species or tissue specific.

### D. Processing

The initial protein undergoes several posttranslational modifications to yield the active peptide, including glycosylation, phosphorylation, and cleavage. N-Glycosylation occurs at amino acid site 203 and is preserved across species. Experimental evidence suggests that the protein then undergoes autocatalytic cleavage between Ser-234 and Ala-235, though neither the precise site of cleavage in the native peptide nor the functional significance of this modification has been elucidated.

### E. Structure

The active CRH-BP consists of 322 amino acids, including a 24-residue leader sequence and 10 conserved cysteine groups, creating five sequential disulfide bridges. Additionally, there is one putative N-glycosylation site, as mentioned previously. The sequence and structure of CRH-BP is well preserved across species. There is 84% sequence homology between rat and human proteins, with all cysteine residues and the putative N-glycosylation site preserved. The absence of any extended hydrophobic regions supports the conclusion that this peptide is not a transmembrane receptor for CRH.

### F. Binding Affinity

Though it binds more slowly, CRH-BP binds CRH with comparable if not greater affinity, compared to CRH-R1, as determined by dissociation constants ($K_i = 0.17$ vs 3.2 n$M$, respectively). Likewise, CRH-BP binds Ucn with equally high affinity ($K_i = 0.1$ n$M$). However, preferential binding across species first differentiated CRH-BP from the CRH receptor. Recombinant rat and human CRH-BP bind rat and human CRH with equal affinity but bind ovine CRH, which differs by seven amino acids, at a $10^3$-fold lower affinity. CRH-R1 makes no such distinction, suggesting quite different structural requirements for binding.

### G. Determinants of Affinity

CRH residues 9–28 are essential for ligand binding. More specifically, residues 22–25, located within the central domain, are of critical importance. Within this short span, ovine CRH differs from human CRH at residues 22, 23, and 25, accounting for the diminished binding affinity of ovine CRH. Structurally, disruption of the disulfide bridges extinguishes CRH binding, indicating that the bridges are also essential for activity.

## V. UROCORTIN, UROCORTIN II, AND UROCORTIN III

The recent identification of a family of structurally related peptides has uncovered a broad CRH network that includes a specific peripheral regulatory system. Ucn, the first of these peptides identified in 1995, is a 40-amino-acid peptide that shares 45% homology with CRH (Fig. 1).

### A. Localization

Although Ucn has a more limited distribution in the CNS as compared with CRH, expression occurs principally in the CNS, with lower levels of expression in the periphery (Table 1). The dominant regions of Ucn expression are the Edinger–Westphal nucleus and lateral superior olivary nucleus. Ucn immunoreactivity is relatively weak in the paraventricular nucleus, where CRH is highly expressed. Instead, Ucn is expressed in projections that descend from the Edinger–Westphal nucleus and terminate in CRH receptor-rich regions such as the nucleus of the solitary tract (NTS), which in turn innervates autonomic nuclei in the brain stem and spinal cord. Ucn is also present in several autonomic and motor nuclei of the brain stem. This pattern of expression suggests that the primary function of Ucn is related to autonomic control rather than to serve as a mediator of the HPA axis. Ucn expression in the periphery varies across species. Ucn is expressed in a wide variety of both rat and human peripheral tissues; however, in the mouse, it has been demonstrated only in the brain.

### B. Genetics and Structure

The genetic organization and the posttranslational modifications of Ucn are similar to those of CRH. The mouse gene is located on chromosome 5, whereas in humans it resides on chromosome 2p23–p21. Like CRH, the gene is composed of two exons separated by a single intron, with the entire coding region residing on the second exon. In addition, the Ucn and CRH genes share in common several transcription factor binding sites within their promoter regions. The full-length translated prohormone is 122 amino acids, with little sequence similarity to pro-CRH. However, both prohormones are subject to the same posttranslational modifications. Cleavage occurs at a dibasic site preceding the active peptide sequence-in the case of Ucn this is at Arg$^{79}$-Arg$^{80}$. The Ucn C-terminus is also composed of Gly-Lys, residues that are targets of cleavage and subsequent amidation to yield the mature, active peptide. The greatest homology between CRH and Ucn is in the region between amino acids 4 and 16 (Fig. 1). Additionally, in the C-terminal portion, Ala-31 and Asn-34 are strictly conserved between Ucn and all currently identified CRH-related peptides in vertebrates.

### C. Binding Affinity

The importance of the N-terminal region for receptor activation is reiterated in Ucn, because deletion of the first seven amino acids converts the peptide into a potent CRH receptor antagonist. Like CRH, stabilization of the Ucn α-helical conformation is crucial to binding. As discussed previously, Ucn and CRH display similar binding affinity ($K_i$) and receptor activation to CRH-R1 (Table 3). In contrast, binding affinity of Ucn to CRH-R2 is 40-fold greater than that of CRH. Ucn also shows approximately 10-fold greater potency than CRH in inducing intracellular cAMP in CRH-R2-bearing cells. In relation to the CRH-R2 splice variants, Ucn reveals only a slightly higher affinity for CRH-R2β than for the α- and γ-subtypes.

### D. Urocortins II and III

Two additional members of the CRH peptide family—Ucn II and Ucn III-have recently been identified in mice; their respective human prohormone orthologues, stresscopin-related peptide and stresscopin, have also been identified. Both mature peptides consist of 38 amino acids and are more closely related to Ucn (42%) and to each other (40%) than to CRH (Ucn II, 34%; Ucn III, 26%).

TABLE 3 Relative Ligand–Binding Affinity[a]

| Peptide | CRH-R1 | CRH-R2α | CRH-R2β | CRH-R2γ |
|---|---|---|---|---|
| CRH | +++ (0.95–4.5) | +/++ (13–57) | +/++ (17–65) | ++ (25) |
| Ucn | +++ (0.12–0.4) | +++ (0.58–8.9) | +++ (0.4–8.8) | +++ (1.4) |
| Ucn II | − (>100) | +++ (1.7–2.1) | +++ (0.5–0.66) | NT |
| Ucn III | − (>100) | +++ (1.8–13.5) | NT | NT |

[a] Numbers in parentheses reflect the range of $K_i$ (n$M$) across species. NT, Affinities not tested.

However, like Ucn, Ucn II and Ucn III bind CRH-R2 with high affinity and exhibit similar potencies in receptor activation (Table 3). The key feature distinguishing Ucn II and Ucn III from Ucn is their high degree of selectivity for the CRH-R2 receptor. Ucn II and Ucn III show no appreciable binding affinity for CRH-R1 ($K_i > 100\,\text{n}M$) nor do they exhibit significant potency in activating CRH-R1 as measured by intracellular cAMP accumulation, thus supporting their candidacy as endogenous ligands for CRH-R2. Although there remains some overlap, the tissue distribution of these two peptides is quite distinct from that of Ucn (Table 1). In the mouse, distribution of both Ucn II and Ucn III within the CNS is found to be predominantly subcortical.

In the mouse periphery, Ucn II has been identified in the heart and posterior pituitary and Ucn III has been localized to the skin and small intestine. In humans, detailed CNS localization has yet to be accomplished. However, the human orthologue of Ucn II, stresscopin-related peptide, is widely expressed in the periphery, with highest expression levels found in the heart, adrenals, and peripheral blood. Likewise, the Ucn III orthologue, stresscopin, has been detected in most peripheral tissues, with highest concentrations found in the digestive tract, skeletal muscle, thyroid, adrenal, and pancreas. Either one or both of these novel peptides may be the endogenous ligand for the activation of this peripheral CRH receptor system.

## VI. CONCLUSION

The CRH family of peptides and receptors represents a complex, far-reaching regulatory network spanning the CNS and the periphery. Each distinct peptide and receptor subtype has a unique tissue distribution and a selective pharmacology, and all act in concert to provide a system for neurohormonal and autonomic responses to central and peripheral stressors. Although the functional significance of each component interaction as well as the broader physiological and clinical significance of this regulatory system are not completely understood, the recent identification and pharmacological characterization of additional members of the CRH family have expanded our understanding of a broad and encompassing system.

## Glossary

**adrenocorticotropic hormone** Adrenocorticotropic hormone (ACTH) is produced in the pituitary gland and is released following hypothalamic stimulation. ACTH acts directly on the adrenal glands to stimulate glucocorticoid production.

**G-protein-coupled receptor** A family of seven transmembrane-spanning receptors characterized by their activation of intracellular signaling pathways via a GTP-binding protein.

**hypothalamic–pituitary–adrenal axis** Represents the hierarchical organization of the physiologic response to stress by the neuroendocrine system. Activation of the pituitary gland by the hypothalamus leads to subsequent stimulation of the adrenal glands to produce systemic glucocorticoids.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Corticotropin-Releasing Hormone (CRH) • Corticotropin-Releasing Hormone Receptor Signaling • Corticotropin-Releasing Hormone, Stress, and the Immune System • Endocrine Rhythms: Generation, Regulation, and Integration • Glucocorticoid Effects on Physiology and Gene Expression • Stress**

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# Corticotropin-Releasing Hormone Receptor Signaling

Bhawanjit Brar, Marilyn H. Perrin, and Wylie W. Vale

*Salk Institute, La Jolla*

I. INTRODUCTION
II. CRH RECEPTORS
III. ADENYLYL CYCLASE, CAMP, AND PROTEIN KINASES
IV. CALCIUM SIGNALING
V. MITOGEN-ACTIVATED PROTEIN KINASES
VI. CGMP
VII. EXTRACELLULAR FACTORS THAT REGULATE CRH RECEPTOR SIGNALING
VIII. SUMMARY

**The primary response to a stressful event is activation of the hypothalamic–pituitary–adrenal axis. The stress response results in the synthesis and release of corticotropin-releasing hormone (CRH) from the hypothalamus, followed by an increased release of proopiomelanocortin peptides (adrenocorticotropic hormone, melanocyte-stimulating hormones, and endorphins) from the pituitary. Adrenocorticotropic hormone (ACTH) acts on the adrenal gland to cause release of glucocorticoids. The initial isolation of CRH, a 41-amino acid peptide, was based on its function as the major secretagogue of ACTH. Three more mammalian CRH-related ligands, urocortin, urocortin II, and urocortin III, have now been cloned. The importance of the CRH system as a major integrator of behavioral, autonomic, and endocrine responses to stress is underscored by its effects on learning and memory and appetite and weight control and its possible role in affective disorders such as melancholic depression.**

## I. INTRODUCTION

A cascade of responses to the CRH family of peptides is initiated at the cellular level by binding of CRH ligands to integral membrane protein receptors. The complexity of the signaling pathways activated by CRH ligands has become increasingly obvious as an increasing number of CRH receptor (CRH-R) subtypes and CRH-related peptide ligands have been identified and shown to exhibit diverse tissue- and cell-type-specific distributions. The activation of specific CRH-Rs in distinct cell types by receptor-selective CRH peptides may activate selective signaling pathways. Cross talk between signaling pathways may in turn affect cell function and may activate physiological processes that are essential for the maintenance of homeostasis and survival during stress.

Early studies of anterior pituitary cells demonstrated that CRH stimulates cyclic adenosine monophosphate (cAMP) and activates protein kinase A. Subsequent studies of CRH signaling have focused predominantly on CRH in brain, heart, myometrial tissue, skin, circulatory system, and gut cells. CRH-R activation by CRH peptides results in coupling to G-proteins such as $G_{\alpha\sigma}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha z}$. Signaling pathways stimulated by G-proteins include adenylyl cyclase (AC)/cAMP, protein kinases A, B, and C (PKA, PKB, PKC), intracellular calcium ($[Ca^{2+}]_i$), cyclic guanosine monophosphate (cGMP), and mitogen-activated protein kinase (MAPK) pathways. The cAMP/PKA pathway has been extensively studied with respect to CRH-R activation. Factors affecting CRH-R signaling, such as receptor and ligand expression, as well as cross talk with other neuromodulatory factors, are discussed in the following sections.

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## II. CRH RECEPTORS

The type 1 CRH receptor from mammals, birds, fish, and *Xenopus* has been cloned. The gene for the type 1 human CRH receptor encodes four receptor subtypes, CRH-R1α, CRH-R1β, CRH-R1c, and CRH-R1d. The type 2 CRH receptor from mammals, fish, and *Xenopus* has been cloned and a homologous partial sequence has been reported in a study using chicken cells. The gene for the type 2 human CRH receptor encodes four alternatively spliced receptors: CRH-R2α, CRH-R2α-tr, CRH-R2β, and CRH-R2γ; to date, there are two rodent variants, CRH-R2α and CRH-R2β. The catfish is the only source of a third distinct CRH receptor that has been cloned. The CRH-R1 and CRH-R2 splice variant forms are differentially expressed in the brain and periphery. The receptors belong to the class B G-protein-coupled receptor family that includes receptors for parathyroid hormone, vasoactive intestinal peptide, secretin, growth hormone-releasing factor, calcitonin, glucagon, and insect diuretic hormone.

Receptors for the CRH family of ligands are thought to exert their cellular effects via functional linkage to heterotrimeric G-proteins (Fig. 1), which activate signaling pathways through their α and/or βγ subunits. CRH-Rs couple to $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha z}$, and activation of these specific G-proteins leads to the stimulation of selective intracellular signaling pathways. The functional linkage of G-proteins to the CRH-R is dependent on the cell type and ligand.

Activation of $G_{\alpha s}$ by the CRH-R stimulates AC, which in turn increases cAMP production and activates PKAs. CRH elicits the release and intracellular accumulation of cAMP from cells of various parts of the brain, from pituitary cells, and in a number of cell lines. For example, rat retinal membranes express two populations of CRH binding sites, and incubation of these membranes with an antibody that recognizes the carboxyl terminus of the α subunit of $G_{\alpha s}$ prevents

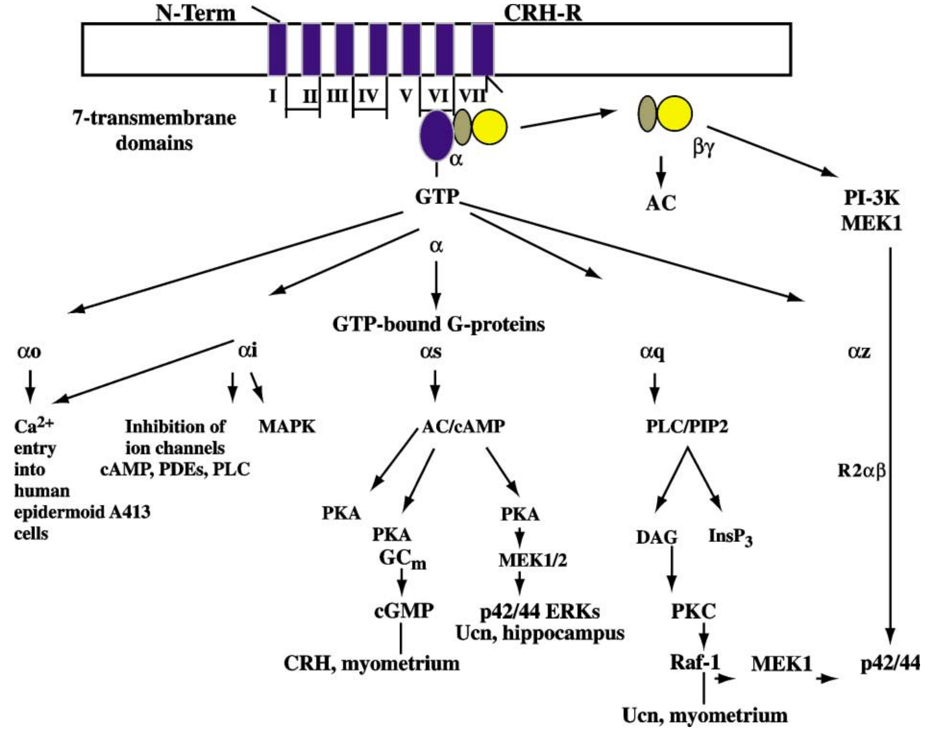
N-Term
CRH-R
I
II
III
IV
V
VI
VII
7-transmembrane domains
α
βγ
AC
PI-3K
MEK1
GTP
α
GTP-bound G-proteins
αo
αi
αs
αq
αz
Ca2+ entry into human epidermoid A413 cells
Inhibition of ion channels cAMP, PDEs, PLC
MAPK
AC/cAMP
PLC/PIP2
R2αβ
PKA
PKA
GCm
PKA
MEK1/2
DAG
InsP3
cGMP
CRH, myometrium
p42/44 ERKs
Ucn, hippocampus
PKC
Raf-1
MEK1
p42/44
Ucn, myometrium

**FIGURE 1** Corticotropin-releasing hormone receptor (CRH-R) activation can result in signaling through a number of different signaling pathways: the choice of pathway appears depend on preferential coupling of the G-protein to the receptor in the specific cell type. CRH-Rs are coupled to five classes of G-proteins, αo, αi, αs, αq, and αz. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CHO-R, Chinese hamster ovary stable cell line expressing CRH receptors; DAG, diacylglycerol; ERK, extracellular signal-related kinase; $GC_m$, guanylyl cyclase (membrane bound); GTP, guanosine triphosphate; $InsP_3$, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MEK1/2 (MEK1), MAPK kinase; PDE, phosphodiesterase; PI-3K, phosphatidylinositol 3-OH kinase; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKA, PKC, protein kinases A and C; PLC, phospholipase C; Ucn, urocortin.

TABLE I Activation of AC and cAMP by CRH, Ucn I, Ucn II, and Ucn III in CHO-R Cells[a]

| Peptide | Binding, hCRH-R1 ($K_i$, n$M$) membranes | Binding, rCRH-R2α ($K_i$, n$M$) membranes | Binding, mCRH-R2β ($K_i$, n$M$) membranes |
|---|---|---|---|
| $h_r$CRH | 0.95 (0.47–2.0) | 13 (7.2–22)* | 17 (10–29) |
| rUcn | 0.32 (0.14–0.77) | 2.2 (0.91–5.4) | 0.62 (0.14–2.8) |
| hUcnII | >100 | 1.7 (0.73–4.1) | 0.50 (0.22–0.16) |
| mUcnII | >100 | 2.1 (0.78–5.4) | 0.66 (0.13–3.3) |
| hUcnIII | >100 | 21.7 (8.2–57) | 13.5 (9.2–9.7) |
| mUcnIII | >100 | 5.0 (4.0–6.3) | 1.8 (0.77–4.1) |

[a] The values were determined from three to six independent experiments using stably transfected CHO cells or their membranes. The $EC_{50}$ and $K_i$ values were determined using prism software. h, human; m, mouse; r, rat. Ucn II and Ucn III are more selective for CRH-R2 than for CRH-R1.

†, SEM; *, 95%, confidence limit.

CRH stimulation of AC. In hippocampal neurons, urocortin (Ucn) activates $G_{\alpha s}$ and $G_{\alpha o}$ and subsequently the AC/cAMP pathway and PKA.

CRH-Rs in myometrial cells are coupled to $G_{\alpha s}$, $G_{\alpha o}$, $G_{\alpha i}$, and $G_{\alpha q}$; selective activation of $G_{\alpha q}$ by Ucn results in MAPK activation. Ucn preferentially activates $G_{\alpha q}$ with 10-fold greater potency compared to CRH and induces inositol 1,4,5-trisphosphate ($InsP_3$) production. Both CRH and Ucn stimulate cAMP production in myometrial cells, suggesting that both activate receptor coupling to $G_{\alpha s}$. Furthermore, CRH stimulates vasodilation in the human myometrium via a nitric oxide (NO)/cGMP-dependent pathway mediated by CRH-R1, with a rank order for activation of G proteins as follows: $G_{\alpha s} > G_{\alpha o} > G_{\alpha q} > G_{\alpha i} > G_{\alpha z}$. The differential activation of G-proteins by CRH and Ucn in human myometrial cells suggests that the peptides have distinct biological roles in these cells via activation of specific CRH-Rs and signaling cascades.

CRH-R1 is highly expressed within the rat cerebral cortex and is coupled to AC. This receptor is capable of activating $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha o}$, and $G_{\alpha z}$, with subsequent stimulation of two intracellular signaling cascades: (1) CRH predominantly activates $G_{\alpha s}$, resulting in an increase in cAMP, and (2) $G_{\alpha q}$ activation by CRH stimulates PLC, which increases $InsP_3$ and $Ca^{2+}$. The hydrolysis of InsPs can lead to the formation of several distinct messengers, such as diacylglycerol (DAG), raising the possibility of simultaneous CRH-mediated activation of multiple signaling pathways.

In the rat cerebral cortex, Ucn stimulates all but one of the G-proteins with a potency similar to that of CRH; the exception is $G_{\alpha q}$, for which Ucn is more potent than CRH. Incorporation of $GT^{32}P$-labeled amino acids in $G_{\alpha s}$ occurs at a 10-fold lower concentration of CRH than is required to stimulate AC, and may imply that a threshold number of $G_{\alpha s}$ molecules is required to induce activation of AC or that not all of CRH-R is coupled to AC. The $G_{\alpha z}$ and $G_{\alpha i}$ proteins are weakly coupled to the CRH-R in the cerebral cortex. It has been shown in other receptor systems that $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha q}$ can stimulate PLC and increase $InsP_3$s. CRH activation of $G_{\alpha i}$ may also lead to liberation of the βγ subunits and to activation of selective ACs. This may explain how pertussis toxin (PTX), an inhibitor of $G_{\alpha i}$ and $G_{\alpha o}$, can abolish the basal and CRH-stimulated AC activity in cerebral cortical membranes. The CRH-R can also activate $G_{\alpha o}$ and $G_{\alpha i}$, and these G-proteins have been implicated in the inhibition of AC and in the modulation of cation channels in human epidermoid A413 cells.

Therefore, preferential activation of specific G-proteins coupled to the CRH-Rs regulates the downstream intracellular signaling pathways that are activated by CRH or Ucn. The effects of Ucn II and Ucn III on CRH-R G-protein activation have not been extensively studied.

## III. ADENYLYL CYCLASE, CAMP, AND PROTEIN KINASES

All CRH peptides increase intracellular cAMP concentrations following activation of $G_{\alpha s}$, which activates AC. An increase in intracellular cAMP can lead to activation of PKA and in some cases PKC, which in turn stimulate the activation of other signaling components within a cell. Activated PKA and PKC can feedback to regulate cAMP production by regulating AC.

The $G_{\alpha s}$/AC/cAMP pathway is the most extensively studied signaling cascade activated by the CRH-R subtypes and has been used to discriminate

| **cAMP, hCRH-R1 (EC$_{50}$, n$M$)** | **cAMP, rCRH-R2$\alpha$ (EC$_{50}$, n$M$)** | **cAMP, CRH-R2$\beta$ (EC$_{50}$, n$M$)** |
|---|---|---|
| 0.26 (±0.05)[†] | 5.3 (±2.4)[†] | 3.0 (±1.0)[†] |
| 0.15 (0.03–0.64) | 0.063 (0.014–0.28) | 0.087 (0.017–0.43) |
| >100 | 0.26 (0.11–0.61) | 0.42 (0.16–1.1) |
| >100 | 0.14 (0.04–0.43) | 0.05 (0.02–0.12) |
| >100 | 0.16 (0.09–0.28) | 0.12 (0.06–0.20) |
| >100 | 0.073 (0.052–0.01) | 0.081 (0.08–0.80) |

subtype agonist selectivity. The relative potencies of the CRH peptides in binding to and activating CRH-Rs in Chinese hamster ovary (CHO) cells expressing CRH-R1 and CRH-R2 subtypes is summarized in Table 1. The universal activation of AC following stimulation of the CRH-Rs is observed in a diverse range of cell types. In most cell systems studied, CRH-R stimulation of AC is coupled through $G_{\alpha s}$, but CRH-R1 expressed in neutrophils of the spleen does not seem to function through $G_{\alpha s}$, as indicated by the inability of CRH to stimulate cAMP production. Also, CRH action in Leydig cells is exerted through direct or indirect activation of PKC at the level of the catalytic subunit of AC, and this effect has not been shown in any other cell type. The CRH-R in Leydig cells does not couple to $G_{\alpha s}$. In Y-79 human retinoblastoma cells, CRH-stimulated AC is amplified by $Mg^{2+}$ and is inhibited by submicromolar $[Ca^{2+}]$. Therefore, in these cells specific CRH-Rs stimulate cAMP formation by interacting with $G_{\alpha s}$ and by affecting a $Ca^{2+}$-inhibitable form of AC.

CRH stimulates accumulation of cAMP and release of ACTH from primary cultures of anterior pituitary (AP) cells and the mouse corticotroph cell line, AtT-20. Corticosteroids depress the CRH-mediated increase in cAMP by down-regulating CRH-R expression and, hence, inhibit secretion of ACTH from these cells. Therefore, the glucocorticoid inhibition of CRH-mediated ACTH secretion from the AP may be regulated by depletion of cAMP levels.

PKC has been shown to regulate AC activity in AP cells with both inhibitory and stimulatory actions. An increase in PKC activity by phorbol ester potentiates CRH stimulation of ACTH secretion and cAMP production in AP cells. This effect was not seen in intermediate pituitary (IP) or in AtT-20 cells treated with CRH. However, long-term treatment of cultures with phorbol ester, which depletes cell stores of PKC, causes a dramatic attenuation of ACTH release by CRH, but has no significant effect on cAMP production.

Both human (h) and rat (r) Ucn peptides promote secretion of ACTH from rat AP. Intravenous (iv) administration of Ucn causes an increase in ACTH and corticosterone secretion *in vivo* and an increase in proopiomelanocortin (POMC) and CRH-R1 expression in the AP. Ucn has a more potent and longer term effect than CRH. In AP cultures, Ucn stimulation of ACTH release is seven times greater than that of CRH and the effect is enhanced by phorbol esters that activate PKC, but is reduced by PKA inhibitors. Hence activation of PKC has a synergistic effect on Ucn-induced ACTH release, similar to the synergism between CRH and PKC.

CRH-Rs are found in heart, skin, and myometrial (during pregnancy) cells and in lymphocytes. The rat heart expresses CRH-R2β. CRH peptides stimulate cAMP levels in isolated rat neonatal cardiac myocytes. In human myometrial cells CRH-Rs are functionally coupled to AC and increase intracellular cAMP levels; cAMP is a well-known myometrial relaxant. The positive inotropic effect of CRH in these cells is due to activation of cAMP and prostaglandin (PG) pathways, whereas, chronotropic action is solely due to cAMP signaling. In skin cells, CRH-R1 is more efficient than CRH-R2 at transducing the CRH signal, with greater accumulation of cAMP. In human immortalized HaCaT keratinocytes, the relative potencies for activating AC through CRH-R1 are CRH = Ucn > sauvagine > urotensin I. It is likely that CRH and Ucn bind to distinct domains within the receptors, leading to conformational changes that facilitate coupling to G-proteins and activation of intracellular signaling molecules.

In human peripheral blood mononuclear cells (hPBMCs), CRH significantly increases cAMP in human monocytes, but not in lymphocytes. A CRH-R antagonist, α-helical CRH(9-41), inhibits this effect. Moreover, the CRH-stimulated cAMP levels are reduced to baseline by the intracellular $Ca^{2+}$ antagonist HA1004. This indicates cross talk between $Ca^{2+}$ and cAMP signaling in these cells. Hence, cAMP and/or $Ca^{2+}$ play a role in the CRH transduction pathway in human lymphocytes.

CRH-R stimulation activates PKA, PKB, and PKC. The activation, regulation, and physiological response of these protein kinases is dependent on cell type, ligand, and receptor (Fig. 2).

## A. PKA

CRH-R1 and CRH-R2 are positively coupled to increases in cAMP, which activates PKA by triggering the dissociation of the catalytic and regulatory subunits of the holoenzyme. The catalytic subunit of PKA directly phosphorylates and activates target proteins; phosphorylation of transcription factors such as $Ca^{2+}$/cAMP response element binding protein (CREB) at Ser-133 yields phospho-CREB (P-CREB), which regulates transcription of genes containing the $Ca^{2+}$/cAMP response element (CRE). PKA is involved in mediating the CRH/Ucn-stimulated increase in ACTH secretion and POMC and CRH-R1 expression in the AP.

PKA can directly regulate other intracellular signaling components, such as AC, MAPK, $Ca^{2+}$, PKC, and cGMP. For example, the CRH-mediated cAMP increase in hPBMCs is attenuated by a PKA inhibitor. Regulation of MAPK by PKA is seen in hippocampal neurons that express CRH-R1. Hippocampal neurons are protected from oxidative and excitotoxic cell death by Ucn and CRH, which has a 10-fold lower potency than Ucn, but not by Ucn II. Inhibition of PKA, MAPK kinase (MEK1), and PKC abrogates the Ucn-mediated protective effect. PKA regulates CRH-R-mediated phosphorylation of MAPK in normal ovine AP corticotrophs and the CRH-mediated inhibition of MAPK activity in AtT-20 cells. Also, the cell-differentiating action of CRH in immortalized noradrenergic neuronal CATH.a cells is mediated by PKA.

PKA can also influence CRH-R-stimulated $Ca^{2+}$ currents. For example, the large CRH-induced oscillatory increase in cytosolic [$Ca^{2+}$] in corticotrophs is largely dependent on cAMP activation of PKA. PKA regulates the increase in cytosolic [$Ca^{2+}$] by phosphorylation and activation of voltage-sensitive

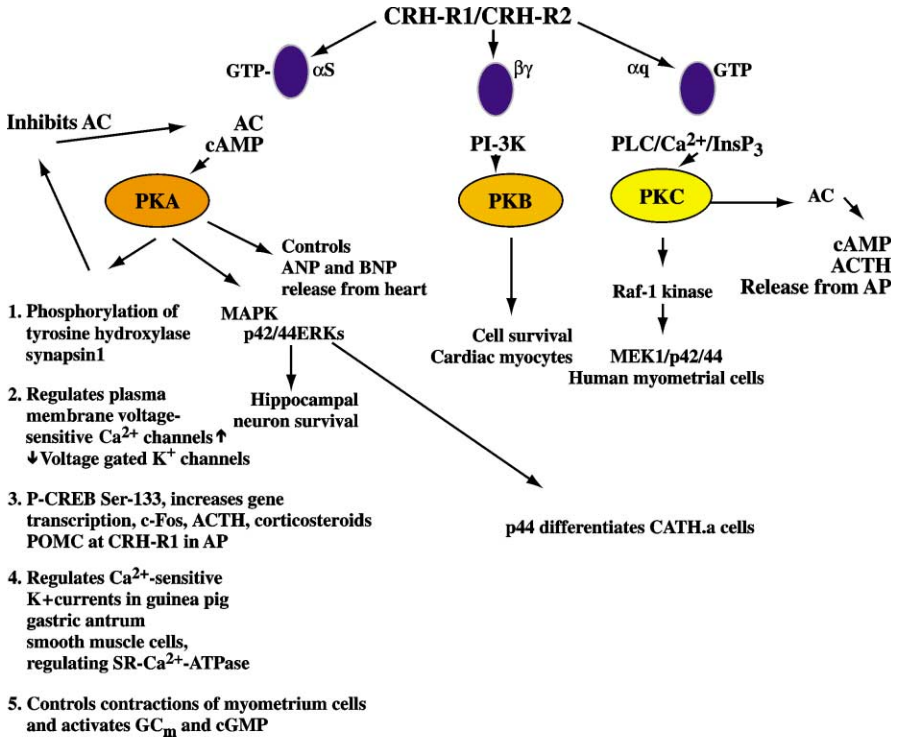
CRH-R1/CRH-R2
GTP-
αS
βγ
αq
GTP
Inhibits AC
AC
cAMP
PI-3K
PLC/$Ca^{2+}$/$InsP_3$
PKA
PKB
PKC
AC
Controls
ANP and BNP
release from heart
cAMP
ACTH
Release from AP
Raf-1 kinase
1. Phosphorylation of
tyrosine hydroxylase
synapsin1
MAPK
p42/44ERKs
Cell survival
Cardiac myocytes
MEK1/p42/44
Human myometrial cells
2. Regulates plasma
membrane voltage-
sensitive $Ca^{2+}$ channels↑
↓Voltage gated $K^+$ channels
Hippocampal
neuron survival
3. P-CREB Ser-133, increases gene
transcription, c-Fos, ACTH, corticosteroids
POMC at CRH-R1 in AP
p44 differentiates CATH.a cells
4. Regulates $Ca^{2+}$-sensitive
K+currents in guinea pig
gastric antrum
smooth muscle cells,
regulating SR-$Ca^{2+}$-ATPase
5. Controls contractions of myometrium cells
and activates $GC_m$ and cGMP

FIGURE 2 Corticotropin-releasing hormone receptor (CRH-R) stimulation can activate all three protein kinases, PKA, PKB, and PKC. Upstream activation of these kinases varies, depending on the G-protein and the cell type. Downstream signaling from the protein kinases leads to different physiological processes in different cell types. ACTH, adrenocorticotropic hormone; ANP, BNP, atrial and brain natriuretic peptides; SR, sarcoplasmic reticulum (other abbreviations as in Fig. 1).

$Ca^{2+}$ channels, thus regulating $[Ca^{2+}]_i$ directly. Therefore, CRH-R activation results in an increase in PKA activation, change in electrical excitability, and $[Ca^{2+}]$ mobilization, which, in turn, result in ACTH secretion from corticotrophs.

In guinea pig gastric antrum smooth muscle cells, Ucn increases $Ca^{2+}$-sensitive $K^+$ currents and does not affect the kinetics and voltage dependence of inward $Ca^{2+}$ current. The Ucn-mediated increase of $Ca^{2+}$-sensitive $K^+$ currents is regulated by PKA. The PKA-dependent increase of $Ca^{2+}$ concentration near the plasma membrane is due to enhanced release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores. In rat tail arteries, Ucn is a potent endothelium-independent dilator, and Ucn-mediated CRH-R activation of PKA causes a reduction in the sensitivity of the contractile apparatus for $Ca^{2+}$.

PKA can also modulate receptor-mediated $Ca^{2+}$ responses. In CATH.a cells, submaximal concentrations of pituitary AC-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP, and carbachol produce transient increases in $[Ca^{2+}]_i$. This effect is abolished by prior treatment of the cells with $1 \mu M$ CRH. The activation of PKA similarly abolishes the increase in $[Ca^{2+}]_i$ by these peptides. This study demonstrates that CRH inhibits receptor-mediated $[Ca^{2+}]_i$ responses in a locus coeruleus cell line, possibly by activation of PKA. This modulation could be important in controlling neuronal function *in vivo* in stressful situations in which the levels of CRH are increased in the locus coeruleus. Therefore, the physiological role for the inhibition of $Ca^{2+}$ mobilization by CRH might be to modulate neuronal function by alteration of $Ca^{2+}$-dependent processes such as gene transcription, which would otherwise be stimulated by PACAP, VIP, or carbachol.

CRH increases the amplitude of and decreases the frequency of contractions in preparations of myometrium from pregnant rats, and this effect is mediated by PKA. In human myometrial cells, acute CRH administration activates CRH-R1 and stimulates the membrane-bound guanylate cyclase ($GC_m$), which in turn increases cGMP and contractility. PKA inhibition reduces CRH-stimulated cGMP production. Therefore, CRH-stimulated $GC_m$ activity is partially dependent on the PKA that may phosphorylate the kinase homology domain essential for $GC_m$ activity. Ucn stimulates the release of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) from neonatal rat cardiac myocytes, and this process is abrogated by inhibiting PKA. Ucn stimulates cAMP and $[^3H]$leucine uptake and enhances endothelin-1-induced increase of $[^3H]$leucine uptake. Hence CRH-R2 activation by Ucn induces ANP and BNP secretions from cardiac myocytes, at least in part via a PKA pathway during cardiac hypertrophy.

## B. PKC

PKA, $Ca^{2+}$, cAMP, and PI (DAG and $InsP_3$s) activate PKC. Prolonged activation of PKC can down-regulate CRH-R expression and reduce signaling in human myometrial cells; also in these cells, Ucn activates PKC and Raf-1 kinase and, subsequently, MAPK.

PKC has been shown to activate MAPK in hippocampal neurons. In human myometrial cells, in contrast to PKA, PKC inhibits CRH-induced $GC_m$ activity. PKC has been shown to down-regulate the expression of CRH-Rs and PKC may target multiple components of the intracellular signaling pathway to reduce the CRH-induced cGMP response in these cells.

## C. PKB/Akt

PKB/Akt functions to promote cell survival by inhibiting apoptosis. Ucn is expressed in the heart and binds with high affinity to CRH-R2β. Ucn promotes cardiac myocyte survival against hypoxia reoxygenation (HR) injury, and this survival involves activation of the MAPK pathway (MEK1/2 p42/44 MAPK). Also, Ucn stimulates the phosphorylation of PKB/Akt via phosphatidylinositol 3-OH kinase (PI-3K) in cardiac myocytes. Inhibiting MEK1, PI-3K, or PKB/Akt with genetic and chemical inhibitors inhibits the Ucn-cardioprotective effect in HR.

Additionally, exogenous CRH provokes changes in the cellular shape of invertebrate immunocytes from the mollusk *Mytilus galloprovincialis*. These changes in cell shape are mediated by PI-3K, PKB/Akt, PKC, and PKA, indicating cross talk between these pathways.

# IV. CALCIUM SIGNALING

CRH also signals through the second messenger $Ca^{2+}$, and a majority of these effects are regulated by PKA. CRH-R-mediated $Ca^{2+}$ signaling has been demonstrated in human and rat pituitary corticotrophs, arterial cells, smooth muscle cells, skin cells, and neuronal cell lines.

In pituitary corticotrophs, a change in the cytosolic free $[Ca^{2+}]_i$ is important in controlling ACTH secretion. CRH-R stimulation by CRH results in an increase in the cell firing frequency of corticotroph action potentials and induces an oscillatory increase in cytosolic $[Ca^{2+}]_i$. CRH also increases membrane depolarization, which is associated with the generation of action potentials in quiescent corticotrophs and an increase in action potential frequency in spontaneously active corticotrophs. The increase in $[Ca^{2+}]_i$ is due to an increase in frequency and amplitude of cytosolic $Ca^{2+}$ transients into the cell; the $Ca^{2+}$ transients are completely dependent on voltage-sensitive L-type $Ca^{2+}$ channels and are regulated by P-type $Ca^{2+}$ channels that are activated by CRH-induced membrane depolarization. The voltage-dependent $Na^+$ channel depolarizes the membrane, thereby activating the voltage-gated $Ca^{2+}$ channels, and is therefore involved in the rapid initial component of the CRH-induced action potential. Inhibition of PKA does not inhibit CRH-induced membrane depolarization, but attenuates the CRH-induced increase in action potential frequency and $[Ca^{2+}]_i$ transients. The phosphorylation targets for PKA have not been identified; however, PKA is known to phosphorylate L-type voltage-sensitive $Ca^{2+}$ channels. Also, CRH induces inhibition of $K^+$ currents that maintain a negative resting membrane potential, which is believed to contribute to the membrane depolarization and increased membrane excitability. In rat corticotrophs, a CRH-induced inhibition of an identified $Ba^{2+}$-sensitive inwardly rectifying $K^+$ channel is believed to contribute to the membrane depolarization and to increases in firing frequency. The effects of CRH on dorsal raphe neurons revealed that, in the presence of $Ca^{2+}$, CRH rapidly and reversibly increases firing rates of serotonergic neurons located in the ventral portion of the dorsal raphe nucleus. Similar to its effect on the AP, CRH at physiological concentrations increases $[Ca^{2+}]_i$ in isolated single beta cells of rat islets via the L-type $Ca^{2+}$ channel by activating the cAMP/PKA pathway.

CRH and Ucn are endogenous vasodilators in rat mesenteric, cerebral, and femoral arteries as well as in rat aorta and in rat basilar and uterine arteries. The CRH-R2-mediated vasodilatory effect in rat aorta and in fetal–placental circulation is endothelium dependent and most likely is mediated by NO release and cGMP. In the rat basilar artery, Ucn antagonizes vascular contraction mainly via AC stimulation and subsequent activation of $Ca^{2+}$-dependent $K^+$ channels. Ucn is also a potent endothelium-independent dilator of rat tail arteries, an effect likely to be mediated by PKA, causing a reduction in the sensitivity of the contractile apparatus for $Ca^{2+}$.

Alterations of $[Ca^{2+}]_i$ are responsible for the vasodilatory effect of Ucn in guinea pig gastric antrum smooth muscle cells. In these smooth muscle cells Ucn causes a concentration-dependent increase of $Ca^{2+}$-sensitive $K^+$ currents and does not affect the kinetics and voltage dependence of inward $Ca^{2+}$ currents. This results in an increase in $[Ca^{2+}]$ at the plasma membrane. Emptying of intracellular $Ca^{2+}$ stores, as well as activity of blockers of AC and PKA, antagonize the effect. Therefore, Ucn binds at physiological concentrations to the CRH-R and activates AC/cAMP/PKA. The final target for these biochemical events is most probably the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase or another integral SR membrane protein that is functionally involved in the regulation of the SR $Ca^{2+}$ pump.

In contrast to smooth muscle cells, the vasodilatory effect of CRH and Ucn on the isolated rat heart appears not to be mediated by $Ca^{2+}$, but by prostaglandins (PGs). Finally, Ucn stimulation of ANP and BNP from neonatal rat cardiac myocytes is mediated by PKA and a voltage-dependent $Ca^{2+}$ channel.

In a number of skin cell types, CRH-R-mediated stimulation of $[Ca^{2+}]_i$ is largely due to an influx of $Ca^{2+}$ through the extracellular membrane. High $[Ca^{2+}]_i$ in malignant melanocytes is due to CRH-R activation. CRH-R-mediated changes in $[Ca^{2+}]_i$ have antiproliferative effects on Cloudman melanoma cells, on B16 melanoma growth *in vivo*, on human immortalized keratinocytes (HaCaT), and on hamster melanomas (SK-MEL188, AbCl). In A431 cells, CRH increases $[Ca^{2+}]_i$ by stimulating $Ca^{2+}$ influx through channels coupled to $G_{\alpha i}$ and $G_{\alpha o}$ and by mobilization through production of $InsP_3$'s. Treatment of CHO cells stably expressing CRH-R1α and CRH-R2β with sauvagine results in an increase of intracellular $Ca^{2+}$, both by mobilization of $Ca^{2+}$ from intracellular stores and by influx across the plasma membrane.

## V. MITOGEN-ACTIVATED PROTEIN KINASES

The activity of three main families of MAPKs can be regulated by several G-proteins, including $G_{\alpha s}$, $G_{\alpha q}$, and $G_{\beta\gamma}$. CRH peptides activate the MEK1/ERK1/2/p42/44 pathway and differentially activate p42/44 MAPKs in a number of cell lines and tissues (Fig. 3).

MAPK is activated by sauvagine in CHO cells stably expressing rat CRH-R1, CRH-R2α, and CRH-R2β. Sauvagine activation of p42/44 MAPK results in phosphorylation of Elk1, which can bind to the serum response element (SRE). Phosphorylated Elk1, together with a serum response factor protein, can increase transcription of immediate early genes containing the SRE, such as the gene for c-Fos. The p42/44 MAPKs are partially regulated through PI-3K

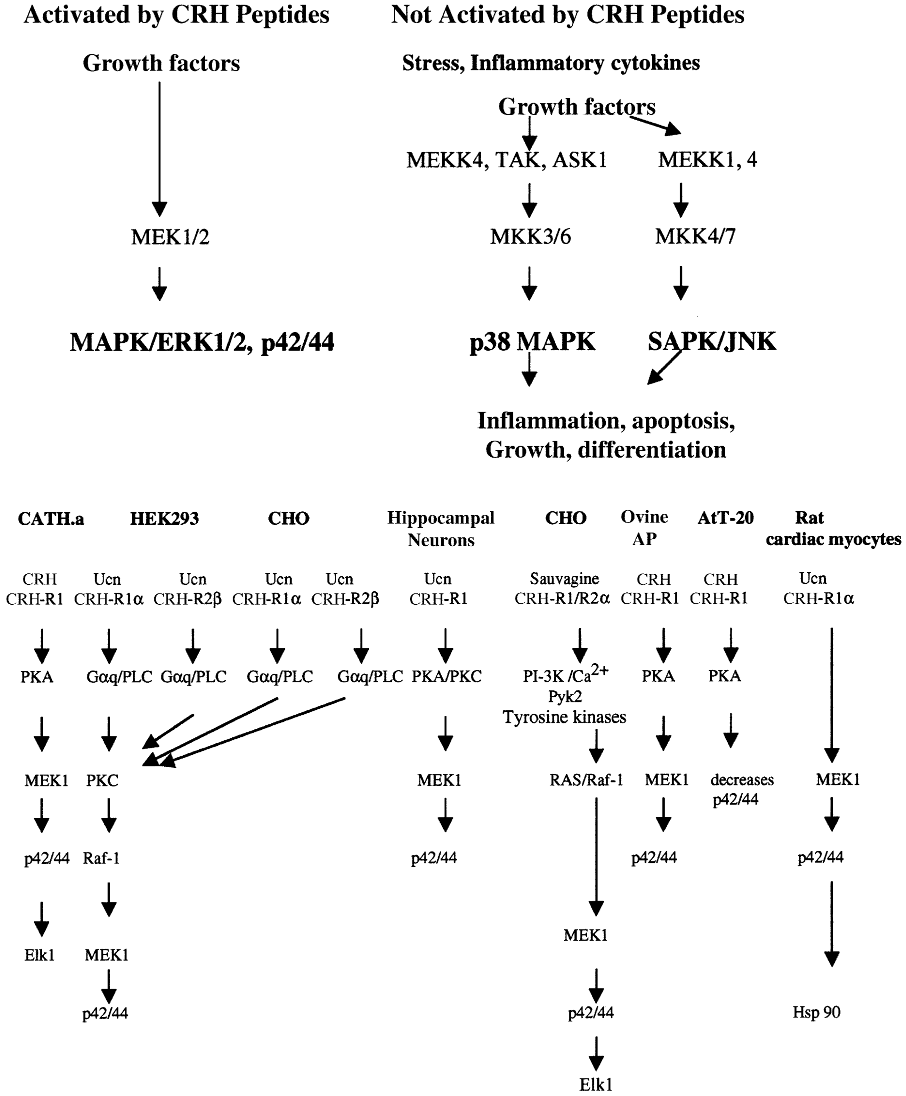
Activated by CRH Peptides
Growth factors
MEK1/2
MAPK/ERK1/2, p42/44
Not Activated by CRH Peptides
Stress, Inflammatory cytokines
Growth factors
MEKK4, TAK, ASK1
MEKK1, 4
MKK3/6
MKK4/7
p38 MAPK
SAPK/JNK
Inflammation, apoptosis,
Growth, differentiation
CATH.a
HEK293
CHO
Hippocampal Neurons
CHO
Ovine AP
AtT-20
Rat cardiac myocytes
CRH CRH-R1
Ucn CRH-R1α
Ucn CRH-R2β
Ucn CRH-R1α
Ucn CRH-R2β
Ucn CRH-R1
Sauvagine CRH-R1/R2α
CRH CRH-R1
CRH CRH-R1
Ucn CRH-R1α
PKA
Gαq/PLC
Gαq/PLC
Gαq/PLC
Gαq/PLC
PKA/PKC
PI-3K /Ca2+ Pyk2 Tyrosine kinases
PKA
PKA
MEK1
PKC
MEK1
RAS/Raf-1
MEK1
decreases p42/44
MEK1
p42/44
Raf-1
p42/44
p42/44
p42/44
Elk1
MEK1
MEK1
p42/44
p42/44
Hsp 90
Elk1

FIGURE 3 Multiple pathways of activation in a number of cell systems, mediated by corticotropin-releasing hormone receptor (CRH-R) stimulation. CRH-R stimulation can result in activation of the MEK1/2, MAPK/ERK1/2, p42/44 pathway; activation depends on the CRH-R subtype stimulated, the ligand, and the cell type. ASK1, apoptosis signal-regulating kinase; JNK, JUN N-terminal kinase; SAPK, stress-activated protein kinase; TAK, transforming growth factor-B-activated kinase (other abbreviations as in Fig. 1).

and through PI-specific phospholipase C (PLC) of the $Ca^{2+}$-sensitive proline-rich tyrosine kinase (Pyk2). Pyk2 can then activate tyrosine kinases and MAPKs. Activation of MAPK by sauvagine is independent of PKA and of cAMP, but is dependent on $Ca^{2+}$ in the CHO CRH-R1α and CRH-R2α cell lines.

As is observed in the transfected CHO cell lines, activation of MEK1 by Ucn in primary cultures of rat cardiac myocytes results in the phosphorylation of p42/44 MAPKs. In the heart, the MEK1 pathway is involved in the cardioprotective effect of Ucn against HR injury by reducing apoptosis and necrotic cell death. Ucn causes an increase in expression of the cytoprotective heat-shock protein 90 (Hsp 90) via MEK1 activation. Ucn, but not Ucn II, promotes survival of hippocampal neurons via CRH-R1 activation. Inhibition of PKA, MEK1, and PKC inhibits the Ucn-mediated protective effect. MEK1 is also a substrate for PKC and PKA, suggesting that crosstalk between signaling pathways is required for the protective effect of Ucn in these hippocampal cells.

CRH acts as a differentiation factor in CATH.a cells, and blocking either the ERK pathway or PKA inhibits neurite outgrowth. The transcription factors activated by PKA and p42/44 MAPK were investigated and it was shown that PKA inhibition inhibits CRH-mediated phosphorylation of the p44 isoform in CATH.a cells. CREB is phosphorylated and activated by PKA and Elk is phosphorylated by p42/44 MAPK. ERG-1 is involved in neuronal differentiation and CRH activates the ERG-1 promoter, possibly via activation of MEK1 and PKA.

CRH differentially regulates MAPK activity in normal ovine anterior pituitary cells, as compared to regulation in AtT-20 cells. Incubation of ovine interior pituitary cells with CRH increases MAPK activity mediated by PKA. In contrast, incubation of AtT-20 cells with CRH inhibits MAPK activity mediated by PKA. Interestingly, ANP reverses the effects of MAPK activity in both cell types, suggesting that ANP is involved in MAPK regulation in the AP.

In myometrial cells from pregnant humans, Ucn, but not CRH, activates the MEK1 p42/44 MAPK pathway. These cells express CRH-R2β, CRH-R1α, CRH-R1β, CRH-R1c, and CRH-R1d, with CRH-R1 being the prominent receptor subtype. In HEK293 and CHO cells stably expressing these receptors CRH fails to activate p42/44 MAPK. Ucn activates both CRH-R1α and CRH-R2β, and not the other receptor subtypes, resulting in MAPK activation by stimulating $G_{\alpha q}$/PLC/$InsP_3$, DAG/PKC/Raf-1 kinase/MEK1/p42/44. In comparison to the MAPK pathway, CRH and Ucn activate cAMP at all receptor types. This study suggests that Ucn and CRH have distinct biological roles in the myometrium. CRH-R1α, which binds Ucn and CRH with equal affinity, can differentially activate MAPK. CRH receptors selectively activate MAPK, but all of the receptors activate AC.

## VI. CGMP

cGMP is another molecule implicated in CRH receptor signaling. In response to G-protein-coupled receptor (GPCR) activation, cGMP is synthesized by membrane-bound guanylate cyclase ($GC_m$) and soluble guanylate cyclase ($GC_s$). In cultured rat AP cells and immortalized GH3 cells, CRH-R activation by CRH increases cAMP and cGMP. Inhibition of PKA inhibits CRH-R-mediated activation of $GC_s$ in pituitary cells. CRH-R activation by CRH modulates $GC_s$ signaling though $G_{\alpha s}$, AC, and cAMP.

$$\mathrm{CRF} + \mathrm{CRF\text{-}R} \rightarrow G_{\alpha s} \rightarrow \mathrm{AC} \rightarrow \mathrm{cAMP} \rightarrow \mathrm{PKA} \rightarrow \mathrm{GCs} \rightarrow \mathrm{GMP}$$

In myometrial cells from pregnant humans, CRH stimulates vasodilation through an NO/cGMP-dependent pathway by activating CRH-R1. The actions of NO in the myometrium are mediated, at least partly, via activation of $GC_s$ and increased production of cGMP, which reduces myosin light chain phosphorylation. CRH, but not Ucn II or Ucn III, has a dual effect on the myometrial nitric oxide synthase (NOS)/GC pathway, with a short-term effect mediated by PKA (acute) and a long-term effect mediated by increased NOS expression. Acute stimulation of these cells with CRH results in the activation of the cGMP pathway by enhancing $GC_m$ and to a lesser extent $GC_s$. This effect is independent of NOS but is partially dependent on PKA. PKC inhibits the CRH-induced $GC_m$ activity, possibly by down-regulating the expression of CRH-Rs. Long-term treatment with CRH up-regulates endothelial NOS (eNOS) and brain NOS (bNOS) mRNA. The increase in eNOS and bNOS expression results in an increase in activity of $GC_s$ and cGMP.

## VII. EXTRACELLULAR FACTORS THAT REGULATE CRH RECEPTOR SIGNALING

CRH-R and CRH-R ligand expression levels, CRH-binding protein (CRH-BP), and other neuromodulators can all regulate CRH-R signaling. In the heart, CRH-R2β mRNA decreases in response to Ucn. Lipopolysaccharide (LPS) increases plasma ACTH levels, which in turn decreases heart CRH-R2β mRNA. Corticosterone, dexamethasone, and physical restraint-induced stress decrease CRH-R2β mRNA in the heart and aorta. Tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-1α decrease CRH-R2β in A7R5 aortic smooth muscle cells. Systemic IL-1α and TNF administration significantly down-regulate CRH-R2 mRNA in mouse heart. *In vivo*, inflammatory mediators such as LPS and/or cytokines increase Ucn, which in turn down-regulates CRH-R mRNA levels. Because CRH and Ucn increase cardiac contractility and coronary blood flow, impaired CRH-R2 function during systemic inflammation may ultimately diminish the adaptive cardiac response to adverse conditions. Within the human heart, CRH-R signaling may be modulated in different pathological conditions, such as left ventricular hypertrophy, in which Ucn levels increase and CRH-R2β mRNA levels decrease. Both CRH-R1 and CRH-R2 are expressed in different regions within the human heart, but CRH-R1 has not been reported in mouse or rat heart. With the discovery of new CRH-R-selective peptides and differential heart receptor

**TABLE 2 Summary of Signaling Components Activated by CRF Ligands at CRF-Rs in Specific Tissues and Cell Types**

| Tissue/cell[a] | CRF[b] | Ucn[b] | Ucn II[b] | Ucn III[b] | Sauvagine[b] |
|---|---|---|---|---|---|
| Anterior pituitary corticotrophs (r/h) | PKC, PKA, AC/cAMP, MAPK, $Ca^{2+}$, $K^+$, cGMP | PKC, PKA, AC/cAMP | cAMP | No response | cAMP |
| AtT-20 cells (m) | cAMP, PKA/MAPK | cAMP | cAMP response at high doses | No response | cAMP |
| Hippocampal neurons(r) | cAMP, PKA | $G_{\alpha s}$,$G_{\alpha o}$/AC/cAMP/PKA, MEK1, MAPK, PKC | No cAMP response | —[c] | —[c] |
| Cerebral cortex (r) | $G_{\alpha s}$/AC/cAMP, $G_{\alpha q}$/11/PLC/ $InsP_3$/DAG/$Ca^{2+}$ | $G_{\alpha s}$/AC/cAMP, $G_{\alpha q}$/11/PLC/ $InsP_3$/DAG/$Ca^{2+}$ | —[c] | —[c] | —[c] |
| Heart cells (r/m) | cAMP | MAPK, cAMP, PGs, ANP/BNP, release, PKA, PI-3K, PKB/Akt, voltage-dependent $Ca^{2+}$ channel | —[c] | —[c] | —[c] |
| Skin cell types | AC/cAMP, $Ca^{2+}$ influx via $G_{\alpha i}$, $G_{\alpha o}$, $InsP_3$s | AC/cAMP | —[c] | —[c] | AC/cAMP |
| Guinea pig gastric smooth antrum muscle | AC/cAMP | $Ca^{2+}$-sensitive $K^+$ currents, AC/cAMP, PKA/SR-$Ca^{2+}$-ATPase | —[c] | —[c] | —[c] |
| Retina(r) | $G_{\alpha s}$/cAMP | —[c] | —[c] | —[c] | —[c] |
| Myometrium (h) | $InsP_3$,AC/cAMP, $PG_s$, $GC_m$-PKA, cGMP, PKC, eNOS/bNOS, NO/$GC_s$ cGMP | Gαq/PKC, PLC/$InsP_3$, PKC, Raf-1 kinase, MAPK, AC/cAMP | —[c] | —[c] | —[c] |
| Tail artery(r) | PKA, $Ca^{2+}$ | PKA, $Ca^{2+}$ | —[c] | —[c] | —[c] |
| Basilary artery (r) | AC/cAMP, PKA $Ca^{2+}$ | AC/cAMP, PKA $Ca^{2+}$ | —[c] | —[c] | —[c] |
| CATH.a cells | PKA, MAPK $Ca^{2+}$ | —[c] | —[c] | —[c] | —[c] |
| CHO-R1α | cAMP | cAMP | cAMP response at high doses | No response | cAMP, MAPK, $Ca^{2+}$ |
| CHO-R2β/α | cAMP | cAMP | cAMP | cAMP | cAMP, MAPK, $Ca^{2+}$ |
| Blood cells | cAMP/$Ca^{2+}$ PKA | —[c] | —[c] | —[c] | —[c] |

[a] Tissues and cells were from rats (r), humans (h), or mice (m).
[b] All MAPK was P42/44 MAPK.
[c] Data not available.

expression, Ucn may exhibit differential signaling pathways in different areas of the heart.

*In vivo*, during sustained stress, elevated CRH levels result in a reduction of cAMP and ACTH release by rat corticotroph cells, due to down-regulation in expression of CRH-R1. A similar desensitization effect has been seen in other cell lines. The G-protein receptor kinases (GRK2/3) are involved in homologous desensitization of this receptor in a number of cell lines. An increase in PKC also decreases CRH-R expression and sensitivity to ligand in pituitary and myometrial cells. In human myometrial cells, oxytocin reduces CRH binding to CRH-Rs and AC activity (at term, during pregnancy, but not preterm) via PKC activation. PKC directly phosphorylates and desensitizes the CRH-R in human myometrial cells. In CATH.a cells, in addition to PLC and PKC, cAMP down-regulates CRH-R1 mRNA.

CRH-BP may selectively inhibit CRH and Ucn actions by inhibiting access of these ligands to their receptors. CRH-BP maintains appropriate levels of these peptides in the central nervous system and therefore controls their signaling.

CRH and Ucn interact with other signaling pathways. CRH and Ucn inhibit feeding by acting downstream of the leptin signaling pathway, and CRH modulates the immune system by regulating the secretion of cytokines and neuropeptides of purified macrophage and lymphocytes. Ucn inhibits LPS-induced increases in TNF and IL-1α *in vivo* and TNF release from Kupffer cell cultures. This supports the function of Ucn as an anti-inflammatory agent. CRH and Ucn are vasodilatory and have inotropic effects on the rat heart. Inhibiting cyclooxygenase inhibits the vasodilatory effect of Ucn, suggesting that Ucn regulates PG production in the rat heart and providing evidence that there is cross talk between pathways.

In the human myometrium, CRH controls symmetrical contractility, whereas IL-1α and oxytocin stimulate production of PGs. CRH partially inhibits IL-1α- and oxytocin-stimulated prostaglandin E2 (PGE2) production. This effect is dependent on the CRH-R and not on cyclooxygenases involved in PG synthesis. At term in pregnancy, there is a reduction in the functional ability of CRH to stimulate cAMP due to a reduction of the number of $G_{\alpha s}$ subunits and an inhibitory action of oxytocin, which activates PKC, leading to phosphorylation and desensitization of the CRH-R. In the human myometrium, a dose-dependent increase of trophoblast ACTH or PGE2 secretion is induced by Ucn and is inhibited by astressin (a CRH-R peptide antagonist). Ucn increases PGE2-induced myometrial contractility.

## VIII. SUMMARY

CRH ligands activate specific intracellular signaling pathways in different cell types (see Table 2 for summary). A great deal of cross talk exists between the different CRH-R-mediated signaling pathways, in particular with regard to the cAMP/PKA pathway. The intricacies of CRH signaling probably reflect the presence of more than one receptor subtype and ligand in cells involved in orchestrating the endocrine and paracrine stress response. In addition, the coupling of these receptors to specific G-proteins provides a diversity of multiple signaling pathways that may be activated following CRH-R stimulation.

## Acknowledgments

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## Glossary

**adenylyl cyclase** Enzyme that catalyzes conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). There are 10 known isoforms of the enzyme; 9 are membrane bound and 1 is soluble. The various forms of adenylyl cyclase (AC) are differentially regulated; however, all membrane-bound forms are stimulated by the stimulatory GTP-binding protein of AC, $G_{\alpha s}$. All but one membrane-bound AC isoform are stimulated by forskolin.

**corticotropin-releasing hormone family of peptides** Isolated in 1981, corticotropin-releasing hormone (CRH) was characterized as the major factor that orchestrates the endocrine stress response in mammals. Urocortin (Ucn) is a mammalian CRH-related peptide; two new members of the CRH peptide family, stresscopin-related peptide/urocortin II (Ucn II) and stresscopin/urocortin III (Ucn III), have recently been identified in humans, mice, and fish.

**corticotropin-releasing hormone receptors** Bind to two types of 7-transmembrane domain (TMD) G-protein-coupled receptors, CRH-R1 (type 1) and CRH-R2 (type 2).

**extracellular signal-related kinases** Belong to one subfamily of mitogen-activated protein kinases (MAPKs) composed of 42- and 44-kDa kinases, known as p42-ERK and p44-ERK, respectively. Phosphorylation and activation of p42-ERK and p44-ERK are mediated by MAPK kinase MEK1/2, commonly referred to as MEK1.

**G-proteins** Composed of three subunits, α, β, and γ. Activation of receptors that couple to G-proteins results in GTP exchanging for GDP on the α subunit, a process that can further activate a number of signaling pathways. The βγ subunit can itself activate a number of effectors.

**mitogen-activated protein kinases** Constitute a widely conserved family of serine/threonine protein kinases involved in many cellular programs, including proliferation, differentiation, movement, and death. MAPKs are phosphorylated and activated by MAPK kinases (MAPKKs) such as MEK1. Stress-activated protein kinase (SAPK)/JUN N-terminal kinase (JNK) and p38 are phosphorylated and activated by MAPK kinases, which in turn are phosphorylated and activated by MAPKK kinases (MAPKKKs). The MAPKKKs are activated by a family of small GTPases and/or protein kinases connecting the MAPK module to the cell surface receptor or to external stimuli.

**protein kinase A** Tetrameric holoenzyme that is activated when cAMP binds to its two regulatory subunits, resulting in release of active catalytic subunits. Three catalytic subunits have been identified, designated Cα, Cβ, and Cγ.

**protein kinase B and phosphatidylinositol 3-OH kinase** Phosphatidylinositol 3-OH kinase (PI-3K) consists of an 85-kDa regulatory (p85) and a 110-kDa catalytic (p110) subunit. It catalyzes the phosphorylation of PI to phosphatidylinositol 3-phosphate, PI-4-phosphate to PI-3,4-bisphosphate, and PI-4,5-bisphosphate to PI-3,4,5-trisphosphate. The products of PI-3K act on multiple downstream effectors, including the serine/threonine kinase, PKB/Akt. The activation of PKB/Akt requires phosphorylation on both Thr-308 and Ser-473 and the direct interaction of PI-3K lipid products with a pleckstrin homology domain at its N-terminus. The phosphorylation of Thr-308 is catalyzed by 3-phosphoinositide-dependent kinase-1 and may contribute to the phosphorylation of Ser-473.

**protein kinase C** Family of serine/threonine protein kinases that are activated by $Ca^{2+}$ and/or phospholipids in response to extracellular signals. The isoforms of PKC have been classified into three groups: (1) classical PKCs are $Ca^{2+}$ dependent and are activated by phosphatidylserine, diacylglycerol (DAG), and phorbol esters (such as phorbol myristic acid); (2) "novel" PKCs are $Ca^{2+}$ independent; and (3) atypical PKCs do not require DAG and $Ca^{2+}$ for their activation.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Calcium Signaling • Corticotropin-Releasing Hormone (CRH) • Corticotropin-Releasing Hormone Pharmacology • Corticotropin-Releasing Hormone, Stress, and the Immune System • Protein Kinases • Stress**

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# Corticotropin-Releasing Hormone, Stress, and the Immune System

Susan E. Murray and Mary P. Stenzel-Poore
*Oregon Health and Science University, Portland*

I. INTRODUCTION
II. STRESS-ACTIVATED PATHWAYS
III. IMMUNE SYSTEM
IV. HPA ACTIVATION AND IMMUNE EFFECTS
V. HPA-INDEPENDENT EFFECTS ON IMMUNITY
VI. CONCLUSIONS AND PERSPECTIVES

**The neuroendocrine and immune systems are activated during times of threat to survival. The former is activated during psychological or physical stress, and both are activated during infection, or immunological stress. Thus, stress-activated pathways and immune responses are coregulated and intimately affect one another. Corticotropin-releasing hormone is a principal regulator of the stress response through activation of the hypothalamic–pituitary–adrenal axis as well as through stress-responsive pathways in the central nervous system.**

## I. INTRODUCTION

This article focuses primarily on the effects that stress and corticotropin-releasing hormone (CRH)-activated pathways have on immunity. Anecdotal and clinical evidence suggest that major life stress may dispose individuals to increased risk for illness, including infectious disease. This has led to the idea that compromised immunity may play a role in stress-related diseases. In fact, various measures of immune function have been shown to be altered during chronic stress, such as during long-term care giving or following the death of a family member. For instance, antibody responses to vaccination and several measures of cellular immunity are correlated negatively with life stress. However, the relationship between altered immune parameters and increased susceptibility to infectious disease in humans is difficult to define. Although gross immunosuppression (such as during human immunodeficiency virus infection) clearly increases susceptibility, it is unknown if smaller alterations in individual immune parameters bear significant consequence for infectious disease in otherwise healthy adults. Furthermore, a limited number of immune functions can be measured in humans. Such difficulties have driven the development of animal models to address these questions. Thus, much of the following discussion is derived from studies of animal models of stress.

## II. STRESS-ACTIVATED PATHWAYS

Stress-activated systems originate in the brain. To regulate the immune system, signals generated in the central nervous system (CNS) must be translated to the periphery. This is accomplished through two

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## II. STRESS-ACTIVATED PATHWAYS

Stress-activated systems originate in the brain. To regulate the immune system, signals generated in the central nervous system (CNS) must be translated to the periphery. This is accomplished through two primary systems—the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS). The central components of these systems are located in the paraventricular nucleus (PVN) of the hypothalamus, which produces CRH, and the locus coeruleus (LC) in the brain stem, a site of catecholamine production (Fig. 1).

CRH is a 41-amino-acid neuropeptide that is produced in numerous brain regions, although the name CRH reflects stimulatory actions on adrenocorticotropic hormone (ACTH). CRH produced by neurons of the PVN travels the hypophyseal portal system and binds CRH type 1 receptors on pituitary corticotrophs, which causes secretion of ACTH into the bloodstream. ACTH, in turn, drives release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex (Fig. 1). Peripheral SNS effects are mediated primarily by catecholamine release from peripheral nerves, although some fibers release neuropeptides. Postganglionic SNS fibers that originate in the brain stem innervate peripheral organs and, for the most part, release norepinephrine. Additionally, preganglionic fibers innervate the adrenals and cause the release of epinephrine and, to a lesser extent, norepinephrine (Fig. 1).

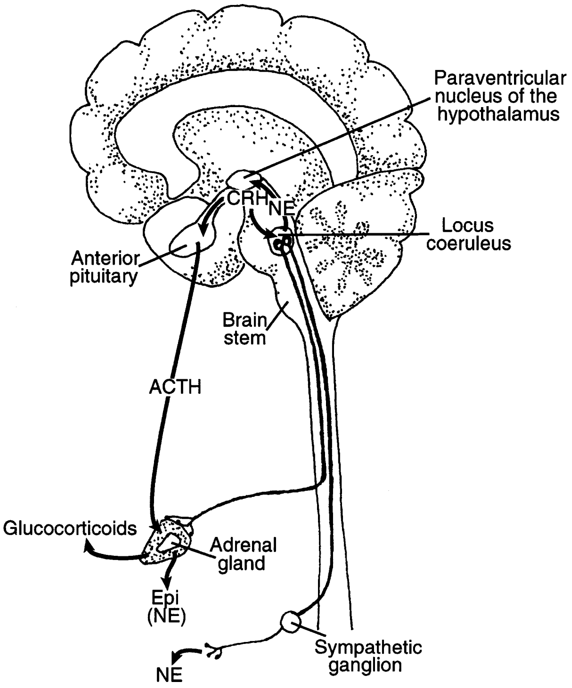

**FIGURE 1** Components of the stress system. Two major stress-responsive systems are shown, the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system. Corticotropin-releasing hormone (CRH)-producing neurons of the paraventricular nucleus of the hypothalamus and norepinephrine (NE)-producing neurons of the locus coeruleus positively regulate one another. CRH from the paraventricular nucleus causes adrenocorticotropic hormone (ACTH) release from the anterior pituitary, which then induces glucocorticoid release from the adrenal cortex. Sympathetic nerves innervate the adrenal directly or synapse at a sympathetic ganglion, causing peripheral release of epinephrine (Epi) and norepinephrine (NE).

The CRH and LC-norepinephrine systems are enjoined in a mutual positive regulation. Thus, hypothalamic CRH activates not only the HPA axis, but also the SNS through release of CRH from projections to the brain stem. Conversely, catecholaminergic (primarily norepinephrine) fibers that project from the LC to the hypothalamus activate CRH (Fig. 1).

## III. IMMUNE SYSTEM

The immune system defends the body against invading bacterial, viral, and parasitic pathogens as well as malignancies. For simplicity, the immune system can be divided broadly into two arms, the innate and the adaptive. The innate immune system is a rapid, nonspecific first line of defense against infection. Innate effector cells include phagocytes such as macrophages, monocytes, dendritic cells, and neutrophils, which bind and ingest pathogens nonspecifically and in some cases release microbicidal molecules. Additionally, granular lymphocytes known as natural killer (NK) cells contribute to innate immunity by recognizing surface changes on virus-infected and malignant cells and targeting them for destruction. The innate immune response also causes release of acute-phase proteins, which aid directly in pathogen clearance and may promote host survival by inducing fever and adaptive behavior, i.e., sickness behavior (malaise, appetite suppression).

In contrast, the adaptive immune system is slower to respond but is more specific and allows for the development of immunologic memory. Lymphocytes, which express unique antigen receptors, must bind the infectious agent and undergo multiple rounds of proliferation. A portion of the expanded population persists as memory cells, which respond more quickly and robustly if the same pathogen is encountered again. T lymphocytes, responsible for “cellular” immunity, recognize small peptides from pathogenic proteins that are presented on the surface of cells harboring a pathogen. When stimulated, T lymphocytes produce cytokines to aid macrophages or B lymphocytes, or directly kill the antigen-presenting cell. B lymphocytes are responsible for “humoral” immunity. Stimulation through the B-cell antigen receptor induces secretion of pathogen-specific antibodies that cause uptake, destruction, or neutralization of a pathogen.

The innate and adaptive arms of the immune system do not operate in isolation, but regulate one another. Importantly, the innate immune system produces cytokines, which help recruit lymphocytes to the site of injury and alert the adaptive immune system of danger. Cytokines fall generally into two "helper" categories, $T_H1$ and $T_H2$. $T_H1$ cytokines are proinflammatory and preferentially activate cellular immunity. $T_H2$ cytokines, in contrast, are anti-inflammatory and preferentially activate humoral immunity.

## IV. HPA ACTIVATION AND IMMUNE EFFECTS

Glucocorticoids, a downstream product of HPA activation, exert a wide array of immunomodulatory effects. A vast literature documents effects of glucocorticoids on various immune parameters; however, much of this research has been done *in vitro* or *in vivo* using synthetic glucocorticoids (i.e., dexamethasone, prednisolone) at supraphysiologic concentrations. Thus, the discussion here focuses on immune alterations observed with endogenous glucocorticoids produced in response to stress or CRH activation.

### A. Inflammation

A major role of glucocorticoids is to limit inflammation during an immune response. The proinflammatory cytokines interleukin-1β (IL-β), IL-6, and tumor necrosis factor α (TNFα) are released very early during an immune response. These cytokines act as signals of immunologic stress, activating the HPA axis at the level of the pituitary and hypothalamus. Glucocorticoids produced in response to hypothalamic–pituitary activation then inhibit cytokine production from leukocytes (Table 1). This negative regulatory loop is crucial during intense inflammatory processes, such as sepsis; without this feedback, elevated cytokine levels cause fever, vascular leakage, and subsequent organ failure, leading to death. Thus, linking the HPA axis with immune activation is an adaptive response, designed to control excess inflammation during immune responses. Clinicians take advantage of this property of glucocorticoids to treat chronic inflammatory diseases such as asthma and rheumatoid arthritis. However, a certain degree of inflammation is necessary for immune activation to signal the host of an invading pathogen. Thus, overactivity of the HPA axis during a non-immune-type stress (e.g., starvation, predator attack, social stress) may alter how the immune system responds to a subsequent pathogenic insult.

**TABLE 1 Effects of Glucocorticoids on the Immune System**

| Target | Effect |
|---|---|
| Precursor lymphocytes | Apoptosis |
| Pro-inflammatory cytokines | Decreased |
| Th 1/Th 2 balance | Shift toward Th 2 |
| Cytotoxic T lymphocytes | Decreased cytotoxicity |
| DTH response/leukocyte traffic to skin | Augmented (acute); inhibited (chronic) |
| Neutrophils | Increased numbers |
| B lymphocytes | Decreased IgG/memory responses |

### B. Leukocyte Populations

One well-studied immunomodulatory effect of HPA activation is lymphocyte death. B and T lymphocytes alike are sensitive to glucocorticoid-induced apoptosis, with immature lymphocytes being most sensitive (Table 1). Extended periods of HPA activation cause a striking decrease in thymus cellularity as well as more modest changes in spleen and lymph node size—the largest decrement occurring in lymphocyte populations.

However, it is important to appreciate that the effects of glucocorticoids are dependent on cell type and duration of exposure. For instance, glucocorticoids increase neutrophil numbers by increasing their release from the bone marrow and lengthening their half-life. In addition, short periods of stress affect lymphocyte populations through trafficking rather than cell death (Table 1). Glucocorticoids released during acute stress decrease most populations of circulating leukocytes, but these cells are redistributed to the skin and lymphoid organs (spleen and lymph nodes). This redistribution is thought to be adaptive because injury and/or infection are likely to accompany acute stress; thus, mobilization of immune cells to the site of pathogen entry (the skin) and to the sites of lymphocyte activation (peripheral lymphoid organs) prepares the host for possible infection.

### C. Leukocyte Function

Glucocorticoids can also affect immune function, both directly and through leukocyte trafficking as mentioned previously. For instance, the leukocyte redistribution during acute stress correlates with enhanced T-cell-mediated immune responses in the skin [delayed-type hypersensitivity (DTH) reaction].

This again demonstrates the idea that acute and chronic stress will often exert distinct immunomodulatory effects, because the same stressor (restraint in this case) applied chronically induces the opposite effect—the DTH response is decreased significantly (Table 1). This effect of chronic stress is due primarily to glucocorticoids, which may also inhibit cellular immunity by shifting the balance of cytokine response from a $T_H1$ to a $T_H2$ type. Additionally, glucocorticoids produced during chronic stress diminish the cytolytic function of cytotoxic T lymphocytes (Table 1). This impairment likely bears relevance to disease progression because it correlates with herpes virus reactivation during chronic social stress.

The effects of glucocorticoids on B lymphocyte function are less clear. Stress has been shown to decrease antibody responses to pathogens or protein antigens in both human and animal studies. Generally, immunoglobulin G (IgG) and secondary (memory) responses are more severely diminished, whereas IgM and primary responses are minimally affected. Some evidence indicates that the impairment may stem from lack of adequate T-cell help, rather than direct effects on B lymphocytes. However, few studies that examined the effect of stress on antibody production have determined the mediators of this suppression. In one case, SNS activation appears important, but in transgenic mice overexpressing CRH, elevated levels of glucocorticoids are likely involved.

## V. HPA-INDEPENDENT EFFECTS ON IMMUNITY

### A. Effects of SNS Activation

Activation of the SNS is also involved in immunomodulation. Effects can be mediated by circulating catecholamines released from the adrenals or by catecholamines released from nerve fibers that innervate lymphoid organs (Fig. 1). SNS activation accounts, in large measure, for stress- and CRH-mediated suppression of NK activity as well as some degree of T lymphocyte cytotoxicity (Table 2). In addition, catecholamines favor development of $T_H2$-type cytokines by the immune response (Table 2). It should be noted that distinct immune compartments may respond differently to these stress mediators; for example, splenic T-cell responses are suppressed by catecholamines whereas blood T-cell responses are unaffected. Also, in many circumstances immune effects of stress or CRH result from a combination of HPA and SNS activation, with partial effects seen when one pathway is blocked.

**TABLE 2 Non-HPA Effects of Stress on the Immune System**

| Catecholamines | Peripheral CRH/Ucn I, II, III |
|---|---|
| Shift toward Th 2 cytokines | Augment inflammation |
| Decrease cytotoxic responses | Increase pro-inflammatory cytokines |
| Decrease Ig responses | Augment mast cell degranulation |

### B. Direct Effects of CRH

CRH expression is most robust in the CNS and rarely detected in the circulation. However, CRH is also found peripherally, where it is produced locally in peripheral tissues or released from peripheral nerves. In addition, the discovery of several neuropeptides related to CRH adds to the family of ligands that can bind CRH receptors. The first of these, urocortin I (Ucn I), was originally identified in mammals by homology with the fish peptide, urotensin, which is involved in osmotic regulation. Subsequently, Ucn II and Ucn III (in humans also referred to as stresscopin-related peptide and stresscopin, respectively) were discovered based on sequence similarity with Ucn I. All three urocortins are expressed in the CNS as well as in the periphery, including immune compartments. Ucn II and Ucn III are selective agonists for the CRH type 2 receptor, and, unlike the CRH type 1 receptor, CRH type 2 receptor is highly expressed outside of the CNS. Therefore, immune actions previously attributed to CRH may, in fact, be mediated by a related peptide. Several types of leukocytes (e.g., T lymphocytes, neutrophils, and monocytes) express CRH receptors and, in the case of neutrophils, CRH receptor expression increases on immunologic or psychological stress. Most studies on direct effects of CRH in the immune system have been performed *in vitro* and demonstrate that CRH stimulates various leukocyte populations to increase proliferation or cytokine production. Studies that have shown a physiologic effect of endogenously produced CRH have primarily examined inflammatory processes.

In contrast to its action via glucocorticoids, peripheral CRH appears to be proinflammatory, increasing exudate in models of aseptic inflammation and enhancing proinflammatory cytokine production (Table 2). This effect may be due in part to vasodilation and increased vascular permeability, but leukocyte populations are probably direct targets as well. In addition, CRH induces mast cell degranulation via direct actions on CRH receptors expressed by these cells (Table 2). Histamine released from mast cell degranulation mediates acute inflammation and allergic responses. The precise source of CRH during these inflammatory processes is not certain, but inflamed tissue can show immunoreactivity for CRH, as can infiltrating leukocytes. Furthermore, peptidergic nerve fibers are associated closely with mast cells in perivascular spaces and lymphoid tissues.

## VI. CONCLUSIONS AND PERSPECTIVES

Stress clearly exerts profound influence over immunity through activation of CRH-responsive pathways. We are beginning to define these pathways and their immune targets, but a major challenge remains in determining the consequences of stress-induced immunomodulation on human health. It is simplistic, perhaps, to view stress as immunosuppressive. Activation of stress pathways results in an array of specific immune alterations; some cell populations or functions are augmented while others are inhibited. In acute stress, many of these changes are likely adaptive, regulating the immune response to avoid overactivation or directing effector cells to susceptible sites. Chronic activation of these pathways, however, threatens immune function and leads to serious impairments in health and survival against pathogens.

## Glossary

**catecholamines** A class of neurotransmitters, including epinephrine and norepinephrine, involved in autonomic nervous system actions.

**cytokines** Leukocyte-secreted proteins that affect the behavior of other cells.

**glucocorticoids** A class of adrenal-gland-secreted steroid hormones involved in regulating metabolism and immune activation.

**immunoglobulins** Also known as antibodies; a secreted form of B lymphocyte antigen receptors that bind to pathogens.

**leukocytes** Cells of hematopoietic origin; i.e., white blood cells.

**lymphocytes** Leukocytes that express antigen receptors encoded by rearranged gene segments.

**sympathetic nervous system** A division of the autonomic nervous system that mediates responses to stress, e.g., increased heart rate, blood pressure, and energy mobilization.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Corticotropin-Releasing Hormone (CRH) • Corticotropin-Releasing Hormone Pharmacology • Corticotropin-Releasing Hormone Receptor Signaling • Glucocorticoids and Autoimmune Diseases • Sex Hormones and the Immune System • Stress • Stress and Reproduction**

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# CRH

See *Corticotropin-Releasing Hormone*

# Cripto

Caterina Bianco[*], Nicola Normanno[†], David S. Salomon[‡], and Fortunato Ciardiello[*]

[*]*Università di Napoli "Federico II"* • [†]*Istituto Nazionale Tumori Napoli* • [‡]*National Cancer Institute, Maryland*

I. INTRODUCTION
II. THE EGF–CFC FAMILY IN EMBRYONIC DEVELOPMENT
III. BIOLOGICAL EFFECTS OF EGF–CFC PROTEINS IN MAMMALIAN CELLS
IV. CR-1 RECEPTOR AND DOWNSTREAM SIGNALING PATHWAYS
V. EXPRESSION OF CR-1 IN HUMAN CARCINOMAS AND PREMALIGNANT LESIONS

**Cripto is a glycoprotein member of the epidermal growth factor (EGF) related family of growth factors and has a length of 188 amino acids. Cripto protein is produced in large amounts by human undifferentiated teratocarcinoma cells and the murine P9 teratocarcinoma cell line. Cripto transcripts are detected only in undifferentiated cells and disappear from the cell after its differentiation induced by treatment with retinoic acid and hexamethylenbisacetamide. Cripto can function as a dominantly acting oncogene because its over expression in mammary epithelial cells can lead to transformation *in vitro*.**

## I. INTRODUCTION

The EGF (epidermal growth factor)–CFC (cysteine-rich domain) family consists of extracellular, cell-associated proteins that have been identified in several vertebrate species. Members of this family of genes include human Cripto-1 (CR-1) (also known as teratocarcinoma derived growth factor-1/TDGF-1) and criptin, mouse cripto-1 (Cr-1) and *cryptic*, chicken Cripto, *Xenopus laevis* FRL-1, and zebrafish *one-eyed pinhead* (*oep*).

Multiple copies of cripto-related sequences are present in the human and mouse genomes. At least five other human Cripto-1 (CR-1)-related pseudogenes and two mouse cripto-1 (Cr-1) pseudogenes have been identified. The EGF (epidermal growth factor)–CFC (cysteine-rich domain) family of proteins has a general profile consisting of a potential amino-terminal signal peptide, a variant EGF-like motif, a conserved CFC region, and a short hydrophobic carboxy-terminus that is essential for membrane anchorage by a glycosylphosphatidylinositol moiety. The overall sequence identity between these proteins is only 22–32%, whereas in the EGF-like domain the sequence similarity is nearly 60–70% and in the CFC domain is 35–48%. The variant EGF-like domain is a region of approximately 40 amino acids containing six cysteine residues that can form three intramolecular disulfide bonds. Although in the canonical EGF-like domain three loops (A, B, and C) can be recognized, the variant EGF-like domain in the EGF–CFC proteins lacks the A loop, possesses a truncated B loop, and has a complete C loop. This differentiates the EGF–CFC proteins from other members of the EGF family of growth factors such as EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), and heregulins. Since members of the EGF–CFC family lack the A loop, this suggests that these proteins do not directly bind to any of the *erb* B type I tyrosine kinase receptors, including the epidermal growth factor receptor (EGFR), *erb* B-2, *erb* B-3, and *erb* B-4. EGF–CFC proteins range from 171 to 230 amino acids with a core protein of 15–21 kDa. Native mouse and human cripto proteins range in size from 24 to 36 kDa but additional proteins ranging in size from 14 to 60 kDa have been identified by gel electrophoresis. This variation in size could be due to the removal of the hydrophobic signal peptide and to posttranslational modifications of the core protein. In fact, all the members of the EGF–CFC family, except for *oep*, are glycoproteins that contain a single N-glycosylation site and potential O-glycosylation sites. Recently, a single O-linked fucosylation site has been found between the second and the third cysteines in the EGF-like domain of all the EGF–CFC proteins. This rare modification is also present in the EGF domain of secreted proteins involved in blood coagulation or clot dissolution such as blood coagulation factor VII and urokinase plasminogen activator and in *Drosophila melanogaster* and mammalian Notch1, and it is critical for cell signaling and receptor binding. EGF–CFC proteins also contain several potential myristylation sites and consensus sequence for potential phosphorylation on serine and threonine by protein kinase C, cyclic AMP-dependent protein kinase A (PKA), and casein kinase II.

## II. THE EGF–CFC FAMILY IN EMBRYONIC DEVELOPMENT

During early mouse embryogenesis, low levels of Cr-1 mRNA expression can first be detected by reverse transcription–polymerase chain reaction in the inner cell mass of the postimplantation mouse blastocyst at day 4 of development. By *in situ* hybridization, Cr-1 mRNA expression is restricted to the epiblast cells of the primitive streak, to the developing mesoderm during gastrulation, and later to the myocardium of the truncus arterious of the developing heart. After day 8, very little expression of Cr-1 can be detected in the embryo, with the exception of the developing heart. Cr-1 protein expression has also been detected in the developing embryo by Western blot analysis. In concordance with Cr-1 mRNA expression, Cr-1 protein is found in the developing mesoderm at day 6.5 and in the myocardium of the developing heart at later fetal stages. The presence of Cr-1 in the developing cardiac outflow suggests that it may play an essential role during embryonic cardiac development and differentiation. In this respect, Cr-1-deficient pluripotential embryonic stem cells lose their ability to differentiate *in vitro* in cardiomyocytes, whereas they maintain the ability to differentiate in other cell types. Germ-line knockout of mouse Cr-1 is embryonically lethal, with homozygous embryos lacking a primitive streak and embryonic mesoderm. In particular, homozygous embryos are composed mostly of anterior neuroectoderm with a severe loss of mesoderm and endoderm. Furthermore, mouse Cr-1 is necessary for the correct orientation of the anterior–posterior (A–P) axis, since in homozygous embryos markers of the head organizer are localized in the distal visceral endoderm and markers of the primitive streak are located in the proximal epiblast. In contrast, heterozygous embryos are normal, suggesting that Cr-1 produced by the wild-type host is able to rescue the mutant phenotype. Genetic studies have also demonstrated that zebrafish *oep* and mouse cryptic are involved in the establishment of the left–right (L–R) embryonic axis. In this regard, mutation of *oep* in zebrafish and targeted disruption of cryptic in the mouse result in several L–R laterality defects including randomization of cardiac looping and atrial–ventricular septal defects, right pulmonary isomerization, inverted situs of the spleen, pancreas, and stomach, hyposplenia, and heterotaxia. Moreover, embryos that lack both maternal and zygotic activity of *oep* display a severe phenotype characterized by cyclopia, lack of head, trunk, mesoderm, and endoderm, and altered orientation of the A–P axis. Recent data have demonstrated that zebrafish *oep* is an essential component of the signaling pathway of the zebrafish Nodal-related genes *cyclops* and *squint* and can function as a possible co-receptor or competence factor for Nodal-related proteins. Nodal-related proteins and *oep* subsequently signal through the downstream activin type IIB and type I (ALK4) receptors that induce Smad-2 and Smad-4 activation. In fact, mutations in *oep*, *cyclops*, and *squint* display a similar phenotype, suggesting that they act in the same pathway. The *oep* mutant phenotype can be rescued by expression of a full-length or secreted carboxy-terminal truncated *oep* protein, overexpression of activin, or activation of downstream components in the activin signaling pathway, such as ALK4 or Smad-2. Recently, mouse Cr-1 has been shown to bind to the ALK4/ActRIIB receptor complex and to induce Smad-2 phosphorylation in a Nodal-dependent fashion when ectopically expressed in *Xenopus* oocytes.

## III. BIOLOGICAL EFFECTS OF EGF–CFC PROTEINS IN MAMMALIAN CELLS

The first evidence of the biological activity associated with cripto-1 in mammalian cells was provided by studies that demonstrated the ability of human CR-1 to transform mouse NIH 3T3 fibroblasts, mouse NOG-8 mammary epithelial cells, and mouse CID-9 mammary epithelial cells *in vitro*. However, NOG-8- and CID-9-transformed cells are unable to form tumors in nude mice, suggesting that additional genetic alterations are necessary to complete the tumorigenic phenotype *in vivo*. In rat CREF embryo fibroblasts or rat FRLT-5 thyroid epithelial cells that have been transformed by either c-Ha-*ras* or c-Ki-*ras* proto-oncogenes, respectively, expression of rat Cr-1 is up-regulated. Reversion of the transformed phenotype by overexpression of a *ras* suppressor gene, K*rev*-1, results in a loss of expression of rat Cr-1, suggesting that Cr-1 might significantly contribute to cellular transformation induced by p21 *ras*. Overexpression of mouse Cr-1 in primary mouse mammary epithelial cells induces lateral ductal branching and clonal expansion of ductal hyperplasias when these cells are reintroduced into the cleared mammary fat pad of syngeneic, ovariectomized virgin mice. However, mammary carcinomas have not been observed in these hyperplastic outgrowths even after several *in vivo* transplantations. When inserted into the fourth inguinal mammary gland of ovariectomized mice, slow-release Elvax pellets containing a peptide corresponding to the EGF-like domain of

CR-1 produce a 20-fold increase in DNA synthesis in the adjacent epithelial cells and a 3-fold increase in lateral ductal branching in the areas surrounding CR-1 pellets. A comparable *in vitro* effect has also been observed with recombinant human CR-1 protein or with different mouse mammary epithelial cells overexpressing mouse Cr-1. In fact, NMuMG, TAC-1, and EpH4 mouse mammary epithelial cells overexpressing Cr-1 when cultured in a three-dimensional type 1 collagen gel matrix are able to form branching tubules. Furthermore, all the Cr-1-transfected mouse mammary epithelial cells and human Caski cervical carcinoma cells overexpressing CR-1 exhibit an increase in migratory potential in Boyden chamber studies and in wound healing assays. Exogenous, recombinant CR-1 protein is also able to stimulate chemotaxis of wild-type EpH4 cells and can induce scattering of NOG-8 mammary epithelial cells grown at low density as colonies on plastic. The ability of Cr-1 to stimulate both scattering and branching morphogenesis in collagen gels is similar to the effects induced by hepatocyte growth factor or transforming growth factor-β1 (TGF-β1) in mammary epithelial cells. The scattering effect is characterized by a change in the morphology of the epithelial cells to a more fibroblastic-like phenotype and is characterized by a decrease in cell–cell adhesion. In this respect, loss of expression in β-catenin or an increase in tyrosine phosphorylation of β-catenin can inhibit its association with E-cadherin, facilitating cell scattering and migration. In fact, Cr-1-overexpressing mouse mammary epithelial cells exhibit an enhanced basal level of tyrosine phosphorylation of β-catenin, resulting in a decreased association with E-cadherin.

Another property associated with CR-1 is the ability to modulate milk protein expression in HC-11 mouse mammary epithelial cells. HC-11 mouse mammary epithelial cells express the milk protein β-casein at confluency after exposure to the lactogenic hormones dexamethasone, insulin, and prolactin (DIP). Similar to other EGF-related peptides, pretreatment of HC-11 cells with CR-1 during logarithmic growth induces a competency response to DIP with respect to the induction of the milk protein β-casein. In contrast, simultaneous treatment of HC-11 cells with CR-1 in the presence of DIP inhibits β-casein expression. This inhibitory effect of CR-1 on β-casein expression in response to DIP is not unique to this mouse mammary epithelial cell line, since β-casein expression is also inhibited by CR-1 in primary mouse mammary explant cultures established from midpregnant mice. The ability of CR-1 to block lactogenic hormone-induced expression of β-casein is mediated through a p21$^{ras}$-dependent, phosphatidylinositol 3-kinase (PI3K) pathway. CR-1 can also induce apoptosis in HC-11 mouse mammary epithelial cells when they are grown under serum-restricted and survival factor-deprived conditions. Apoptosis is mediated through the induction of a caspase-3-like protease and down-regulation of the expression of the anti-apoptotic protein Bcl-$X_L$. However, the effects of CR-1 on apoptosis in some tumor cell lines are completely different. In this respect, CR-1 can function as a survival factor protecting human cervical carcinoma cells from apoptosis through a PI3K/AKT-dependent signaling pathway.

## IV. CR-1 RECEPTOR AND DOWNSTREAM SIGNALING PATHWAYS

Human CR-1 is not able to directly bind to the EGFR or to *erb* B-2, *erb* B-3, or *erb* B-4 type I receptor tyrosine kinases when they are ectopically expressed either alone or in various pairwise combinations in Ba/F3 mouse pro-B lymphocytes or mouse 32D myeloid cells. Rather, CR-1 binds to a specific high-affinity saturable receptor that does not interact with other EGF-related peptides. Interestingly, CR-1 can indirectly enhance the specific tyrosine phosphorylation of *erb* B-4 in several different mouse and human mammary epithelial cells either by facilitating heterodimerization of *erb* B-4 with a novel CR-1 receptor or by indirectly stimulating *erb* B-4 tyrosine phosphorylation through a soluble *src*-like tyrosine kinase. Finally, chemical cross-linking of $^{131}$I-CR-1 to mouse and human mammary epithelial cells identifies two bands of 60 and 120 kDa, suggesting the presence of a novel multicomponent receptor complex for CR-1. Recently, using an immunoglobulin CR-1 fusion protein, specific binding and localization of CR-1 receptor were detected in mouse mammary epithelial cells and in the developing mouse mammary gland. Interestingly, the immunoglobulin–CR-1-binding pattern in the mouse mammary gland significantly correlates with the expression of the mouse Cr-1 protein, suggesting that Cr-1 may function in an autocrine pathway with its putative receptor during development of the mammary gland.

CR-1, on binding to its unknown receptor, can activate the *ras*/*raf*/mitogen-activated protein kinase (MAPK) pathway in several types of nontransformed and malignant human mammary epithelial cells. In particular, CR-1 full-length protein or a p47 refolded peptide corresponding to the EGF-like domain can induce a rapid and transient increase in the tyrosine phosphorylation of p66, p52, and p46 isoforms of the adapter protein Shc (Src homology and collagen), facilitating its association with growth factor receptor-binding protein 2 and leading to the subsequent downstream activation of p42 and p44 isoforms of MAPK. Furthermore, CR-1 is able to stimulate the tyrosine phosphorylation of the p85 regulatory subunit of PI3K and induce the phosphorylation of AKT and glycogen synthase kinase 3β in HC-11 mouse mammary epithelial cells and in SiHa human cervical carcinoma cells. Although CR-1 is involved in a Nodal signaling pathway during embryonic development, activation of an activin/TGF-β signaling pathway in mammalian cells by CR-1 has not been demonstrated yet.

## V. EXPRESSION OF CR-1 IN HUMAN CARCINOMAS AND PREMALIGNANT LESIONS

CR-1 is overexpressed, relative to noninvolved adjacent tissue, in approximately 50–90% of carcinomas that arise in the colon, breast, stomach, pancreas, gallbladder, ovary, endometrium, and cervix. Additionally, enhanced expression of CR-1 has also been detected in premalignant lesions in the breast, colon, stomach, and gallbladder, suggesting that CR-1 overexpression might be an index of precancerous changes in some of these tissues. For example, the frequency and the level of CR-1 expression in colon adenomas or intestinal metaplasia in the stomach are directly correlated with the size, histological subtype, and degree of dysplasia in these lesions. The gradual increase in CR-1 expression that is observed in the multistage process that evolves from colon adenoma to carcinoma suggests that CR-1 might be an early marker for malignant transformation in the colon. In this context, immunoreactive CR-1 has been detected in approximately 60% of colon mucosa specimens from individuals with a high risk of colorectal carcinoma, but in only 20% of colon mucosa from low-risk individuals. A statistically significant correlation has also been found between a high proliferation rate and increased expression of CR-1 in the mucosa specimens from high-risk individuals. Finally, expression of CR-1 in the adjacent noninvolved colon epithelium surrounding colon tumors is significantly correlated with increased lymph node involvement and with a higher rate of recurrence of colorectal cancer.

Since CR-1 is expressed at high levels in several different types of human malignancies, different approaches have been successfully used to impair CR-1 expression in several human carcinoma cells. In fact, the *in vitro* growth of human colon, breast, ovarian, and teratocarcinoma cell lines is significantly inhibited following treatment with specific anti-CR-1 antisense oligonucleotides or transfection with antisense expression vectors directed against CR-1. Furthermore, inhibition of CR-1 expression in GEO colon cancer cells significantly reduces their tumorigenicity *in vivo* in nude mice. Data from several studies suggest that CR-1 cooperates with other EGF-related peptides in sustaining the proliferation of human cancer cells through autocrine pathways. Therefore, a combination of different antisense oligonucleotides directed against various EGF-related growth factors has been used in human cancer cells. In fact, treatment of human colon, breast, and ovarian carcinoma cells with a combination of antisense oligonucleotides directed against AR, TGF-α, and CR-1 results in a more efficient growth inhibition *in vitro* when compared with treatment with a single oligonucleotide. In addition, a combination of the three antisense oligonucleotides directed against AR, TGF-α, and CR-1 is able to induce programmed cell death in human breast cancer cells, whereas treatment with a single oligonucleotide does not increase apoptosis, suggesting that EGF-related growth factors cooperate in the regulation of cell survival. Finally, a combination of AR, TGF-α, and CR-1 antisense oligonucleotides significantly inhibits the growth of human colon xenografts *in vivo* in nude mice. A significant reduction in microvessel count can also be observed in mice treated with a combination of the three antisense oligonucleotides. Furthermore, interesting results have been obtained using combinations of CR-1 antisense oligonucleotide with conventional chemotherapeutic drugs in colon cancer cells. In a clonogenic assay, an additive *in vitro* growth inhibitory effect has been observed when GEO cells are pretreated with different concentrations of 5-fluorouracil, adriamycin, *cis*-platinum, or mitomycin followed by treatment with a CR-1 antisense oligonucleotide. Recently, promising results have been obtained using a combination of a novel mixed-backbone CR-1 antisense oligonucleotide with agents that block intracellular signal transduction pathways, such as humanized anti-EGFR monoclonal antibody (MAb C225) and 8-Cl-cAMP, an inhibitor of type I PKA. Low doses of each agent produce only a 15–35% growth inhibition in GEO colon cancer cells *in vitro*. In contrast, a combination of CR-1 antisense oligonucleotide with either MAb C225 or 8-Cl-cAMP induces a synergistic anti-proliferative effect *in vitro* in GEO cells. Furthermore, when the three agents are combined, a nearly complete inhibition of GEO growth *in vitro* is observed. Combination of the three agents also induces apoptosis, whereas a single treatment or combination of two agents does not have any apoptotic effect.

## Glossary

**cripto** Cripto is a novel growth factor that is normally expressed during embryonic development in a select group of fetal tissues. Cripto is also expressed at high levels in several types of human cancers.

**embryogenesis** The development of an individual from a fertilized ovum; the process of embryo formation.

**nucleotide** The hydrolysis product of a nucleic acid, consisting of a purine or pyrimidine base combined with a ribose or deoxyribose sugar and a phosphate group; a phosphate ester of a nucleoside.

## See Also the Following Articles

**Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor**

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# Crosstalk of Nuclear Hormone Receptors with STAT Factors

Carrie S. Shemanko* and Bernd Groner†

*University of Calgary • †Institute for Biomedical Research, Frankfurt*

I. INTRODUCTION
II. STAT SIGNALING PATHWAY
III. ESTROGEN RECEPTOR PLUS STAT5B
IV. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS PLUS STAT5B
V. GLUCOCORTICOID RECEPTOR PLUS STAT1 AND PU.1
VI. GLUCOCORTICOID RECEPTOR PLUS STAT3
VII. GLUCOCORTICOID RECEPTOR PLUS STAT5
VIII. CONCLUSIONS

**Nuclear hormone receptors interact with signal transduction and transcription (Stat) molecules to regulate genes. Crosstalk, the convergence of different signaling pathways, occurs between the receptors and the Stat family of proteins**

anti-proliferative effect *in vitro* in GEO cells. Furthermore, when the three agents are combined, a nearly complete inhibition of GEO growth *in vitro* is observed. Combination of the three agents also induces apoptosis, whereas a single treatment or combination of two agents does not have any apoptotic effect.

## Glossary

**cripto** Cripto is a novel growth factor that is normally expressed during embryonic development in a select group of fetal tissues. Cripto is also expressed at high levels in several types of human cancers.

**embryogenesis** The development of an individual from a fertilized ovum; the process of embryo formation.

**nucleotide** The hydrolysis product of a nucleic acid, consisting of a purine or pyrimidine base combined with a ribose or deoxyribose sugar and a phosphate group; a phosphate ester of a nucleoside.

## See Also the Following Articles

**Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor**

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# Crosstalk of Nuclear Hormone Receptors with STAT Factors

CARRIE S. SHEMANKO* AND BERND GRONER†

*University of Calgary* • †*Institute for Biomedical Research, Frankfurt*

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VIII. CONCLUSIONS

**Nuclear hormone receptors interact with signal transduction and transcription (Stat) molecules to regulate genes. Crosstalk, the convergence of different signaling pathways, occurs between the receptors and the Stat family of proteins**

**during gene transcription and provides an additional level of regulation. The molecular mechanisms by which crosstalk is accomplished depend on the nuclear hormone receptor, the Stat protein, and the promoter sequence.**

## I. INTRODUCTION

### A. Transcriptional Regulation by Nuclear Receptors as Ligand-Dependent Transcription Factors and as Cofactors

The classic model of transcriptional regulation by nuclear hormone receptors involves binding of monomers, homodimers, or retinoid X receptor (RXR) heterodimers to DNA regulatory sequences in the promoters of target genes. The DNA response element for monomers is a single hexameric site. Homodimers or heterodimers bind to two hexameric motifs arranged as palindromes, inverted palindromes, or direct repeats. The arrangement and spacing of the DNA response element help to determine binding specificity. Some hexameric response elements can mediate the transcriptional response from more than one ligand–receptor complex, resulting in transcriptional control of target genes from overlapping signals (e.g., thyroid hormones and retinoic acid).

Dimerization is mediated by the multifunctional ligand-binding domain (LBD) as well as the DNA-binding domain (DBD) of the nuclear hormone receptors (NHRs). The DBD zinc fingers mediate specific and high-affinity DNA binding as well as DNA-based receptor dimerization. There are three types of heterodimeric complexes: unoccupied (unliganded) heterodimers, nonpermissive heterodimers, and permissive heterodimers. Permissive heterodimers, such as peroxisome proliferator-activated receptor/RXR (PPAR/RXR), can be activated by ligands of either RXR or its partner and are synergistically activated in the presence of both ligands. In nonpermissive heterodimers, RXR often acts as a silent partner. Dimers can also bind negative response elements and cause gene repression. These sites have been found for the glucocorticoid receptor (GR) and thyroid hormone receptor (TR). Negative or positive regulation of gene transcription usually results from the interaction of NHRs with corepressors or co-activators.

A second, nonclassic mode of nuclear hormone receptor function has been recently discovered. This mechanism of transcriptional regulation is based on specific protein–protein interactions and allows the integration of seemingly unrelated signaling pathways. Crosstalk was initially suspected when the simultaneous activation of two transcription factors resulted in a different qualitative or quantitative regulation of genes compared to a situation in which only one of the factors was activated. The effect could be observed even in the regulation of genes that contained a DNA response element in their promoter region for only one of the two transcription factors. The crosstalk of different families of transcription factors with NHRs has been investigated in detail. Examples of this regulatory principle include the functional interaction between the GR and activator protein 1 (AP-1) and also the between the GR and signal transducers and activators of transcription (Stats). Other transcription factor families, such as nuclear factor κB (NF-κB)/Rel, Oct, and CCAAT/enhancer-binding protein (C/EBP), have also been shown to participate in crosstalk with members of the NHR family. The comprehensive molecular mechanisms for the negative or positive crosstalk that can ensue have not been fully elucidated, though many important aspects have been described. Here the focus is on data concerned with crosstalk between the NHR and the Stat family of transcription factors.

Transcriptional crosstalk can be the consequence of different molecular interactions (Fig. 1). First, it has been shown that two transcription factors with specific DNA-binding potential interact, but that only one of them is bound to DNA via its DNA-binding domain—the second factor is recruited to the promoter through protein–protein interactions. Second, each transcription factor can recognize different elements in the same promoter of the target gene. Third, transcription factor binding sites are arranged so that they are neighboring or overlapping each other, and that proximity influences the activity or binding of the other protein. It enables direct protein–protein interactions, but can also cause competition for DNA binding sites or limiting amounts of cofactors. The effect of this third mode of crosstalk is often manifested in gene repression. Elucidation of the different modes of receptor action, classic and transcriptional crosstalk mediated, have been aided by the utilization of various ligands or gene mutations.

## II. STAT SIGNALING PATHWAY

In mammalian cells, seven genes encode seven Stat family transcription factors: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. Diversity of function is further enhanced by the production

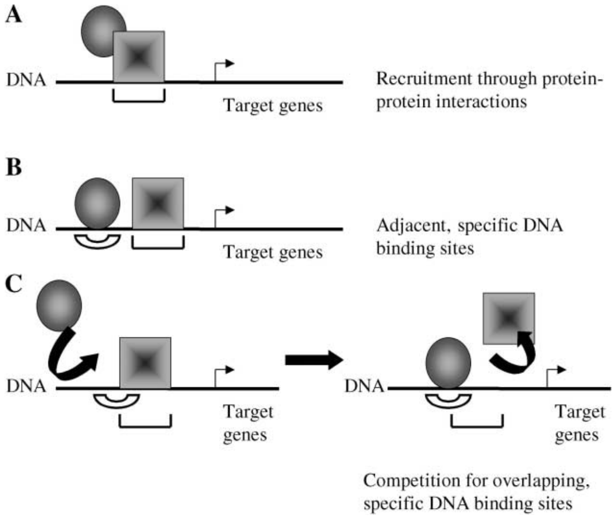
A
DNA
Target genes
Recruitment through protein-protein interactions
B
DNA
Target genes
Adjacent, specific DNA binding sites
C
DNA
Target genes
DNA
Target genes
Competition for overlapping, specific DNA binding sites

**FIGURE 1** Transcriptional crosstalk. (A) Protein–protein interactions result in the recruitment of two transcription factors to the promoter sequence, only one of which is bound to a specific DNA sequence. (B) Each transcription factor recognizes its own element in the gene promoter, with the possibility of direct or indirect contact. (C) Specific DNA binding sites can involve overlapping sequences. The binding of factors is determined by relative affinity and can result in mutual interference. An example of this (not shown) is the binding of YY1 and Stat5 to the β-casein promoter, where, as long as YY1 is bound, the promoter is repressed. On Stat5 activation, YY1 is replaced by Stat5.

of alternatively spliced variants and proteolytic posttranslational processing. Stat1, Stat3, and Stat4 mRNAs have been found to be alternatively spliced and Stat5 can be proteolytically processed to yield alpha (long) and beta (short) forms. The short forms lack transcription-activating domains and may act as naturally occurring dominant negative proteins. Cytokines and/or growth factors can specifically activate Stat molecules. Specific ligand binding initially causes receptor activation and tyrosine phosphorylation by the associated Janus kinase (JAK) (Fig. 2). Stat molecules, present in a latent state in the cytoplasm, interact with the phosphotyrosyl sites in the intracellular domain of the receptor through their SH2 domains and are in turn tyrosine phosphorylated by JAK. These activated molecules now dimerize and translocate to the nucleus, where they regulate the transcription of target genes. The target genes are involved in a wide range of cellular processes, including growth, differentiation, and apoptosis. The diversity of the signals that mediate their effects through Stat factors and the various functions affected by Stat factors in different cell types make it reasonable to assume that combinatorial mechanisms are at work to mediate the cellular responses.

## III. ESTROGEN RECEPTOR PLUS STAT5B

Estrogen receptor-α (ER-α) and ER-β bind the steroid hormone estrogen and xenoestrogens, and are also activated by peptide growth factor signals to mediate effects on growth and development in a wide range of tissues. ERs can crosstalk with other DNA-bound transcription factors in order to regulate gene expression. This can cause up-regulation of genes with promoter binding sites for Sp-1 and AP-1 (differential effects with the two ERs) and can negatively affect genes with regulatory sites for NF-κB, GATA-1, and C/EBP. The receptors bind additional cofactors in order to integrate multiple signals.

Crosstalk can also be observed between the ER and Stat5. This crosstalk results in an estrogen-dependent inhibition of Stat5-mediated transcriptional induction and is accompanied by decreased Stat5 DNA binding and tyrosine phosphorylation. The latter two events also occur independently of ER ligand. These observations, however, have not been consistently made in all cell types (Fig. 3A). Synergism between ER-α or ER-β and Stat5b was also reported. Transcription induced by prolactin-activated Stat5b was enhanced by the expression of either ER, though with a larger quantitative effect through ER-β, in a mammary epithelial cell line. The synergism required activation of Stat5b, but not ligand activation of the ERs. The DNA-binding domain and the hinge of the ERs were essential for the functional interaction with Stat5b, though it was not determined if the effect was mediated by a direct physical interaction. Recruitment of additional cofactors or binding to the promoter might be required.

It has also been determined that other members of the steroid hormone receptor family crosstalk with Stat5. Mineralocorticoid, progesterone, and glucocorticoid receptors synergize with Stat5 in the induction of transcription from a Stat-responsive promoter. Conversely, Stat5 negatively interferes with transcription from promoters carrying DNA response elements for those receptors. The androgen receptor does not appear to interact with Stat5.

## IV. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS PLUS STAT5B

The NHR subfamily of PPARs consists of three receptor types, PPAR-α, PPAR-δ, and PPAR-γ, each

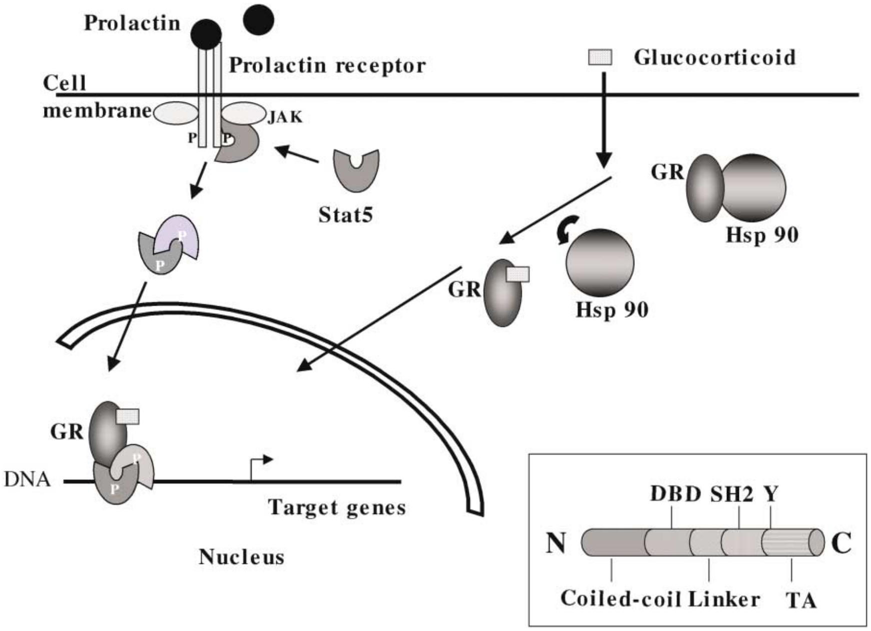

**FIGURE 2** The prolactin–JAK–Stat pathway. Left-hand pathway: Prolactin or another cytokine binds to its receptor and induces receptor and Janus kinase (JAK) activation. This creates a docking site for latent Stat5 or for the Stat appropriate to the signal. Stat is in turn phosphorylated and activated, whereupon it heterodimerizes or homodimerizes and translocates to the nucleus to regulate gene transcription. Right-hand pathway: Glucocorticoid activates the glucocorticoid receptor (GR), which is held in a responsive state by heat-shock protein 90 (Hsp 90), and the ligand/GR unit translocates to the nucleus to bind to promoters carrying a glucocorticoid response element, or to promoters dependent on Stat5 for transcription. Inset: Functional domains of the Stat proteins, showing the coiled-coil domain, which is important for protein–protein interactions; the DNA-binding domain (DBD); the flexible linker region; the SH2 domain; the transactivation domain (TA); and the tyrosine residue (Y), which, when phosphorylated, represents Stat activation.

of which is developmentally and tissue-specifically expressed. They are ligand-activated transcription factors that recognize PPAR response elements (PPRE) when heterodimerized with RXR. PPARs can repress gene transcription by negatively interfering with NF-κB, and AP-1.

PPAR-α mediates hepatic peroxisome proliferation after induction by nongenotoxic carcinogens. This response is inhibited by growth hormone (GH), a peptide hormone that stimulates the activation of Stat1, Stat3, Stat5a, and Stat5b. It was experimentally shown that the GH inhibition of PPAR-α reporter gene transcription was mediated specifically by Stat5b. Stat5b tyrosine phosphorylation, its DBD, and its transcription activation domain plus the amino-terminal activation function-1 (AF-1) transactivation domain of PPAR-α were essential to mediate the inhibition. Direct protein–protein interactions were not detected (Fig. 3B). It is possible that Stat5b competes with PPAR-α for an essential co-activator, though p300 and steroid receptor co-activator-1 (SRC-1) were shown not to be involved in this case. This negative crosstalk is most likely the molecular basis for the regulation of PPAR-α-dependent responses by GH. This could be important in the modulation of inflammatory responses by leukocytes, which have an intact GH-JAK-Stat pathway, or by inflammatory cells, which have the capacity to synthesize and secrete GH. GH may also inhibit tumor development by suppressing the PPAR-α response to peroxisome proliferators that have been linked to carcinogenesis. GH-activated Stat5b also inhibited the transcriptional activity of PPAR-γ, PPAR-δ, and thyroid hormone receptor.

## V. GLUCOCORTICOID RECEPTOR PLUS STAT1 AND PU.1

Inflammatory and immune responses are signaled through interferon γ (IFNγ) and Stat1 as well as corticosteroids, which activate the GR. These signals converge on the Fcγ receptor I (FcγRI) gene promoter region through crosstalk between Stat1 and the GR. Monocyte-specific transcription of this gene is

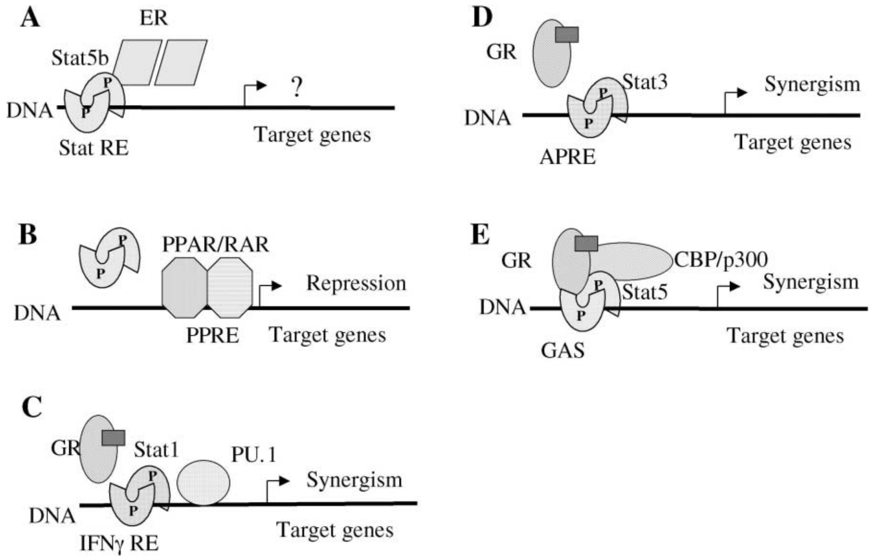

**FIGURE 3** Various mechanisms of transcriptional crosstalk between NHRs and Stats. (A) Stat5b is able to affect gene regulation by its interaction with the estrogen receptor (ER) (Stat RE, Stat response element). (B) Stat5b, in the absence of direct contact with a peroxisome proliferator-activated receptor (PPAR-α, -γ, or -δ), causes inhibition of transcription on a promoter with a PPAR response element (PPRE). (C) The glucocorticoid receptor (GR) does not interact with Stat1 but promotes transcription, with PU.1 providing cell type specificity (IFNγ RE, interferon γ response element). (D) The GR acts indirectly with Stat3 to enhance transcriptional response on the interleukin-6 response element of the rat $\alpha_2$-macroglobulin promoter response element (APRE). (E) The GR and Stat5 interact to promote transcription on a promoter with Stat binding sites (CBP, $Ca^{2+}$/cAMP response element-binding protein; GAS, interferon γ-activated sequence).

mediated by a second response element that binds PU.1, an Ets family transcription factor, and activation of this gene is dependent on both Stat1 and PU.1. Action of the GR results in an enhancement of Stat1-dependent gene activation. Crosstalk between Stat1 and the GR does not appear to involve direct physical interaction between the two proteins (Fig. 3C). The use of GR variants showed that the functional crosstalk does not depend on the interaction of the GR with this promoter, although its DNA-binding domain was important, as was its hormone-dependent transactivation function. The DNA-binding function of active Stat1 was essential. It was shown that although the GR activated transcription on the Stat1-responsive promoter, Stat1 did not affect GR-dependent transcription. The requirement of protein synthesis in the process indicates that the GR may enhance transcription by inducing synthesis of transcriptional coregulators. Recruitment of cofactors could not be ruled out, although CREB-binding protein (CBP), an important co-activator for both GR and Stat5, was not a rate-limiting component. Up-regulation of the-high affinity Fcγ receptor I in monocytes is important for endocytosis of immune complexes and in antibody-mediated cytotoxic reactions. The GR integrates its signal differently with Stat1 and Stat5 in order to regulate gene expression that results in coordinated cytokine and glucocorticoid signaling.

## VI. GLUCOCORTICOID RECEPTOR PLUS STAT3

Synergism of the GR with Stat3 has been documented on the Stat3-responsive promoter, $\alpha_2$-macroglobulin. The synergism is dependent on the binding of interleukin-6 (IL-6)/JAK-activated Stat3 to the DNA response element, $\alpha_2$-macroglobulin promoter response element (APRE). Testing two other Stat3-responsive promoters containing a dual Stat3 response element plus a cAMP-responsive element did not result in synergism with the GR, indicating specificity for the APRE. There was no detectable physical interaction between the GR and Stat3 (Fig. 3D), although the synergism appears to involve an undefined co-activator process, and not secondary gene expression. IL-6 activation of acute-phase proteins such as $\alpha_2$-macroglobulin is important in host defense mechanisms and relies on glucocorticoid for maximal expression.

## VII. GLUCOCORTICOID RECEPTOR PLUS STAT5

The most extensively characterized crosstalk between a NHR and a Stat factor is that between the GR and Stat5 (Fig. 2). An inverse regulatory mechanism has been described through which the two transcription factors modulate each other's function. The GR can participate in the induction of Stat5-responsive promoters and enhance the extent of Stat5 induction (Fig. 3E). Stat5 can inhibit GR-mediated transcription from a GR-responsive promoter. It was shown that this interaction depends on the specific DNA binding of Stat5, whereas the GR DNA-binding domain does not seem to be important. Activation of Stat5 is essential, with the stimulus provided by prolactin or IL-2 on respective responsive promoters. Ligand binding of the GR was nonessential, but its AF-1 transactivation domain was critical. Both Stat5a and Stat5b were shown to interact with GR *in vivo*, independent of lactogenic hormone signaling in mammary epithelial cells and at all stages of mammary gland development. After lactogenic hormone induction, the GR was identified as part of the Stat5/DNA-bound complex, indicating a functional coupling between Stat and NHR pathways. The GR appears to promote the active conformation of C/EBPβ as well as prolong the activated state of Stat. p300/CBP is an important co-activator for Stat5 and the GR, but was not rate limiting in the repression mediated by Stat5 on a GR-responsive promoter. The *in vivo* interaction between the GR and Stat5 was also detected in liver extracts and in NIH 3T3 cells. Mice in which the GR has been inactivated through homologous recombination die shortly after birth. Mice homozygous for a GR mutation that disrupts dimerization and DNA binding are viable, indicating that the mutant GR still functions in the mice. One possible explanation is that crosstalk with Stat5, and possibly other transcription factors, is responsible for the survival of the mice.

Stat5 and the GR also synergize in the regulation of the whey acidic protein gene promoter, another milk protein gene. It is possible that a different mechanism is involved, because this regulation depends on the presence of the nuclear factor I (NF-I) response element, the Stat5 site, and clustered GR half-sites. NF-IB2 was found to participate in full synergism with Stat5 and GR. NF-IA4, NF-IB2, and NF-IX1 are developmentally regulated in the mammary gland but interact with the DNA response element on this promoter with differential specificity. A common GR-based mechanism conveys crosstalk with Stats and cytokine signaling in coordination with cell type-specific gene expression. GR crosstalks with Stat1, Stat3, Stat5a, Stat5b, and Stat6 in order to add additional levels of regulation to cytokine/growth factor/peptide hormone signaling.

## VIII. CONCLUSIONS

The interaction of nuclear hormone receptors with Stat molecules is a common mechanism for specific gene regulation that is shared among many different family members, such as estrogen, glucocorticoid, mineralocorticoid, progesterone, and thyroid hormone receptors and PPAR-α, PPAR-γ, and PPAR-δ. It is likely that other members of the NHR family will be found to interact with one of the Stat family members in order to achieve transcription crosstalk. Although the crosstalk of NHRs with Stats to regulate gene transcription is a common theme, the molecular mechanisms appear to depend on the Stat, the NHR, and the promoter sequences involved. Physical interaction of the NHR with the Stat is not always detectable (in Fig. 3, compare B–D to 3A and 3E, showing interaction), nor is DNA binding of both partners commonly found (Fig. 3). Another important question to be answered concerns the qualitative and quantitative effects of crosstalk. Two transcription factors that act positively in gene induction on their own can result in gene repression on simultaneous activation and complex formation.

NHRs mediate their transcriptional regulatory function through at least two qualitatively different classes of cofactors—co-activator and corepressor molecules and their associated chromatin remodeling components. The recruitment of these cofactors is tightly regulated, ordered, and necessary for transcription initiation and attenuation of hormone-regulated genes. How multiple cofactors are recruited into large transcription complexes and what determines the balance between positively and negatively acting factors are the focus of current research. The potential of Stats to recruit co-activators and co-repressors with their associated chromatin remodeling functions makes them formally quite similar to TRs and RARs, members of the NHR family. Through direct physical interactions or through adjacent DNA binding sites Stats might be able to influence the timing of NHR binding to the DNA, the recruitment of chromatin remodeling complexes, assembly of the transcription preinitiation complex, and recruitment of cofactors, and thus modify the cellular response to nuclear hormones.

## Glossary

**co-activator** Protein or protein complex that can act as a bridging factor to recruit other cofactors to the DNA-bound transcription factor, can act to bridge DNA-bound factors to the basal transcription machinery, or can acetylate nucleosomal histones or transcription factors.

**corepressor** Can bind transcription activators and inhibit formation of active complexes, or can recruit histone deacetylases, causing transcriptional repression.

**crosstalk** The point at which two signaling pathways converge to provide an additional level of regulation.

**DNA response element** The DNA nucleotide sequence of a gene promoter that confers specific interaction with a signal-activated DNA-bound transcription factor.

**transcriptional crosstalk** The crosstalk of two signaling pathways converging on a gene promoter.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Crustacean Endocrine Systems

Dave Borst

*Illinois State University*

I. INTRODUCTION
II. ECDYSTEROIDS AND THE CONTROL OF CRUSTACEAN MOLTING
III. METHYL FARNESOATE, A CRUSTACEAN JUVENILE HORMONE
IV. NEUROENDOCRINE COMPOUNDS
V. ANDROGENIC GLAND HORMONE
VI. SUMMARY

**Crustaceans produce a number of endocrine compounds, including neuropeptides, steroids, and terpenoids. Some of these compounds are**

## Glossary

**co-activator** Protein or protein complex that can act as a bridging factor to recruit other cofactors to the DNA-bound transcription factor, can act to bridge DNA-bound factors to the basal transcription machinery, or can acetylate nucleosomal histones or transcription factors.

**corepressor** Can bind transcription activators and inhibit formation of active complexes, or can recruit histone deacetylases, causing transcriptional repression.

**crosstalk** The point at which two signaling pathways converge to provide an additional level of regulation.

**DNA response element** The DNA nucleotide sequence of a gene promoter that confers specific interaction with a signal-activated DNA-bound transcription factor.

**transcriptional crosstalk** The crosstalk of two signaling pathways converging on a gene promoter.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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VI. SUMMARY

**Crustaceans produce a number of endocrine compounds, including neuropeptides, steroids, and terpenoids. Some of these compounds are**

**secreted by glandular tissues such as the Y-organ, the mandibular organ, and the androgenic gland. Others are secreted by neurosecretory centers, such as the sinus gland-X-organ complex and the pericardial organs, or by cells in the gut and brain. These compounds regulate a wide range of essential physiological processes in this large and diverse group of anthropods.**

## I. INTRODUCTION

Crustaceans form a diverse, monophyletic group of over 50,000 known species. Although most crustaceans live in marine environments, many species live in freshwater and terrestrial habitats. Currently, this group is divided into 11 classes, 6 of which contain only a few (<100) species. The 5 largest crustacean classes are the *Brachiopoda*, which includes fairy shrimp, brine shrimp (*Artemia*), and water fleas (*Daphnia*); the *Ostracoda*, which includes seed shrimp; the *Copepoda*, which includes both parasitic and free-living species, some of the latter being major contributors to marine plankton; the *Cirripedia*, which includes the barnacles and several crustacean parasites (*Sacculina*); and the *Malacostraca*, which contains over half of the known crustaceans. This last class has several important orders, including the *Isopoda* (woodlice and pillbugs), *Amphipoda* (sand fleas and whale lice), and *Decapoda* (lobsters, crabs, and shrimp). Because of their large size, commercial importance, and relative ease in handling, the endocrine systems of decapods have been most thoroughly studied.

Crustaceans use a variety of endocrine compounds, including neuropeptides, steroids, and terpenoids. Some of these compounds are produced by endocrine systems that show complex (second and third order) levels of regulation. As shown in Fig. 1, the major crustacean endocrine tissues include the following:

1. The sinus gland–X-organ complex of the eyestalk (the source of several neuroendocrine peptides);
2. the Y-organ (the source of ecdysteroids);
3. the mandibular organ (the source of methyl farnesoate);
4. the pericardial organs (the source of peptides that regulate cardiac function); and
5. the androgenic gland (the source of androgenic hormone).

However, other tissues (e.g., the gonads, gut, brain, and thoracic ganglia) also produce hormones. Together, these endocrine compounds regulate a wide range of important physiological processes, including molting, metamorphosis, reproduction, osmoregulation, metabolism, coloration, and behavior.

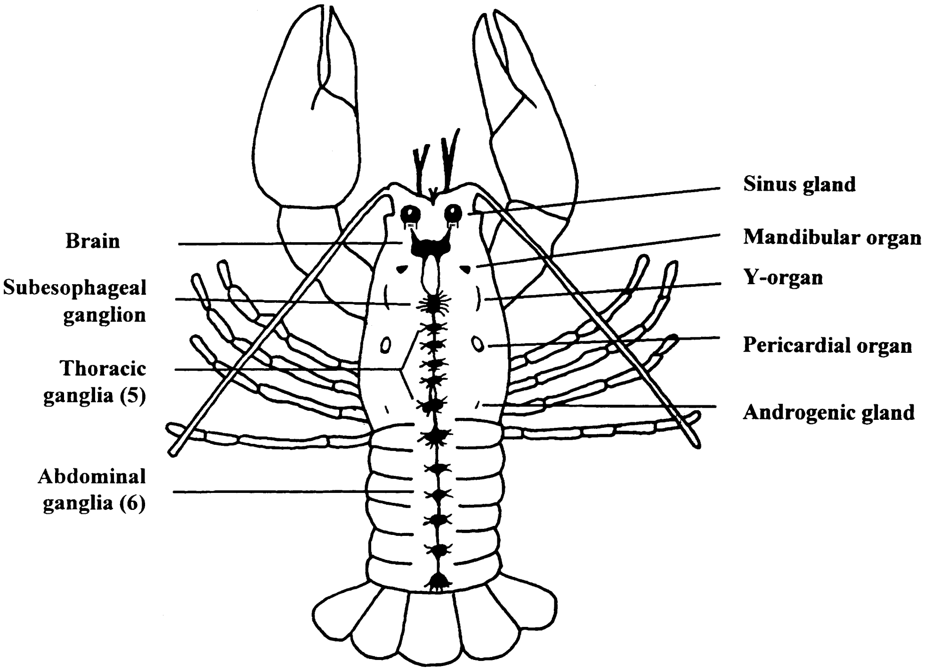


FIGURE 1 The location of the major endocrine and neuroendocrine tissues in crustaceans.

## II. ECDYSTEROIDS AND THE CONTROL OF CRUSTACEAN MOLTING

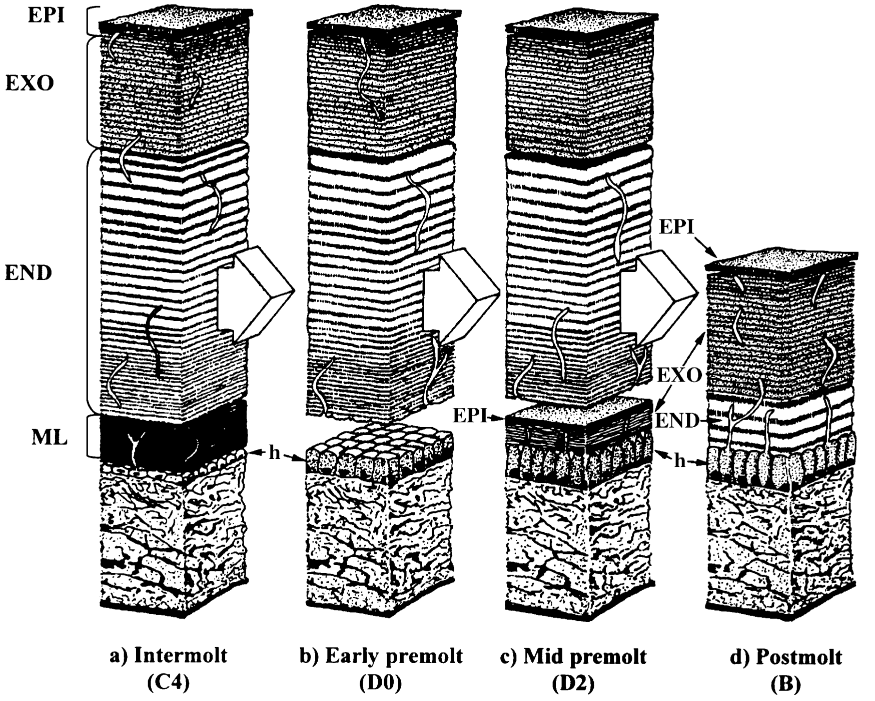
### A. The Molt Cycle

Arthropods are covered by an exoskeleton that provides a framework for physical support, muscle attachment, and protection from trauma and pathogens. The benefits of the exoskeleton are offset by the limitations it places on somatic growth, requiring the periodic molting of the old exoskeleton and its replacement with a larger one. Thus, crustaceans and other arthropods show an incremental rather than a continuous increase in body size, although the growth of internal tissues to fill the new exoskeleton continues throughout the molt cycle. In some crustaceans (e.g., lobsters and crayfish), growth is indeterminate, and molting continues throughout the life of the animal, albeit at a slower pace as an individual grows larger. In other species (e.g., many crabs), growth is determinate, and molting ends when an individual reaches sexual maturity. The elaboration of a new exoskeleton is metabolically expensive, so the processes involved in the molt cycle tend to dominate the activities of crustaceans. In addition, the soft shell of a freshly molted animal limits its movement and makes it more vulnerable to predation.

The structure of the exoskeleton during intermolt (stage C4) is shown in Fig. 2a. The exoskeleton is produced by a single layer of epidermal cells (the hypodermis) lying directly below it. The exoskeleton has four major layers: the outermost epicuticle, the exocuticle, the endocuticle, and the inner membranous layer. These layers are composed of various combinations of lipids, chitin (a carbohydrate polymer), protein, and calcium salts. Some parts of the exoskeleton (e.g., the arthrodial membranes that join leg and body segments) are not calcified to maintain their flexibility. The hypodermis secretes the layers of the exoskeleton sequentially during different stages of the molt cycle, allowing the stages to be identified by the morphological characteristics of the exoskeleton. The stages of the molt cycle are typically divided into postmolt (metecdysis, stages A, B, and $C_{1-3}$), intermolt (anecdysis, stage $C_4$), premolt (proecdysis, stage $D_{0-3}$), and molting (ecdysis, stage E).

### B. The Ecdysteroids

Among the more important hormones regulating molting are the ecdysteroids. Several ecdysteroids



a) Intermolt (C4) b) Early premolt (D0) c) Mid premolt (D2) d) Postmolt (B)

FIGURE 2 Changes in the crustacean exoskeleton during the molt cycle: EPI, epicuticle; EXO, exocuticle; END, endocuticle; ML, membranous layer. The epithelial cells in the hypodermis (h) hypertrophy when the exoskeleton is being synthesized. Reprinted with permission from Aiken, D. E., and Waddy, S. L. (1992). The growth process in crayfish. *Rev. Aquat. Sci.* 6, 335–381. Copyright CRC Press, Boca Raton, Florida.

have been detected in crustaceans, including ecdysterone and ponasterone A (see Fig. 3). The most common form appears to be ecdysterone (20-hydroxyecdysone; β-ecdysone, crustecdysone), the same compound that regulates molting in insects. The rise in ecdysteroid levels from the low or undetectable levels of the intermolt stage heralds the onset of premolt. During premolt, ecdysteroid levels rise, stimulating the hypodermis to secrete hydrolytic enzymes that dissolve the membranous layer, separating the hypodermis from the exoskeleton (apolysis, stage D0; see Fig. 2b). As premolt continues, these hydrolytic enzymes digest the inner layers of the exoskeleton, allowing the recovery of its components (e.g., sugars and amino acids) and causing it to become thinner and weaker. During premolt (stages D1–D3), the hypodermis also produces and secretes the materials that form the epicuticle and exocuticle layers of the new exoskeleton, which remains soft and pliable inside the old exoskeleton (Fig. 2c).

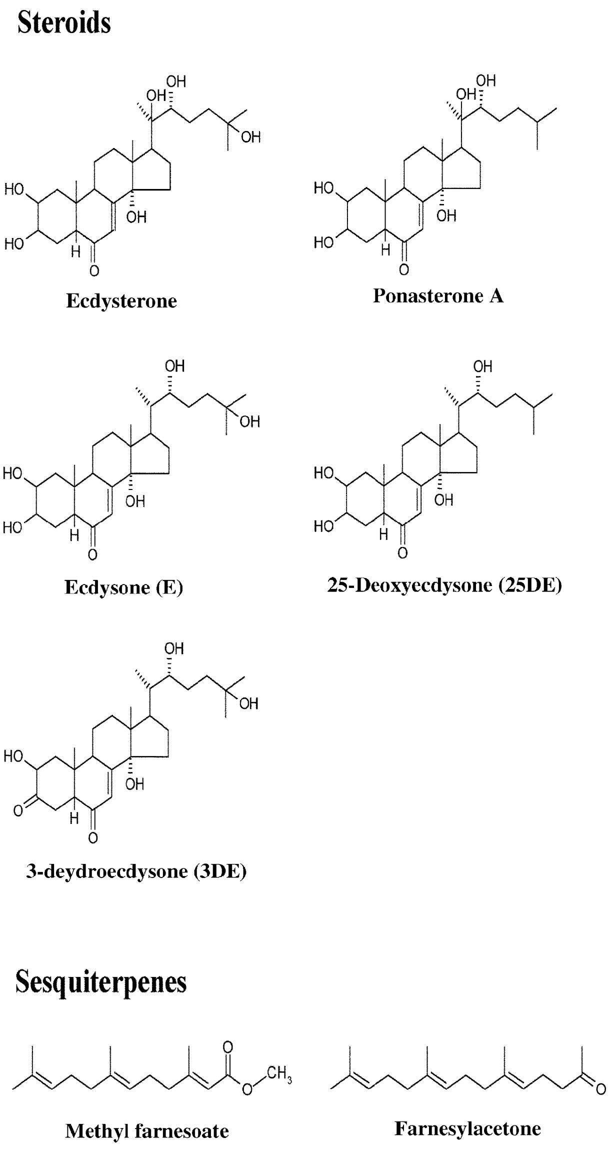


FIGURE 3 Structures of some crustacean steroid hormones and sesquiterpene hormones.

Immediately prior to molting, ecdysteroid levels fall to very low levels. During this period, water is absorbed, increasing the hydrostatic pressure within the old exoskeleton. This pressure ruptures the thoracicoabdominal membrane, allowing the individual to withdraw from the old exoskeleton (ecdysis, stage E). Molting is rapid and typically requires only a few minutes. The newly molted animal absorbs more water, which expands the new exoskeleton to a volume approximately 50% greater than before molting. The exoskeleton is then hardened by tanning and calcification (stage A). During the postmolt period (stages B–$C_2$), ecdysteroid levels remain low or undetectable, and their hypodermis secretes the endocuticle (Fig. 2d). At the end of the postmolt period (stage $C_3$), the hypodermis secretes the membranous layer, completing the exoskeleton. The intermolt period (stage $C_4$) is typically the longest phase of the molt cycle and is used to accumulate metabolic reserves. However, in rapidly molting individuals, it may be curtailed or absent.

In adults, the intermolt period is often used for reproduction. Indeed, the metabolic demands of reproduction and molting in females usually make these processes mutually exclusive. In species with indeterminate growth (e.g., the American lobster), females often have a 2-year cycle, molting 1 year and reproducing the next. In contrast to reproduction, mating in many decapods is synchronized with molting. Sexually mature individuals have complex courting behaviors that begin a few days prior to the female molt, and most matings occur within 24 h after the female molts. The male's spermatophore is carried by the female until spawning occurs.

In addition to the hypodermis, other tissues are affected by the molt cycle. For example, there are extensive changes in the muscle tissue of crustaceans during the molt cycle. This is quite obvious in the American lobster, in which the muscle of the large claws (first pereiopods) is much larger in diameter than the proximal segments of the appendage. Up to 60% of the claw mass may be lost during premolt, facilitating its withdrawal during the molt. Molting also provides a time for the regeneration of lost limbs. Limb loss usually occurs along a predesignated breaking point (the autotomy plane), followed by the rapid formation of the autotomy membrane and a scab. Within a few days, a blastema forms on the stump of the appendage. During premolt, this differentiates into a limb bud, and at molting, the limb bud will expand to form a replacement limb or claw.

### C. The Y-Organ and Its Regulation

In decapods, ecdysteroids are produced by the Y-organs, which are located in the anterior region of the cephalothorax. Ecdysteroids are synthesized from dietary cholesterol, which circulates in the hemolymph bound to high-density lipoprotein (HDL). The cholesterol–HDL complex binds to HDL receptors on the Y-organ and enters the cells by endocytosis. The Y-organs convert the cholesterol to ecdysteroid precursors that are further converted to the active steroids by peripheral tissues. The identities of these precursors vary somewhat by taxonomic group. In the crab *Cancer antennarius*, the Y-organs secrete ecdysone (E) and 3-dehydroecdysone (3DE) (or 3-oxo-ecdysone). These are converted in peripheral tissues by 3-reductase (which converts 3DE to E) and ecdysone 20-monooxygenase (which converts E to ecdysterone). In contrast, Y-organs from the green crab *Carcinus maenas* secrete E and 25-deoxyecdysone, which are converted peripherally to ecdysterone and ponasterone A, respectively, by ecdysone 20-monooxygenase (see Fig. 3).

The production of ecdysteroids by the Y-organ is regulated in part by molt-inhibiting hormone (MIH), a peptide hormone produced by the sinus gland–X-organ complex of the eyestalk (see below). MIH inhibits the production of ecdysteroids by the Y-organ, so eyestalk ablation often (but not always) causes an animal to precociously molt. MIH binds to high-affinity binding sites on Y-organ membranes, increasing cytosolic levels of cyclic nucleotides [either cyclic AMP (cAMP) or cyclic GMP (cGMP), depending on the species]. For example, treatment of Y-organs from *Car. maenas* with MIH causes cGMP levels to rise 60-fold. Elevated levels of cyclic nucleotides cause a decrease in both HDL uptake and the conversion of cholesterol to ecdysteroids. Ecdysteroid production is stimulated by extracellular calcium, in part by increasing the activity of cyclic nucleotide phosphodiesterase, thereby lowering levels of cAMP or cGMP. In some species, calcium has also been reported to stimulate the activity of protein kinase C, which then increases the production of ecdysteroids.

In addition to MIH, there appear to be other compounds that regulate Y-organ activity. One source of these compounds is the regenerating limb bud, which appears to contain compounds that accelerate and delay molting. For example, removal of multiple limbs from the land crab *Gecarcinus lateralis* during intermolt can stimulate the precocious entry of an individual into premolt, allowing the lost limbs to be more rapidly regenerated. This appears to be due to a stimulatory compound produced by the limb bud. In contrast, removal of limbs during early premolt before a critical period delays molting until the limb buds are sufficiently developed to allow their regeneration. This appears to be due to an inhibitory compound released by the regenerating limb.

### D. Ecdysteroid Receptors

Ecdysteroids affect target tissues by binding to nuclear receptor proteins that act as transcription factors. The ecdysteroid receptors (EcRs) belong to a large superfamily of related proteins that includes receptors for vertebrate steroid hormones, vitamin D, retinoic acids, thyroid hormones, and ecdysteroids. The amino acid sequences of these proteins are highly conserved in regions that are critical for receptor function. Thus, the amino acid sequences of the DNA-binding domain and the ligand-binding domain of the EcR in the fiddler crab *Uca pugilator* and the fruit fly *Drosophila melanogaster* are 89 and 59% identical, respectively. In addition, insect EcR forms a heterodimer with ultraspiracle (USP), a homologue of the vertebrate retinoid-X receptor (RXR). Crustacean tissues also contain a RXR receptor protein that has a high degree of similarity to the USP protein.

## III. METHYL FARNESOATE, A CRUSTACEAN JUVENILE HORMONE

Juvenile hormone (JH) is a family of sesquiterpene compounds found in insects. JH regulates a number of processes, most notably metamorphosis, reproduction, and caste formation in social insects. Methyl farnesoate (MF) is the only member of this family that has been detected in crustaceans, and it appears to have analogous functions in these species (see Fig. 3). Administration of MF to larvae of the lobster *Homarus americanus* and the prawn *Macrobrachium rosenbergii* has been shown to retard their growth and development. Likewise, hemolymph levels of MF in the spider crab *Libinia emarginata* are elevated in males that are reproductively mature, suggesting a role in male reproduction and morphogenesis. MF levels have been correlated with ovarian development in several species, and treatment of *Procambarus clarkii* with MF stimulates ovarian maturation.

MF has also been shown to stimulate ecdysteroid synthesis by Y-organs *in vitro*, suggesting a role in the molt cycle. Finally, MF levels in the green crab *Car. maenas* have been shown to rise chronically in response to osmotic stress, suggesting a possible role in osmoregulation.

In decapods, MF is synthesized in the mandibular organ. The final step in the MF pathway is the activity of the enzyme farnesoic acid O-methyl transferase, which has been characterized and cloned. The activity of this enzyme and the overall production of MF by the mandibular organ are regulated in part by a neuroendocrine peptide(s) from the sinus gland–X-organ complex that inhibits the activity of the mandibular organ [mandibular organ-inhibiting hormone (MO-IH); see below]. Evidence for this peptide came from observations that eyestalk ablation caused the hypertrophy of the mandibular organ and increased production of methyl farnesoate. MO-IH appears to inhibit MF synthesis by increasing intracellular levels of cyclic nucleotides (cAMP or cGMP, depending on the species).

MF is hydrophobic, causing it to bind to hemolymph proteins. In some species, there appear to be preferred proteins for this binding, and these may be analogous to the JH-binding proteins found in insect hemolymph. Unlike insects, many of which have JH-specific esterase in their hemolymph, crustaceans do not have hemolymph esterases that degrade MF. Even so, the half-life of MF in hemolymph appears to be quite short (ca. 45 min), probably a consequence of its adsorption by cellular membranes.

## IV. NEUROENDOCRINE COMPOUNDS

Neuroendocrine cells have been identified in the eyestalk, the brain, the subesophageal ganglion, the thoracic ganglia, and other parts of the central nervous system. Some of these cells release their products in neurohemal organs, collections of enlarged nerve terminals that store and release neuroendocrine peptides. In decapod crustaceans, there are several neurohemal organs, the best studied being the pericardial organs and the eyestalk sinus glands.

### A. Pericardial Organs

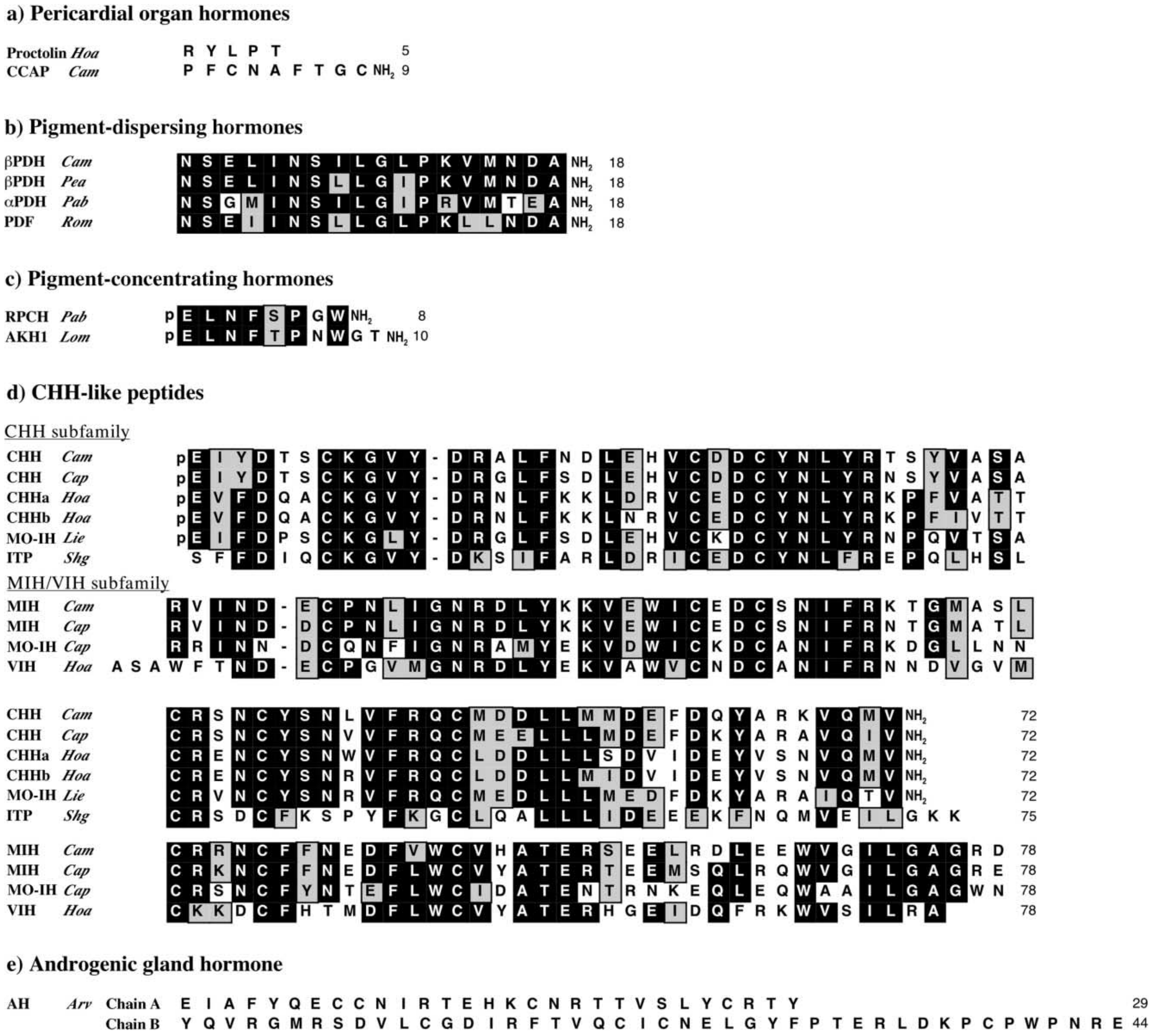
The pericardial organs (POs) are found in the pericardial cavity near the heart and are formed by axons projecting from the thoracic ganglia. As implied by their location, the POs control cardiac function by releasing peptides (see Fig. 4a) and biogenic amines (e.g., serotonin, octopamine, and dopamine). The neuropeptides released by the POs include crustacean cardioactive peptide (CCAP, a nonapeptide, variants of which are found in several invertebrate phylla) and proctolin (a pentapeptide, first isolated as a factor that stimulates hindgut contractions in the cockroach). These affect the rate and amplitude of heart muscle contractions, respectively. In some species, CCAP may also stimulate respiratory rhythm. Proctolin-like immunoactivity has been found widely throughout the nervous system, suggesting that it may have additional functions.

### B. The Sinus Gland

The sinus glands (SGs) are formed from axons projecting from the X-organ, a cluster of neurosecretory neurons located in the most proximal (the medulla terminalis) of the three neural ganglia in the eyestalk. These axons project distally to the juncture of the medulla interna and the medulla externa, where they form the sinus gland (see Fig. 5). This structure stores and releases two major classes of neuroendocrine peptides: pigment-effector hormones, which regulate the movement of pigment granules in chromatophores and retinal cells, and the crustacean hyperglycemic hormone (CHH) family of peptides, which regulate a wide variety of physiological processes including glucose levels, osmoregulation, reproduction, and molting. Some of these SG neuropeptides are also found in other tissues, where they may be systemically released to act as hormones or locally released to act as neuromodulators.

### C. Pigment-Effector Hormones

These neuropeptides control pigment granule movement in chromatophores and retinal pigment cells. Chromatophores are integumental cells with cytoplasmic extensions that contain pigment granules (the most common colors are red, white, yellow, and black). Most chromatophores are monochromatic and appear colored when the granules are dispersed and are largely colorless when the granules are condensed. These cells are responsible for the rapid and reversible changes in the color of crustaceans. Retinal pigment cells control the amount of light that illuminates the eye. These cells include the distal pigment cells, which lie along the length of each ommatidium (the functional subunit of the crustacean compound eye). In light-adapted eyes, hormones disperse the pigment granules in these cells,



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a) Pericardial organ hormones

Proctolin Hoa     R Y L P T                5
CCAP      Cam     P F C N A F T G C NH2    9

b) Pigment-dispersing hormones

βPDH  Cam    N S E L I N S I L G L P K V M N D A NH2  18
βPDH  Pea    N S E L I N S L L G I P K V M N D A NH2  18
αPDH  Pab    N S G M I N S I L G I P R V M T E A NH2  18
PDF   Rom    N S E I I N S L L G L P K L L N D A NH2  18

c) Pigment-concentrating hormones

RPCH  Pab    pE L N F S P G W NH2        8
AKH1  Lom    pE L N F T P N W G T NH2   10

d) CHH-like peptides

CHH subfamily
CHH    Cam        pE I Y D T S C K G V Y - D R A L F N D L E H V C D D C Y N L Y R T S Y V A S A
CHH    Cap        pE I Y D T S C K G V Y - D R G L F S D L E H V C D D C Y N L Y R N S Y V A S A
CHHa   Hoa        pE V F D Q A C K G V Y - D R N L F K K L D R V C E D C Y N L Y R K P F V A T T
CHHb   Hoa        pE V F D Q A C K G V Y - D R N L F K K L N R V C E D C Y N L Y R K P F I V T T
MO-IH  Lie        pE I F D P S C K G L Y - D R G L F S D L E H V C K D C Y N L Y R N P Q V T S A
ITP    Shg           S F F D I Q C K G V Y - D K S I F A R L D R I C E D C Y N L F R E P Q L H S L
MIH/VIH subfamily
MIH    Cam             R V I N D - E C P N L I G N R D L Y K K V E W I C E D C S N I F R K T G M A S L
MIH    Cap             R V I N D - D C P N L I G N R D L Y K K V E W I C E D C S N I F R N T G M A T L
MO-IH  Cap             R R I N N - D C Q N F I G N R A M Y E K V D W I C K D C A N I F R K D G L L N N
VIH    Hoa       A S A W F T N D - E C P G V M G N R D L Y E K V A W V C N D C A N I F R N N D V G V M

CHH    Cam        C R S N C Y S N L V F R Q C M D D L L M M D E F D Q Y A R K V Q M V NH2      72
CHH    Cap        C R S N C Y S N V V F R Q C M E E L L L M D E F D K Y A R A V Q I V NH2      72
CHHa   Hoa        C R E N C Y S N W V F R Q C L D D L L L S D V I D E Y V S N V Q M V NH2      72
CHHb   Hoa        C R E N C Y S N R V F R Q C L D D L L M I D V I D E Y V S N V Q M V NH2      72
MO-IH  Lie        C R V N C Y S N R V F R Q C M E D L L L M E D F D K Y A R A I Q T V NH2      72
ITP    Shg        C R S D C F K S P Y F K G C L Q A L L L I D E E E K F N Q M V E I L G K K    75

MIH    Cam        C R R N C F F N E D F V W C V H A T E R S E E L R D L E E W V G I L G A G R D  78
MIH    Cap        C R K N C F F N E D F L W C V Y A T E R T E E M S Q L R Q W V G I L G A G R E  78
MO-IH  Cap        C R S N C F Y N T E F L W C I D A T E N T R N K E Q L E Q W A A I L G A G W N  78
VIH    Hoa        C K K D C F H T M D F L W C V Y A T E R H G E I D Q F R K W V S I L R A        78

e) Androgenic gland hormone

AH  Arv  Chain A   E I A F Y Q E C C N I R T E H K C N R T T V S L Y C R T Y                                 29
         Chain B   Y Q V R G M R S D V L C G D I R F T V Q C I C N E L G Y F P T E R L D K P C P W P N R E   44
```

FIGURE 4 Structures of crustacean peptide hormones. The amino acid sequences of several crustacean endocrine peptides and related insect peptides. Most of these peptides have been sequenced in several species but space permits the display of only a few examples. Residues on a black background are identical; those on a gray background have similar chemical properties. Some peptides contain a modified N-terminal amino acid (pE, pyroglutamate) and/or C-terminal amide ($NH_2$). (a) Peptides present in the pericardial organs. Both peptides are also found in insects. (b) Pigment-dispersing hormones (α and β) and the related insect peptide PDF. (c) Pigment-concentrating hormone and the related insect peptide AKH. (d) CHH-like peptides, including the related insect peptide ITP. The CHH-like peptides are grouped into two subfamilies, the CHH subfamily (which includes lobster CHHa, the lobster MIH) and the MIH/VIH subfamily. The sequences of subfamily members are compared in this figure. (e) Androgenic gland hormone, which contains two peptides (the A chain and the B chain) connected by disulfide bonds (not shown). AH, androgenic gland hormone; AKH1, adipokinetic hormone; CCAP, crustacean cardioactive peptide; CHH, crustacean hyperglycemic hormone; ITP, ion transport peptide; MIH, molt-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; PDF, pigment-dispersing factor; PDH, pigment-dispersing hormone; RPCH, red pigment-concentrating hormone; VIH, vitellogenin (gonad)-inhibiting hormone. *Arv*, *Armadillidium vulgare*; *Cam*, *Carcinus maenas*; *Cap*, *Cancer pagurus*; *Hoa*, *Homarus americanus*; *Lie*, *Libinia emarginata*; *Lom*, *Locusta migratoria*; *Pab*, *Pandalus borealis*; *Pea*, *Penaeus aztecus*; *Rom*, *Romalea microptera*; *Shg*, *Shistocerca gregaria*.

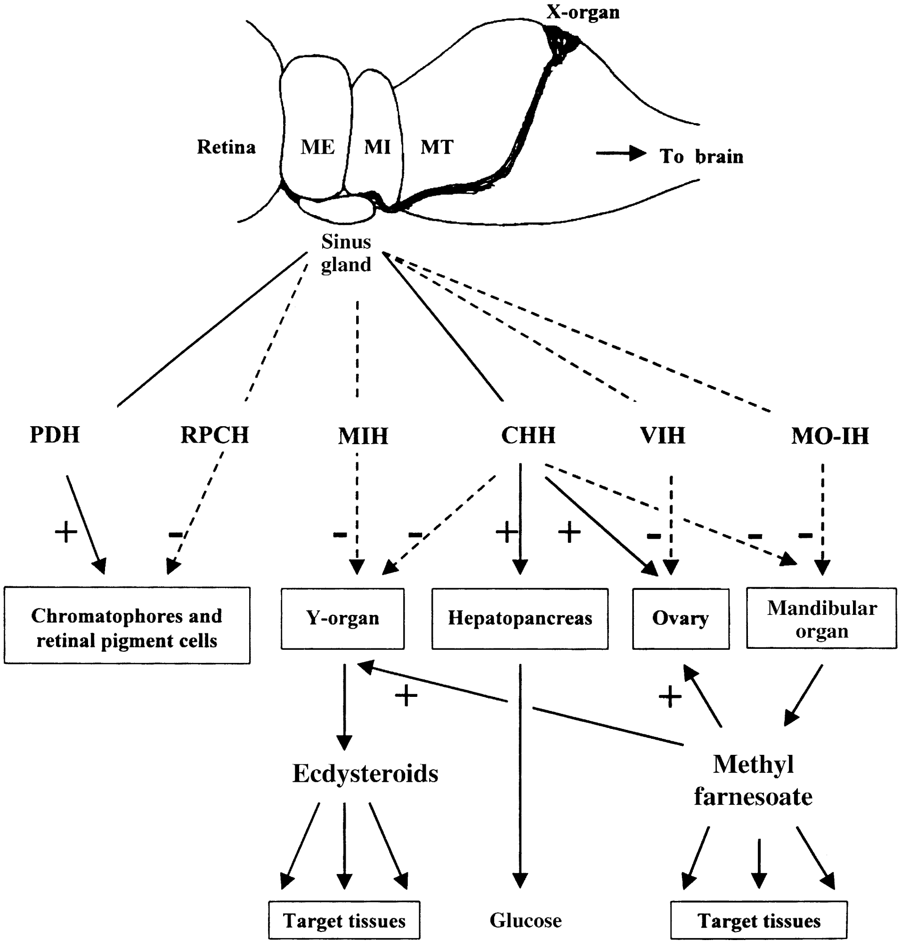
X-organ
Retina
ME
MI
MT
To brain
Sinus gland
PDH
RPCH
MIH
CHH
VIH
MO-IH
+
-
-
-
+
+
-
-
-
Chromatophores and retinal pigment cells
Y-organ
Hepatopancreas
Ovary
Mandibular organ
+
+
Ecdysteroids
Methyl farnesoate
Target tissues
Glucose
Target tissues

FIGURE 5 Neuroendocrine compounds released by the eyestalk sinus gland. Hormones with a stimulatory effect (including pigment granule dispersion) have solid arrows and a plus sign, hormones with an inhibitory effect (including pigment granule concentration) have dashed arrows and a minus sign. ME, medulla externa; MI, medulla interna, MT, medulla terminalis; PDH, pigment-dispersing hormone; RPCH, red pigment-concentrating hormone; MIH, molt-inhibiting hormone; CHH, crustacean hyperglycemic hormone; VIH, vitellogenesis (or gonad)-inhibiting hormone; MO-IH, mandibular organ-inhibiting hormone.

screening the photoreceptor of the ommatidium from light entering through neighboring ommatidia. In dark-adapted eyes, the pigment granules are concentrated, allowing more light to reach the photoreceptor.

Although the sinus gland is one of the richest sources of pigment-effector hormones, these hormones have a widespread distribution in the crustacean nervous system, where they act as neuromodulators. These peptides have been isolated from many crustaceans, and their eyestalks usually contain two types of mutually antagonistic factors. Peptides related to pigment-dispersing hormone (PDH) cause pigment dispersion in chromatophores and light adaptation pigment movements in retinal pigment cells. In contrast, hormones such as red pigment-concentrating hormone (RPCH) cause pigment concentration in chromatophores and dark adaptation in retinal pigment cells.

### *1. PDH*

The chromatographic separation of eyestalk extracts usually reveals multiple peaks of PDH activity. These peptides form a family with two related branches (α-PDH and β-PDH), and each branch contains multiple variants (see Fig. 4b). The β-PDH variants appear to be more widely distributed. PDH peptides show substantial sequence similarity, including the same length (18 residues), conserved residues at seven positions, and conservative amino acid substitutions at other positions. The precursors for β-PDH have been identified by cDNA sequencing and have similar structures, including a signal peptide of 20–23 residues and a precursor-related peptide with 33 or 34 residues. PDH is closely related to a family of insect peptides known as PDFs (pigment-dispersing factors), named initially for their effects on crustacean chromatophores (see Fig. 4b). PDFs are involved in insect biological rhythms and PDF immunoreactivity co-localizes with PER (the clock gene) immunoreactivity in *D. melanogaster.*

### *2. RPCH*

Chromatographic analysis of eyestalk extracts indicates that at least two pigment-concentrating hormones are present in eyestalk extracts. Only RPCH has been isolated and sequenced (see Fig. 4c). This octapeptide has a pyroglutamate at the N-terminal end and an amide at the C-terminal end. The identical peptide appears to be present in over 15 species, indicating that it is highly conserved among crustaceans. RPCH is closely related to adipokinetic hormone (AKH), an insect family of decapeptides (see Fig. 4c). RPCH is found in other tissues, including the lobster stomatogastric ganglion, where it acts as a neuromodulator. Cloning studies show that the precursor of RPCH in the green crab *Car. maenas* contains a signal peptide of 25 amino acids and a precursor-related peptide of 74 amino acids.

## D. The CHH Family of Neuroendocrine Peptides

The sinus gland contains a number of neuropeptides with 70–80 residues that regulate a wide variety of physiological functions. These peptides are related to CHH, and some (but not all) of these peptides have hyperglycemic activity. In addition to their similarity in size, they share similarities in their amino acid sequences, including the conserved locations of 6 cysteine residues that form three disulfide bridges. In addition to CHH, they include MIH, MO-IH, and vitellogenesis-inhibiting hormone [(VIH) also known as gonad-inhibiting hormone (GIH)].

### *1. CHH*

CHH is the best-studied member of this family. CHHs have 72 or 73 residues, a pyroglutamate at the N-terminus, and an amide at the C-terminus (see Fig. 4d). CHH is synthesized as a preprohormone of several peptides arranged from the N-terminal end in the following order: a 26-amino-acid signal peptide, a precursor-related peptide of variable length (33–38 amino acids), an N-terminal dipeptide fragment, CHH, and a C-terminal fragment with 2–4 residues. Many species contain multiple CHH peptides that have closely related amino acid sequences. In addition, some species have stereoisomers of the same peptide that differ in the D- or L-configuration of the third amino acid (phenylalanine). These stereoisomers are due to the posttranslational modification of the protein. Both types of variants can have different activities and functions. The amino acid sequences of CHHs from different species are highly conserved and show greater than 55% identity. CHH is also related (40% identity) to the ion transport peptide, which stimulates chloride transport in the hindgut of the desert locust, *Shistocerca gregaria* (see Fig. 4d).

CHH regulates many physiological functions, including hemolymph levels of both glucose and lipids, digestive enzyme secretion by the hepatopancreas, osmoregulation by the gills, and ovarian development during reproduction. These pleiotropic effects of CHH may reflect the activity of different isoforms. Its hyperglycemic effects have received the most attention. Hemolymph levels of glucose are normally quite low, but treatment with CHH will cause a transient rise in glucose levels. This effect is due to the binding of CHH to membrane receptors on the hepatopancreas and abdominal muscle cells, which increases their levels of cAMP and cGMP. These cyclic nucleotides bind to protein kinases that activate phosphorylase and inhibit glycogen synthase. Recent studies have shown that stress (e.g., hypoxia and parasitic infection) causes a rise in the hemolymph levels of CHH and glucose. CHH also appears to have a role in osmoregulation, and perfusion of gills with this peptide causes a rapid and reversible increase in the transepithelial potential and the $Na^+$ influx by gill tissue. CHH also appears to have a stimulatory effect on oocyte growth (see discussion of VIH below). In addition to the sinus gland, CHH is present in the second roots of the thoracic ganglia. It is also found in the paraneurons of the foregut and hindgut immediately prior to molting. These cells release large amounts of CHH that increase water uptake during the molting process.

### *2. MIH*

MIH regulates molting by inhibiting the production of ecdysteroids by the Y-organ (see above). The MIH peptides in crabs, such as *Car. maenas* and *Cancer pagurus*, form a separate subgroup of the CHH family, the MIH/VIH subfamily (see Fig. 4d). These peptides have 78 amino acids and unblocked N- and C-termini and are synthesized as a 113-amino-acid prohormone with a 35-amino-acid signal peptide. The amino acid sequences of crab MIHs are highly conserved. In *Car. maenas* and *Can. pagurus*, these peptides show approximately 80% identity. In contrast, MIH peptides from these species show far less similarity (approximately 30% amino acid identity) to their own CHH peptides. In other species, isoforms of CHH have MIH activity. MIH in *H. americanus* appears to be a CHH isoform (CHH-A) and has both hyperglycemic and MIH activity. CHH has MIH activity in other species, including *Car. maenas* and *P. clarkii*, even when a separate MIH peptide is also present. In *Car. maenas*, CHH and MIH can act synergistically to inhibit Y-organ production of ecdysteroids.

### 3. MO-IH

MO-IH inhibits the production of MF by the mandibular organ (see above). In *Can. pagurus*, MO-IH has 78 amino acids and unblocked N- and C-termini and is a member of the MIH/VIH subfamily of CHH-like peptides (see Fig. 4d). It is synthesized as a prohormone with a 34-amino-acid signal peptide. The MO-IH of *Can. pagurus* is closely related to MIH (approximately 60% amino acid identity) and less related to its own CHH (approximately 20% identity). In other crustaceans, it appears that CHH is the MO-IH (see Fig. 4d). In *L. emarginata*, the MO-IH has 72 amino acids and has hyperglycemic activity. Its sequence is closely related to that of CHH (approximately 80% identity to CHH in *Can. pagurus*) and is less closely related to that of MO-IH in *Can. pagurus* (approximately 20% identity).

### 4. VIH

VIH (or GIH) inhibits gonadal development. Eyestalk ablation of females in the right physiological status accelerates gonadal maturation rather than molting, suggesting the presence of an eyestalk peptide that inhibits gonadal growth. In females, VIH inhibits oocyte growth, by interfering with the uptake of vitellogenin (the major yolk protein found in oocytes) or by inhibiting the synthesis of this protein. This peptide is also found in males, but its function is unclear. VIH in *H. americanus* has 77 amino acids, has unblocked N- and C-termini (see Fig. 4d), and is related to the MIH/VIH subfamily of CHH-like peptides. It is synthesized as a prohormone with a 34-amino-acid signal sequence. Lobster VIH shows approximately 50% sequence identity to MIH from crabs and approximately 42% sequence identity to MO-IH from *Can. pagurus* (see Fig. 4d). Of particular interest is the observation that VIH is synthesized in the same neuroendocrine cells that produce CHH, suggesting that they may both be involved in oogenesis. Measurement of the hemolymph levels of these two peptides appears to confirm this idea. Hemolymph levels of VIH are high during immature and previtellogenic stages of ovarian growth and levels of CHH are high during stages of oocyte maturation. This finding and other evidence suggest that CHH may be a gonad-stimulating hormone, acting in opposition to VIH.

## V. ANDROGENIC GLAND HORMONE

The androgenic glands have been identified in most orders of the Malacostraca, but many recent studies have used amphipods and isopods. The androgenic gland is associated with the terminal region of the sperm duct and in many species can be easily removed. This tissue produces androgenic gland hormone, which can masculinize individuals. However, the response to this hormone varies with the species and the specific tissue. Ablation of the gland in the isopod *Armadillidium vulgare* causes feminization of young males, resulting in the development of oocytes, an oviduct, and female external morphology. Implantation of androgenic glands into young genetic females of this species will masculinize both their primary and secondary sexual characteristics, including behavior, creating functional males that can produce progeny. These data suggest that the undifferentiated crustacean gonad will autodifferentiate into ovarian tissue unless the androgenic gland hormone induces male morphogenesis.

The androgenic glands contain several compounds that affect gonadal function. In *Car. maenas*, this tissue produces $C_{18}$ isoprenoid ketones, such as farnesylacetone, that inhibit ovarian protein synthesis (see Fig. 3). However, these compounds do not appear to be responsible for the effect of this gland on sexual differentiation. A second product of this tissue is a peptide hormone, androgenic gland hormone (see Fig. 4e). In *A. vulgare*, this hormone is an 11 kDa glycopeptide with two subunits (A and B with 29 and 44 amino acids, respectively) that are stabilized by both intra- and interchain disulfide bridges. In a manner analogous to the synthesis of insulin, the androgenic hormone is produced as a preprohormone from which a 21-residue signal peptide and a 46-residue C peptide (located between the N-terminal B subunit and the C-terminal A subunit) are removed to yield the mature hormone.

The androgenic glands appear to be regulated by neurohormones from the eyestalk. Eyestalk ablation in immature crabs causes hypertrophy of the androgenic gland and precocious spermatogenesis.

Ablation of the protocerebrum in isopods has the same effect. Preliminary evidence suggests that this inhibitory compound may be CHH. There is also evidence for the presence of a stimulatory factor in the ventral ganglion, but this compound(s) has not been characterized.

## VI. SUMMARY

Although the past decade has brought considerable progress in identifying the endocrine compounds in crustaceans, the endocrine systems of these species are still poorly characterized. More information would be welcome, since it would allow us to compare crustacean endocrine systems to those of other arthropods. In addition, such information may have direct application in the cultivation of species that are important as food (lobsters, shrimp, and crabs) or in suppressing the growth of species that have destructive effects (barnacles). Finally, crustacean endocrine systems may be useful for monitoring and predicting the effects of chemical pollutants that could disrupt the physiology of other crustaceans (and thus the life-webs in which they play a role) and possibly humans and other vertebrates.

## Glossary

**androgenic gland** Strands of cells associated with the male gametic duct. These cells produce androgenic gland hormone, which induces primary and secondary male sexual characteristics.

**ecdysteroids (molting hormone)** A family of steroid hormones regulating molting in arthropods. The most common form is ecdysterone (20-hydroxyecdysone, β-ecdysone).

**mandibular organ** The site of methyl farnesoate synthesis. In decapod crustaceans, this compact gland is located in the thoracic region attached to the mandible adductor tendon.

**methyl farnesoate (MF)** A sesquiterpenoid compound that is a member of the insect family of juvenile hormones (JH). MF has been detected in crustaceans and may function as their JH.

**molting (ecdysis)** The shedding of the old exoskeleton and its replacement by a new exoskeleton. This periodic process has specific stages leading up to and following the molt that make up the molt cycle.

**sinus gland/X-organ complex** A neurosecretory complex in the eyestalk of decapod crustaceans. Several important peptide hormones are produced and released by this complex.

**Y-organ** Paired glands of ectodermally derived cells that produce ecdysteroids.

## See Also the Following Article

**Ecdysteroids, Overview**

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# CSF

See *Colony-Stimulating Factor-1*

# CXC Chemokines

Philip M. Murphy

*National Institute of Allergy and Infectious Diseases, National Institutes of Health, Maryland*

I. INTRODUCTION
II. CLASSIFICATION
III. STRUCTURAL BIOLOGY
IV. SIGNALING PATHWAYS
V. INFLAMMATORY CXC CHEMOKINES
VI. HOMEOSTATIC CXC CHEMOKINES
VII. CXC CHEMOKINES AND ANGIOGENESIS
VIII. VIRAL CXC CHEMOKINES
IX. SUMMARY

**The CXC chemokines constitute a functionally diverse division of the chemokine superfamily defined by the presence of the eponymous CXC motif (Cys–any amino acid–Cys) near the N-terminus of the protein sequence. Three other principal chemokine divisions have been defined, based on other arrangements of conserved cysteines in this location. They are named accordingly C, CC, and CX3C. At the functional level, chemokines are signaling molecules that act primarily at seven-transmembrane domain G-protein-coupled receptors. Their main targets are hematopoietic cells, and their main biologic functions are immunoregulation, host defense, and inflammation, although additional roles in cancer, human immunodeficiency virus infection, wound healing, and other settings have been described. Apart from the CXC motif, there is no property common to all CXC chemokines that distinguishes them from all other types of chemokines. Still, this system of organization is useful since all the chemokines that attract neutrophils and/or regulate angiogenesis have the CXC motif. During the immune response, CXC chemokine functions are coordinated with those of the other chemokine families.**

## I. INTRODUCTION

Half of the known human CXC chemokines, CXCL1–3 and CXCL5–8, are important neutrophil chemoattractants and pro-angiogenic factors and act in the setting of innate immunity, sometimes redundantly. Members of a smaller functionally related cluster, CXCL9–11, regulate Th1 immune effector function and are angiostatic. The other six family members have more unique and nonoverlapping functions. CXCL12 is critical for hematopoiesis and embryogenesis and its receptor, CXCR4, is used by human immunodeficiency virus (HIV) to enter target cells. CXCL13 regulates B-cell and T-cell migration to B-cell zones of secondary lymphoid tissue. CXCL14–16 have only recently been identified and their properties are not yet well understood. CXCL4 is the least "chemokine-like" since it is not chemotactic; instead, it has strong prothrombotic and angiostatic activity. In addition to these human chemokines, some herpesviruses encode CXC chemokines and chemokine receptors, and one of these, viral G-protein-coupled receptor (vGPCR) of human herpesvirus 8, appears to play an important role in the pathogenesis of Kaposi's sarcoma (KS). The CXC chemokine receptors CXCR2 and CXCR3 have emerged as attractive targets in immunologically mediated inflammatory diseases and CXCR4 may be an important target in HIV/acquired immune deficiency syndrome (AIDS). CXC chemokines themselves have potential applications in wound healing, cancer, HIV/AIDS, and inflammation and as vaccine adjuvants.

## II. CLASSIFICATION

CXC chemokines can be divided by structure into two main subgroups according to the presence or absence of the tripeptide sequence ELR (glutamic acid–leucine–arginine) N-terminal to the first cysteine (Table 1). The seven human ELR CXC chemokines target a common neutrophil receptor and are angiogenic. Non-ELR CXC chemokines differentially regulate T and B lymphocytes, pre-B cells, monocytes, and dendritic cells (DCs) through a separate set of differentially expressed GPCRs, and several of them

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TABLE 1 The CXC Chemokine Family: Nomenclature and Organization

| Systematic name | Common names |  | Human chromosome | Structural subgroup | Receptors | General phenotypes |  |
|---|---|---|---|---|---|---|---|
|  | Human | Mouse |  |  |  | Immune | Vascular |
| CXCL1 | Groα/MGSA | MIP-2, KC | 4q21.1 | ELR | CXCR2 | Inflammatory | Angiogenic |
| CXCL2 | Groβ | MIP-2, KC | 4q21.1 | ELR | CXCR2 | Inflammatory | Angiogenic |
| CXCL3 | Groγ | MIP-2, KC | 4q21.1 | ELR | CXCR2 | Inflammatory | Angiogenic |
| CXCL4 | PF-4 | PF-4 | 4q21.1 | Non-ELR | ND | ND | Angiostatic |
| CXCL5 | ENA-78 | GCP-2, LIX | 4q21.1 | ELR | CXCR2 | Inflammatory | Angiogenic |
| CXCL6 | GCP-2 | GCP-2, LIX | 4q21.1 | ELR | CXCR1 & 2 | Inflammatory | Angiogenic |
| CXCL7 | NAP-2 | NAP-2 | 4q21.1 | ELR | CXCR2 | Inflammatory | Angiogenic |
| CXCL8 | IL-8 | Unknown | 4q21.1 | ELR | CXCR1 & 2 | Inflammatory | Angiogenic |
| CXCL9 | Mig | Mig | 4q21.1 | Non-ELR | CXCR3 | Inflammatory | Angiostatic |
| CXCL10 | IP-10 | IP-10/CRG-2 | 4q21.1 | Non-ELR | CXCR3 | Inflammatory | Angiostatic |
| CXCL11 | I-TAC | I-TAC | 4q21.1 | Non-ELR | CXCR3 | Inflammatory | Angiostatic |
| CXCL12 | SDF-1 α/β | SDF-1/PBSF | 10q11.21 | Non-ELR | CXCR4 | Homeostatic | Angiogenic |
| CXCL13 | BCA-1 | BLC | 4q21.1 | Non-ELR | CXCR5 | Homeostatic | ND |
| CXCL14 | BRAK | BRAK | 5q31.1 | Non-ELR | ND | ND | ND |
| (CXCL15) | Unknown | Lungkine |  | Non-ELR | ND | ND | ND |
| CXCL16 |  |  | 17p13 | Non-ELR, Multimodular | CXCR6 | Homeostatic | ND |

*Note.* ND, not determined.

are angiostatic. CXC chemokines can also be divided into two main immunoregulatory subgroups designated "homeostatic" and "inflammatory", depending on whether they regulate leukocyte migration mainly during development and surveillance or during the effector phase of the immune response.

CXCL16 deserves special mention because it is a non-ELR CXC chemokine with a complex multimodular structure shared not with other CXC chemokines but with CX3CL1, the sole CX3C family member. Both molecules have transmembrane domains, which tether them to the plasma membranes of the producing cells, mucin-like stalks, which extend the chemokine domains from the plasma membrane, and cytoplasmic domains of unknown function. Since CX3CL1 is a direct cell–cell adhesin, it is expected that CXCL16 will also have this activity.

Several unconventional CXC elements have also been identified, including the nonsignaling CXC chemokine-binding protein Duffy on red blood cells, which may function as a chemokine scavenger, and virus-encoded CXC chemokines, chemokine mimics, chemokine receptors, and chemokine scavengers.

## III. STRUCTURAL BIOLOGY

Chemokines range from ~7 to 16 kDa in molecular mass and appear to fold in the same general manner. Three anti-parallel β-sheets are arranged in the shape of a Greek key and are connected by loops and stabilized by disulfide bonds between the first and third and between the second and fourth conserved cysteines. The domain N-terminal to the first cysteine is unordered, and the C-terminal domain forms an α-helix draped over the Greek key domain.

Seven-transmembrane domain (7TM) receptors have an extracellular N-terminus and an intracellular C-terminus, which together with the transmembrane domains define three extracellular loops (ECLs) and three intracellular loops (ICLs). The protein sequences of CXC chemokine receptors are co-linear and ~340–360 amino acids in length, and their amino acid identity ranges from 25 to 80%. The least conserved amino acids cluster on the extracellular face of the receptor. Other common features include an acidic and relatively short N-terminal segment, the sequence DRYLAIVHA or a variation of it in ICL2, a short basic ICL3, and a cysteine in each of the four extracellular domains.

The three-dimensional structure of chemokine receptors is unknown; however, a reasonable model can be constructed based on analogy with the crystal structure of rhodopsin. The model contains two disulfide bonds, one linking the N-terminus with ECL3 and the other linking ECL1 and ECL2, which constrain the transmembrane domains (TMDs) like staves in a barrel arranged in a counterclockwise orientation relative to the cell interior. The ICLs form a large docking surface for heterotrimeric G-proteins and potentially other independent downstream effectors.

Most CXC chemokines can bind to glycosaminoglycans (GAGs) *in vitro* and therefore *in vivo* may be tethered by GAGs on endothelial cells and in the extracellular matrix during presentation to leukocyte receptors. The receptor appears to have two functionally distinct ligand-binding subsites. The first provides a docking site for the folded domain of the chemokine monomer, whereas the second, formed by the TMDs, accepts the chemokine's N-terminus when the first subsite is occupied and is used for triggering. Subsite 1 accounts for high-affinity binding, which is the sum of multiple low-affinity interactions involving multiple extracellular determinants on the receptor. The exact location of the binding determinants may differ for different chemokines and receptors. Unlike many cytokine receptors, chemokine receptors are composed of a single polypeptide chain. Some GPCRs form dimers as part of the signaling mechanism, but whether this is required for chemokine signaling is controversial.

## IV. SIGNALING PATHWAYS

CXC chemokines activate G-protein-dependent and G-protein-independent pathways involving diverse intracellular effectors including phospholipases (PL) A2, C, and D and phosphatidylinositol-3 kinase $\gamma$ (PI-3K$\gamma$). They may also activate effectors for classic growth factor pathways, including protein tyrosine kinases and phosphatases, low-molecular-weight GTPases, and mitogen-activated protein (MAP) kinases, even in nondividing cells such as neutrophils. The phospholipases and lipid kinases induce extensive remodeling of plasma membrane phospholipid and generate lipid second messengers. Different chemokines and the different cellular responses induced by chemokines—migration, cytotoxicity (degranulation and oxidant production), and proliferation—involve both common and distinct signaling pathways. Moreover, different chemokines may activate different signaling pathways via the same receptor in the same cell. Most of the information about CXC chemokine signaling is based on studies of neutrophil activation by CXCL8 and T-cell activation by CXCL12 (also known as stromal cell-derived factor-1 or SDF-1).

Pertussis toxin-sensitive $G_i$-proteins are the main G-protein subtypes coupled to all CXC chemokine receptors in all types of leukocytes. The precise $\alpha$-, $\beta$-, and $\gamma$-subunit subtypes have not been unequivocally delineated. Studies in phagocytes suggest that the main signaling subunit for the chemotactic pathway is $\beta\gamma$, not $\alpha$, and that $\beta\gamma$ must come specifically from $G_i$. This indicates that $\beta\gamma$ is necessary but not sufficient to support chemokine functional responses.

Beyond $G_i$, CXC chemokine signaling pathways become much more diverse and complex. The best-characterized immediate downstream effectors activated by $\beta\gamma$ in leukocytes are phospholipase C (PLC) subtypes $\beta2$ and $\beta3$, and PI-3K$\gamma$, all of which catalyze the formation of distinct plasma membrane-associated lipid second messengers from phosphorylated phosphatidylinositol (PI) substrates. PLC generates diacylglycerol and an intracellular calcium flux, which are important for CXCL8 induction of superoxide production in neutrophils but not for chemotaxis. PI-3K$\gamma$ generates phosphatidylinositol-3,4, 5-trisphosphate, which is thought to act by recruiting proteins containing pleckstrin homology (PH) or Phox domains to the plasma membrane. Four PH domain-containing targets—Akt/PKB and the guanine nucleotide exchange factors for the Rho family members Rac 2, Rho, and cdc42—have been shown to modulate distinct phases of cell movement in various model systems. Analysis of PI-3K$\gamma$ knockout mice has indicated that this enzyme plays a differential role in the chemotactic responses of different leukocyte subpopulations to chemoattractants. However, CXCL8 is the only chemokine among the chemoattractants evaluated thus far using cells from these mice. Neutrophils from Rac2 knockout mice are defective in CXCL8-induced chemotaxis and actin polymerization.

$G_i$-independent signaling mechanisms may include the Ras–Raf MAP kinase pathway and various nonreceptor tyrosine kinases such as the focal adhesion-associated protein Pyk2. However, the importance of these pathways may differ for different chemokines and leukocytes. For example, pharmacologic blockade of the MAP kinase pathway has been reported to disrupt CXCL12 activation of T-cell chemotaxis but not CXCL8 activation of neutrophil chemotaxis. CXC chemokines have been reported to cross talk with other signaling systems, including priming of CXCL8 by hematopoietic growth factors and inhibiting CXCL12 by signaling through B-cell antigen receptors, ephrin-B, and the neuronal guidance factor Slit through its neutrophil receptor, Robo.

## V. INFLAMMATORY CXC CHEMOKINES

### A. CXCL8 and Related ELR CXC Chemokines in Innate Immunity

CXCL8 is the prototypical inflammatory chemokine and the first chemokine shown to have leukocyte chemotactic activity. The CXCL8 gene is normally silent in most cells, but can be rapidly induced in most if not all cell types by diverse stimuli, including pro-inflammatory cytokines [e.g., interleukin-1 (IL-1) and tumor necrosis factor (TNF)], oxidant stress, viruses, and bacterial products such as lipopolysaccharide (LPS). In contrast, glucocorticoids and immunoregulatory cytokines (e.g., IL-10 and both type 1 and type 2 interferons) suppress CXCL8 production. Key nuclear factors that mediate CXCL8 gene expression include nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein β (C/EBP), activator protein-1 (AP-1), and NF–IL-6. In a blister model of acute inflammation in human, endogenous CXCL8 peaks at nanomolar concentrations at ~24 h, whereas C5a and leukotriene B4, which unlike CXCL8 do not require new gene expression but instead are formed enzymatically from precursor substrates, appear earlier. This suggests that orderly sequential expression of chemoattractants may control the evolution of the inflammatory response.

The neutrophil appears to be the major chemotactic target of CXCL8 *in vitro* and *in vivo*. Intradermal injection of CXCL8 in human causes rapid (< 30 min) and selective accumulation of large numbers of neutrophils in perivascular regions of the skin, and many neutrophil-mediated human diseases have been associated with the presence of CXCL8, including psoriasis, gout, acute glomerulonephritis, acute respiratory distress syndrome (ARDS), rheumatoid arthritis, reperfusion injury, and urinary tract infection. Systemic administration of neutralizing anti-CXCL8 antibodies is protective in diverse models of neutrophil-mediated acute inflammation in the rabbit (skin, airway, pleura, glomeruli), providing proof of concept that CXCL8 is a nonredundant mediator of innate immunity and acute pathologic inflammation in these settings (see Table 2). *In vitro*, neutrophils can also produce CXCL8, suggesting that it may work in an autocrine and paracrine manner to amplify neutrophil accumulation at inflamed sites.

There is therefore great interest in developing drugs that block CXCL8 action. However, selecting a target is complicated by the fact that CXCL8 binds to two high-affinity, structurally related [78% amino acid (aa) identity] chemotactic receptor subtypes, CXCR1 and CXCR2, which are both constitutively co-expressed at high levels on human neutrophils (see Table 3). Moreover, all six other human ELR CXC chemokines bind CXCR2 with high affinity and CXCR1 with substantially lower affinity and could function redundantly with CXCL8. Studies using selective blocking agents have disagreed on the relative importance of CXCR1 and CXCR2 in CXCL8-induced chemotaxis of primary human neutrophils. Unfortunately, rodents cannot be used to settle the issue genetically because mice lack counterparts of both CXCL8, and rat CXCR1 is expressed in macrophages, not neutrophils. Recently, SB-265610, a nonpeptide small-molecule antagonist with exquisite selectivity for CXCR2, has been shown to completely block CXCL8-induced chemotaxis of human neutrophils *in vitro* and to prevent neutrophil accumulation in the lungs of hyperoxia-exposed newborn rats. Still, other data suggest that CXCR1 cannot be ignored. For example, a patient study found that CXCR1 expression levels on neutrophils, possibly modulated by genetic polymorphism in the CXCR1 coding region, may be an important risk factor in bacterial pyelonephritis.

CXCR2 appears to play key roles in neutrophil-mediated acute inflammation and anti-microbial host defense in the mouse. CXCR2 knockout mice are less susceptible to acute urate crystal-induced gouty synovitis and are more susceptible to *Escherichia coli* pyelonephritis, *Onchocerca volvulus* keratitis, *Aspergillus fumigatus* and *Nocardia asteroides* pneumonia, gram-negative bacterial brain abscess, and *Toxoplasma gondii* encephalitis.

CXCL8 and its receptors may also have functions that extend beyond neutrophil migration and innate immunity. CXCL8 receptors are also expressed on T cells, monocytes, natural killer (NK) cells, immature dendritic cells, and basophils, as well as on nonhematopoietic cell types, including endothelial cells, neurons, and keratinocytes. Interestingly, CXCL8 can induce monocyte adhesion to endothelial cells but not monocyte chemotaxis. The biological significance of this is borne out by the ability of adoptively transferred CXCR2$^{-/-}$ bone marrow to protect mice in a model of atherosclerosis. In addition, CXCL8 has been found in human atheroma and may also promote lesions through effects on angiogenesis.

An unsuspected role for CXCL8 in adaptive immunity was suggested by the ability of neutralizing anti-CXCL8 antibody administration to block delayed-type hypersensitivity reaction to tuberculin in rabbit skin. A role in hematopoiesis *in vivo* was revealed in CXCR2 knockout mice, which have massive expansion of neutrophils and B cells throughout the hematopoietic system. This occurs only when the mice are derived in pathogen-free, not germ-free, environments, which suggests that normal hematopoiesis is regulated by a balance of CXCR2

TABLE 2 **Some Biological Roles Found for CXC Chemokines and CXC Chemokine Receptors in Patient Studies or in Animal Models by Molecular Targeting (Gene Knockout, Chemokine Neutralization, or Receptor)**

| Chemokine | Biological roles | Species |
|---|---|---|
| CXCL1 | • Melanoma and other cancers | Human |
| KC, MIP-2 | • Bleomycin-induced pulmonary fibrosis | Mouse |
| | • Acute neutrophil-mediated inflammation (many models) | |
| | • Antimicrobial host defense (many models) | |
| CXCL4 | • Autoimmune heparin-induced thrombocytopenia | Human |
| CXCL8 | • Acute neutrophil-mediated inflammation (many models) | Rabbit |
| | • Cancer-associated angiogenesis | Human |
| CXCR2 | • Wound healing | Mouse |
| | • Antimicrobial host defense (many models) | |
| CXCL9/(CXCR3) | • Resistance to mouse CMV infection | Mouse |
| CXCL10/CXCR3 | • Cardiac allograft rejection | Mouse |
| CXCL12/CXCR4 | • HIV infection | Human |
| | • Bone marrow myelopoiesis | Mouse |
| | • B-cell lymphopoiesis | |
| | • Cardiovascular development | |
| | • Cerebellar development | |
| | • Breast cancer metastasis | |
| CXCL13/CXCR5 | • B-T positioning in secondary lymphoid tissue | Mouse |
| CXCL15 | • Resistance to *Klebsiella pneumoniae* | Mouse |

inhibitory signals and undefined enhancers induced by environmental factors. The mechanism may involve in part direct inhibition of mouse hematopoietic progenitor cell proliferation by signaling through CXCR2. CXCR2 knockout mice also revealed a role for this receptor in wound healing.

The expression of other ELR CXC chemokines has both features that it shares with the expression of CXCL8 and features that are unique, and together these chemokines may act redundantly in some contexts but not in others. For example, in most cell types, CXCL1–3 (also known as Groα, Groβ, and Groγ, respectively), CXCL5 (also known as epithelial cell-derived neutrophil activator-78 or ENA-78) and CXCL6 (also known as granulocyte chemotactic protein-2 or GCP-2) are all regulated at

TABLE 3 **Differential Expression of CXC Chemokine Receptors on Leukocyte Subpopulations**

| | Receptor | | | | | |
|---|---|---|---|---|---|---|
| Leukocyte subpopulation | CXCR1 | CXCR2 | CXCR3 | CXCR4 | CXCR5 | CXCR6 |
| Red blood cells | | | | | | |
| Platelets | | | | + | | |
| Neutrophils | + | + | | + | | |
| Eosinophils | | + | | | | |
| Basophils | | + | | + | | |
| Macrophages | + | + | | + | | |
| Mature DCs | | | | + | | |
| Immature DCs | + | + | | + | | + |
| Naive T cells | + | + | | + | | |
| Memory T cells | | | + | + | + | |
| Th1 cells | | | + | + | + | + |
| Th2 cells | | | | + | + | |
| B lymphocytes | | | + | + | + | |
| NK cells | + | + | + | + | | + |
| $CD34^+$ cells | | | | + | | |
| Thymocytes | | | | | + | |

the level of transcription and subsequent protein production after induction with most of the same inflammatory stimuli that turn on CXCL8 production. Yet neutrophils stimulated with fibrinogen and formylmethionyl-leucyl-phenylalanine (fMLF) express massive amounts of only CXCL8; melanoma cells selectively express CXCL1, which has been implicated as an important growth and angiogenic factor for this and other tumors; and CXCL5 is selectively expressed in unstimulated colonic epithelial cells. In mouse, TNF-induced neutrophil extravasation requires neutralization of both MIP-2 and KC, the CXCL1–3 homologues of mice, indicating redundant function in this setting. In contrast, MIP-2 is the major chemokine that attracts neutrophils into herpes simplex virus 1 (HSV-1)-infected mouse cornea, perhaps due to the fact that resident corneal cells and inflammatory cells contribute to MIP-2 synthesis, whereas KC production is restricted to corneal cells.

ELR CXC chemokines have been evaluated as therapeutic agents in diverse disease settings. For example, they can accelerate hepatic regeneration when administered systemically in an acetaminophen-induced hepatotoxicity mouse model. CXCL10 has similar activity but acts by inducing CXCR2 on hepatocytes. They have also been tested as DNA vaccines. In mouse, a MIP-2-encoding plasmid given mucosally with a plasmid encoding gB of HSV induces a Th1 immune response and renders recipients more resistant to HSV vaginal infection.

CXCL1, CXCL7 (also known as neutrophil-activating peptide 2 or NAP-2), and CXCL8 also bind to Duffy. The concentration of Duffy in blood is far higher than the concentration of CXCR1 or CXCR2, so that it may serve as an effective scavenger to maintain effective gradients from tissue to blood. This could also explain in part why systemic administration of these chemokines is well tolerated.

## B. Platelet-Specific Chemokines CXCL4 and CXCL7

CXCL4 (also known as platelet factor-4 or PF-4) and CXCL7 are considered together because of their shared structure and their unusual distribution and regulation. They are 50% identical at the amino acid level, less homologous to all other chemokines, and restricted primarily to platelets, where unlike CXCL8 and other inflammatory chemokines, they are regulated posttranslationally. Paradoxically, despite high amino acid sequence identity, these chemokines are members of different CXC subclasses, which has important implications for their function. CXCL4, the first chemokine ever discovered, lacks the ELR motif and may be the only chemokine that is not a leukocyte chemoattractant or a ligand for a GPCR. In contrast, CXCL7 is a typical ELR CXC chemokine, able to induce neutrophil chemotaxis by activating CXCR2. Yet both chemokines are stored in platelet α granules and are released at extremely high concentrations (micromolar) upon platelet activation.

Upon release, CXCL4 aggregates to form tetramers that are critical for binding to chondroitinsulfate proteoglycans, their major known binding sites. In contrast, CXCL7 is activated by sequential proteolysis of its N-terminus. The prepropeptide form, platelet basic protein (92 aa), is trimmed during platelet maturation to produce the 85-amino-acid major stored form, connective tissue activating peptide-III (CTAP-III), which is inactive on neutrophils. CTAP-III is further processed during platelet release to 81-amino-acid β-thromboglobulin, which is also inactive. This is then cleaved by a cell surface-bound, cathepsin G-like enzyme on neutrophils to form 70-amino-acid CXCL7. CXCL7 may function as an immediate-early mediator of neutrophil recruitment released from platelets at sites of inflammation. An alternative possibility is that the massive release of CXCL7 from platelets may provide a mechanism for immobilizing neutrophils at the vessel wall under conditions of high shear through down-regulation of CXCR2. Compared to ELR CXC chemokines, CXCL4 does not induce chemotaxis or degranulation of lysosomal enzymes. It does, however, induce secondary granule exocytosis and release of matrix-degrading enzymes, which may facilitate neutrophil penetration of inflamed tissues. Moreover, it mediates strong adhesion of neutrophils to endothelial cells by functional activation of lymphocyte function-associated antigen-1 (LFA-1), unlike the ELR CXC chemokines, which induce adhesion by up-regulating the adhesion molecule Mac-1.

A common function reported for CXCL4, CXCL7, and CXCL8 is to increase the survival of normal hematopoietic precursors and to protect them from the toxicity of chemotherapeutic agents. A more unique function for CXCL4 is its strong prothrombotic activity, due to its ability to bind heparin. Unfortunately, this sometimes results in the clinical condition heparin-induced thrombocytopenia, which is characterized by formation of antibodies to a complex of CXCL4 and heparin. This is a well-recognized risk factor for thromboembolic complications that occurs in approximately 1–5% of patients treated with heparin.

### C. CXCL15 and Innate Immunity

CXCL15 (lungkine) is a non-ELR mouse chemokine that lacks a defined human counterpart. The selective and constitutive expression of CXCL15 mRNA in fetal and adult mouse lung, particularly in epithelial cells, is distinct from that of all other known chemokines, which suggested at first that it may have a developmental or homeostatic function. However, the CXCL15 knockout mouse has normal lung development. Instead, it has increased susceptibility to *Klebsiella pneumoniae*, indicating that CXCL15 functions in innate immunity. Consistent with this finding, CXCL15 is secreted into the air spaces, induces *in vitro* and *in vivo* migration of neutrophils, and is up-regulated in lung inflammation models.

### D. CXCL9, CXCL10, and CXCL11: Non-ELR Inflammatory CXC Chemokines in Adaptive Immunity

CXCL9, CXCL10, and CXCL11 (also known as Mig, IP-10, and I-TAC, respectively) form a structurally and functionally related subgroup of non-ELR CXC chemokines that regulate the migration of Th1-polarized effector T cells to inflamed sites during the adaptive immune response. Two key properties common to all three of these chemokines account for their involvement in Th1 responses. First, all three are induced at the transcriptional level in diverse cell types by the Th1 immunoregulatory cytokine interferon-γ (IFN-γ), but not by the Th2 cytokine IL-4. Second, all three signal via the same receptor, CXCR3, which is invariant among chemokine receptor combinations that mark activated Th1-polarized $CD4^+$ T cells. Thus, in Th1 immunity, there is a positive feedback loop in which IFN-γ induces the production of these chemokines, which then recruit T cells that produce IFN-γ.

Other factors may differentially regulate CXCL9, CXCL10, and CXCL11 expression, which may account for their specialized biological roles. CXCL9 is the most strongly dependent on IFN-γ for expression. Nevertheless, it can also be induced by TNF-dependent factors in high endothelial venules, which results in monocyte migration to lymph nodes draining inflamed tissues. CXCL10 is constitutively expressed in stromal cells of lymphoid organs (spleen, thymus, and lymph nodes), suggesting a potential role in T-cell development and effector function. Detailed study of CXCL9 and CXCL10 during viral and protozoan infections of mice has revealed overlapping but distinct patterns of expression. CXCL11 is the most potent CXCR3 agonist in chemotaxis assays and preferentially induces transepithelial migration of T cells and down-regulation of the receptor.

CXCL10 appears to be the most powerful chemokine mediator of acute cardiac allograft rejection. In human, this chemokine and leukocytes expressing CXCR3 are detected in rejecting human cardiac allografts, and in a mouse model, rejection does not occur if the recipient mouse, treated with a brief, subtherapeutic course of cyclosporin A, comes from a CXCR3 knockout background or if the donor heart is from a CXCL10 knockout background. Moreover, wild-type mice treated with an anti-CXCR3 monoclonal antibody show prolonged allograft survival. Apparently, CXCL9 and CXCL11 do not compensate for the absence of CXCL10 because they are expressed later and at lower levels. CXCL10 neutralization is also protective in experimental autoimmune encephalomyelitis.

Likewise, in mouse cytomegalovirus infection, which causes hepatitis, a chemokine-to-cytokine-to-chemokine cascade selectively involving CXCL9 is required for antiviral host defense. In particular, hepatic CCL3 (MIP-1α) production is required for recruitment of NK cells to the liver. NK cells are the major source of IFN-γ in this model, and IFN-γ induces CXCL9, which is required for protection.

Consistent with these observations, CXCL9 and CXCL10 have been associated mainly with Th1-polarized human diseases such as psoriasis, tuberculoid leprosy, and sarcoidosis. In addition, virtually all T cells in rheumatoid arthritis synovial fluid and in various inflamed tissues, such as in ulcerative colitis, chronic vaginitis, and sarcoidosis, express CXCR3, particularly in perivascular regions. CXCR3 is also consistently detected on transformed B cells from chronic lymphocytic leukemia (CLL) patients. Thus, CXCR3 and its ligands are attractive clinical targets in Th1-polarized immunologically mediated diseases and possibly cancer. CXCL9 and CXCL10 have also shown efficacy as anti-tumor agents in mouse models, delivered directly as protein or by genetic vectors. Moreover, CXCL10 has been used therapeutically in a Th2-polarized mouse model of airway inflammation, resulting in conversion to a Th1 immune response and reduced disease progression.

## VI. HOMEOSTATIC CXC CHEMOKINES

### A. CXCL12

CXCL12, the only chemokine essential for life, is constitutively expressed in most tissues and is a major regulator of hematopoiesis and embryonic development. The gene is alternately spliced to encode two functionally indistinguishable variants, CXCL12$\alpha$ and CXCL12$\beta$, which differ by a 4-amino-acid extension at the C-terminus. Originally isolated from bone marrow stromal cells, CXCL12 is the only known endogenous ligand for CXCR4, the most broadly expressed and highly conserved of all chemokine receptors. In addition to cells in many tissues, CXCR4 is found on all types of leukocytes and unlike many other chemokine receptors it does not require cell activation for expression on freshly isolated T cells from peripheral blood. Accordingly, CXCL12 is a powerful pan-leukocyte chemoattractant *in vitro*, although specific roles in the inflammatory response, lymphocyte homing, and recirculation have not yet been delineated.

Genetic disruption of CXCL12 and CXCR4 in the mouse gives the same phenotype, suggesting that they make up a monogamous signaling unit *in vivo*. The animals die of unclear causes in the perinatal period, with abnormal bone marrow myelopoiesis and defective B-cell, but normal T-cell, lymphopoiesis. Together with the fact that CXCL12 is the most highly expressed chemokine in bone marrow, these data suggest that CXCL12 may promote hematopoiesis in part by regulating the trafficking and positioning of hematopoietic precursor cells in this organ. Consistent with this finding, human stem cell engraftment has been reported to be regulated by CXCR4 in non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice. CXCL12 may also be important in thrombopoiesis and platelet activation. It induces megakaryocyte migration and is the only known chemokine that induces platelet aggregation, mediated through CXCR4. Since CXCL12 is expressed by smooth muscle cells, endothelial cells, and macrophages in human atherosclerotic plaques but not in normal vessels, it may play a role in the pathogenesis of atherosclerosis and thrombo-occlusive diseases. CXCL12 and CXCR4 knockout mice also have ventricular septal defects, defective gastric vasculogenesis, and defective cerebellar granule cell positioning, indicating a broad role in development of multiple nonhematopoietic cell lineages.

The CXCL12/CXCR4 axis may also be important in cancer through diverse mechanisms, including promotion of angiogenesis, recruitment of cancer-associated leukocytes, and direct guidance of cancer cells to metastatic sites. In this regard, human breast cancer cells but not normal breast epithelium express CXCR4, and preferred target organs for breast cancer metastasis (bone marrow, lymph node, liver, and lung) are rich in CXCL12. In a mouse model, the blockade of CXCR4 prevented metastasis of breast cancer to liver and lymph node. Roles in melanoma and ovarian cancer have also been suggested. Furthermore, high CXCR4 levels on cancer cells have been associated with extramedullary organ infiltration in childhood acute lymphoblastic leukemia.

Along with CCR5, CXCR4 is a major HIV co-receptor that facilitates the infection of CD4$^+$ target cells. HIV strains that use CXCR4 selectively, known as X4 strains, typically do not transmit disease but emerge in a subset of patients late in the course of infection. CXCL12 can specifically inhibit X4 HIV replication by steric blockade as well as by inducing internalization of CXCR4. X4 HIV gp120 has additional diverse CXCR4-dependent effects on cells that have interesting implications for mechanisms of HIV pathogenesis. It is able to induce monocyte migration, which may be important for recruitment of additional target cells. It can induce apoptosis of human neurons, which may be relevant to the pathogenesis of HIV encephalitis and AIDS dementia. In addition, it can stimulate macrophages to induce apoptosis of CD8($^+$) T cells, suggesting a co-receptor mechanism of cytotoxic T-lymphocyte suppression of viral replication.

Several small molecules and peptides, including some originally identified in HIV drug discovery programs, have been shown to selectively block chemokine receptor and/or HIV co-receptor activities of CXCR4. They include CXCL12-derived peptides, the polyarginine ALX40-4C, and the bicyclam AMD3100. The last substance is currently in clinical trials for HIV/AIDS. CXCR4 has also been blocked with intrakines, which are modified forms of CXCL12 delivered by gene therapy that remain in the endoplasmic reticulum and block the surface expression of newly synthesized CXCR4. Clinical development of CXCR4-blocking agents in HIV infection will have to confront safety questions as to whether the virus will evolve to use other co-receptors and whether one or more of the phenotypes seen in CXCR4 knockout mice will occur.

### B. CXCL13

CXCL13 [also known as B-cell-attracting chemokine 1 (BCA1)] and CXCR5 comprise a monogamous ligand–receptor pair critical for the homeostatic organization of B-cell zones of secondary lymphoid tissue. CXCR5 has been detected on all peripheral blood and tonsillar B cells, but on only a fraction of cord blood and bone marrow B cells. A third subset of CD4$^+$ memory cells, designated follicular helper T cells ($T_{FH}$), is defined by CXCR5 expression and the absence of CCR7. These cells do not produce Th1 or Th2 cytokines upon activation but are able to provide help for B-cell maturation and antibody production. Consistent with this finding, they constitute the majority of CD4$^+$ memory cells in follicular zones of inflamed tonsils. Like other chemokine receptors, CXCR5 is dynamically regulated on T cells. Following TCR stimulation, CXCR5 is up-regulated on memory/effector T cells, whereas IL-2 causes down-regulation.

CXCL13, the major chemokine active on mature B cells, is constitutively expressed in secondary lymphoid organs on follicular high endothelial venule (HEV) and therefore is properly positioned to focus CXCR5$^+$ B and T cells from the blood into follicles. Consistent with this finding, CXCR5 knockout mice have a severe defect in normal B-cell migration and localization and have aberrant lymphoid development, including absent inguinal lymph nodes, absent or phenotypically abnormal Peyer's patches, and ectopic development of germinal centers in T-cell zones of the spleen. CXCL13 is also required for B1-cell homing, natural antibody production, and body cavity immunity. CCL19/CCL21–CCR7 play an analogous role for T-cell–dendritic cell positioning in T-cell zones.

### C. CXCL14

CXCL14, also known as BRAK (breast and kidney chemokine), is a recently identified non-ELR CXC chemokine with three unusual properties. First, it is constitutively expressed in diverse human tissues (heart, brain, placenta, lung, intestine, liver, skeletal muscle, and pancreas) and cell types (squamous epithelium, keratinocytes, dermal fibroblasts, and lamina propria cells of the intestine). Second, like CXCL12 and unlike all other chemokines, it is highly conserved among species (two amino acid differences between human and mouse). Third, it is the only chemokine that chemoattracts only monocytes. *In vivo*, macrophages were reported to colocalize with CXCL14-producing fibroblasts, suggesting that it may regulate the homeostatic recruitment and development of tissue macrophages. Resting leukocytes do not express CXCL14; however, tumor-infiltrating leukocytes and LPS-stimulated B cells and monocytes are strongly positive, suggesting additional potential roles in host–tumor interactions and inflammation. In striking contrast to normal tissue, most cancers do not express CXCL14.

### D. CXCL16

CXCL16 is distantly related to other CXC chemokines. Its unique hybrid structure is described in Section I. CXCL16 is expressed on the surface of antigen-presenting cells (B cells, macrophages, and dendritic cells in lymphoid organ T-cell zones) and by cells in the splenic red pulp. Functional CXCL16 is also shed from macrophages. The CXCL16 receptor CXCR6 is expressed preferentially on memory T cells and on activated Th1 and Tc1 effector T-cell subsets. The exact biological role of the CXCL16/CXCR6 axis is unknown; however, reasonable hypotheses include attraction of activated T-lymphocyte subsets during inflammation, facilitation of immune responses via cell–cell contact, and guidance of T-cell trafficking in the splenic red pulp. CXCL16 is also expressed in the thymic medulla and in some nonlymphoid tissues, suggesting roles in thymocyte development. Like CXCR4, CXCR6 functions as an HIV co-receptor and is able to support cell entry by simian immunodeficiency virus and both X4 and R5 strains of HIV. Its precise role in HIV pathogenesis has not been defined.

## VII. CXC CHEMOKINES AND ANGIOGENESIS

There is growing evidence that angiogenesis is regulated in part by the balance of angiogenic and angiostatic chemokines in tissue microenvironments. CXCL12 and all ELR CXC chemokines are angiogenic in rat corneal micropocket and related assays, whereas CXCL4 and the CXCR3 ligands CXCL9–11 are angiostatic. CXCR2 is expressed on endothelial cells and appears to mediate angiogenic signals, whereas angiostatic chemokines appear to work not by GPCRs but by binding to glycosaminoglycans on endothelial cells, which prevents docking of pro-angiogenic growth factors such as basic fibroblast growth factor (bFGF). A functional CXCL10-specific receptor expressed by epithelial and endothelial cells that is neither CXCR3 nor glycosaminoglycan must also be considered.

The balance of angiogenic and angiostatic CXC chemokine expression has been proposed to regulate wound healing, fibrotic diseases, and cancer growth and spread. More generally, the overall balance of these chemokines may regulate the extent of injury and the rate of repair in disease. In addition to direct effects on endothelial cells, a countercurrent principle of chemokine action has been proposed in which paraneoplastic expression of ELR CXC chemokines promotes tumorigenesis by favoring a pro-angiogenic environment in part by inducing the release of pro-angiogenic factors such as gelatinase B from recruited neutrophils. This indicates that blocking pro-angiogenic chemokines or administering angiostatic chemokines may be beneficial in appropriately selected cancers. This has been borne out in several instances, including human non-small-cell lung carcinoma (NSCLC), in which the ratio of ELR to non-ELR CXC chemokine expression has been reported to be high, and in a SCID mouse model in which either administration of exogenous CXCL9 or neutralization of endogenous tumor-derived CXCL8 could inhibit tumor growth and metastasis via a decrease in tumor-derived vessel density. Importantly, CXCL8 did not act as an autocrine growth factor for NSCLC proliferation, and in general, for reasons that are unclear, tumors appear to have surprisingly small numbers of leukocytes given the amount of chemokines that they make. Thus, their major effects on tumorigenesis may be at the level of angiogenesis.

Chemokines may also have immunologically mediated effects on cancer. In this regard, induction of tumor-protective $CD8^+$ cellular immunity by IL-12 in a murine neuroblastoma model has been reported to depend entirely on endogenous CXCL10, and exogenous CXCL10 has been reported to block establishment of tumor in a thymus-dependent manner. CXCL9 and CXCL10 have also been reported to enhance the anti-tumor effects of IL-2 and IL-12 in mouse models. Genetic fusion of CXCL10 to a self tumor antigen induces protective, T-cell-dependent anti-tumor immunity; however, the mechanism appears to involve recruitment of antigen-presenting cells. With regard to fibrotic disorders, neutralization of endogenous MIP-2 has been shown to be protective in bleomycin-induced pulmonary fibrosis in mice.

Taken together, CXC chemokines deserve attention as potential therapeutic agents in cancer. This concept has been tested clinically in the case of CXCL4, which produces striking anti-tumor effects in animal models; however, responses have thus far been disappointing in humans.

## VIII. VIRAL CXC CHEMOKINES

HIV and many herpesviruses and poxviruses deploy chemokine mimics, which are used for diverse purposes including immune evasion and cell entry. Among the herpesviruses, these mimics are typically acquired by copying host chemokine and chemokine receptor genes. Three herpesvirus CXC chemokines and two CXC chemokine receptors have been identified to date. Human cytomegalovirus (HCMV) has two adjacent open reading frames (ORFs) that encode CXC chemokines vCXC-1 (ORF UL146) and vCXC-2 (ORF UL147). The function of vCXC-2 has not been reported; however, vCXC-1 is a potent neutrophil chemoattractant acting specifically at CXCR2. This may account for the presence of neutrophils at sites of HCMV infection and could function to recruit more target cells for viral dissemination. Of note, MCK-2, a CC chemokine encoded by mouse CMV, appears to work in this manner. Specifically, it induces inflammation early at the site of infection and later promotes dissemination of the virus to the salivary gland.

Marek's disease virus (MDV), an α herpesvirus of chickens that infects the feather follicle epithelium and causes a variety of syndromes, including generalized immunosuppression, acute neuronal symptoms, paralysis, T-cell lymphomas, and, rarely, atherosclerosis, encodes a CXC chemokine designated vIL-8. Like host chemokine genes, and unlike other known viral chemokine genes, vIL-8 is encoded by multiple exons, suggesting that it was captured from genomic DNA or nuclear mRNA. It is expressed with late ($\gamma 2$) kinetics. Baculovirus-expressed vIL-8 is secreted and directly chemoattracts chicken peripheral blood mononuclear cells but not heterophils. Direct effects of this chemokine on inflammation *in vivo* have not been defined. Instead, through undefined mechanisms, recombinant MDV lacking vIL-8 has been reported to support a decreased level of lytic infection and is less oncogenic.

Herpesvirus saimiri is also an oncogenic herpesvirus, but from the γ herpesviridae. It does not encode chemokines but instead encodes a receptor, ECRF3, encoded by ORF 74, which can be activated by human CXCL1, CXCL7, and CXCL8. This gene does not appear to be required for transformation and its actual biological role is undefined. ECRF3 is syntenic with ORF 74 of the related $\gamma$ herpesvirus HHV8, which encodes vGPCR. HHV8 is thought to be necessary although not sufficient for development of Kaposi's sarcoma and is thus also known as the Kaposi's sarcoma-associated herpesvirus. Transgenic mice expressing vGPCR under the control of the CD2 promoter develop multicentric, angioproliferative Kaposi's sarcoma-like lesions, although the receptor is actually expressed in a minority of proliferating cells. At the biochemical level, vGPCR constitutively induces NIH 3T3 cell transformation and angiogenesis *in vivo*, as well as pro-inflammatory cytokine, chemokine, and growth factor gene expression *in vitro*, and the activity can be modulated by chemokine agonists such as CXCL1 and by inverse agonists such as CXCL10 and CXCL12. The ability to respond to agonists appears to be important *in vivo* since KS-like tumors in mice do not occur when agonist recognition is abolished by receptor mutagenesis. The basic role of this receptor in the normal life cycle of HHV8 is not known.

## IX. SUMMARY

The CXC chemokines, initially studied for their potent effects on neutrophils, are now generally recognized to have diverse pleiotropic activities, covering hematopoiesis, immunoregulation, and both innate and adaptive immune effector function and extending to hemostasis, organ development, angiogenesis, cancer, HIV pathogenesis, and herpesvirus infection. Emerging evidence suggests that cancers may grow and spread in part according to the balance of endogenous pro-tumorigenic and anti-tumorigenic CXC chemokines, which may have direct effects on cell proliferation and migration as well as modulate angiogenesis. These biological connections have come from systematic programs of chemokine and chemokine receptor discovery and expression. In contrast, the connection of CXC chemokines to HIV/AIDS and facilitation of other infectious diseases was completely unpredicted. CXCR2, CXCR3, and CXCR4 are now attractive therapeutic targets in inflammation, cancer, and HIV/AIDS, and selected chemokine ligands have potential applications as anti-tumor agents and as adjuvants in DNA vaccines. The stage is now set to discover whether the basic science reviewed here can be translated into clinical benefits.

## Glossary

**angiogenesis** Blood vessel formation.

**chemokine** The term is derived from "chemotactic cytokine", which was coined to identify members of a family of structurally defined proteins, most of which directly chemoattract leukocytes. Collectively, chemokines and chemokine receptors coordinate specific leukocyte trafficking and are therefore critical to the development and function of the immune system.

**chemotaxis** Migration of a biological entity toward the source of a chemical stimulus. In the case of leukocytes, migration requires adhesion to a substrate, polarization of the cell's sensing surface, and cytoskeletal remodeling, which leads to protrusion of a leading edge and retraction of a trailing edge.

**G-protein** A guanine nucleotide-binding protein involved in intracellular signaling.

**immune system** The physiologic system consisting mainly of white blood cells that specializes in protecting the organism from infection and clearing debris after tissue injury.

**leukocyte** Any kind of white blood cell, divided into two main subgroups: phagocytes and lymphocytes. Phagocytes, which include neutrophils, monocytes, eosinophils, and dendritic cells, directly engulf microbes and other particulate matter. Lymphocytes, which include T cells and B cells, attack targets either directly, in the case of cytotoxic T cells, or indirectly, in the case of plasma cells, a terminally differentiated B cell specialized to produce antibodies.

**receptor** A cell-associated molecule able to activate the cell upon interaction with an extracellular stimulus. Chemokine receptors are all members of the G-protein-coupled receptor superfamily, which have seven conserved plasma membrane-spanning domains.

## See Also the Following Articles

**Angiogenesis • Anti-Inflammatory Actions of Glucocorticoids • CC, C, and CX$_3$C Chemokines • GPCR (G-Protein-Coupled Receptor) Structure • Pro-Inflammatory Cytokines and Steroids**

## Further Reading

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Encyclopedia of Hormones.

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Adrian J. Dunn and David E. Scarborough
*Louisiana State University Health Sciences Center*

I. INTRODUCTION
II. EFFECTS OF CYTOKINES ON THE HPA AXIS
III. GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I
IV. THYROID-STIMULATING HORMONE
V. LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE
VI. PROLACTIN
VII. SUMMARY

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**Cytokines are proteins or glycoproteins that are secreted by cells of the immune system. They play numerous important roles in coordinating immune system function and are the major messengers between immune cells. In this role, they are the hormones of the immune system. However, it has long been recognized that cytokines may also be synthesized and secreted by nonimmune cells and that they exert important effects on nonimmune cells.**

## I. INTRODUCTION

Cytokines comprise families of proteins formerly known as lymphokines (from lymphocytes), monokines (from monocytes), and the interleukins. The cytokine family includes 18 interleukins (ILs), numbered accordingly (IL-1 through IL-18), the interferons, and other molecules named for the function for which they were first discovered, such as tumor necrosis factor α (TNFα), transforming growth factor (TGF-α and TGF-β), leukemia inhibitory factor (LIF), macrophage-migrating inhibitory factor (MIF), and granulocyte/macrophage colony-stimulating factor (GM-CSF). A summary of the nature and activities of the principal cytokines follows.

### A. Interleukin-1

IL-1 exists in two forms, IL-1α and IL-1β. The two forms are closely related but differ significantly in their amino acid sequences within species, and substantial differences exist in the amino acid sequences between species. There are two receptors for IL-1, type I and type II, but only the type I receptor is thought to be involved in physiological responses. The type II receptor does not contain an internal signaling sequence and has been postulated to function as a "decoy" receptor. Physiologically this may be equivalent to the presence of binding proteins or soluble receptors that buffer the free concentrations of important messengers. Both IL-1α and IL-1β bind on type I (and type II) receptors, and although IL-1α is somewhat less potent than IL-1β, both induce similar responses. There is an endogenously produced antagonist for the receptor to IL-1 (the IL-1 receptor antagonist, IL-1ra). IL-1 is

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IL-1 was initially characterized as an endogenous pyrogen, i.e., a substance that induces fever. However, it was soon discovered that purified recombinant IL-1β administered to rats potently activated the hypothalamic–pituitary–adrenocortical (HPA) axis. This activity was confirmed in other species and is not confined to the HPA axis. It is now known that IL-1 has a plethora of diverse effects on different cells in the body. It is recognized as a major proinflammatory cytokine—it is produced rapidly after damage to many different cell types and it signals other cells to initiate inflammatory responses.

### B. Interleukin-2

IL-2 is produced by T helper ($T_H1$) lymphocytes and is a growth-promoting factor that is critical for the clonal proliferation of T cells.

### C. Interleukin-6

IL-6 is a glycoprotein and a proinflammatory cytokine produced in large quantities by monocytes early in immune responses. It is considered to be responsible for the acute-phase response, a critical step in immune activation. The acute-phase response involves induction of fever, activation of the HPA axis, and production of a number of circulating proteins (α1-acid glycoprotein, $\alpha_2$-macroglobulin, β-fibrinogen, C-reactive protein, serum amyloid A protein, and transferrin); the acute-phase response also results in an increase in the number of peripheral leukocytes, especially neutrophils. It is important in B-cell proliferation. Many of the functions of IL-6 overlap with those of IL-1. Plasma concentrations of IL-6 are elevated dramatically and rapidly after infections and following many other kinds of biological stress.

### D. Transforming Growth Factor-β

TGF-β exists in five isoforms (TGF-β1–TGF-β5). The functions of these isoforms overlap. TGF-β is produced in large quantities by platelets and certain other tissues and is a potent growth inhibitory factor for many kinds of cells, but especially for T lymphocytes.

### E. Tumor Necrosis Factor α

As its name implies, TNFα has the ability to kill cells by inducing cytotoxicity. It is considered to be the third major proinflammatory cytokine. It is thought to be responsible for apoptosis and cachexia, the life-threatening state associated with severe infections.

### F. Interferons

The interferons (IFNα, IFNβ, and IFNγ) are regarded as antiviral agents. IFNγ is produced by $T_H1$ cells and enhances natural killer (NK) cell activity.

### G. Cytokines and the Pituitary Gland

The presence of certain cytokine receptor messenger RNAs (mRNAs) and, in some cases, of the cytokine receptors (especially receptors for IL-1, IL-2, and IL-6) has been demonstrated in various preparations of anterior pituitary cells. Cytokine mRNAs and cytokines have also been found (IL-1, IL-1ra, IL-2, and IL-6). Unfortunately, although the presence of mRNA is suggestive, it should not be assumed that gene products are properly formed and are functional. Generally, an abundance of cytokines and their receptors has been found only in abnormal preparations, in cell lines derived from the anterior pituitary, or in *ex vivo* preparations. Nevertheless, limited amounts of these cytokines and their receptors may exist under normal physiological conditions, and they certainly appear to be induced during sepsis and other pathological conditions. There has been much speculation that cytokines can directly affect the release of anterior pituitary hormones, but there are few if any convincing data indicating a physiological role. An important exception is IL-6, which may act directly on the pituitary to elicit HPA activation (see later).

The following discussions review and summarize the known actions of cytokines impacting the secretion of hormones of the anterior pituitary. Table 1 provides a summary of the known actions of cytokines on anterior pituitary hormones.

## II. EFFECTS OF CYTOKINES ON THE HPA AXIS

### A. The HPA Axis

The HPA axis is a hierarchy in which activation of neurons in the paraventricular nucleus (PVN) of the hypothalamus, which contains corticotropin-releasing hormone (CRH), results in the secretion of CRH in the median eminence region of the hypothalamus. CRH is secreted into the portal blood supply and is carried to the anterior pituitary; there it acts on specific receptors on corticotrophs, resulting in the

**TABLE I Summary of Cytokine Actions on Anterior Pituitary Hormone Secretion**

| Hormone | Cytokine | Activity[a] |
|---|---|---|
| Adrenocorticotropic hormone | Interleukin-1 | ↑ ↑ ↑ |
| | Interleukin-6 | ↑ |
| | Tumor necrosis factor α | ↑ |
| Growth hormone | Interleukin-1 | ↑ ↓ |
| | Interleukin-6 | ↑ ↓ |
| | Tumor necrosis factor α | ↑ ↓ |
| Luteinizing hormone | Interleukin-1 (icv)[b] | ↓ ↓ ↓ |
| | Interleukin-6 | ↑ ↓ |
| | Tumor necrosis factor α | ↑ ↓ |
| Prolactin | Interleukin-1 | ↑ ↓ |
| | Interleukin-6 | ↑ |
| | Tumor necrosis factor α | ↑ ↓ |
| Thyroid-stimulating hormone | Interleukin-1 | ↓ ↓ ↓ |
| | Interleukin-6 | ↓ |
| | Tumor necrosis factor α | ↓ ↓ ↓ |

[a] The relative strength of increase or decrease in activity is indicated respectively, by up or down arrow. Reports of both increases and decreases are also indicated ( ↑ ↓ ).

[b] icv, Intracerebroventricular.

secretion of adrenocorticotropic hormone (ACTH) into the general circulation. ACTH is transported throughout the body, but as it passes through the adrenal cortex it binds to specific receptors that stimulate the synthesis and subsequent secretion of adrenal glucocorticoids (corticosterone in rodents, cortisol in humans and many other species). Circulating glucocorticoids are ultimately responsible for a host of metabolic and regulatory responses. Compared to intact animals, glucocorticoid-deficient animals and humans are profoundly more likely to succumb to a wide variety of illnesses and stresses, especially infections and trauma. Routinely, glucocorticoid concentrations are markedly elevated in severe infections and injuries. Thus, the mechanism for inducing a glucocorticoid response to infection and injury is of major physiological importance.

#### *1. IL-1-Induced Activation of the HPA Axis*

A particularly critical observation is that intraperitoneal (ip) administration of purified recombinant human IL-1β to rats potently activates the HPA axis. This phenomenon has been confirmed by a number of researchers and in other species, including humans. The activity is not confined to IL-1 or to the HPA axis, although IL-1 is the most potent stimulator of this axis. The mechanism of this effect is still somewhat controversial, and multiple mechanisms exist. Apparently, IL-1 can act at multiple levels of the HPA axis, i.e., the brain, the hypothalamus, the anterior pituitary, and the adrenal cortex. It may well be that the most important mechanism varies with the physiological state of the animal. However, the weight of the evidence suggests that under normal physiological circumstances the primary mechanism involves hypothalamic CRH.

### B. IL-1 Action on the Adrenal Cortex

IL-1 has been shown to stimulate the secretion of corticosterone from the adrenal cortex *in vitro* in some studies, but not in others. A direct effect on the adrenal cortex is unlikely to explain the normal *in vivo* elevation of plasma concentrations of corticosterone because IL-1 administration also elevates plasma ACTH in both rats and mice. Also, IL-1 fails to induce increases in plasma corticosterone in hypophysectomized rats and mice (one rat study, however, did show some increase). Moreover, the corticosterone response to IL-1 in rats and mice is largely prevented by pretreatment with an antibody to CRH.

### C. IL-1 Action on the Anterior Pituitary

An early report indicated that IL-1 induces ACTH secretion from AtT-20 cells, but studies of abnormal cells may not be relevant in intact animals. Several reports have indicated that IL-1 stimulates ACTH release from the anterior pituitary *in vitro*, but many other investigators have failed to find effects of IL-1 on the *in vitro* pituitary secretion of ACTH. It is important to note that *in vitro* studies cannot answer questions concerning *in vivo* mechanisms. Interestingly, the conflicting results might be explained by the observation that prolonged incubation appears to increase the sensitivity of pituitary ACTH secretion to IL-1, while decreasing the response to CRH. A direct pituitary effect appears to be excluded as the normal physiological mechanism because lesions of the hypothalamic PVN and pretreatment with CRH antibodies prevent the ACTH and glucocorticoid responses to IL-1.

### D. IL-1 Action via the Hypothalamus

A hypothalamic site of action of peripherally administered IL-1 is suggested because lesions of the PVN prevent increases in plasma ACTH and corticosterone. Also, IL-1 increases the electrophysiological activity of CRH neurons *in vivo*. In support of a hypothalamic involvement, IL-1 stimulates CRH release from hypothalamic slabs *in vitro*, although such *in vitro* studies cannot provide definitive conclusions. CRH is implicated by the observation that peripherally administered IL-1 elevates concentrations of CRH in portal blood and depletes CRH from the median eminence, both actions presumably reflecting increased release of CRH. Also, *in vivo* immunoneutralization of CRH prevents increases in plasma ACTH and corticosterone in response to IL-1 in rats and mice. Moreover, CRH knockout mice (mice lacking the gene for CRH and thus unable to produce it) show only a very small increase in plasma corticosterone after IL-1 administration.

The evidence for direct actions on the pituitary and adrenal glands derives largely from *in vitro* experiments and is therefore susceptible to artifact. The *in vivo* evidence strongly favors a role for hypothalamic CRH as the major mechanism in normal healthy animals. However, in studies of mice treated with antibody to CRH, there were small increases in plasma corticosterone following intraperitoneal IL-1, and similar small increases were also observed in CRH knockout mice. This suggests that when the HPA axis is impaired, the pituitary or adrenal cortex may gain the ability to mount a modest glucocorticoid response. This may have pathological significance in that a glucocorticoid response may be conserved when the pituitary or hypothalamus fails to respond. It is to be noted that an IL-1- and lipopolysaccharide (LPS)-induced secretion of ACTH and corticosterone can be observed in young rats at a time when HPA responses to stressors are minimal or absent.

It should not be assumed that the action of IL-1 is exerted directly on the hypothalamus, even though intrahypothalamic injections of IL-1 can activate the HPA axis. There is some evidence that IL-1 may act on the median eminence, on the organum vasculosum laminae terminalis (OVLT), and/or on the area postrema. All three structures are circumventricular organs that lack a blood–brain barrier, thus the entry of IL-1 would not be hindered. There is also substantial evidence for the involvement of brain noradrenergic systems. Peripheral administration of IL-1β activates brain noradrenergic neurons, especially in the hypothalamus. These neurons may be activated in the nucleus tractus solitarius of the brain stem, the site of origin of ascending noradrenergic neurons that innervate the hypothalamus, including the PVN. The activation may be local via the area postrema or indirectly via vagal afferents from the periphery. In rats, lesions of this ascending noradrenergic projection impair the HPA response. A subdiaphragmatic vagotomy, which lesions the vagal afferents that project to the nucleus tractus solitarius, also inhibits the noradrenergic response and the increase in plasma ACTH. However, the increase in plasma corticosterone is significantly less affected.

It is entirely possible that more than one mechanism is involved in the IL-1-induced activation of the HPA axis and that the precise mechanism may depend on the site in which IL-1 is produced or the route of its injection. The HPA response to intravenous (iv) IL-1 occurs very rapidly after injection, dissipates rapidly, and is quite sensitive to cyclooxygenase (COX) inhibitors. By contrast, the response to ip IL-1 is sensitive to COX inhibitors only in the early phase of the response (which is much more prolonged than the response to iv IL-1). The latter may be more dependent on the noradrenergic system, which responds in a manner that closely parallels the increases in plasma corticosterone.

The elevation of glucocorticoids by IL-1 (and IL-6 and TNFα) has been speculated to provide feedback, limiting immune responses during inflammatory responses. Perhaps more importantly, it may serve to limit inflammatory responses by confining them to the damaged or infected sites in the body.

## E. Other Cytokines

### *1. Interleukin-2*

Although a few reports suggest that IL-2 can activate the HPA axis, most indicate very little effect, unless IL-2 is infused chronically.

### *2. Interleukin-6*

IL-6 administration has HPA-activating activity, increasing plasma concentrations of ACTH and corticosterone in rats, mice, and humans. The HPA response to ip IL-6 is fast and short-lived compared to the response to IL-1. IL-6 is significantly less potent in elevating plasma ACTH and corticosterone compared to IL-1, requiring doses an order of magnitude higher. Moreover, the maximum response is well below that elicited by IL-1, and even microgram doses fail to induce maximal plasma concentrations of corticosterone in mice. However, in humans, plasma cortisol is readily elevated by relatively small doses of IL-6.

The HPA-activating effect of IL-6 in rats has been reported to be sensitive to an antibody to CRH. However, IL-6 induces relatively normal ACTH responses in CRH knockout mice. IL-6 is known to have a direct stimulatory effect on ACTH secretion from the pituitary and, unlike IL-1, stimulates Fos expression in the pituitary gland, but not in the PVN. These latter results suggest a pituitary site of action for IL-6 in elevating plasma ACTH and corticosterone, consistent with the rapid time course. IL-6 may be important in mediating ACTH responses in the absence of CRH. The HPA response to IL-6 is not sensitive to COX inhibitors.

### 3. *Tumor Necrosis Factor α*

Administration of TNFα to rats at doses that fail to affect blood pressure, food consumption, or plasma prolactin concentrations causes significant elevations of plasma ACTH within 20 min. Most reports have found TNFα like IL-6, to be significantly less potent than IL-1 in rats and mice, although perhaps with a more prolonged effect. However, one group found human TNFα to be almost equipotent with human IL-1β in rats. Activation of the HPA axis by TNFα is abolished by lesions of the PVN and appears to be mediated at least partially by CRH. Treatment with a CRH antibody completely blocks the ACTH response, whereas the corticosterone response is only partially inhibited. Indomethacin blocks the ACTH response to TNFα in rats in a dose-dependent manner.

### 4. *Interferons*

Intraperitoneal administration of human or mouse IFNα to mice fails to alter plasma ACTH or corticosterone. Administration of IFNα to humans, however, elevates plasma cortisol. Most studies have shown that IFNγ has little effect on the HPA axis.

### 5. *Leukemia Inhibitory Factor*

LIF appears to play a role in HPA activation. LIF knockout mice show impaired HPA responses to stress and immune stimuli, and this deficit is reversed by LIF administration.

## III. GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I

Growth hormone (GH) plays a major role in assuring the proper growth of long bones during maturation; it also modulates carbohydrate and fat metabolism at all ages and provides an ongoing stimulus to a wide variety of tissues in adulthood. GH-deficient adults suffer loss of bone mineral density, loss of muscle mass, and decreased ability to maintain blood glucose levels. GH also appears to have a variety of effects on immune cells. Most of these are supportive or stimulatory. Because systemic cytokine concentrations are elevated in response to major tissue trauma and serious infection, cytokine modulation of GH secretion may be useful in coordinating a metabolic response to the starvation that accompanies major illness or injury and in supporting the immune and inflammatory aspects of the host response. Many actions of GH are believed to be mediated by its secondary hormone insulin-like growth factor-I (IGF-I). In the circulation IGF-I is associated with a number of binding proteins that may modulate its activity. Thus, assessing the full physiological action of GH is quite complex and simple measurements of GH levels may be misleading.

Acute LPS administration stimulates GH secretion in humans and in sheep but suppresses it in rats, chickens, and cattle. In humans, many stressors induce increases in GH secretion, including exogenous pyrogen and bacterial sepsis. These increases are associated with declines in the levels of IGF-I, suggesting resistance to the usual GH stimulation of IGF-I. GH has some direct actions, especially the mobilization of fatty acids from fat stores, which likely circumvent the resistance phenomenon. In chronic critical illness, GH secretion declines to below normal levels over the initial 2 weeks and stays low until recovery occurs. Recent studies of exogenous GH treatment in prolonged critical illness have shown some improvement in nitrogen balance, but have not shown improved patient survival. Administration of growth hormone-releasing hormone (GHRH) in such patients improves GH secretion, suggesting that suppression of GH secretion is at the hypothalamic level.

The precise role of cytokines in these changes in GH secretion and action is still unclear. Hypothalamic regulators of pituitary GH secretion include stimulation by GHRH and inhibition by somatostatin. IL-1β, TNFα, and IL-6 have been shown to stimulate somatostatin release from rat hypothalamic cells *in vitro*. IL-1 also stimulates GHRH release. In some studies, IL-1 administered into the third ventricle of male rats increased GH secretion, but other investigators have found the opposite effect. IL-6 given iv to rats did not alter GH secretion. *In vitro*, IL-1 stimulates GH release from rat hemipituitary and dispersed pituitary cell cultures. TNFα inhibits basal release and GHRH-stimulated GH secretion from dispersed rat anterior pituitary cell cultures. Thus, in rat cells *in vitro* there are opposing effects of different cytokines on GH secretion. The inhibitory effect of TNFα on GH release and the stimulatory effects of IL-1, IL-6, and TNFα on somatostatin would be consistent with the tendency of LPS to suppress GH in the rat. The stimulatory effects of IL-1 on GH in the rat model *in vitro* and *in vivo* run counter to the *in vivo* effect of LPS. In sheep, LPS stimulates GH secretion *in vivo*. *In vitro* in dispersed sheep pituitary cells, IL-1 stimulates basal GH secretion, but not GHRH-stimulated GH secretion. TNFα inhibits GHRH-stimulated GH secretion, but not basal GH release. IL-2 and IFNγ are without effect.

In sum, there are major species differences in the response of GH to inflammatory mediators. It is unclear whether the down-regulation of GH observed in prolonged human critical illness is harmful or helpful, and whether any cytokine effects at the level of the hypothalamus or pituitary are key elements of that down-regulation. Cytokine stimulation of hypothalamic somatostatin and TNFα inhibition of pituitary GH release are consistent with the dominant GH changes seen in humans.

## IV. THYROID-STIMULATING HORMONE

In humans with severe illnesses, there are multiple abnormalities in thyroid hormone economy, including low plasma triiodothyronine and normal or low plasma thyroxine. Collectively, these changes are called the euthyroid sick syndrome, meaning that the patient has no intrinsic disease of the thyroid and that the changes seen are temporary and entirely secondary to the nonthyroidal illness. Clinically, these patients lack signs and symptoms of hypothyroidism. Even so, these changes could have survival value in that the catabolic state that usually accompanies severe illness may be moderated by the reduction in thyroid hormone activity and gluconeogenesis may be facilitated. Treatment to increase the abnormally low thyroid levels has not proved beneficial. Ordinarily, low levels of triiodothyronine (T3) and tetraiodothyronine (T4, or thyroxine) result in stimulation of pituitary thyroid-stimulating hormone (TSH) secretion. However, TSH levels in inflammation and sepsis are more commonly low or normal, suggesting that TSH secretion is inhibited. Pituitary TSH secretion is stimulated by hypothalamic thyrotropin-releasing hormone (TRH) and inhibited by hypothalamic somatostatin. Because stalk section results in loss of TSH secretion, TRH is believed to be the more important regulator.

Pharmacologic doses of glucocorticoids are known to suppress TSH secretion. Because cytokines are strong stimulators of ACTH and glucocorticoid secretion, one possibility is that the suppression of TSH seen with cytokine stimulation is secondary to elevation of glucocorticoids. Against this, at least one study comparing heart attack patients with septic patients found suppression of TSH only in the sepsis group, despite both groups having equivalent elevations of ACTH and cortisol.

Bacterial endotoxin given to hypothyroid subjects with basally elevated TSH levels produces a rapid decline in TSH levels. In both rats and humans the major inflammatory cytokines (IL-1, TNFα, and IL-6) suppress TSH secretion *in vivo*. In general, cytokines appear to mediate most of the effects of endotoxin on neuroendocrine systems. Although this is likely to be true for endotoxin effects on TSH, neutralization of TNFα with a fusion protein and blockade of IL-1 with the IL-1 receptor antagonist did not block the suppression of TSH produced by endotoxin given to human volunteers. IL-1β inhibits hypothalamic TRH gene expression in the rat hypothalamus and in rat hypothalamic cell cultures. TNFα treatment reduces hypothalamic TRH content in rats. IL-1β, TNFα, and IL-6 also stimulate somatostatin secretion by hypothalamic cell cultures. IL-1β stimulates somatostatin release from hypothalamic slices. Either inhibition of TRH or stimulation of somatostatin would tend to suppress pituitary TSH secretion. Most *in vitro* studies of cytokine actions on cultured pituitary cells have not found significant changes in either basal or TRH-stimulated TSH secretion.

In sum, infection and inflammation affect thyroid hormone metabolism at multiple levels. The major neuroendocrine change is suppression of TSH secretion, which seems likely to be mediated by cytokine inhibition of hypothalamic TRH or stimulation of hypothalamic somatostatin.

## V. LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE

Gonadal function is regulated in an unusually complex manner. Multiple gonadal hormones interact with the pulsatile release of hypothalamic gonadotropin-releasing hormone (GnRH) to control secretion of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH secretion is specifically controlled by modulation of the pulsatile release of the hypothalamic peptide GnRH, interacting with the feedback effects of gonadal hormone levels. [GnRH is also called luteinizing hormone-releasing hormone

(LHRH). Some investigators prefer the latter term on the grounds that there may be separate specific hypothalamic regulators of FSH secretion.] FSH regulation is further complicated in that members of a family of peptide hormones, the activins and inhibins, are secreted by the gonads and interact with gonadal hormone levels and GnRH activity in the control of pituitary FSH secretion. Severe illness and stress generally inhibit gonadal function. As with the thyroid axis, cytokines and severe illness produce direct effects, lower circulating concentrations of the end-organ hormones without the expected increase in pituitary tropic hormone secretion. Cytokines can regulate some steroidogenic enzymes, and the direct effects of cytokines on both male and female gonads have been the subject of numerous studies. Early in critical illness in males, LH and FSH rise as testosterone falls, suggesting primary failure of the testis. Soon LH and FSH decline to a healthy, normal range. Analogous to the euthyroid sick syndrome, normal levels of gonadotropin in the face of low testosterone levels suggest inhibition of gonadotropin secretion. From a teleological perspective, how low testosterone would benefit the organism in conditions of stress or tissue injury is not readily apparent. In chronic stress, the anabolic effects of androgen on muscle mass would seem to be preferred to unopposed catabolism. Androgens have been used therapeutically in the cachexia associated with advanced AIDS, but otherwise there are very few data concerning their utility in illnesses associated with elevated cytokine concentrations.

A brief period of lower testosterone with elevated LH is observed in human males following acute administration of IL-6, paralleling the early response to endotoxin administration. In human males with prolonged critical illness and elevated plasma IL-6 and TNFα, pulsatile LH secretion and mean LH concentrations are suppressed when testosterone and estradiol levels are low. Administration of pulsatile GnRH substantially enhances pulsatile LH secretion, but this effect wanes over a 5-day period and is accompanied by only a partial, transient rise in testosterone levels. This suggests that the gonadal axis suppression associated with this kind of chronic critical illness operates at hypothalamic, pituitary, and gonadal levels.

Additional evidence that major illness operates at the hypothalamic and pituitary levels comes from studies of LH and FSH in severely ill, postmenopausal human females. LH and FSH rise markedly after the menopause, following the cessation of ovarian function. In hospitalized, ill postmenopausal women, LH and FSH levels are often inappropriately in the premenopausal normal range, or even frankly low. In females, inhibition of ovulation during infection or chronic illness, or inhibition of the gonadal hormone needed to continue a pregnancy during infection, makes more intuitive sense than does inhibition of testosterone in males. Here again, however, there are few or no direct data to support this intuitive notion. Estrogen administration to stressed, ill, hypogonadal females has not been actively studied.

Again, analogous to the situation with the thyroid axis, high glucocorticoid concentrations might be a primary mechanism for suppression of the reproductive axis. A number of observations argue against this. In castrated male rats, destruction of the PVN does not prevent the suppression of LH secretion observed following footshock. Peripheral administration of IL-1β provokes marked activation of the HPA axis, but does not suppress LH secretion. For example, 1 μg of IL-1β ip does not significantly alter LH over 4 h in male rats, a dose that markedly stimulates corticosterone secretion. HPA activation and LH suppression are thus dissociable, suggesting that LH suppression does not depend on elevation of glucocorticoids.

In contrast to the lack of effect of peripheral administration, intracerebroventricular (icv) administration of IL-1 into the third ventricle inhibits pulsatile secretion of GnRH and suppresses LH secretion. CRH appears to mediate the effect of many stressors on LH secretion, and IL-1 certainly activates central CRH, but CRH probably does not mediate the effect of cytokines on gonadotropin secretion. CRH antagonists in rat models can block most of the effects of stress and some effects of cytokines, such as the pyrogenic effect of IL-1β. However, even when administered into the third ventricle, these antagonists appear unable to block IL-1-induced suppression of GnRH neuron activity or suppression of LH secretion. An exception to this was observed in a study of rhesus monkeys, suggesting that there may be species differences in this mechanism.

Central IL-1 decreases GnRH mRNA in the cell bodies of GnRH neurons, as well as c-fos expression, suggesting that these GnRH cell bodies are a key target of IL-1 activity. The effects of central IL-1 on GnRH and LH secretion are blocked by naloxone, suggesting a role for opioid agonists in this effect. The central IL-1 effect is most pronounced with IL-1β, IL-1α being less effective. Some effect is also seen with TNFα but IL-6 is relatively ineffective.

Because systemic cytokine administration appears to have minimal effects on LH secretion *in vivo*, and because systemic cytokines probably have ready access to both the median eminence and the pituitary gland, it seems unlikely that direct cytokine effects on the pituitary gland are major regulators of LH secretion. *In vitro* studies of cytokine effects on pituitary LH release have yielded variable results. IL-6 stimulates LH from dispersed pituitary cell cultures and perifused pituitaries. TNFα inhibits GnRH-stimulated LH secretion from 3-day-old dispersed rat anterior pituitary cell cultures, but not basal LH release.

In sum, severe illness and stress generally suppress the reproductive axis in both males and females. While stress of many kinds may involve activation of the inflammatory cytokines, experimental work suggests that circulating cytokines are not key to the suppression of the gonadal axis. Rather, central cytokines acting via the suppression of GnRH neuron cell bodies seem to be the major pathway. Whether this suppression has survival value remains to be investigated.

## VI. PROLACTIN

Prolactin (PRL) serves to prepare the breast for lactation late in pregnancy and has some effects on fuel metabolism and immune function. These latter effects appear to be relatively minor in most circumstances. PRL is unique among the anterior pituitary hormones in that stalk section increases its secretion. Thus, the dominant regulatory effect from the hypothalamus is inhibitory. Hypothalamic dopamine is inhibitory to PRL secretion and TRH is stimulatory. Blocking TRH often has little effect on plasma PRL, tending to confirm the dominance of dopamine in prolactin regulation. Other hypothalamic factors, such as vasoactive intestinal polypeptide (VIP), have been implicated in PRL regulation, but their importance *in vivo* is unclear.

PRL is regarded as a stress hormone in that blood concentrations are increased in response to numerous stressors. The value of this response is not clear. Prolactin resembles GH in both structure and activity; PRL- and GH-secreting cells derive from the same lineage in pituitary development. Combined PRL/GH tumors are the most common type of multi-hormone-secreting pituitary adenoma. The effects of PRL on immune functions resemble those of GH, being supportive or stimulatory to immune activity in most experimental settings. PRL is elevated in some patients with lupus and Reiter's syndrome, raising the possibility that excessive immune activation and hyperprolactinemia might be causally related. There is thus some circumstantial evidence to suggest that PRL stimulation by cytokines could be biologically useful.

Administration of IL-1, TNFα, and IFNγ iv and icv usually have little effect on PRL secretion *in vivo*, although stimulation is sometimes observed. Similarly, 1 μg of IL-1 ip did not significantly alter PRL over 4 h in male rats, despite a marked increase in corticosteroid secretion. In dispersed pituitary cell cultures *in vitro*, various results have been reported: IL-6 stimulates PRL secretion, IL-1 has no effect or is inhibitory, IL-2 is stimulatory, TNFα has no effect or is stimulatory, and IFNγ both stimulates and inhibits. Overall, current evidence does not support any consistent effect of cytokines on PRL secretion, or that any such effect is of major physiological importance.

## VII. SUMMARY

Certain cytokines can alter the secretion of hormones of the anterior pituitary. The literature suggests that such activities are most commonly observed with the proinflammatory cytokines, IL-1, IL-6, and TNFα. However, this conclusion may be attributed to the fact that these have been the most well studied cytokines. Whether cytokines exert direct effects on the anterior pituitary is still controversial. Although cytokines and their receptors have been reported to be present in the anterior pituitary, most of the physiological evidence indicates that the hypothalamus is critically involved in the secretory process, using the normal releasing and release-inhibiting factors. An important exception may be IL-6, which may act directly on the pituitary to stimulate ACTH secretion. With the important exception of IL-1, the physiological significance of these effects of cytokines is unresolved. The IL-1-induced activation of the HPA axis is considered an important mechanism for triggering the stress response to cell damage. It appears to be a universal response and functions in ontogeny before the HPA axis responds normally to other stressors. The subsequent glucocorticoid suppression of immunity is speculated to suppress widespread inflammatory responses in favor of local processes that can deal with the damage or infection. Suppression of some aspects of thyroid axis function is common in inflammatory states and cytokines appear to play a role in this, particularly at the level of hypothalamic regulation of the pituitary secretion. Suppression of reproductive function is also common in inflammatory states, with the best current evidence implicating cytokine effects at the hypothalamus in this modulation of pituitary function. The biological value of these changes in thyroid and gonadal function is unclear. Given the lack of good physiological evidence for most of the known cytokines, our understanding of the involvement of cytokines in the orchestration of neuroendocrine responses to infection and inflammation is very incomplete and is likely to be subject to revision as new data accumulate.

## Glossary

**corticotropin** Synonymous with adrenocorticotropic hormone.

**cytokine** A protein or glycoprotein synthesized and secreted by immune cells; plays roles in communication within the immune system.

**gonadotropin-releasing hormone** The hypothalamic decapeptide that stimulates the pituitary to secrete LH and FSH; usually used as a synonym for luteinizing hormone-releasing hormone.

**gonadotropins** A generic term for the pituitary hormones that regulate or stimulate the gonads, i.e., luteinizing hormone and follicle-stimulating hormone.

**growth hormone-releasing hormone** The hypothalamic peptide, found in several molecular forms, that stimulates the pituitary to secrete growth hormone.

**hypothalamic-pituitary-adrenal axis** Cells and pathways that form the communications between the hypothalamus, the pituitary, and the adrenal gland. The major hypothalamic factors are corticotropin-releasing hormone and vasopressin, both of which stimulate the release of pituitary adrenocorticoticopic hormone, which in turn stimulates the release of glucocorticosteroids from the adrenal cortex (principally corticosterone and cortisol).

**lipopolysaccharide** A group of related compounds released when the cell walls of gram-negative bacteria are degraded. Also known as endotoxin, lipopolysaccharide is a potent stimulator of the immune system and the vasculature, acting on the specific receptors (toll-like receptor 4) on B cells, macrophages, and endothelial cells.

**negative feedback** In the context of pituitary function, the extremely common phenomenon by which hormones modulate the activity of their own regulatory apparatus (e.g., insulin-like growth factor-I, which is induced by growth hormone, is able to act on the hypothalamus and the pituitary to reduce growth hormone secretion).

**somatostatin** A 14- or 28-amino-acid peptide, found in the hypothalamus and elsewhere, that inhibits pituitary secretion of growth hormone and, to a lesser degree, other pituitary hormones, such as thyroid-stimulating hormone.

**thyrotropin (thyroid-stimulating hormone)** The pituitary hormone that stimulates the thyroid gland to produce thyroxine and triiodothyronine.

**thyrotropin-releasing hormone** The hypothalamic tripeptide that stimulates the pituitary to secrete thyroid-stimulating hormone and, to a lesser degree, prolactin.

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Gonadotropin-Releasing Hormone (GnRH) • Growth Hormone (GH) • Growth Hormone-Releasing Hormone (GHRH) • Insulin-like Growth Factor (Igf) Signaling • Interferons • Luteinizing Hormone (LH) • Monoaminergic and Cholinergic Control of the Anterior Pituitary • Prolactin • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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# Cytokinins

Fabien Nogué, Martine Gonneau, and
Jean-Denis Faure
*Institut National de la Recherche Agronomique, Versailles*

I. INTRODUCTION
II. STRUCTURE OF NATURAL AND SYNTHETIC CYTOKININS
III. CYTOKININ BIOSYNTHESIS AND METABOLISM
IV. CYTOKININ SIGNALING
V. BIOLOGICAL ROLES
VI. CONCLUSIONS

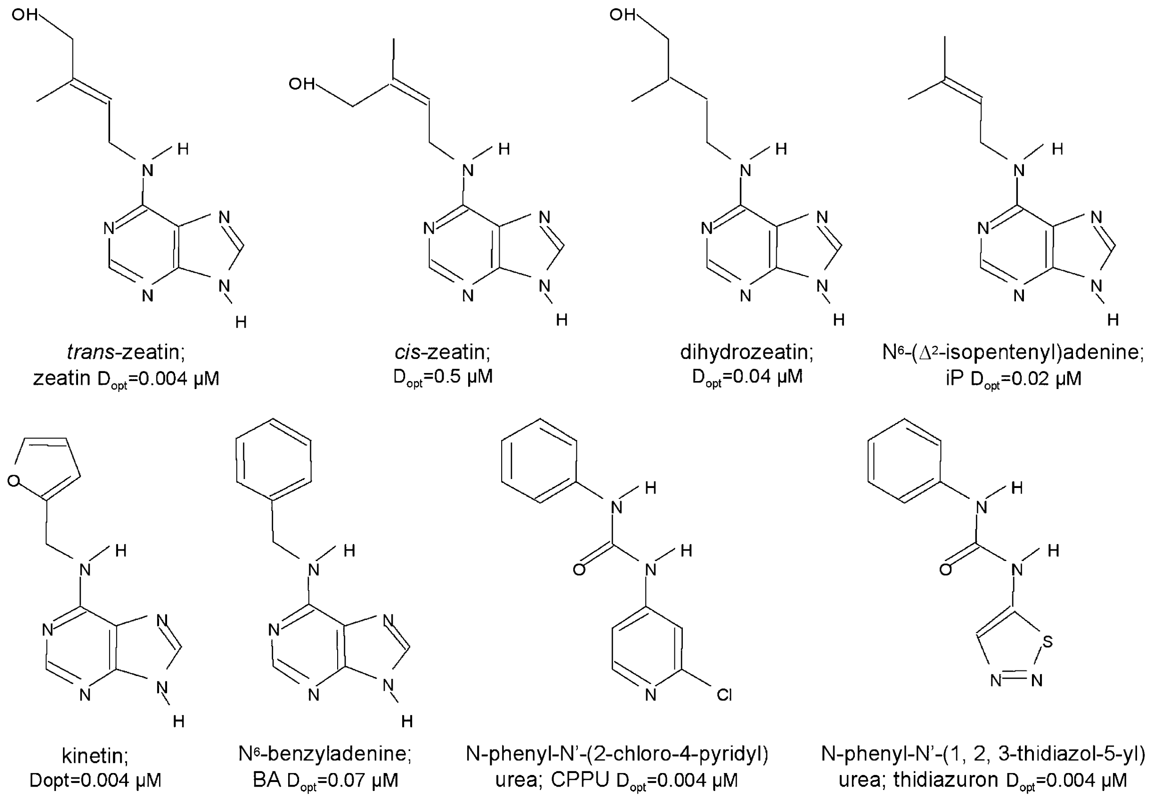
**Cytokinins were first described as molecules promoting cell division and differentiation in plants. It was later discovered that cytokinins regulate a wide range of responses involved in plant development, including shoot meristem maintenance, leaf formation, root growth, chloroplast biogenesis, and senescence.**

## I. INTRODUCTION

Miller and co-workers discovered the first cytokinin, kinetin, in 1955. Kinetin, purified from autoclaved herring sperm, was a compound that restored normal division of tobacco stem pith tissue cells cultured in the presence of auxin. Kinetin does not occur naturally in plants but results from rearrangement of heated DNA. A large group of molecules structurally related to kinetin have now been identified in plants as endogenous active cytokinins (CKs).

## II. STRUCTURE OF NATURAL AND SYNTHETIC CYTOKININS

CKs that occur naturally in plants are adenine derivatives (Fig. 1). The most common is zeatin, which was the first natural cytokinin to be identified.



*trans*-zeatin; zeatin $D_{opt}$=0.004 μM

*cis*-zeatin; $D_{opt}$=0.5 μM

dihydrozeatin; $D_{opt}$=0.04 μM

$N^6$-($\Delta^2$-isopentenyl)adenine; iP $D_{opt}$=0.02 μM

kinetin; Dopt=0.004 μM

$N^6$-benzyladenine; BA $D_{opt}$=0.07 μM

N-phenyl-N'-(2-chloro-4-pyridyl) urea; CPPU $D_{opt}$=0.004 μM

N-phenyl-N'-(1, 2, 3-thidiazol-5-yl) urea; thidiazuron $D_{opt}$=0.004 μM

FIGURE 1 Structures of natural and synthetic adenine and synthetic phenylurea cytokinins. $D_{opt}$, optimum concentration for the maximal response in tobacco callus growth bioassay (Skoog test).

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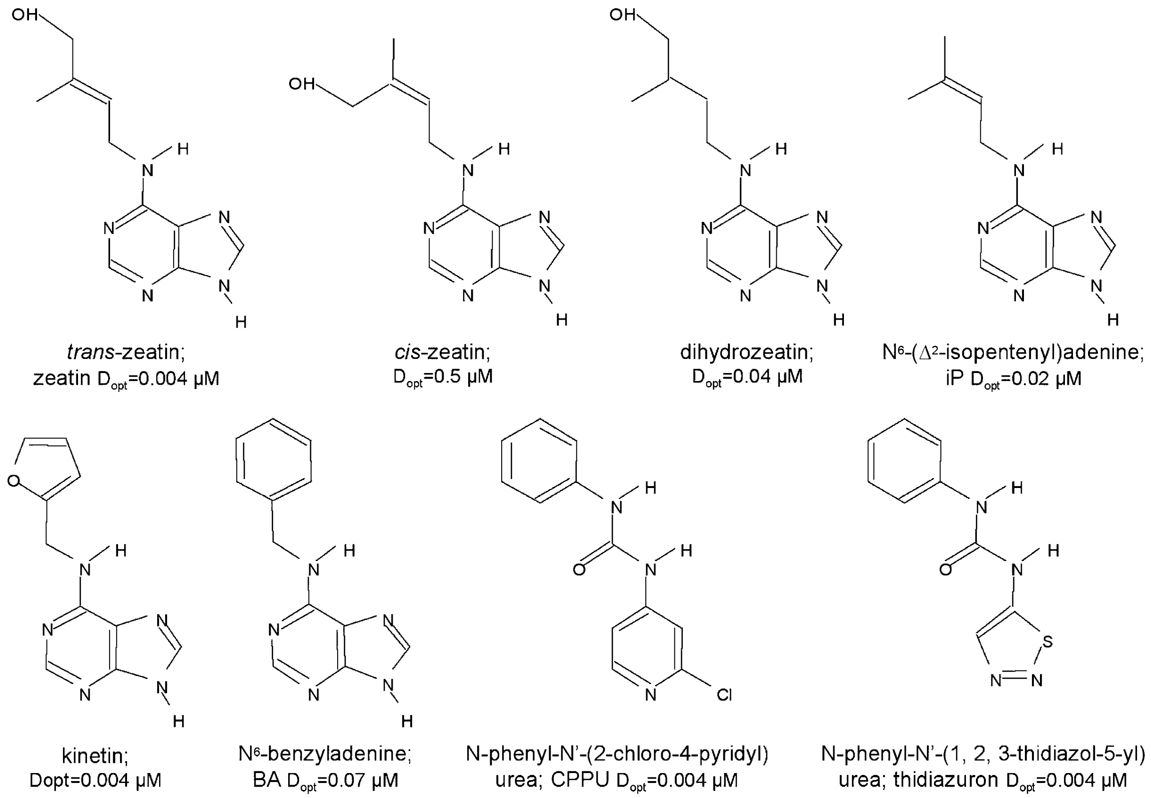
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*cis*-zeatin; $D_{opt}$=0.5 μM

dihydrozeatin; $D_{opt}$=0.04 μM

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N-phenyl-N'-(1, 2, 3-thidiazol-5-yl) urea; thidiazuron $D_{opt}$=0.004 μM

**FIGURE 1** Structures of natural and synthetic adenine and synthetic phenylurea cytokinins. $D_{opt}$, optimum concentration for the maximal response in tobacco callus growth bioassay (Skoog test).

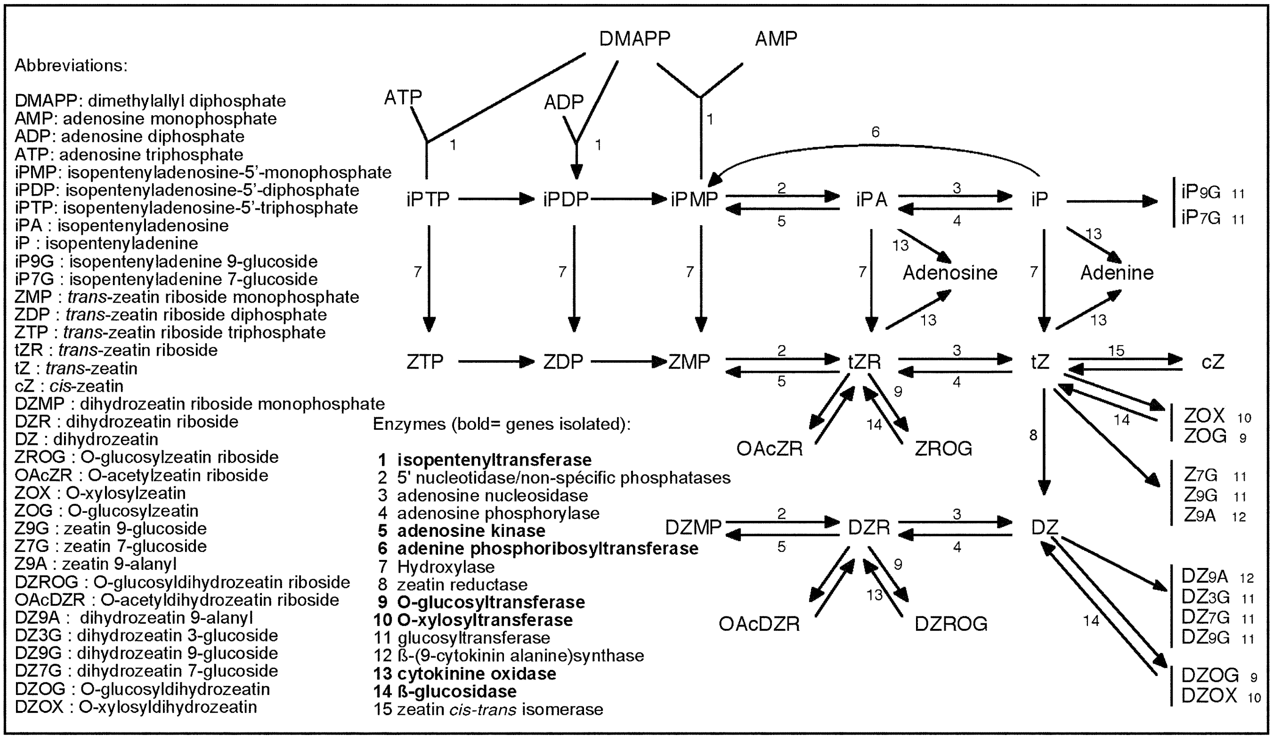
Dihydrozeatin, which is zeatin with a saturated side chain, has also been identified in many species, whereas *cis*-zeatin and isopentenyladenine are generally minor components. These molecules can also exist as ribosides, which have also been shown to be active in plants.

Synthetic analogues of adenine that have cytokinin activity have been generated. These include kinetin and benzyladenine, which have been commonly used for *in vitro* tissue culture work. Synthetic urea derivatives can also act as CKs. Diphenylurea (DPU) was the first urea thought to possess cytokinin activity, which was discovered in coconut milk and was at one time used as a growth substance for tissue culture work. In fact, DPU probably did not contribute significantly to the CK activity because zeatin is the active native cytokinin molecule in the milk. Nevertheless, urea-type molecules do show potential as synthetic CKs (Fig. 1). A level of cytokinin activity similar to that of zeatin has been described for *N*-phenyl-*N*′-(2-chloro-4-pyridyl)-urea (CPPU). Thidiazuron, a urea-type molecule used as a cotton defoliant, also acts as an effective cytokinin. In contrast to zeatin, these active phenylureas are highly stable.

There is no evidence that phenylurea-type CKs occur naturally in plant tissues. Since of this, only the adenine-type cytokinin metabolism is described here. Cytokinin metabolism has been largely elucidated and genes have been identified for some enzymes involved. Regulation of active cytokinin levels in plants may occur by control of synthesis, reversible conjugation, or irreversible inactivation.

## III. CYTOKININ BIOSYNTHESIS AND METABOLISM

CKs are present in most plant tissues, but synthesis is believed to be localized to root tips, shoot meristems, and immature seeds. Since several CKs are found as components of transfer RNA (tRNA), a central element of protein biosynthesis, it had been speculated for many years that tRNA was the primary source of CKs. However, because the major cytokinin found in tRNA is the less active *cis*-zeatin, this pathway is not considered to be the main route of biosynthesis. A second pathway is the adenosine monophosphate (AMP) pathway (Fig. 2). In this pathway an isopentenyl group is transferred from dimethylallyl diphosphate (DMAPP) to the $N^6$ of AMP, resulting in the



Abbreviations:

DMAPP: dimethylallyl diphosphate
AMP: adenosine monophosphate
ADP: adenosine diphosphate
ATP: adenosine triphosphate
iPMP: isopentenyladenosine-5'-monophosphate
iPDP: isopentenyladenosine-5'-diphosphate
iPTP: isopentenyladenosine-5'-triphosphate
iPA : isopentenyladenosine
iP : isopentenyladenine
iP9G : isopentenyladenine 9-glucoside
iP7G : isopentenyladenine 7-glucoside
ZMP : *trans*-zeatin riboside monophosphate
ZDP : *trans*-zeatin riboside diphosphate
ZTP : *trans*-zeatin riboside triphosphate
tZR : *trans*-zeatin riboside
tZ : *trans*-zeatin
cZ : *cis*-zeatin
DZMP : dihydrozeatin riboside monophosphate
DZR : dihydrozeatin riboside
DZ : dihydrozeatin
ZROG : O-glucosylzeatin riboside
OAcZR : O-acetylzeatin riboside
ZOX : O-xylosylzeatin
ZOG : O-glucosylzeatin
Z9G : zeatin 9-glucoside
Z7G : zeatin 7-glucoside
Z9A : zeatin 9-alanyl
DZROG : O-glucosyldihydrozeatin riboside
OAcDZR : O-acetyldihydrozeatin riboside
DZ9A : dihydrozeatin 9-alanyl
DZ3G : dihydrozeatin 3-glucoside
DZ9G : dihydrozeatin 9-glucoside
DZ7G : dihydrozeatin 7-glucoside
DZOG : O-glucosyldihydrozeatin
DZOX : O-xylosyldihydrozeatin

Enzymes (bold= genes isolated):

**1 isopentenyltransferase**
2 5' nucleotidase/non-spécific phosphatases
3 adenosine nucleosidase
4 adenosine phosphorylase
**5 adenosine kinase**
**6 adenine phosphoribosyltransferase**
7 Hydroxylase
8 zeatin reductase
**9 O-glucosyltransferase**
**10 O-xylosyltransferase**
11 glucosyltransferase
12 ß-(9-cytokinin alanine)synthase
**13 cytokinine oxidase**
**14 ß-glucosidase**
15 zeatin *cis-trans* isomerase

FIGURE 2 Cytokinin biosynthesis and metabolism. The identity of the main substrate for isopentenyltransferase (AMP, ADP, or ATP) needs to be determined.

production of isopentenyladenosine-5′-monophosphate (iPMP). This reaction is thought to be catalyzed by a DMAPP:AMP isopentenyltransferase. The most well-characterized isopentenyltransferase (IPT) is the IPT enzyme of the gall-forming bacteria *Agrobacterium tumefaciens*. Completion of the *Arabidopsis* genome has allowed for the identification of nine putative genes coding for isopentenyltransferases in the model plant. Expression of these genes in *Escherichia coli* resulted in DMAPP:AMP isopentenyltransferase activity in crude bacterial extracts. Recently, another cytokinin pathway has been demonstrated in *Arabidopsis* in which *trans*-zeatin riboside monophosphate (ZMP) is synthesized independently of iPMP (Fig. 2). Interestingly, one *Arabidopsis* IPT enzyme, AtIPT4, was shown to have DMAPP:ATP/ADP isopentenyltransferase activity, instead of either DMAPP:AMP or DMAPP:tRNA isopentenyltransferase activity. The realization that zeatin-type compounds are the predominant CK in plants has led to the hypothesis that isopentenylation of ATP and/or ADP is one of the key steps in cytokinin biosynthesis in plants.

## A. Modification of the Adenine Ring

Following synthesis, CKs can be metabolized via one of three reactions: conversion to the base/nucleoside/nucleotide forms, modification of the adenine ring, or modification of the $N^6$-isoprenoid side chain (Fig. 2). Depending on whether these reactions are reversible, the final products are considered to be either storage or inactivated forms of CKs. Although the conversions between base/nucleoside/nucleotide seem primarily related to purine metabolism and only incidental to cytokinin metabolism, the fact that, at least in tobacco cell suspension cultures, only the base form is active confirms the importance of these conversions to the activity.

The enzymes involved in these interconversions generally display a higher affinity for adenine, adenosine, and AMP than for the corresponding CKs. This is the case for the 5′-nucleotidase that converts cytokinin nucleotides to bases, for the adenosine nucleosidase that converts cytokinin nucleosides to bases, for the adenine phosphorylase that converts cytokinin bases to nucleosides, and for the adenosine kinase that converts cytokinin nucleosides to nucleotides. Two genes encoding the adenine phosphoribosyltransferase that converts cytokinin bases to nucleotides have been isolated from *Arabidopsis*. The adenine phosphoribosyltransferase 1 (APT1) enzyme shows a much higher affinity for adenine than for benzyladenine, whereas the other enzyme, APT2, has threefold higher affinity for benzyladenine than for adenine. A gene encoding an adenosine kinase has been cloned from moss, but its substrate specificity has not yet been reported.

Most adenine-type CKs can also undergo other types of conversions, restricted only by steric hindrance. For example, the adenine ring can be glucosylated at the 3, 7, or 9 position and conjugated to alanine at the 9 position. A glucosyltransferase that catalyzes the formation of 7- and 9-glucosides has been isolated from radish. Also, a transferase that converts zeatin to its 9-alanyl derivative has been purified from lupine seeds. The exact role of those conjugates is still unclear and, with the exception of the 3-glucosides, they exhibit low or no cytokinin activity in bioassays.

## B. Modification of the $N^6$-Isoprenoid Side Chain

Modifications of the $N^6$-isoprenoid side chain of CKs have pronounced effects on cytokinin activity. For example, the zeatin side chain can be reduced to produce the aforementioned dihydrozeatin, conjugated to O-glycosides, converted to *cis*-zeatin, or cleaved by cytokinin oxidases (Fig. 2). The side chain on dihydrozeatin can also be conjugated to O-glycosides but it is resistant to cytokinin oxidases. A zeatin reductase that converts *trans*-zeatin to dihydrozeatin has been isolated from *Phaseolus vulgaris* seeds. Because dihydrozeatin is resistant to cytokinin oxidases, zeatin reductase may play an important role in cytokinin homeostasis.

Cytokinin O-glycosyltransferases are highly specific to CKs. Two O-glycosyltransferases have been isolated, an O-xylosyltransferase from *P. vulgaris* and an O-glucosyltransferase from *Phaseolus lunatus*. Those two enzymes are specific for zeatin and dihydrozeatin, with no activity toward *cis*-zeatin and cytokinin ribosides. Besides showing high specificity for particular CKs, these enzymes also show specificity for the sugar donor, leading to the hypothesis that O-glycosylation of CKs is tightly regulated in plants.

A gene encoding a *cis*-zeatin O-glucosyltransferase that does not recognize *trans*-zeatin or dihydrozeatin has been isolated from maize. In addition, an enzyme specific for *cis*-zeatin, called *cis*–*trans*-zeatin isomerase, has been partially purified from *Phaseolus*. This enzyme preferentially converts the cis isomer to the trans isomer and may play an important role in regulating cytokinin activity because it converts the less active *cis*-zeatin to the highly active *trans*-zeatin. The existence of *cis*-zeatin *O*-glucosyltransferase and *cis-trans*-zeatin isomerase increases the complexity of the cytokinin metabolism scheme and emphasizes the potential role of *cis*-zeatin derivatives in cytokinin homeostasis.

A major modification of the cytokinin $N^6$-isoprenoid side chain is a reduction by cytokinin oxidase, which results in formation of adenine-type compounds from cytokinin bases and nucleosides, but not from nucleotides. Because of its crucial role, cytokinin oxidase has been extensively studied and purified from a variety of plants, with genes representing cytokinin oxidases from maize and *Arabidopsis* being recently cloned. These enzymes share homology with flavin adenine dinucleotide (FAD)-dependent oxidases. Kinetic analyses have shown that urea-type CKs are strong competitive inhibitors of cytokinin oxidase activity. Benzyladenine and kinetin are not substrates for this enzyme.

## IV. CYTOKININ SIGNALING

The first step of cytokinin signaling is recognition and binding by specific receptors. For many years, several groups have employed biochemical strategies in order to identify cytokinin-binding proteins that may represent cytokinin receptors. Some of these proteins demonstrate the properties expected for receptors but their physiological functions have never been established. Genetic and genomic approaches have allowed for important progress in the field of plant hormone perception and signaling, including the mechanism of CK signal transduction. Although our knowledge is still limited, the transduction pathway that is initiated by cytokinin has recently begun to be elucidated.

To identify components of the CK signaling pathway, *Arabidopsis* mutants were screened for those that demonstrated altered cytokinin responses, including rapid cell proliferation and shoot formation in tissue culture. Mutants that qualified were subsequently called *CK response* and *CK independent* (*cre1*, *cki1*, and *cki2*). Wild-type hypocotyl explants, when exposed to increasing levels of active cytokinin (kinetin, benzyladenine, isopentenyladenine, and the phenylurea-type CKs), respond with rapid cell proliferation and with greening and formation of shoots. The *cki1* mutant displayed typical CK-dependent phenotypes in the absence of exogenous hormone, whereas *cre1* mutant explants were less responsive. The structures of the predicted CRE1 and CKI1 proteins are similar to those of the sensors of two-component regulators, which are important signaling systems commonly found in prokaryotes and lower eukaryotes.

### A. CK Receptors Are Histidine Protein Kinase

A typical prokaryotic two-component system is composed of a sensor histidine kinase (also termed sensor) and a response regulator (Fig. 3). Sensors included a variable input domain responsible for the perception of external stimuli and a conserved histidine kinase domain (transmitter). Response regulators consist of a receiver domain and an output domain. The signal perception by the input domain results in autophosphorylation of a conserved histidine residue in the histidine kinase domain of the sensor. The phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain of a cognate response regulator. In some two-component systems, a multistep phosphorelay is utilized, involving a sequential phosphorylation of intermediate histidine phosphotransmitter proteins (HPts) and, finally, transfer of the phosphoryl group to a response regulator. Typical eukaryotic two-component systems comprise a sensor histidine kinase domain fused to a receiver domain and are therefore termed hybrid two-component regulators. CRE1 is a predominantly hydrophobic two-membrane-spanning domain in the N-terminal region, with an extracellular loop that can

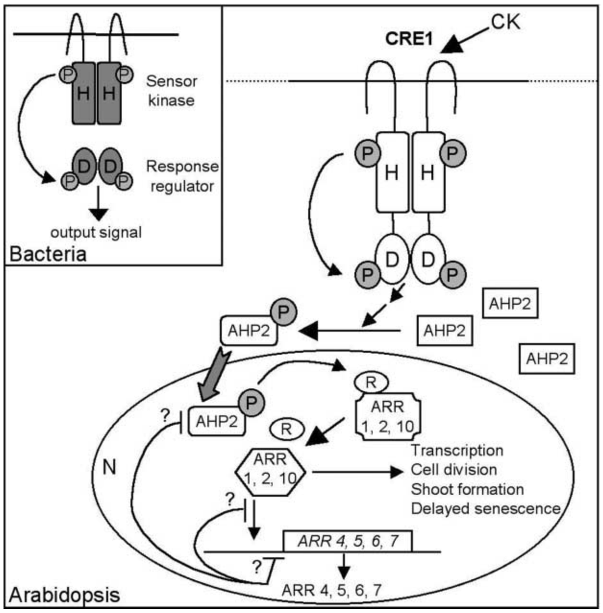


**FIGURE 3** Two-component signaling in *Arabidopsis*. Model of the cytokinin signal transduction pathway. Adapted from Hwang and Sheen (2001).

bind a ligand. The C-terminal region is probably cytoplasmic and consists of a histidine kinase domain and an unusual tandem of two receiver domains. By contrast, the ethylene receptor (ETR1) family and the putative *Arabidopsis thaliana* histidine kinase (AtHK1) osmosensor have only a single receiver domain.

Elegant experimental approaches using yeast complementation have demonstrated that CRE1 functions as the cytokinin receptor. Heterologous complementation of a yeast histidine kinase sensor mutant by CRE1 successfully restores growth of the mutant but only if active CKs are present. CRE1 (AtHK4) binds a variety of natural and synthetic CKs and diphenylurea derivatives with a high affinity ($K_d = 5 \times 10^{-9}\ M$), indicating that the receptor is responsive to physiological concentrations of the hormone. This demonstrates that, in yeast, cytokinin-dependent CRE1 activation leads to SSK1-mediated mitogen-activated protein kinase (MAPK) inactivation via an existing signaling pathway. Similar approaches in *Schizosaccharomyces pombe* and *Escherichia coli* demonstrated that CRE1 (AtHK4) as well as closely related *Arabidopsis* sensor histidine kinases (AtHK2 and AtHK3) function as cytokinin-responsive sensors. In contrast, the CKI1 histidine kinase that was identified from an *Agrobacterium*-mediated activation tag mutant screen does not bind CKs and its role in CK signaling remains to be elucidated.

## B. Cytokinin Signaling Involves Histidine Phosphotransfer Proteins and Response Regulators

Two-component regulator signaling requires a phosphotransfer that initiates with a histidine kinase and either terminates with a receiver domain or continues through a phosphororelay that includes an HPt protein and an *Arabidopsis* response regulator (ARR). All of the elements of two-component signaling systems are present in plants. Analysis of the *Arabidopsis* genomic system reveals at least 11 histidine kinase sensors; five ethylene receptors; three cytokinin receptors, AtHK2, AtHK3, and AtHK4 (CRE1); and one osmosensor (AtHK1). Five genes (*AHP1–AHP5*) encode typical histidine phosphotransmitters and *Arabidopsis* possesses at least 20 genes encoding proteins similar to response regulators (the ARR series). *ARR* genes have been classified into two distinct subtypes. Members of the A-type have been found to be rapidly induced by cytokinin. *ARR4* and *ARR5* transcripts accumulate within 10 min after application of either adenine or phenylurea-type cytokinin. The ARR5 protein is expressed primarily in root and shoot meristems, which is consistent with a role for CK in the regulation of cell division *in vivo*. The B-type ARRs act as transcriptional factors.

A transient expression assay based on transcriptional activation of a cytokinin primary response gene, *ARR6*, in protoplasts of *Arabidopsis* leaves has been developed to dissect the regulatory components of the cytokinin signal transduction pathway. This assay allows for analysis of the role of two-component regulators and begins to decipher their involvement in cytokinin signaling. It appears that histidine kinase activities are linked to various *Arabidopsis* histidine phosphotransmitter (AHP) proteins that serve as cytoplasmic/nuclear shuttles to distinct ARR proteins in the nucleus. The last steps are an ARR-dependent transcriptional activation of target genes and a negative feedback loop through cytokinin-inducible *ARR* genes. CRE1 transcriptionally activates *ARR6* only after application of CK, whereas CKI1 activity is CK independent, indicating that it may continuously activate the pathway. Histidine protein kinase activity is required because mutations in the critical histidine and aspartate residues of CRE1 and CKI1 have a dominant negative effect. AHP proteins are not limiting factors in the cytokinin signaling pathway and are transferred into the nucleus in a cytokinin-dependent manner. In this transient expression assay, B-type ARRs were capable of activating cytokinin responses, including A-type ARR expression. CK-induced A-type ARR proteins in turn act to repress their own expression. Ectopic expression of one cytokinin-related *ARR* (*ARR2*) results in senescence delay, a typical cytokinin response. ARR2 is thought to act downstream in the pathway because it is independent of CKI1 activity and blocks CK signaling if expressed as a mutated dominant negative allele.

From these results, a hypothetical model of the cytokinin signal transduction pathway in *Arabidopsis* can be developed (Fig. 3). In this model, the cytokinin signal is externally perceived by multiple histidine protein kinases located at the plasma membrane. On perception of a cytokinin stimulus, the histidine protein kinases initiate a phosphorelay signaling cascade that results in the nuclear translocation of phosphorylated AHP proteins from the cytosol. Activated AHP proteins can then release B-type ARR proteins from a putative repressor complex in the nucleus, the nature of which remains to be elucidated. The B-type ARR proteins (ARR1, ARR2, and ARR10) can then act as activators of transcription of various target genes that control shoot formation and senescence. The cytokinin-inducible A-type *ARR* genes are directly up-regulated by B-type *ARR* and are involved in a negative feedback loop.

## V. BIOLOGICAL ROLES

CKs are involved in many processes throughout plant growth and development (Fig. 4). However, the precise roles of CKs have been difficult to analyze, mainly because (1) many of the classic CKs responses are also controlled by other hormones (auxin, abscisic acid, or ethylene) or environmental stimuli (light or carbon/nitrogen source) and (2) development of most of the physiological responses is slow (a few hours to days). Most early work was performed with exogenously supplied CKs, which has the drawback of poor spatial and temporal control of CK levels in plants. Recent advances have allowed local and temporal endogenous CK accumulation to be controlled—for instance, by expressing the *IPT* (isopentenyltransferase) gene, which produces CK, under specific inducible promoters. An alternative is to control CK degradation as a means to decrease endogenous CK concentration.

### A. Cell Division

CK was first described as a necessary factor, in conjunction with auxin, for the promotion of cell proliferation *in vitro*. CK was found to regulate the cell cycle machinery directly at the $G_1$–S transition through the specific induction of cyclin D3 (CycD3) mRNA in *Arabidopsis* cell culture. *CycD3* is expressed in *Arabidopsis* dividing tissues such as shoot meristems, young leaf primordia, axillary buds, procambium, and vascular tissues. Exogenously supplied CKs enhance *CycD3* expression in these different tissues but do not lead to ectopic expression. CK-regulated *CycD3* is a key step in cell division because constitutive expression of *CycD3* in transgenic leaf explants allows induction and maintenance of cell divisions in the absence of exogenous CKs. CKs also control cell division at the $G_2$–M transition: *Nicotiana plumbaginifolia* cell cultures deprived of CKs arrest in $G_2$ and present a highly tyrosine-phosphorylated cyclin-dependent kinase (CDK, also named p34cdc2). In yeast and mammals, CDK dephosphorylation of a specific tyrosine by the phosphatase cdc25 is required for progression through mitosis. This result suggests that CKs might stimulate a cdc25-like activity required for the $G_2$–M transition.

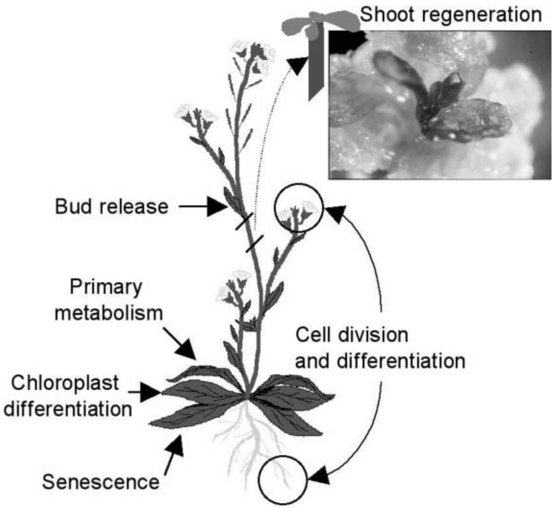

Shoot regeneration
Bud release
Primary metabolism
Cell division and differentiation
Chloroplast differentiation
Senescence

**FIGURE 4** Cytokinin responses in plants.

Cell division in meristems is controlled by a specific set of transcription factors, including the homeobox-related factors encoded by *KNOTTED-like1* (*KNAT1*) and *SHOOT MERISTEMLESS* (*STM*). *STM* is required for the maintenance of the meristematic stem cells and *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. Both genes are up-regulated by CKs. Conversely, overexpression of the maize *KNOTTED1* (*KN1*) gene in tobacco and overexpression of *KNAT1* in lettuce lead to CK accumulation. Ectopic expression of maize *KN1* in cultured tobacco tissues results in growth independent of exogenously supplied cytokinin. In the case of lettuce, *KNAT1* overexpression shifts leaf determinate growth to a shootlike indeterminate growth. These data suggest the existence of a regulatory loop between this class of homeobox transcription factor genes and cytokinin accumulation.

To identify genes involved in control of cell division by cytokinin, mutants showing ectopic cell proliferation specifically enhanced by CKs were isolated in *Arabidopsis*. The apical portions of the mutant seedlings were transformed into a callus-like structure by the application of CKs. Cells of the mutant seedlings were found to dedifferentiate and lose intercellular adhesion. In weak alleles, plants could be grown to the adult stage and showed spontaneous and local cell proliferation as well as cell invasiveness leading to organ fusion. These phenotypes, reminiscent of mammalian tumor development, were linked to three genes called

*PASTICCINO1*, *PASTICCINO2*, and *PASTICCINO3* (*PAS1, PAS2*, and *PAS3*). *PAS1* was found to encode a protein similar to FK506-binding proteins from the immunophilin family. This family of proteins has been found to be involved in the regulation of activities of protein complexes in various signaling pathways, probably due to their foldase activity.

## B. Cell Differentiation

Soon after its discovery, CK was found to be necessary and sufficient for shoot development for *in vitro* tissue regeneration of many plant species (Fig. 4). This role has been confirmed *in vivo* by numerous studies using local or systemic CK accumulation. In almost every case, the main effect of cytokinin is reduction of apical dominance by the growth of axillary buds.

Several mutant screens were performed to identify the genes involved in CK-induced shoot production. The screens allowed isolation of the two-component histidine kinase, CKI1 and CRE1/AtHK4. A loss-of-function screen was also undertaken in *Arabidopsis* to identify mutants that grow as callus on hormone-free medium and can form shoots. Six lines that were identified (called *shooty callus*) were sterile and thus could not be analyzed genetically. Nonetheless, the mutants showed an increase in *CKI1*, *KNAT1*, and *STM* expression, suggesting that cytokinin signaling was modified. Similar *Arabidopsis* mutants that respond more sensitively than the wild type to CKs were isolated and were designated *ckh1* and *ckh2*, for cytokinin-hypersensitive. The calli produced from *ckh* mutants exhibit typical cytokinin responses, including rapid proliferation and chloroplast development in response to lower levels of CKs than is seen in the wild type. The cytokinin levels in all these mutants were not increased.

To analyze the effect of decreased CK levels on plant development, the *Arabidopsis* cytokinin oxidase gene was overexpressed in transgenic tobacco. Cytokinin-deficient plants developed stunted shoots with smaller apical meristems. The time between new leaf initiation was prolonged, and leaf cell production was only 3–4% of that of wild type, indicating an absolute requirement of CKs for leaf growth. In contrast, root meristems of transgenic plants were enlarged and gave rise to faster growing and more branched roots. These results suggest that CKs are an important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots.

Cks are also involved in other differentiation processes, such as vascular tissue development, as illustrated by the mutations in one of the cytokinin receptors (CRE1/AtHK4). The developmental ontogeny of the vascular system (consisting of xylem, phloem, and procambium) is specified early in the *Arabidopsis* root meristem. The absence of the CK receptor CRE1/AtHK4 leads to the absence of phloem and procambium, which are established through a set of asymmetric cell divisions. This demonstrates that CKs are required for phloem and procambium development in the root.

## C. Senescence and Carbon/Nitrogen Metabolism

Leaf senescence is an active process involving remobilization of nutrients from senescing leaves to other parts of the plant. Whereas senescence is accompanied by a decline in leaf cytokinin content, supplemental cytokinin, such as in *IPT* or *KN1* overexpressors, delays senescence.

Although cytokinin and ethylene have opposite roles in the control of senescence, they elicit similar responses in early seedling development. Dark-grown *Arabidopsis* seedlings treated with low levels of CKs show the characteristic ethylene-mediated triple response. Cytokinin-insensitive mutants (*cin*) were isolated based on the lack of a triple response in the presence of cytokinin. The *CIN5* gene was identified as a member of the *Arabidopsis* gene family that encodes 1-amino-cyclopropane-1-carboxylate synthase. This first enzyme in the ethylene biosynthesis pathway is mainly responsible for the sustained rise in ethylene biosynthesis observed in response to low levels of cytokinin. This provides direct evidence for the existence of cross talk between CKs and ethylene.

CKs were originally thought to affect carbon/nitrogen metabolism by promoting the “sink” effect. A plant organ becomes a “sink” when import of photoassimilants exceeds export. A large number of genes involved in carbon and nitrogen metabolism have been found to be regulated by CKs at both the transcriptional and the posttranscriptional levels. Several genes encoding ribulose 1,4-bisphosphate carboxylase, chlorophyll *a* and *b* binding proteins, or phosphoenolpyruvate decarboxylase were up-regulated by CKs. CKs also control expression of the regulatory components of carbon metabolism, such as the members of the SNF1-related protein kinase family. Less is known about regulation of nitrogen metabolism by cytokinin, although the nitrate reductase transcript level is up-regulated by CKs. CK can mimic some light-induced effects in dark-grown seedlings, including induced chloroplast differentiation and greening. Interestingly, CKs repress expression of the photoreceptor phytochrome, probably through a negative feedback regulatory loop.

## VI. CONCLUSIONS

A complex biosynthetic and degradation network controls the levels of the large family of cytokinin molecules found in plants. The mode of action of each cytokinin is determined by a vast spectrum of molecules and interconnecting metabolic pathways. Cytokinins were the second plant hormone (after ethylene) for which receptors were discovered. As was the case for ethylene, the cytokinin receptors were found to be histidine kinase sensors. Interestingly, the two-component signaling system employed by cytokinins in the plant kingdom seems to be absent from the animal kingdom. The diversity of cytokinins and their biological responses, associated with a relatively simple transduction cascade, constitute a paradigm that represents the next challenge for understanding the role of this fundamental plant growth factor.

## Glossary

**activation tag mutant** Produced by *Agrobacterium tumefaciens*-mediated transfer DNA integration, which randomly inserts into the plant genome a DNA fragment that harbors at one extremity a transcriptional enhancer element. After DNA integration, expression of neighboring genes is increased, leading in some cases to mutants with a gain-of-function dominant phenotype.

***Agrobacterium tumefaciens*** Plant pathogen causing crown gall tumors on susceptible plants. Tumor induction results from the transfer of a small piece of DNA, called transfer DNA, from the bacterium to the plant cell during infection. The transfer DNA becomes integrated into plant cell nuclear DNA and expression of genes on this segment causes the plant cell to differentiate into a tumor cell.

**differentiation** Acquisition by an unspecialized plant cell (meristematic cell) of a specialized function.

**meristem** A group of undifferentiated and proliferating cells that continuously give rise to all plant organs and tissues throughout the plant life cycle.

**two-component system** A signal perception and transduction system constituted by a sensor histidine kinase and a response regulator. Sensors include a variable-input domain, which is responsible for perception of external stimuli, and a conserved histidine kinase domain. Response regulators consist of a receiver domain and an output domain. This signaling system is found in prokaryotes, lower eukaryotes, and plants.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Ethylene • Gibberellins • Jasmonates • Systemins • Salicylic Acid**

## Further Reading

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# Decidualization

THOMAS G. KENNEDY
*University of Western Ontario, Canada*

I. INTRODUCTION
II. MORPHOLOGICAL CHANGES
III. FUNCTIONS OF THE DECIDUA
IV. ENDOMETRIAL RECEPTIVITY AND SENSITIZATION
V. LOCAL MEDIATORS OF DECIDUALIZATION
VI. SUMMARY

**In mammalian species with invasive implantation, the endometrium undergoes substantial morphological and functional changes that are collectively referred to as decidualization; the endometrial stromal cells proliferate and differentiate into decidual cells, which ultimately form the maternal component of the placenta.**

## I. INTRODUCTION

The process of blastocyst implantation in mammals is remarkably variable, especially in the extent of trophoblast invasion of the endometrium. In species with invasive implantation, the endometrium undergoes substantial morphological and functional changes, referred to as decidual transformation of the endometrium. In most species, the transformation primarily involves the endometrial stroma; the fibroblastic stromal cells proliferate and differentiate into decidual cells that ultimately give rise to the maternal component of the placenta. Decidualization during pregnancy in most species is localized to sites of blastocyst implantation. An increase in endometrial vascular permeability, also localized to sites of blastocyst implantation, precedes the proliferation and differentiation of stromal cells into decidual cells and is thought to be an essential prerequisite for the transformation.

In many species, a nonspecific stimulus can result in an increase in endometrial vascular permeability and subsequent decidual transformation of the endometrium, provided that the stimulus is applied at the appropriate time. This artificially induced decidualization has been a useful experimental tool that allows the investigation of decidualization without the complications arising from the presence of the conceptus.

Decidualization in the human differs from that in other species in at least two respects. First, a conceptus does not have to be present to initiate the process; it begins “spontaneously” in the late luteal phase of the menstrual cycle with the differentiation of “predecidual cells.” Exposure of the endometrium to progesterone beyond the length of the normal nonpregnant luteal phase results in decidual transformation of the endometrium. Second, decidualization in the human endometrium is generalized, rather than being localized to the site of blastocyst implantation. Presumably, this is a consequence of the fact that a signal from the conceptus is not required for the initiation of this process in the human.

## II. MORPHOLOGICAL CHANGES

The changes in morphology associated with decidualization have been studied in detail in laboratory rodents but are thought to be basically similar in most species. The earliest macroscopically identifiable event is an increase in endometrial vascular permeability that results in the development of stromal edema. It is localized to implantation sites but is generalized if an artificial deciduogenic stimulus is applied along the full length of the uterus. The permeability response is followed by an increase in alkaline phosphatase activity. The differentiating stromal cells become enlarged with rounded nuclei. Subsequently, the cells become rounded and typically have two or more polyploid nuclei. Ultrastructurally, the differentiation of decidual cells is characterized by the accumulation of glycogen and lipid droplets and increased amounts of filament proteins. The content of polyribosomes and rough endoplasmic reticulum also increases.

In laboratory rodents, in response to either an implanting blastocyst or an artificial stimulus, decidualization begins in the anti-mesometrial pole of the endometrium and, over time, spreads to involve the mesometrial stromal cells. Why this is the case is poorly understood. It is possible that the lumenal epithelial cells that have an essential transductive role in the initiation of decidualization differ depending on their location in the endometrium. Alternatively, the anti-mesometrial and mesometrial stromal cells may differ. During pregnancy, the definitive placenta is formed at the mesometrial pole and the anti-mesometrial decidual cells undergo apoptosis to form the decidua capsilaris. The anti-mesometrial and mesometrial decidual cells are morphologically and functionally distinct. For example, rat anti-mesometrial decidual cells produce and/or express insulin-like growth factor-binding protein-1, follistatin, prolactin, and interleukin-11 (IL-11), whereas mesometrial decidual cells produce and/or express prolactin receptors, $\alpha_2$-macroglobulin, and basic fibroblast growth factor.

## III. FUNCTIONS OF THE DECIDUA

Several functions have been ascribed to the decidual cells. These include a nutritional role for the conceptus until formation of the definitive placenta, a role in limiting trophoblast invasion, an immunoprotective role, and an endocrine role.

The putative nutritional function follows from the observations of accumulation of glycogen granules and lipid droplets in the cytoplasm of decidual cells. Proteins are also secreted into the implantation chamber and may be utilized by the conceptus.

The decidua may protect the uterus from the actively invading trophoblastic cells. At extrauterine sites, trophoblastic cells are similar to malignant cells in their invasive properties. In the uterus in the absence of decidualization, trophoblastic cells can perforate the uterus. Decidual cells may limit invasion by establishing a structural barrier and/or by producing factors that act locally to restrict invasion. The extensive remodeling of the extracellular matrix that occurs during decidualization may protect the endometrium against trophoblast invasion. In addition, decidual cells express proteins, such as tissue inhibitor of matrix metalloproteinase, cystatin C, and plasminogen activator inhibitors, that are able to inhibit the activity of proteinases produced by trophoblastic cells, thereby restricting invasion.

The decidual tissue may provide immunological protection for the conceptus by suppressing the maternal immune response, thereby allowing successful completion of pregnancy. The mechanism by which this is accomplished is unknown.

The fact that decidual cells produce prolactin and/or prolactin family members suggests that the decidual tissue has an endocrine function. The physiological roles of these decidual products are unknown but it seems likely that they may be involved in the regulation of maternal adaptations to pregnancy. There is evidence of a paracrine, rather than endocrine, role for decidual prolactin in the rat. Prolactin produced by anti-mesometrial decidual cells acts on mesometrial stromal/decidual cells to regulate their function.

## IV. ENDOMETRIAL RECEPTIVITY AND SENSITIZATION

Implantation and subsequent decidualization can be initiated by a blastocyst only during a limited time when the endometrium is said to be receptive. In laboratory animals that respond to stimulation of the endometrium, there is likewise a transient period when decidualization is obtained, provided that the stimulus is relatively nontraumatic. During the responsive period, the endometrium is said to be sensitized for the decidual cell reaction. Endometrial receptivity and sensitization are thought to refer to identical states, and the terms will be used interchangeably in this article. The timing and endocrine control of receptivity and sensitization are similar and absolutely dependent on progesterone. The requirement for estrogen along with progesterone varies with species. In some animals (e.g., mouse and rat), estrogen is essential, but in others (e.g., hamster and rabbit), it is not. In addition, the amount of estrogen that the uterus is exposed to prior to the onset of receptivity and sensitization is crucial. In ovariectomized rats, for example, estrogen at low doses acts synergistically with progesterone to induce endometrial sensitization; estrogen at high doses is inhibitory.

At the molecular level, the mechanisms by which the steroid hormones bring about endometrial receptivity and sensitization are unknown, but presumably involve changes in gene expression. Studies in this area are complicated by the fact that the endometrium is a heterogeneous tissue consisting of numerous cell types including lumenal epithelium, glandular epithelium, stromal cells, endothelial cells, and bone marrow-derived cells. Decidualization almost certainly involves interactions between cell types. For example, the importance of the lumenal epithelium has been established by experiments demonstrating that if the lumenal epithelium is destroyed or removed, decidualization cannot be obtained in response to stimuli that otherwise would be deciduogenic. The results are consistent with the hypothesis that the lumenal epithelium responds to natural and artificial deciduogenic stimuli with the production of a compound that then acts on the stromal cells to trigger decidualization. Thus, at least in theory, the changes in gene expression regulating receptivity and sensitization could be limited to the lumenal epithelium and related to its ability to produce the triggering compound. This seems unlikely, however, because endometrial stromal cells from nonsensitized uteri undergo decidualization to a lesser extent *in vitro* than cells from sensitized uteri. This difference presumably indicates that gene expression in stromal cells from sensitized and nonsensitized uteri differs.

In general, two approaches have been taken to investigate the mechanisms underlying receptivity and sensitization. The first involved the study of putative mediators of the process of decidualization on the premise that the receptive/sensitized state may be explainable in terms of the synthesis and/or action of the mediators. This approach will be reviewed in a later section. The second and more recent approach has been the use of gene expression screening techniques, such as mRNA differential display or gene array chips, to detect changes in gene expression in the peri-implantation period. Many of the data obtained in these studies have been correlative in nature. In addition, some of the studies have compared implantation sites with interimplantation sites, and the altered gene expression so identified may be the consequence of the initiation of blastocyst implantation and the associated decidualization, rather than being related to receptivity and sensitization. Finally, some of the identified differentially expressed genes encode cell surface or cell adhesion molecules in the lumenal epithelium. Although these molecules may be very important for blastocyst implantation by facilitating the attachment of the blastocyst to the epithelium, they are considered to be outside the scope of this article.

## A. Leukemia Inhibitory Factor

Although uterine expression of leukemia inhibitory factor (LIF) was not discovered by a screening process, it is now well known to have an essential role in uterine receptivity, at least in the mouse. LIF is a secreted glycoprotein that has multiple activities in various *in vitro* culture systems. In the mouse, LIF expression is markedly up-regulated in the endometrial glands just prior to the onset of implantation and then declines to low levels for the remainder of pregnancy. In LIF-deficient mice, implantation does not occur and the uterus does not respond to the intrauterine injection of oil with decidualization. The administration of LIF can override the inhibition of implantation and artificially induced decidualization. Thus, LIF is essential in the mouse for the onset of uterine receptivity and sensitization. It acts predominantly on the lumenal epithelium of the endometrium by a signal transduction pathway involving phosphorylation and nuclear transfer of signal transducer and activator of transcription 3 (Stat3). Interestingly, the lumenal epithelial cells are only transiently responsive to LIF despite the presence of constant levels of LIF receptor throughout the peri-implantation period. Consequently, uterine receptivity appears to be regulated by both the onset of LIF expression in the endometrial glands and its ability to activate Stat3.

The essential role of LIF in regulating endometrial receptivity may not be limited to mice. Uterine LIF expression has been investigated in a number of species and the findings have consistently suggested that LIF may be involved in preparing the endometrium for implantation.

## B. Calcitonin

By using a gene expression screening technique, calcitonin was found to be transiently expressed in rat endometrial glandular epithelial cells in the peri-implantation period, reaching a peak on day 4 of pregnancy, the day before the initiation of implantation, and declining thereafter. Subsequent studies demonstrated that the down-regulation of calcitonin expression by the administration of antisense oligonucleotides targeted against calcitonin mRNA substantially reduced the implantation rate. At present, it is not known whether calcitonin acts on the embryos or the endometrium; there is evidence from *in vitro* studies that calcitonin can accelerate blastocyst differentiation. The effects of calcitonin on endometrial receptivity and sensitization have not yet been reported.

Calcitonin is also expressed in human endometrium during the midsecretory phase of the menstrual cycle that corresponds with the putative timing of endometrial receptivity.

## C. HOX Genes

Homeobox (HOX) genes, which encode highly conserved, developmentally regulated transcription factors used to establish body plans, are usually thought to be expressed only during embryonic development. However, HOX gene expression has been observed in the reproductive tracts of adult mice and humans. The importance of this expression has been demonstrated by targeted disruption of the genes; there is implantation failure as well as resorption of embryos in mice deficient in either

Hoxa10 or Hoxa11. In addition, the intrauterine administration of antisense oligonucleotides targeted against Hoxa10 mRNA decreases the implantation rate. Investigation of the mechanisms underlying the uterine deficits in Hoxa10 mice has found inappropriate regulation of prostaglandin $E_2$ receptor subtypes $EP_3$ and $EP_4$. Down-regulation of endometrial Hoxa10 expression by an antisense strategy has been shown to decrease lumenal epithelial pinopod formation. Since pinopods develop transiently on the lumenal epithelial cells at the time of uterine receptivity, this suggests that Hoxa10 expression is required for the acquisition of receptivity.

### D. Other Genes

The expression of several additional genes has been reported to change in association with the onset of uterine receptivity and sensitization. These include interleukin-15 in human endometrium, glucose-regulated protein 78, and a novel previously uncharacterized gene designated uterine sensitization-associated gene-1 in rats. However, the functional role, if any, of the products of these genes remains to be established.

## V. LOCAL MEDIATORS OF DECIDUALIZATION

This article will restrict itself to consideration of potential mediators of decidualization that are produced and act within the endometrium. Although it is possible that the blastocyst produces a chemical signal that modifies endometrial function, there is relatively little evidence for this. The fact that a variety of nontraumatic stimuli, such as the intrauterine injection of oil, are very effective at inducing decidualization suggests that the endometrium is able to produce the mediators required for the process. However, it is always possible that the mediators of decidualization during blastocyst implantation differ from those mediating artificially induced decidualization. Because of the evidence for an essential role of the lumenal epithelium in implantation and decidualization, it seems likely that the epithelial cells are a source of essential components of the cascade.

The list of potential mediators of decidualization has increased in length in recent years. Historically, the list of potential mediators has included histamine, blastocyst-produced estrogen, leukotrienes, platelet-activating factor, angiotensin II, and neutrophil-derived factors. The evidence for and against these compounds can be found elsewhere.

### A. Prostaglandins

There is considerable evidence that prostaglandins (PGs) have an obligatory role in implantation and decidualization. Numerous studies have shown that inhibitors of PG synthesis and PG antagonists inhibit or delay the initiation of implantation. Exogenous PGs can reverse, at least partially, the effects of inhibitors of PG synthesis on implantation. During pregnancy, the concentrations of PGs are elevated at implantation sites.

During artificially induced decidualization, uterine concentrations of PGs increase in a temporal pattern consistent with PGs being a cause, rather than a consequence, of increased endometrial vascular permeability. Inhibitors of PG synthesis substantially attenuate the increase in endometrial vascular permeability, attenuate alkaline phosphatase activity, and attenuate the subsequent decidualization that normally occurs in response to an artificial deciduogenic stimulus. These responses can be restored by the intrauterine administration of PGs with a time course not different from that occurring in the absence of the inhibitor. It should be noted that, at least for rats, full restoration of decidualization was obtained only if the PGs were infused into the uterine lumen; the intauterine injection of PGs, although effective at restoring the vascular permeability response, did not bring about full decidualization. These and other observations have been interpreted as indicating that PG levels must be elevated within the uterus throughout decidualization.

The sites of synthesis, types, and sites of action of the PGs within the endometrium remain unknown. PGs are produced from arachidonic acid by cyclooxygenase (COX); this enzyme has two isoforms, COX-1 and COX-2, the products of two different genes. Both isoforms are expressed in the endometrium in the peri-implantation period. In the mouse uterus, COX-1 is expressed in the lumenal epithelium of the endometrium until blastocyst attachment late on day 4 of pregnancy (the day when the vaginal plug first appears is considered day 1) when expression is down-regulated before reappearing some 48 h later in the secondary decidual zone. By contrast, COX-2 is expressed in the lumenal epithelium and subepithelial stromal cells at the anti-mesometrial pole only at the sites of blastocyst attachment for a limited time on days 4 and 5, after which expression is localized to the mesometrial pole. More recently, the essential role of COX-2, and by inference of PGs, in implantation and decidualization has been demonstrated by the findings that these processes are dramatically affected in COX-2 null mice; by contrast, COX-1 null mice are fertile.

Which PG is involved in implantation and decidualization is at present controversial. A number of approaches have been taken to identify the PGs involved. Measurements of PGs have not been informative because all that have been measured are elevated at implantation sites and following the application of an artificial deciduogenic stimulus. Binding sites, presumably representing receptors for prostaglandin $E_2$ ($PGE_2$) but not for prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$), are present in the endometrium. PGE-binding sites were detected in the endometrial stroma but not in the lumenal epithelium of rats, whereas in the human, binding has been localized by autoradiography to stromal cells, glandular epithelium, arterioles, and erythrocytes. More recently, *in situ* hybridization has been used to localize transcripts in the mouse and rat uterus. At least four PGE receptor subtypes ($EP_{1-4}$) exist, whereas only one receptor for each of $PGF_{2\alpha}$ and prostaglandin $I_2$ ($PGI_2$) (receptors FP and IP, respectively) has been identified. In agreement with the binding studies in rats, *in situ* hybridization in mice did not detect FP transcripts in the endometrium on day 4. IP mRNA levels were found to be very low or undetectable in the endometrium of mice in the peri-implantation period, as were $EP_1$ transcripts in mice and rats. By contrast, mRNA expression of $EP_2$, $EP_3$, and $EP_4$ was cell specific and correlated with endometrial preparation for implantation and decidualization. For both mice and rats, $EP_2$ transcripts were localized in the lumenal epithelium, whereas $EP_4$ transcripts were localized in both the lumenal epithelium and the stroma of mice but were restricted to the subepithelial stroma in rats. $EP_3$ transcripts were localized to the glandular epithelium and subepithelial stroma of rats and to stromal cells at the mesometrial pole in mice. The failure to detect PGE-binding sites in membrane preparations from rat endometrial epithelial cells contrasts with the detection of $EP_2$ transcripts in these cells. This apparent discrepancy is almost certainly a consequence of the fact that the binding studies were performed on lumenal epithelium from ovariectomized rats treated with progesterone. $EP_2$ transcripts were detected in lumenal epithelium only after the administration of estradiol to progesterone-treated rats.

It is intriguing that in the rat the transcripts for $EP_2$ and $EP_4$ were concentrated in the anti-mesometrial pole of the uterus. COX-2 expression has not been reported for the rat endometrium, but in the mouse at the equivalent stage, its expression is also at the anti-mesometrial pole. The apparent co-localization of the enzyme that is essential for implantation and decidualization and the two E-prostanoid receptors could facilitate the initiation of decidualization in the anti-mesometrial region. However, it should be noted that blastocyst implantation is not affected in $EP_2$ null mice, suggesting that this receptor is not essential for implantation and decidualization. There does not appear to be information on whether the $EP_4$ receptor is essential for implantation and decidualization. Depending on the genetic background, all or the majority of $EP_4$ null mice die shortly after birth.

Based on studies in COX-2 null mice, it has recently been proposed that $PGI_2$, acting via peroxisome proliferator-activated receptor-δ (PPAR-δ), mediates implantation and decidualization. This proposal is based in part on observations that analogues of $PGI_2$ and PPAR-δ ligands were more effective than $PGE_2$ at inducing implantation and decidualization. Although implantation rates not different from those obtained in wild-type controls were achieved with some treatments, at best only partial restoration of decidualization was reported. This may be a consequence of the mode of administration of the compounds: intrauterine injection in oil or intraperitoneal injection. As mentioned previously, full restoration of decidualization in indomethacin-treated rats requires intauterine infusion of PGs, presumably because the concentrations of PGs need to be elevated within the endometrium throughout the response. Recently, the abilities of $PGE_2$ and some analogues of $PGI_2$ to induce decidualization when infused from Alzet osmotic minipumps into the uterine lumen of indomethacin-treated rats have been compared. $PGE_2$ was more effective than the analogues of $PGI_2$ at increasing endometrial vascular permeability and inducing decidualization.

It seems unlikely that the early events during decidualization could be mediated by PPAR-δ. Based on *in situ* hybridization studies, *PPAR*-δ transcripts are detected in the endometrium of mice 24 h, but not 8 h, after the application of a deciduogenic stimulus. The increase in endometrial vascular permeability is first detectable approximately 4 h after uterine stimulation and is therefore not likely mediated by PPAR-δ. Indeed, PPAR-δ is not essential for implantation and decidualization because *PPAR*-δ null mice are fertile.

## B. Growth Factors and Cytokines

There has been considerable interest in the possibility that growth factors and cytokines may have important roles in implantation and decidualization. This is based in part on observations that these factors and their receptors are expressed within the uterus. In this article, consideration will be limited to possible endometrial roles of the factors as opposed to possible effects of the factors on the embryos.

### 1. *Epidermal Growth Factor Receptor and Its Ligands*

There are a number of ligands that bind to and activate the epidermal growth factor (EGF) receptor; these include EGF, transforming growth factor-α (TGF-α), heparin-binding epidermal growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, and heregulins/neu-differentiating factors. These are expressed in the endometrium of many species, often with temporal and spatial specificity. HB-EGF has received particular attention because its expression is induced in the lumenal epithelium at the site of blastocyst implantation. However, because EGF receptors are expressed in stromal cells but not epithelial cells during implantation, any effect that HB-EGF might have on decidualization would presumably involve actions on stromal cells.

Additional evidence for the involvement of EGF receptor ligands in implantation and decidualization has come from studies of the effects of the ligands. Under very constrained conditions, EGF is capable of substituting for estrogen in terminating delayed implantation in rats. Intrauterine injections of EGF or TGF-α increase the decidual cell reaction in rats and augment progesterone-induced decidualization of cultured human endometrial stromal cells. Beads soaked in HB-EGF and introduced into the uterus of mice induce local responses in the endometrium similar to those produced by blastocysts.

It is not currently known whether maternal EGF receptors are required for implantation and decidualization, because adult EGF receptor null mice have not yet been generated. Depending on the genetic background, EGF receptor null mouse embryos die just before implantation, at midgestation, or 2–3 weeks postnatally. However, the mutant mouse waved-2 has defective EGF receptors that bind ligand but subsequent receptor autophosphorylation is reduced by up to 10-fold. These mice are fertile, suggesting that fully functional EGF receptors are not required for implantation and decidualization. However, because there was some residual signaling via the EGF receptor, these observations do not establish that maternal EGF receptors are not absolutely required.

### 2. *Interleukin-1 Receptor Ligands*

The interleukin-1 (IL-1) receptor system is complex. There are two types of IL-1 receptors, IL-1RI and IL-1RII, with the former transducing signals in response to ligand binding and the latter acting as a “decoy” receptor, binding ligand without transducing a signal. Proteolytic cleavage of IL-1RI and IL-1RII results in the formation of soluble receptors (IL-1sRI and IL-1sRII) that bind ligand without transducing signals. The membrane-bound and soluble forms of both receptors bind all three members of the IL-1 ligand family but with differing affinities. In general, it is believed that IL-1RI is the only signal transducing receptor, whereas IL-1RII and the soluble forms of both receptors act as binding molecules that compete with IL-1RI for ligand. There are three ligands for the IL-1 receptors: IL-1, IL-1, and interleukin receptor antagonist (IL-ra). IL-1α and IL-ra preferentially bind to IL-1RI and IL-1sRI. IL-1β, on the other hand, preferentially binds to IL-1RII and IL-1sRII. IL-ra binds to all four types of IL-1 receptors and competitively inhibits IL-1α and IL-1β binding.

IL-1RI, IL-1α, and IL-1β are expressed in human and mouse endometrium. In mice, the mRNA levels of both IL-1α and IL-1β increase dramatically in response to a deciduogenic stimulus. The function of the IL-1 receptor–ligand signaling system in implantation is controversial. It has been reported that implantation in mice could be prevented by the intraperitoneal administration of IL-1ra. However, it was subsequently found that IL-1RI null mice were fertile. Although there was a 20% decrease in litter size, this was not a consequence of decreased implantation rates. By contrast, the IL-1 receptor–ligand signaling system may have an inhibitory role in decidualization in the human. IL-1β inhibits *in vitro* decidualization of human endometrial stromal cells, whereas IL-1α inhibits stromal cell proliferation.

### 3. *Interleukin-11*

IL-11 signals by binding to a complex of IL-11 receptor-α (IL-11Rα) and gp130. The key role of IL-11 signaling in implantation and decidualization was revealed by the discovery that IL-11Rα null female mice were infertile because decidualization was defective, resulting in the death of embryos at approximately day 8 of pregnancy. The initial stages of decidualization were unaffected, but the subsequent progression of decidualization to involve the mesometrial region did not occur. IL-11 expression is up-regulated at the time of blastocyst implantation, especially in the primary decidual zone, in both mice and rats. By contrast, IL-11Rα expression in the uterus remains unchanged during early pregnancy. IL-11 may also have a role in decidualization of human endometrium. IL-11 mRNA and protein have been detected in human endometrium, with maximum concentrations of immunoreactive IL-11 being found in decidual cells.

## VI. SUMMARY

In response to an implanting blastocyst or an artificial stimulus, endometrial stromal cells proliferate and differentiate into decidual cells that are morphologically and functionally distinct. There is a restricted time in pregnancy or pseudo-pregnancy when this transformation can be initiated, and it is under hormonal control. Cell–cell interactions are very important in the process, and the autocrine/paracrine factors mediating the proliferation and differentiation are now being established. Key mediators are PGs and IL-11.

## Glossary

**decidualization** The collective morphological and functional changes that the endometrium undergoes, in mammalian species with invasive implantation. The endometrial stromal cells proliferate and differentiate into decidual cells that ultimately form the maternal component of the placenta.

**endometrial receptivity** The endometrium will allow a blastocyst to initiate successful implantation for only a limited period of time; during this time, the endometrium is said to be receptive.

**endometrial sensitization** In many species, there is a transient period when decidualization is obtained in response to the application of relatively nontraumatic stimuli; during this responsive period, the endometrium is said to be sensitized.

## See Also the Following Articles

**Calcitonin • Endometrial Remodeling • Endometriosis • Epidermal Growth Factor Family • Implantation • Interleukin-1 (IL-1) • Placental Development • Placental Gene Expression**

## Further Reading

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# Defensins

De Yang and Joost J. Oppenheim

*National Cancer Institute, National Institutes of Health, Maryland*

I. INTRODUCTION
II. CLASSIFICATION AND STRUCTURE
III. CELL SOURCES AND DISTRIBUTION
IV. GENES AND THEIR EXPRESSION, POSTTRANSLATIONAL PROCESSING, AND REGULATION
V. ACTIVITIES AND MODE OF ACTION

**Defensins are widely distributed, small, cationic antimicrobial peptides containing three to four pairs of intramolecular disulfide bonds.**

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**Defensins are widely distributed, small, cationic antimicrobial peptides containing three to four pairs of intramolecular disulfide bonds.**

**They are synthesized predominantly in epithelial and/or phagocytic cells either constitutively or upon induction by proinflammatory agents as prepro forms and are processed into mature forms. Structurally common to all defensins is the spatial separation of their positively charged regions and hydrophobic regions, a characteristic allowing them to interact with the microbial membrane, which contains negatively charged phospholipids. The primary activity of defensins is antimicrobial. Defensins in mammals also participate in augmenting *in vivo* host innate and adaptive immune responses through a receptor-mediated mechanism(s) as well as inhibiting the production of immunosuppressive glucocorticoids.**

## I. INTRODUCTION

Macroorganisms including plants, invertebrates, and vertebrates live in an environment laden with microorganisms that are potentially hazardous to life. To survive, macroorganisms have developed various host defense strategies to protect against microbial invasion and to fight and eliminate microbial invaders. One of the strategies of the macroorganisms is the generation of a number of antimicrobial mediators including microbicidal peptides and proteins.

Defensins consist of a family of host gene-encoded antimicrobial peptides widely distributed in plants, insects, birds, and mammals. They are small, cationic, and characterized by six to eight well-conserved cysteine residues that form three to four pairs of intramolecular disulfide bonds. Approximately 150 defensin molecules have been identified in various species. Defensins are classified into several groups of widely divergent peptides whose evolutionary relationships are uncertain (Table 1). Defensins are produced predominantly by epithelial cells separating the host from the environment and by phagocytes. Defensins are synthesized as precursor forms and processed posttranslationally into mature forms. Some defensins are constitutively expressed, and others are induced by proinflammatory signals. Defensins share some common structural features and their primary activity is antimicrobial action against bacteria and fungi. In addition to directing antimicrobial activity, some mammalian defensins regulate a variety of the host innate defense and adaptive immune responses and thus have intracellular as well as extracellular functions. Low levels of human defensins (≈40 ng/ml) are detected in normal plasma, which can increase to 1000 ng/ml with infection. Consequently, although defensins are not secreted by discrete glands, they can act as intracellular mediators that behave like cytokines and have the potential to act as endocrine hormones.

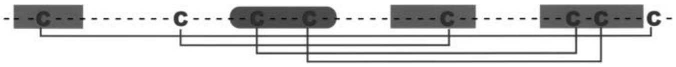
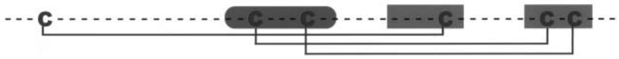
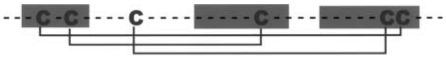
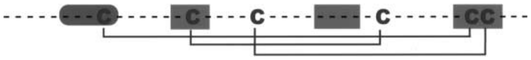
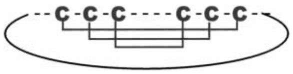
## II. CLASSIFICATION AND STRUCTURE

Based on their sources, defensins are conventionally grouped into three families: plant, insect, and vertebrate defensins (Table 1). Plant defensins, although varying in size (45 to 54 amino acids), share 8 conserved cysteine residues that form four pairs of intramolecular disulfide bridges (C1–C8, C2–C5, C3–C6, and C4–C7). The three-dimensional structures of several plant defensins have been determined by nuclear magnetic resonance (NMR) spectroscopy. The common structure adopted by plant defensins is a βαββ scaffold, consisting of a triple-stranded, antiparallel β-sheet and a single α-helix lying parallel to the β-sheet (Table 1 and Fig. 1). Two disulfide bridges (C3–C6 and C4–C7) connect the CXXXC segment of the α-helix to the CXC segment in the third β-sheet, forming a structural motif known as a cysteine-stabilized α-helix. The disulfide bond C1–C8 connects the amino-terminal short strand of the β-sheet to the carboxyl-terminal cysteine residues, and the disulfide bond C2–C5 connects the second β-sheet to the second cysteine residue.

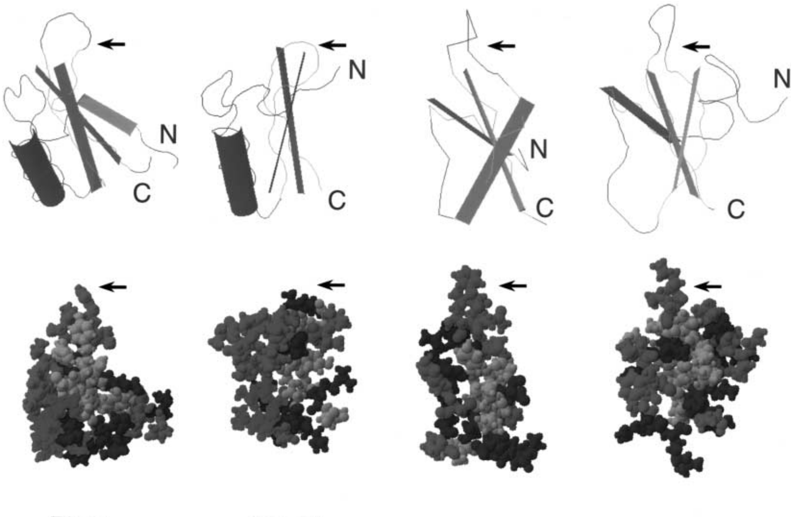
Insect defensins are usually 36 to 46 amino acids long with the exception of the 51-residue bee and bumblebee defensins that possess a 12-amino-acid carboxyl-terminal extension. All insect defensins have 6 conserved cysteine residues that are disulfide-bonded C1–C4, C2–C5, and C3–C6. Insect defensins adopt an αββ scaffold comprising a central α-helix and a double-stranded carboxyl-terminal β-sheet. Similar to plant defensins, the CXXXC segment of the α-helix is connected by two disulfide bridges (C2–C5 and C3–C6) to the CXC segment of the second β-sheet, thus also forming a cysteine-stabilized α-helix. The amino-terminal is brought close to the first β-sheet by the disulfide bond C1–C4 to form an amino-terminal loop.

Vertebrate defensins have been identified in mammals and birds. Similar to insect defensins, vertebrate defensins also have 6 well-conserved cysteine residues. Based on the spacing and disulfide bonding of their six cysteine residues, vertebrate defensins are further classified into α-, β-, and θ-subfamilies (Table 1). The three disulfide bonds of α-defensins are paired C1 to C6, C2 to C4, and C3 to

TABLE 1 **Classification, Source, and Structure of Defensins**

| Classification | | Source (producing cells) | Structure[a] | |
|---|---|---|---|---|
| Family | Subfamily | | Size (aa) | Consensus, disulfide bond, and conformation |
| Plant defensins | – | Seeds, cell wall, epidermis | 45–54 |  |
| Insect defensins | – | Epithelial cells of fat body & trachea, thrombocytoids | 36–46 |  |
| Vertebrate defensins | α | Mammalian phagocytes, Paneth cells | 29–35 |  |
| | β | Mammalian & avian epithelial cells, keratinocytes, phagocytes | 38–42 |  |
| | θ | Primate phagocytes | 18 |  |

[a]The consensus of each defensin group is illustrated by conserved cysteines (C) linked by a dashed line (one dash represents one residue). Disulfide bonds are illustrated by solid lines. Amino acid residues that form β-strands and α-helices are highlighted by rectangular and oval shadows, respectively. The size is the number of amino acid residues (aa) in defensins.



Plant defensin (Rs-AFP1) Insect defensin (defensin A) α-Defensin (RK-1) β-Defensin (HBD2)

FIGURE 1 The three-dimensional structures of selected defensins shown as ribbon-like (top) or space-filling (bottom) models. Shown are the α-helix, the β-strand, and the other structural features including loops, turns, and coils. Arrowheads mark the β-hairpin. N and C indicate the amino- and carboxyl-termini, respectively. In the bottom panel, charged residues are highlighted in black. Rs-AFP, antifungal peptide of *Raphanus sativus* (radish). RK-1, an α-defensin from rabbit kidney. HBD2, human β-defensin 2. [All structures were adapted from the Molecular Modeling Database (MMDB) of the National Center for Biotechnology Information. The MMDB identification numbers for Rs-AFP1, defensin A, RK-1, and HBD2 are 7107, 3486, 16138, and 16324, respectively].

C5, and those of β-defensins are paired C1 to C5, C2 to C4, and C3 to C6. The θ-defensins are cyclic peptides of 18 amino acid residues, with 6 cysteine residues disulfide-bonded C1 to C6, C2 to C5, and C3 to C4. The structures of two human α-defensins (HNP-1 and HNP-3), two rabbit α-defensins (NP-2 and RK-1), one human β-defensin (HBD-2), and one bovine β-defensin (bBD12) have been determined by NMR and/or X-ray crystallography. The backbones of both α- and β-defensins adopt a βββ scaffold, an anti-parallel β-sheet consisting of three β-strands held together by the disulfide bonds (Table 1 and Fig. 1). There is no cysteine-stabilized α-helix in vertebrate defensins. In contrast to α-defensins, β-defensins have a relatively long amino-terminal segment prior to the first β-sheet, within which a short α-helical segment is formed in some β-defensins.

Apart from the evolutionarily conserved cysteine residues, there is no apparent homology among plant, insect, α-, β-, and θ-defensins at the amino acid sequence level. The marked divergence of defensins is probably due to the huge evolutionary pressure exerted by the wide variety of microbial flora that defensins confront. However, the overall structures of defensins have two characteristics in common (Fig. 1). One is that all defensins have a similar structural element, a β-hairpin. In insect defensins, the β-hairpin consists of two β-strands and the interconnecting turn. In plant and vertebrate defensins, the β-hairpin is made of the second and third β-strands as well as the interconnecting turns. The other shared characteristic is that the charged residues (mostly cationic) tend to cluster, allowing the spatial separation of hydrophilic and hydrophobic regions.

## III. CELL SOURCES AND DISTRIBUTION

Defensins are produced primarily by two cell types: epithelial cells separating the host from the external environment and phagocytic cells (Table 1). In plants, defensins accumulate in seeds, in the outer cell wall lining the epidermis of cotyledons, hypocotyls, and endosperm, as well as in the epidermis of seedling and leaf primordia. In insects, defensins are generated by epithelial cells of the fat body and the trachea as well as by thrombocytoids, one type of insect blood cell. Insect defensins can be detected in the hemolymph.

In vertebrates, defensins are produced by epithelial cells of the gastrointestinal, respiratory, urinary, and reproductive systems, Paneth cells, skin keratinocytes, neutrophils, and monocytes/macrophages. Although multiple tissues can express one given defensin, the highest level of gene expression is usually found in one tissue type. In humans, six α-defensins and at least three β-defensins have been identified and cloned (Table 2). HNP1–4 are synthesized by promyelocytic precursors and stored in the granules of phagocytes. HNP1–4 are released upon neutrophil degranulation and can therefore be distributed systemically in the blood. The concentration of HNP1–4 in the plasma of

TABLE 2 The Number, Distribution, Gene Location, Inducibility, and Activities of Human Defensins[a]

| Subfamily | Conventional nomenclature | Expressing cells | Chromosome location | Regulatory element | Inducibility | Activity |
|---|---|---|---|---|---|---|
| α | HNP1 | Neutrophils, monocytes, macrophages | 8p21–p23 | C/EBPα | Constitutive | Antimicrobial, chemotactic, immunomodulatory, antagonistic for ACTH |
| | HNP2 | | | | | |
| | HNP3 | | | | | |
| | HNP4 | | | | | |
| | HD5 | Paneth cells, epithelial cells of the reproductive system | 8p21–p23 | NF–IL-6 | Constitutive | Antimicrobial |
| | HD6 | | | | | |
| β | HBD1 | Epithelial cells, keratinocytes | 8p21–p23 | NF–IL-6 | Constitutive | Antimicrobial, chemotactic |
| | HBD2 | | | NF-κB | Inducible | |
| | HBD3 | | | NF–IL-6, AP-1, GAS | | |

[a] HNP, human neutrophil-derived peptide; HD, human defensin; HBD, human β-defensin; C/EBPα, CCAAT/enhancer-binding protein α; NF, nuclear factor; IL-6, interleukin-6; AP-1, activation protein-1; GAS, IFN-γ activated site; ACTH, adrenocorticotropic hormone.

normal individuals is ~40 ng/ml, but can rise to >1000 ng/ml during severe infection. Human defensin 5 (HD5) and HD6 are produced by Paneth cells. HBD1–3 are predominantly generated by skin keratinocytes and epithelial cells.

The number of defensins varies greatly from one species to another. In contrast to human neutrophils, bovine neutrophils have no α-defensin, but instead store as many as 12 β-defensins. Three additional β-defensins, including tracheal antimicrobial peptide, lingual antimicrobial peptides, and an enteric β-defensin, are generated by bovine tracheal, lingual, and enteric epithelial cells, respectively. Although mouse neutrophils contain neither α- nor β-defensins, mice have at least 6 α-defensins called cryptdins and 6 β-defensins produced by Paneth cells and various epithelial cells, respectively. Rabbits and rats have 6 α-defensins stored in the granules of phagocytes and several β-defensins generated by epithelial cells and keratinocytes.

## IV. GENES AND THEIR EXPRESSION, POSTTRANSLATIONAL PROCESSING, AND REGULATION

The genes of human α- and β-defensins have been relatively well investigated. The genes encoding human α- and β-defensins are localized to a single chromosome region, 8p21–p23 (Fig. 2). The genes encoding HNPs are composed of three exons, with the first exon encoding the 5′-untranslated region (5′-UTR) and the second exon encoding the signal peptide. The genes for HD5, HD6, and HBD1–3 have two exons, with the first exon encoding the 5′-UTR and the signal peptide. In both cases, the last exon encodes the propiece, the mature peptide, and the 3′-UTR. Translation of the defensin mRNA gives rise to preprodefensin, which consists of a signal peptide, a propiece, and the mature defensin. Mature defensin is generated by posttranslational processing or proteolytic removal of the signal peptide and propiece. For HNPs, the processing occurs during transport from the Golgi body to the azurophilic granules, but for the remaining α- and β-defensins, it happens after the extracellular secretion of preprodefensins. The enzymes responsible for the processing of mammalian defensins are not completely understood.

Although HD5–6 and HBD1–3 have their own genes, four HNPs are actually encoded by two genes (Fig. 2). HNP1 and HNP4 are encoded by genes designated *HDEFA1* and *HDEFA4*, respectively. HNP3 arises from a single nucleotide substitution in one of several *HDEFA1* copies, *HDEFA1A*. HNP2 results from the posttranslational removal of the amino-terminal residue from HNP1 and/or HNP3.

The promoter region of human α- and β-defensin genes contains various regulatory elements including CCAAT/enhancer-binding protein α (C/EBPα), nuclear factor–interleukin-6 (NF–IL-6), NF-κB, activation protein-1 (AP-1), and interferon-γ (IFN-γ)-activated site (GAS) (Table 2). The expression of HNP1, HNP3, HNP4, HD5, HD6, and HBD1 is constitutive, whereas that of HBD2 and HBD3

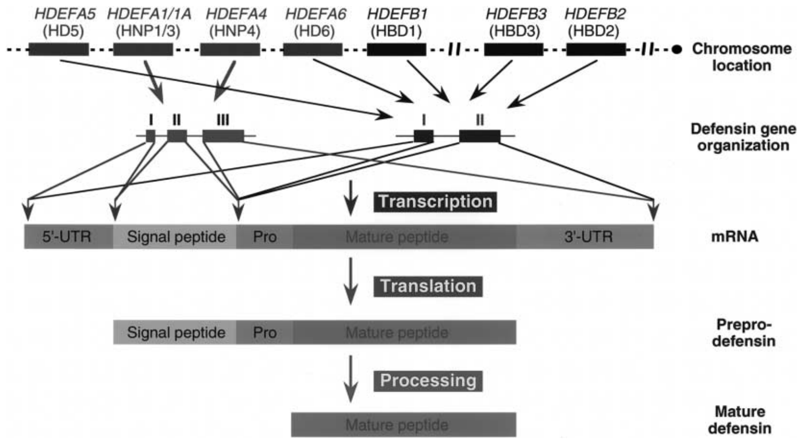


**FIGURE 2** Schematic illustration of the gene location and organization, transcription, translation, and posttranslational processing of human α- and β-defensins. The short arm of human chromosome 8 is shown at the top. The intervening sequences between defensin genes are not drawn to scale. The closed, black circle indicates the centromere. *HDEFA/B*, human α/β-defensin gene. UTR, untranslated region.

is inducible by proinflammatory cytokines (e.g., tumor necrosis factor α, IL-1β, IFN-γ), bacterial products such as lipopolysaccharide, and microorganisms. The inducibility of HBD2 and HBD3 is due to the existence of sites for NF-κB and GAS, respectively, in their promoter regions. C/EBPα, NF–IL-6, and AP-1 sites presumably participate in maintaining the constitutive or basal expression of defensins.

Reports on the gene structure and regulation of expression of defensins distributed in plants, insects, and vertebrates other than humans are still fragmentary; however, the expression patterns fall into three categories: constitutive, inducible, or both. Plant defensins are expressed either in a constitutive, organ-specific manner or in a pathogen-modulated manner. Insect defensins are mostly inducible. For vertebrate defensins, those generated by leukocytes and Paneth cells are, in general, constitutively expressed. Those generated by epithelial cells are mostly inducible with the exception of β-defensin 1 from many species.

## V. ACTIVITIES AND MODE OF ACTION

### A. Antimicrobial Activity

Plant, insect, and vertebrate defensins exhibit antimicrobial activities generally at concentrations higher than 1 μg/ml (Tables 2 and 3). Plant defensins are predominantly antifungal, whereas insect defensins are cytotoxic only to bacteria, especially gram-positive bacteria (Table 3). In contrast, vertebrate α-, β-, and θ-defensins have potent microbicidal activity against fungi and gram-positive and gram-negative bacteria. In addition, some α-defensins are active against enveloped viruses. The antimicrobial activity of defensins is usually measured *in vitro* in low-ionic-strength (10 mM) sodium phosphate buffer, pH 7.4. With the exception of θ-defensins, which are insensitive to high concentrations of salt, the antimicrobial activity of most plant and insect α- and β-defensins is ablated in the presence of high-ionic-strength salt (e.g., 150 mM NaCl). Furthermore, the antimicrobial activity of defensins is also sensitive to divalent cations, especially $Ca^{2+}$, and impaired in the presence of serum. Thus, defensins are more efficient in phagocytic vacuoles and on the surfaces of epithelium and skin than in blood and extracellular fluids.

The antimicrobial spectrum and sensitivity to ionic strength of defensins vary considerably among individual defensin members. For example, both HBD2 and HBD3 are produced by human skin keratinocytes, but their antimicrobial spectra are different. HBD2 is restricted to gram-negative bacteria such as *Escherichia coli* and in particular *Pseudomonas aeruginosa*, as well as yeast such as *Candida albicans*. In contrast, HBD3 is also active against gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*.

The mechanism(s) by which microorganisms are killed by defensins is not completely understood; however, it is generally believed that the killing is a consequence of disruption of the microbial membrane. The polar topology of defensins with spatially separated charged and hydrophobic regions allows them to insert themselves into the phospholipid membranes so that their hydrophobic regions are buried within the lipid membrane interior and their charged (mostly cationic) regions interact with anionic phospholipid head groups and water. Subsequently, some defensins may aggregate to form "channel-like" pores; others may bind to and cover the microbial membrane in a "carpet-like" manner.

TABLE 3 The Antimicrobial Spectrum of Defensins

| Defensin | | Antimicrobial spectrum | | | |
|---|---|---|---|---|---|
| | | | Bacteria | | |
| Family | Subfamily | Viruses | Gram-positive | Gram-negative | Fungi |
| Plant defensin | | − | + | − | +++ |
| Insect defensin | | − | +++ | + | − |
| Vertebrate defensin | α | (+)[a] | +++ | +++ | +++ |
| | β | − | +++ | +++ | +++ |
| | θ | NT | +++ | +++ | +++ |

*Note*. NT, not tested yet.
[a] Active only on some enveloped viruses.

The net outcome is the disruption of membrane integrity and function, which ultimately leads to the lysis of microorganisms. Defensin's cytotoxic effects on microorganisms are relatively selective because the membrane of microorganisms, in contrast to that of host cells, is relatively rich in negatively charged phospholipids.

## B. Chemotactic Activity

Defensins of mammalian species exhibit functional diversification. In addition to their antimicrobial activity, some mammalian defensins promote various host immune responses against microorganisms by having chemotactic activity for host immune cells including dendritic antigen-presenting cells and T lymphocytes, by inducing the production of cytokines such as interleukin-8 by epithelial cells, by degranulating mast cells, and by enhancing *in vivo* immunological reactions.

Human neutrophil-derived α-defensins are chemotactic for human immature dendritic cells (DCs) and peripheral blood naive T lymphocytes. Human β-defensins and mouse β-defensins are chemotactic for human and mouse immature DCs, respectively, as well as human resting memory T cells. The capacity of defensins to induce the chemotaxis of immune cells is optimal at nanomolar concentrations and, unlike their microbicidal activity, is not ablated by the presence of physiologic concentrations of salt (150 mM NaCl) and serum. Defensins are rich at sites of microbial entry as a result of degranulation of recruited neutrophils and/or induced production by local epithelial cells or keratinocytes. Since DCs are the most potent antigen-presenting cells, defensin-mediated recruitment of immature DCs to sites of microbial entry facilitates the uptake, processing, and presentation of microbial antigen and subsequently enhances the initiation of antigen-specific immune responses by DCs that mature and migrate to draining lymph nodes. Indeed, defensins markedly enhance antigen-specific immune responses when administered simultaneously with antigens or as DNA vaccines in which defensin and antigen are linked as a fusion construct.

The effects of defensins on host cells are based on a receptor-mediated mechanism(s). The chemotactic activity of defensins is mediated by a G-protein-coupled seven-transmembrane domain chemotactic receptor(s). One of the receptors that human β-defensins utilize to induce chemotaxis is human CCR6, a CC chemokine receptor that also interacts with human CC chemokine CCL20/LARC/MIP-3α.

The dual (antimicrobial and chemotactic) activities of defensins are not unique since a number of other endogenous antimicrobial peptides such as cathelicidins and azurocidin are also chemotactic for various leukocyte subpopulations. Furthermore, human interferon-γ-inducible chemokines, including MIG, IP-10, and I-TAC, in addition to mobilizing and activating immune cells, also exhibit antimicrobial activities.

## C. Antagonistic Activity for Adrenocorticotropic Hormone

Some mammalian neutrophil-derived α-defensins have the capacity to inhibit the production of immunosuppressive adrenal steroid hormones and thus are also known as "corticostatins." This inhibitory activity is effective at nanomolar concentrations and is achieved by blocking the interaction between adrenocorticotropic hormone and its receptor. During systemic infections, α-defensin levels in plasma can reach up to 100 μg/ml, a concentration sufficient to interfere with the production of adrenal glucocorticoids. Inhibition by α-defensins of immunosuppressive glucocorticoid production facilitates the global immune responses during the early phase of infection.

The importance of defensins in host defense against microbial invasion is supported by many studies. In particular, genetic knockout of the mouse gene encoding matrilysin, which participates in the processing of murine α-defensins, prevents the production of mature murine α-defensins and reduces the resistance of mice to bacterial challenge. Conversely, transgenic overexpression by potatoes of alfAFP, a plant defensin isolated from alfalfa, confers protection against pathogenic fungi to such transgenic potatoes.

## Glossary

**chemotaxis** Directional migration of cells toward a gradient of mediators called chemotactic factors.

**dendritic cells** A type of mammalian leukocyte with dendritic processes and the most potent capacity to take up, process, and present antigens to T lymphocytes.

**keratinocytes** A specialized type of epithelial cell that covers the body surfaces of mammals.

**Paneth cells** A specialized type of secretory cell located at the bottom of small intestine crypts of Lieberkühn.

**regulatory element** A stretch of DNA sequence in the promoter region of genes that when bound by a specific transcription factor results in the regulation of gene transcription.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • CC, C, and CX$_3$C Chemokines • CXC Chemokines • Cytokinins**

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# Deiodinases

Juan Bernal

*Instituto de Investigaciones Biomedicas Alberto Sols, Madrid*

I. INTRODUCTION
II. TYPE I DEIODINASE
III. TYPE II DEIODINASE
IV. TYPE III DEIODINASE
V. SUMMARY

**Deiodination, the most important metabolic pathway of thyroid hormones, is catalyzed by deiodinases, which contain the amino acid selenocysteine in their active center. Three types of deiodinase have been identified in vertebrate tissues: D1, D2, and D3. Deiodinase activity is regulated during development and by physiological factors including the thyroid hormones. D1 and D2 are expressed postnatally and in the adult; D3 is expressed at high levels in placenta, pregnant uterus, and fetal tissues. Deiodinases appear to play a role in regulating intracellular T3 levels in a tissue-specific manner and in coordinating vertebrate growth and development.**

## I. INTRODUCTION

The thyroid gland synthesizes and secretes two hormones that are iodinated amino acids known as iodothyronines: 3,5,3′5′-triiodo-L-thyronine (thyroxine or T4) and 3,5,3′-triiodo-L-thyronine (T3). Under normal conditions, the major circulating form of thyroid hormone is T4. T4 functions primarily as a pro-hormone and can be converted in the tissues to T3, the hormone that is responsible for the majority of thyroid hormone action. Thyroid hormone action is mediated primarily by the interaction of T3 with nuclear receptors, which are ligand-modulated transcription factors that regulate the expression of many genes. Conversion of T4 to T3 in extrathyroidal tissues is a physiologically important, tightly transcription factor results in the regulation of gene transcription.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • CC, C, and CX$_3$C Chemokines • CXC Chemokines • Cytokinins**

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# Deiodinases

JUAN BERNAL
*Instituto de Investigaciones Biomedicas Alberto Sols, Madrid*

I. INTRODUCTION
II. TYPE I DEIODINASE
III. TYPE II DEIODINASE
IV. TYPE III DEIODINASE
V. SUMMARY

**Deiodination, the most important metabolic pathway of thyroid hormones, is catalyzed by deiodinases, which contain the amino acid selenocysteine in their active center. Three types of deiodinase have been identified in vertebrate tissues: D1, D2, and D3. Deiodinase activity is regulated during development and by physiological factors including the thyroid hormones. D1 and D2 are expressed postnatally and in the adult; D3 is expressed at high levels in placenta, pregnant uterus, and fetal tissues. Deiodinases appear to play a role in regulating intracellular T3 levels in a tissue-specific manner and in coordinating vertebrate growth and development.**

## I. INTRODUCTION

The thyroid gland synthesizes and secretes two hormones that are iodinated amino acids known as iodothyronines: 3,5,3′5′-triiodo-L-thyronine (thyroxine or T4) and 3,5,3′-triiodo-L-thyronine (T3). Under normal conditions, the major circulating form of thyroid hormone is T4. T4 functions primarily as a pro-hormone and can be converted in the tissues to T3, the hormone that is responsible for the majority of thyroid hormone action. Thyroid hormone action is mediated primarily by the interaction of T3 with nuclear receptors, which are ligand-modulated transcription factors that regulate the expression of many genes. Conversion of T4 to T3 in extrathyroidal tissues is a physiologically important, tightly regulated reaction, which is catalyzed by specific enzymes known as deiodinases.

Deiodination is also an important metabolic pathway for the inactivation of thyroid hormones. Other pathways in the metabolism of thyroid hormones include modifications of the alanine side chain, conjugation of the phenolic group, and oxidative cleavage of the diphenyl-ether bridge (Fig. 1). These reactions also produce some potentially important metabolites, such as the acetic acid analogues of T3 and T4, 3,5,3′-triidothyroacetic acid (TRIAC) and 3,5,3′,5′-tetraiodothyroacetic acid (TETRAC), and conjugates of T4 and T3 with sulfate and glucuronic acid. TRIAC has an affinity for the nuclear receptors that is one order of magnitude higher than that of T3, but has only a short residence time and its contribution *in vivo* to thyroid hormone action is unknown. The conjugated forms of T4 and T3 with sulfate are present in cord serum, amniotic fluid, and fetal tissues and may constitute hormone reservoirs in tissues with high sulfatase activity, such as fetal liver and brain.

There are two types of iodothyronine deiodination, 5′ deiodination (5′D) and 5 deiodination. 5′D results in the removal of an iodine atom from the 5 (or chemically equivalent 3) position of the outer or phenolic ring of an iodothyronine. Because 5′D of T4 results in the formation of T3, it can be considered an activating system. 5D results in the removal of iodine from the 3 or 5 position of the inner or tyrosyl ring of iodothyronines, and this process inactivates both T4 and T3. Three types of deiodinase have been identified in vertebrate tissues and the general properties of each are listed in Table 1. The interplay among the different deiodinases on the different substrates and intermediates results in a deiodination cascade, illustrated in Fig. 2. The genes encoding the three deiodinases have been identified and their respective cDNAs have been cloned. All three deiodinases contain the rare amino acid selenocysteine (Se-Cys) at their active sites, and selenium is needed for enzyme activity. Se-Cys is encoded by an AUG codon, which, in the presence of a specific element known as a selenocysteine insertion sequence (SECIS), does not function as a terminator of translation but recognizes a specific Se-Cys tRNA. SECIS elements are RNA sequences present in the 3′-untransuntranslated region (3′-UTR) of mRNAs that code for selenoproteins.

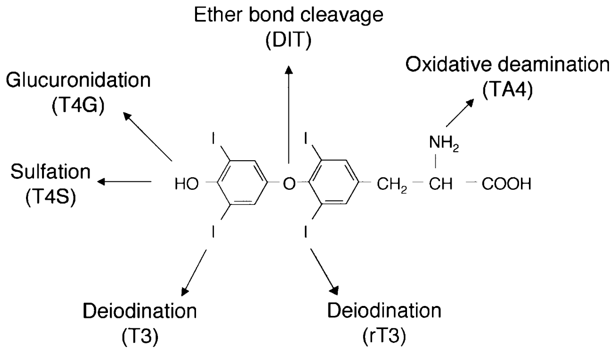

**FIGURE 1** Pathways of thyroid hormone metabolism. Thyroid hormone may be subjected to the following modifications: (1) Oxidative deamination, with the production of tetrac (TA4) from T4 or triac (TA3) from T3. (2) Ether bond cleavage, with the production of diiodotyrosine (DIT). (3) Conjugation of the phenolic group by glucoronic acid (T4G or T3G) or sulfate (T4S or T3S). (4) Deiodination, the loss of iodine atoms that proceeds sequentially as shown in Fig. 2. Modified from T. J. Visser in Thyroid Hormone Metabolism in http://www.thyroidmanager.org, with permission of the authors and Endocrine Education, Inc.

## II. TYPE I DEIODINASE

Type I deiodinase (D1) has both 5′D and 5D activities. T4 and rT3 are the preferred substrates for the 5′D activity while the sulfate conjugates of T4 and T3 are the preferred substrates for the 5D activity. The half-life of the enzyme *in vivo* is about 20–30 h and its activity is inhibited both *in vivo* and *in vitro* by propylthiouracil (PTU) and by aurothioglucose.

*In vitro* D1 needs free sulfhydryl groups for enzymatic activity. It has been proposed that *in vitro*, D1 reacts by a two-substrate mechanism following so-called "ping-pong" kinetics. By virtue of this mechanism, and after interaction with the substrate and deiodination, the enzyme is regenerated by reaction with a thiol co-factor. *In vitro*, dithiothreitol is very effective as a free sulfhydryl compound, but the physiological co-factor has not been identified. *In vivo*, the deiodination reaction might be link to a NADPH-regenerating co-factor system possibly coupled to the pentose phosphate cycle. One candidate for the physiological co-substrate is glutathione, although *in vitro* it is a weak inducer of the enzyme. The possibility remains, however, that the enzymatic reaction *in vivo* proceeds without a thiol co-substrate and that the enzyme is not regenerated after each deiodination cycle, in a sort of suicide reaction.

### A. The D1 Gene

The human gene (*hdio*1) is located at chromosome 1p32–p33, spans 17.5 kb, and contains four exons.

TABLE 1 Properties of Deiodinases

| Property | Type I | Type II | Type III |
|---|---|---|---|
| Activity | 5′D and 5D (outer and inner rings) | 5′D (outer ring) | 5D (inner ring) |
| Deiodination site | Selenocysteine | Selenocysteine | Selenocysteine |
| $K_m$ | $10^{-7}$ M (rT3)<br>$10^{-6}$ M (T4) | $10^{-9}$ M (T4)<br>$10^{-8}$ M (rT3) | $10^{-9}$ M (T3)<br>$10^{-8}$ M (T4) |
| Substrate preference | rT3 > >T4 > T3 | T4 > T3 | T3 > T4 |
| Inhibition by PTU and ATG | Sensitive | Resistant | Resistant |
| Tissue distribution | Liver, kidney, thyroid, brain, pituitary | Rat: brain, puitary, BAT, placenta<br>Human: brain, pituitary, BAT, placenta, thyroid gland, heart, muscle | Placenta, skin, brain, uterus, fetal tissues |
| Physiological role | Provide T3 to plasma; inactivate T3 and T4; degrade T4 and T3 sulfates; degrade rT3 | Provide intracellular T3; Provide plasma T3 in humans | Inactivate T4 and T3 |
| Hyperthyroidism | Increased | Decreased | Increased |
| Hypothyroidism | Decreased | Increased | Decreased |

The UGA codon is present in the second exon. The gene is transcribed from two start sites and produces a 2.2 kb mRNA. Expression of the gene correlates with the presence of a 27 kDa protein (p27) and with D1 activity. The *dio*1 promoter does not contain TATA- or CAAT-boxes, but does contain GC-boxes, which are binding sites for the transcription factor Sp1. In the upstream region of the gene, there are two thyroid hormone-response elements and one retinoic acid-response element. Depending on the presence of these regulatory sites, D1 is stimulated by retinoic acid in undifferentiated cells

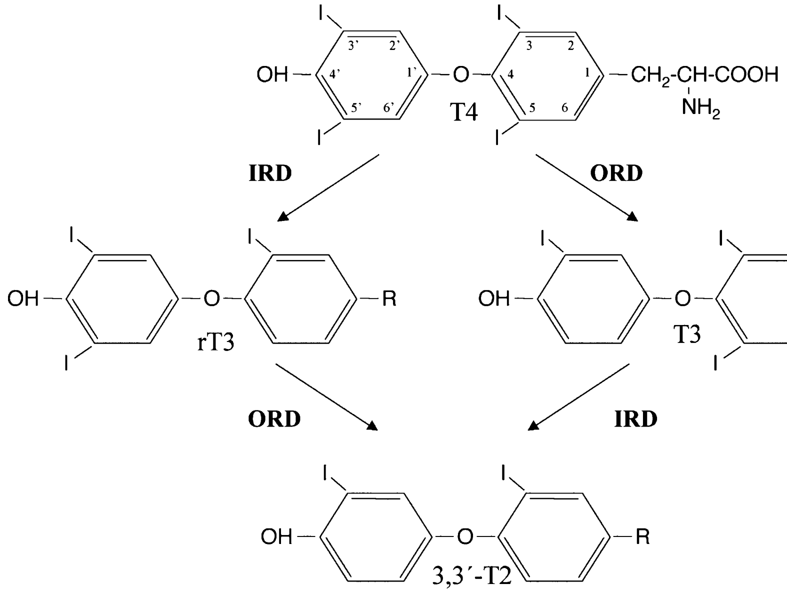

FIGURE 2 The deiodination cascade. T4 may be deiodinated by D1 or D2, with the generation of the active hormone, T3, or by D3 with generation of the inactive compound rT3. T3 and rT3 can be further metabolized to T2, as shown. ORD, outer ring deiodination; IRD, inner ring deiodination. From Thyroid Hormone Metabolism in http://www.thyroidmanager.org, with permission of the authors and Endocrine Education, Inc.

and by T3 in differentiated cells. The mouse gene is on chromosome 4. A mouse strain with decreased D1 expression due to an altered promoter is available.

## B. D1 Expression and Physiological Role

D1 is a cell membrane protein expressed in liver, kidney, thyroid, and pituitary. In the kidney, it is expressed in the S3 segment of the proximal tubule. In the pituitary gland, D1 is expressed in thyrotrophs, lactotrophs, and somatotrophs. Other tissues, such as lung, intestine, muscle, spleen, placenta, mammary gland, white adipose tissue, lymphocytes, and salivary gland, display D1 activity but at relatively low levels. In liver, D1 serves two important functions. On the one hand, it contributes to the pool of circulating T3; indeed approximately 70% of circulating T3 in the rat may be produced in the liver under euthyroid conditions. In addition to providing most of the circulating T3, D1 in the liver degrades rT3 through $5'$-deiodination and catalyzes the 5 deiodination of the sulfate derivatives of T4 and T3.

## C. Regulation of D1 Activity

D1 activity is induced by thyroid hormones, by retinoic acid in thyroid and liver carcinoma cells, and by TSH, via cAMP, in thyrocytes. Basal expression and induction by thyroid hormones require the presence of the β1 isoform of thyroid hormone receptor, which is the main T3 receptor present in liver. Induction by thyroid hormones means that D1 activity is increased in hyperthyroidism and decreased in hypothyroidism.

Expression in liver, pituitary, and thyroid is critical for the compensatory mechanisms afforded by D1 in situations of deficiency or excess of thyroid hormone. In liver, these situations will result in a decreased or an increased degradation of thyroid hormone, respectively. In the pituitary, it is likely that the increased activity of pituitary D1 in hyperthyroidism would increase local conversion of T4 to T3, thereby increasing receptor occupancy and reducing TSH secretion. In the thyroid gland, there is some evidence that D1 is induced in situations of low T4, such as in iodine deficiency, through TSH-induced stimulation of the cAMP pathways. The increased D1 activity would likely result in an increased proportion of hormone secreted as T3 due to increased intrathyroidal conversion of T4 to T3.

The following pathological states are associated with a reduction in D1 activity: starvation, severe illness, bacterial sepsis, surgical interventions, and severe trauma. These conditions are associated with reduced levels of circulating T3, normal T4, and normal or low TSH. The situation is known as "euthyroid sick syndrome," "nonthyroidal illness," or "low T3 syndrome." The mechanism for D1 reduction in these states is not fully understood, but interleukin-1β and TNFα are known to inhibit D1 activity, and the presence of inhibitory cytokine-responsive elements in the human *dio*1 promoter might play a mechanistic role in the pathogenesis of the syndrome.

## III. TYPE II DEIODINASE

Type II deiodinase (D2) has only $5'$D activity. The $K_m$ of this enzyme for T4 and rT3 is three orders of magnitude lower than that of D1, approximately 1 nM for T4 and 10 nM for rT3. One important property that was instrumental in its identification as a second enzyme with $5'$D activity, is that its activity is insensitive to PTU or aurothioglucose, despite the presence of selenocysteine in the active center of the enzyme. *In vitro*, D2 needs high concentrations of free SH groups. The enzymatic reaction follows a "sequential reaction kinetics" typical of multisubstrate reactions, in which both the substrate and the co-factor combine with the enzyme. D2 is inhibited by its substrates, T4 and rT3. Thus, when the concentration of T4 decreases, as in hypothyroidism, the level of D2 activity is increased.

## A. The D2 Gene

The human D2 gene (*hdio*2) is present on chromosome 14q24.3. It contains two exons, separated by a single 7.4 kb intron located at codon 75. The promoter region of the gene contains a strong cAMP-responsive element. The gene is transcribed from two alternative, tissue-specific start sites, giving 6.8 to 7.5 kb mRNAs. Human, rat, mouse, chicken, and frog D2 mRNAs contain an in-frame UGA codon in the deduced amino acid sequence of the active center (position 133 in hD2) and therefore are all considered to be selenoenzymes. Conceptual translation of the human D2 cDNA results in a 31 kDa protein with a high level of similarity to D1 and D3 in the selenocysteine-containing active center of the putative enzyme. A SECIS element has been identified in the extreme $3'$-UTR of the mouse, human, and highly homologous chicken Dio2 genes. D2 is an integral membrane protein located in the endoplasmic reticulum.

## B. D2 Expression and Physiological Role

D2 is expressed in the pituitary gland, central nervous system (CNS), brown adipose tissue (BAT), uterus, and placenta of rats and humans. The highest levels of expression in brain are found in a specialized type of glial cell called tanycytes, which line the walls of the third ventricle and extend processes to the hypothalamus and median eminence. Astrocytes also contain D2 mRNA but at lower concentrations than tanycytes. Expression of D2 in the human is less restricted than in the rat and is also present in skeletal muscle, cardiac muscle, and the thyroid gland. Due to its widespread expression in the human, some authors think that D2 contributes to circulating T3 in addition to D1. As an indication that this may indeed be the case, T3 production in the human is much less inhibited by PTU than rT3.

One important role for D2 is the generation of intracellular T3, especially in tissues such as the CNS, pituitary, and BAT. It can be speculated that these tissues may require a high degree of saturation of the nuclear T3 receptor, and the intracellular production of T3 would allow rapid transport of T3 to the nucleus, which may be facilitated by the subcellular location of D2. Liver and kidney, which express predominantly D1, derive most of the T3 from the plasma, and the physiological saturation of nuclear T3 receptors is approximately 50%. BAT and brain, in contrast, derive more than 50% of intracellular T3 from local deiodination of T4, and the occupancy of the nuclear receptor may reach 80% under physiological conditions. In the pituitary, D2 is involved in the regulation of TSH secretion as a function of serum T4 concentration. Changes in serum T4 influence TSH secretion after conversion to T3, which might be carried out by D1 or D2. The relative contribution of each enzyme might be influenced by the thyroid state. Because D2 is increased in hypothyroidism and decreased in hyperthyroidism, D2 is the predominant enzyme in the pituitary of hypothyroid animals.

D2 activity is detectable in the brain of the rat fetus and increases markedly by the end of pregnancy to reach adult levels. This increased D2 activity is apparently responsible for the 18-fold increase of brain T3 during the same period. Higher than adult levels of D2 activity are transiently achieved during the first month of life in cerebrum and cerebellum. It is believed that high-level expression of D2 may provide a high local concentration of T3 needed for some developmental processes. One of the best examples is the cochlea, in which D2 activity rises dramatically just before the onset of hearing in the neonatal rat.

## C. Regulation of D2 Activity

As stated above, D2 activity is very sensitive to thyroid status and changes inversely with thyroid hormone concentration. Thyroidectomy increases, and thyroid hormone administration decreases, D2 activity in the rat cerebral cortex and other tissues. Changes in D2 activity presumably influence intracellular T3 levels and thus may protect the brain from either hypo- or hyperthyroidism. Under conditions of low iodine intake, increased D2 activity helps to maintain normal T3 concentrations in brain despite greatly reduced T4 concentrations in plasma. Regulation of D2 activity occurs at two levels. The D2 substrates T4 and rT3 rapidly inhibit D2 activity through posttranslational mechanisms involving the ubiquitin-proteasome pathway. In addition, D2 is regulated at the mRNA level.

Mice with a targeted deletion of the D2 gene (D2KO) have been generated recently. The mice have no obvious developmental, physiological, or behavioral abnormalities, and fertility is normal. However, while plasma T3 is normal, plasma T4 and TSH are both increased, suggesting that the pituitary gland of the D2KO mouse is resistant to the feedback effect of plasma $T_4$. The results are consistent with the view that local generation of T3 by pituitary D2 is important in the regulation of TSH secretion. The elevation of plasma T4 is likely due to decreased metabolic clearance as a result of decreased deiodination; whereas a contributing factor may be an increase in the release of T4 from the thyroid as a result of the elevated levels of TSH, plasma T4 is elevated also in thyroidectomized D2KO mice maintained on exogenous T4. In other tissues where local generation of T3 is considered to be important, the results are less clear. As pointed out above, thermogenesis in brown adipose tissue relies on local generation of T3 from T4. However, D2KO mice survive for several days at 4°C, although no basal or cold-inducible deiodinase activity occurs in BAT. It remains to be seen whether the absence of D2 in the brain impairs brain function in otherwise normal animals or whether it potentiates the effects of iodine deficiency.

## IV. TYPE III DEIODINASE

Type III deiodinase (D3) inactivates iodothyronines and represents a potentially important mechanism for the control of intracellular T3 concentrations. D3 has higher affinity for T3 than for T4, and therefore degradation of the active thyroid hormone is favored. The human *dio*3 gene is located at chromosome 14q32. The enzymatic mechanism for type III deiodination might be sequential, and *in vitro* D3 also needs reduced thiols for maximal activity. The reduced thiols might act as activators of the enzyme rather than as co-substrates. D3 activity is not inhibited by PTU or aurothioglucose.

### A. D3 Expression and Physiological Role

The physiological role of D3 is the inactivation of thyroid hormones especially during development. The highest levels of D3 activity are found in the pregnant uterus and placenta. Lower levels of activity are found in fetal tissues, and, with the exception of skin, activity in these tissues decreases after birth. In the adult rat, D3 expression is limited to the skin, brain, and uterus. D3 has been cloned from amphibians and mammals. In the brain, it is expressed in neurons, in contrast to D2, which is expressed in glial cells, as already mentioned.

D3 is thought to play an important role in the control of T3 concentrations in developing tissues. In tadpoles, the tissue profiles of D3 and T3 expression are very similar, and maximal expression of both in a given tissue correlates well with the time of maximal metamorphic change. This suggests the need for very tight regulation of intracellular T3 levels. Indeed, metamorphosis is strongly inhibited by D3 overexpression in transgenic tadpoles, presumably by preventing the tissues from attaining the appropriate level of T3. In mammals, the placenta contains very high levels of D3 activity, and recent studies have demonstrated very high levels of D3 expression in the uterus in the decidual cells surrounding the developing embryo and at later stages in the epithelial cells of the uterus. The maternal/fetal concentration ratio of thyroid hormone is very high and it is thought that the high levels of D3 activity in uterus and placenta protect the embryos from overexposure to maternal thyroid hormone concentrations. In the newborn rat brain, a discrete and intense expression of D3 occurs in neurons located in just a few discrete nuclei, suggesting also that the availability of T3 to these regions is specifically controlled by D3.

## V. SUMMARY

Deiodination is the most important metabolic pathway of thyroid hormones. It is catalyzed by enzymes that contain the amino acid selenocysteine in their active center. There are three types of deiodinases, known as D1, D2, and D3. D1 and D2 remove the iodine atom from the 5 or 3 position of the phenolic ring of iodothyronines, and D1 and D3 remove the iodine from the same positions of the tyrosyl ring. Thus, D1 and D2 can convert T4 to T3, which has a higher affinity than T4 for the thyroid hormone receptor. Therefore, this pathway represents a hormonal activation process. Deiodination of the tyrosyl ring, carried out by D3 and also by D1, degrades T4 and T3 to inactive metabolites. D1 is thought to be primarily responsible for generating T3 for export to plasma T3, and the primary role of D2 is the generation of T3 for local use. The activities of the deiodinases are regulated during development and by physiological factors including the thyroid hormones per se. D3 is highly expressed in placenta, pregnant uterus, and fetal tissues, whereas D1 and D2 are expressed postnatally and in the adult. Evidence suggests that deiodinases play a major role in regulating intracellular T3 levels in a tissue-specific manner and also in coordinating growth and development in vertebrate species.

## Glossary

**lactotrophs** Cells from the anterior pituitary that secrete prolactin.

**retinoic acid response element** Short sequences present in regulatory regions of the genes regulated by retinoic acid that bind the retinoic acid receptor and modulate the rate of gene transcription.

**selenocysteine insertion sequence element (SECIS)** Selenoproteins contain the amino acid selenocysteine. This amino acid is incorporated into proteins by transfer RNAs that use the UGA codon. The UGA codon is usually a stop codon, which signals termination of translation. However, the selenoprotein-encoding mRNAs contain a signal that is able to convert a stop/UGA codon into a selenocysteine codon. This signal is known as a SECIS element and is present in the 3′-untranslated sequence of the mRNA.

**somatotrophs** Cells from the anterior pituitary that secrete growth hormone.

**sulfhydryl group** Chemical group (thiol, -SH) containing sulfur and hydrogen found in cysteine, glutathione, and other molecules.

**TATA-, CAAT-, GC-boxes** Sequences present in the promoter region of eukaryotic genes that recognize and bind ubiquitous transcription factors and allow transcription by RNA polymerase II.

**thyrocytes** The cells from the thyroid gland that produce thyroid hormones.

**thyrotrophs** Cells from the anterior pituitary that secrete thyrotropin.

**TRE** Short sequences present in regulatory regions of the genes regulated by thyroid hormone: that bind the T3 receptor and modulate the rate of gene transcription.

## See Also the Following Articles

**Thyroid Hormone Action on the Heart and Cardiovascular System • Thyroid Hormone Action on the Skeleton and Growth • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH)**

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# Diabetes Type 1 (Insulin-Dependent Diabetes Mellitus)

LISA K. GILLIAM AND ÅKE LERNMARK
*University of Washington*

I. INTRODUCTION
II. HISTORY
III. EPIDEMIOLOGY
IV. ETIOLOGY
V. PATHOGENESIS AND IMMUNOLOGICAL ABNORMALITIES
VI. CLINICAL COURSE

**Type 1 diabetes is the result of an autoimmune process targeting the pancreatic islet beta cells, resulting in insulin deficiency and hyperglycemia. Both genetic and environmental factors are important in the development of this disease. A defect in antigen presentation that leads to the loss of T-cell tolerance is most likely the pathogenic mechanism, although many questions still remain to be answered. The progression to clinical onset of type 1 diabetes is marked by the presence of autoantibodies to glutamic acid decarboxylase 65, IA-2, or insulin.**

## I. INTRODUCTION

Diabetes mellitus is a group of diseases characterized by an increase in blood sugar levels, or hyperglycemia, which occurs when an individual's insulin production does not meet the body's metabolic needs. Diabetes is divided into two major subtypes: type 1, formerly known as juvenile-onset diabetes or insulin-dependent diabetes mellitus (IDDM), and type 2, formerly known as adult-onset diabetes or non-insulin-dependent diabetes mellitus. Type 1 diabetes has traditionally been differentiated from type 2 diabetes based on an individual's age and features of clinical presentation. However, in

**TRE** Short sequences present in regulatory regions of the genes regulated by thyroid hormone: that bind the T3 receptor and modulate the rate of gene transcription.

## See Also the Following Articles

**Thyroid Hormone Action on the Heart and Cardiovascular System • Thyroid Hormone Action on the Skeleton and Growth • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH)**

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# Diabetes Type 1 (Insulin-Dependent Diabetes Mellitus)

Lisa K. Gilliam and Åke Lernmark
*University of Washington*

I. INTRODUCTION
II. HISTORY
III. EPIDEMIOLOGY
IV. ETIOLOGY
V. PATHOGENESIS AND IMMUNOLOGICAL ABNORMALITIES
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**Type 1 diabetes is the result of an autoimmune process targeting the pancreatic islet beta cells, resulting in insulin deficiency and hyperglycemia. Both genetic and environmental factors are important in the development of this disease. A defect in antigen presentation that leads to the loss of T-cell tolerance is most likely the pathogenic mechanism, although many questions still remain to be answered. The progression to clinical onset of type 1 diabetes is marked by the presence of autoantibodies to glutamic acid decarboxylase 65, IA-2, or insulin.**

## I. INTRODUCTION

Diabetes mellitus is a group of diseases characterized by an increase in blood sugar levels, or hyperglycemia, which occurs when an individual's insulin production does not meet the body's metabolic needs. Diabetes is divided into two major subtypes: type 1, formerly known as juvenile-onset diabetes or insulin-dependent diabetes mellitus (IDDM), and type 2, formerly known as adult-onset diabetes or non-insulin-dependent diabetes mellitus. Type 1 diabetes has traditionally been differentiated from type 2 diabetes based on an individual's age and features of clinical presentation. However, in

TABLE 1 **Criteria for the Diagnosis of Diabetes Mellitus**

1. Symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dl (11.1 mmol/liter). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydypsia, and unexplained weight loss.
   <or>
2. FPG ≥ 126 mg/dl (7.0 mmol/liter). Fasting is defined as no calorie intake for at least 8 h.
   <or>
3. 2hpG ≥ 200 mg/dl during an OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

*Note*. Reprinted from Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. (1997). *Diabetes Care* **20**, 1183–1197, with permission. In the absence of unequivocal hyperglycemia with acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use. FPG, fasting plasma glucose; 2hPG, 2 h plasma glucose; OGTT, oral glucose tolerance test; WHO, World Health Organization.

response to advances in the understanding of its pathogenesis, a new approach to the classification of diabetes was instituted by the American Diabetes Association (ADA) in 1997. The ADA guidelines now classify diabetes based on the pathogenic process, rather than the age of the patient and the modality used to treat the disease (insulin-requiring or non-insulin-requiring). According to these guidelines, type 1 diabetes is defined as a disorder in which the destruction of insulin-producing beta cells in the pancreas leads to absolute insulin deficiency. This is usually the consequence of an autoimmune mechanism, in which case it is referred to as type 1a diabetes. Occasionally the beta-cell destruction involves less well defined, non-autoimmune mechanisms, in which case it is referred to as type 1b diabetes. Type 2 diabetes is the result of a combination of defects, including an increase in endogenous hepatic glucose production, impaired insulin secretion by the pancreas, and peripheral tissue insulin resistance. Criteria for the diagnosis of diabetes, as outlined in the 1997 ADA report, are shown in Table 1.

## II. HISTORY

The history of type 1 diabetes can be divided into the preinsulin and insulin eras. In the preinsulin era, the disease was recognized as a wasting syndrome of unknown etiology, in which excessive urination (polyuria) and excessive fluid intake (polydypsia) were key clinical features. The 2nd century Greek physician Aretaeus of Cappadocia first used the term diabetes, meaning “to run through” or “a siphon,” describing the increased thirst and urine output, in one of the earliest recorded descriptions of this mysterious disease:

> Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water, but the flow is incessant, as if the opening of aqueducts. Life is short, disgusting and painful; thirst unquenchable; drinking excessive, which, however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking nor making water. Or if for a time they abstain from drinking, their mouth becomes parched and their body dry; the viscera seems as if scorched up; they are affected with nausea, restlessness and a burning thirst; and at no distant term they expire. [Papaspyros, N. S. (1964). *The History of Diabetes Mellitus*, 2nd ed., pp. 7–8. Georg Thieme Verlag, Stuttgart, Germany]

Prior to the 1920s, type 1 diabetes was universally fatal. More than 100 years ago, it was reported that patients dying from acute-onset diabetes had inflammatory cells in their islets of Langerhans. The identification of the “anti-diabetogenic factor,” insulin, and its first therapeutic use in diabetic individuals in 1922 forever changed the course of this disease. This breakthrough is considered one of the most extraordinary events in the history of disease treatment. With insulin therapy, patients with type 1 diabetes now are able to live for decades after disease onset, rather than months.

## III. EPIDEMIOLOGY

Type 1 diabetes varies in incidence according to age of onset, gender, geography, and ethnicity. This disease is generally diagnosed in youth, with the peak age of onset falling in the 10- to 14-year-old age group. However, the onset of type 1 diabetes can occur at any age, and this disease is being recognized more frequently in older individuals, with the development of assays measuring antibodies that indicate an autoimmune etiology. The incidence of type 1 diabetes also varies geographically, with rates as low as 0.1 individuals per 100,000 per year in China, compared with more than 40 per 100,000 per year in Finland. Like many other autoimmune diseases, the geographic distribution of type 1 diabetes has traditionally been thought of in a "polar–equatorial gradient," in which northern latitude countries suffer higher incidence rates than equatorial countries. However, other environmental and genetic factors confound this generalization. The two countries with the highest incidence rates in the world, Finland and Sardinia, are located at very distant latitudes. Other countries with high incidence rates include Kuwait, Puerto Rico, and several other European countries. Estonia, which neighbors Finland, has an incidence rate of one-quarter that of Finland's, further indicating that factors other than just climate are involved. According to dozens of epidemiological studies over the past 40 years, type 1 diabetes is increasing in incidence. This increase appears to be occurring worldwide, with a greater relative increase in populations with a historically lower incidence.

## IV. ETIOLOGY

Type 1 diabetes is caused by both genetic and environmental factors. This disorder has long been known to run in families. Twin studies have found concordance rates (percentage of cases in which both twins will be affected when one twin has a diagnosis of diabetes) of 0–13% among dizygotic twins and 21–70% among monozygotic twins. In comparison, the risk of developing type 1 diabetes in the general population is 0.4%, and the risk for that of siblings of affected individuals is approximately 6%. The fact that the concordance is not 100% in monozygotic twins, yet monozygotic twins have a higher concordance rate than dizygotic twins, suggests that both genetic and environmental influences play a role in the etiology of type 1 diabetes. Current thinking about the complicated interplay between genes and the environment suggests that an inherited defect in immunomodulation, in combination with a series of modifying environmental influences, such as infections, dietary factors, environmental toxins, sanitation, vaccinations, and health care access, tips the scales in favor of, or against progression to, the development of type 1 diabetes.

### A. Genetic Factors

Significant advances in knowledge about the genes involved in susceptibility to type 1 diabetes have been made in the past 30 years. In the 1970s, investigators identified a strong association between type 1 diabetes and human leukocyte antigen (HLA) alleles, in particular, certain class II molecules. Later, studies mapping genetic susceptibility loci confirmed that the HLA locus on chromosome 6, termed IDDM1, accounts for 45–60% of the genetic risk seen in families. The identification of particular alleles that confer risk has proven technically very difficult because of the strong linkage disequilbrium in that region of the genome. In other words, HLA haplotypes tend to be inherited in complete sets, making it very difficult to establish which of the alleles in that set is the actual diabetogenic factor. By combining data from several population studies, investigators have shown that the DQB1 * 0302-A1 * 0301 haplotype confers the greatest risk, with an additive diabetogenic effect if the DRB1 * 0401 allele is inherited as part of the haplotype. The highest genetic risk is conferred by the DQB1 * 0302-A1 * 0301/DQB1 * 0201-A1 * 0501 genotype. More than 40% of children with new onset type 1 diabetes have this genotype, compared with 3% of healthy children. Other alleles have been shown to have a negative association with risk for diabetes (a protective effect), most notably HLA DQB1 * 0602-A1 * 0102. A summary of the haplotypes associated with risk and protection is shown in Table 2. The mechanism of diabetogenicity of HLA alleles and haplotypes is under active investigation.

**TABLE 2** **Risk of Type 1 Diabetes Associated with DR and DQ Alleles**

| | DQA1 | DQB1 | DRB |
|---|---|---|---|
| High risk | | | |
| | 0301 | 0302 | 0401 |
| | 0301 | 0302 | 0402 |
| | 0301 | 0302 | 0404 |
| | 0301 | 0302 | 0405 |
| | 0501 | 0201 | 0301 |
| Moderate risk | | | |
| | 0401 | 0402 | 0801 |
| | 0101 | 0501 | 0101 |
| | 0301 | 0303 | 0901 |
| Weak protection | | | |
| | 0301 | 0301 | 04 |
| | 0301 | 0302 | 0403 |
| | 0201 | 0201 | 0701 |
| | 0501 | 0301 | 1101 |
| Strong protection | | | |
| | 0102 | 0602 | 1501 |
| | 0101 | 0503 | 1401 |
| | 0201 | 0303 | 0701 |

*Note.* Reprinted from Redondo, M. J., Fain, P.R., and Eisenbarth, G. S. (2001). Genetics of type IA diabetes. *Rec. Prog. Horm. Res.* 56, 69–89, with permission. © The Endocrine Society.

The underlying defect in type 1 diabetes is thought to be a disorder in antigen presentation, which generates self-reactive T helper cells specific for islet cell antigens. The "risk" HLA molecules could theoretically be involved in one of two ways: either they are ineffective at presenting relevant peptides to immature T cells in the thymus, failing to induce tolerance to these antigens, or they are better able than "nonrisk" alleles to present relevant peptides to these self-reactive T cells in the periphery.

Some non-HLA genetic factors have been shown to influence the development of type 1 diabetes. The insulin gene on chromosome 11, IDDM2, has polymorphisms within the noncoding region that confer either risk or protection for developing type 1 diabetes. This locus has been estimated to contribute less than 10% toward disease susceptibility. One proposed mechanism for the pathogenicity of the insulin susceptibility locus suggests that in diabetic individuals, the insulin gene is expressed in reduced amounts in the thymus during formative periods for the immune system, and the development of tolerance fails to occur properly. Another susceptibility locus for which a candidate gene has been cloned is the CTLA-4 gene on chromosome 2q (IDDM12). This gene is relevant to diabetic pathogenesis because the protein normally limits the proliferative response of activated T cells. Population studies have suggested that CTLA-4 gene polymorphisms may be a risk factor for diabetes. Furthermore, animal studies have shown that the recurrence of autoimmune diabetes in pancreatic islet-transplanted Bio Breeding (BB) rats, an animal model for type 1 diabetes, could be abrogated by adenovirus-mediated transfer of the CTLA-4 gene. Although the mechanism is not known, it has been postulated that the transfected CTLA-4 delivers a tolerogenic signal to the T cells, allowing the islet cell graft to survive.

In addition to the HLA complex, the insulin gene, and the CTLA-4 gene, several other loci have been identified in association with type 1 diabetes. Many of these genes have not yet been cloned, but with the rapid advancement in this area of research, a better understanding of the genetic influences on this heterogeneous disorder will likely be attained in the next few years. Defined and putative type 1 diabetes loci are shown in Table 3.

TABLE 3 **Defined and Putative Genetic Factors for Type 1 Diabetes**

| Locus | Chromosome | Marker/candidate genes |
|---|---|---|
| IDDM1 | 6p21.31 | HLA |
| IDDM2 | 11p15.5 | 5′ Insulin VNTR |
| IDDM3 | 15q26 | D15S107 |
| IDDM4 | 11q3 | D11S1917, H0570polyA |
| IDDM5 | 6q24–q27 | D65476–D65448 |
| IDDM6 | 18q21 | D18S64 |
| IDDM7 | 2q31 | D2S152, D2S1391 |
| IDDM8 | 6q27 | D6S1590 |
| IDDM9 | 3q21–q25 | D3S1303 |
| IDDM10 | 10p11–q11 | D10S193, D10S565 |
| IDDM11 | 14q24.3–q31 | D14S67 |
| IDDM12 | 2q33 | CTLA-4 |
| IDDM13 | 2q34 | NRAMPI (D2S164) |
| IDDM15 | 6q21 | D65283 |
| IDDM17 | 10q25.1 | D10S1681 |
| IDDM18 | 5q33–q34 | IL-12B |
| No IDDM | 16q22–q24 | D16S3098 |
| No IDDM | 1q42 | D15617 |

*Note*. Adapted from Redondo, M. J., Fain, P. R., and Eisenbarth, G. S. (2001). Genetics of type IA diabetes. *Rec. Prog. Horm. Res.* **56**, 69–89, with permission. © The Endocrine Society.

## B. Environmental Factors

Environmental triggers, in addition to genetic factors, are thought to play a role in the development of type 1 diabetes. Three of the best studied risk determinants include viral infections, early infant diet (cow's milk versus breast milk), and toxins. Although Finnish studies on diet suggested an association between early introduction of cow's milk components and diabetic risk, trials in the United States and Germany did not support these findings, and this point remains controversial. The concept that viral infections might play a role is based on numerous case-control studies in which infectious outbreaks are temporally associated with a higher incidence of diabetes. Furthermore, congenital infections with rubella and enterovirus have been strongly implicated in the subsequent development of diabetes by infants exposed *in utero*. Candidate viruses and other environmental factors are listed in Table 4. In addition to the possible contribution of congenital viral infections, other unidentified factors in the fetal environment appear to influence the risk of type 1 diabetes in the child. Both increasing maternal age and birth order (higher risk in the first born) have recently been shown to be related to risk of type 1 diabetes in the offspring. Further prospective studies are needed to better define environmental triggers for type 1 diabetes, as these have been very difficult to characterize.

TABLE 4 Viruses Implicated in Type 1 Diabetes

| Virus | Host organism |
|---|---|
| Coxsackie | Human, mouse |
| Rubella | Human, hamster |
| Mumps | Human |
| Cytomegalovirus | Human |
| Rotavirus | Human |
| Encephalomyocarditis | Mouse |
| Meningovirus | Mouse |
| Reovirus | Mouse |
| Lymphocytic choriomeningitis | Rat |
| Kilham strain (mumps virus) | Rat |

## V. PATHOGENESIS AND IMMUNOLOGICAL ABNORMALITIES

The current understanding of the pathogenesis of type 1 diabetes has been aided by findings on autopsy of type 1 diabetic individuals, the presence of concomitant organ-specific autoimmune disease, such as thyroiditis, and measurements of disease markers, such as autoantibodies, in individuals with the disease. Prior to the 1970s, there was an understanding that two different types of diabetes existed; however, they were commonly thought to be gradations of the same basic disease, perhaps based on homozygous versus heterozygous inheritance of an unidentified "diabetes gene." Clues indicating that type 1 diabetes had a distinct, immune-based pathogenesis date back to the early 1900s. At that time, pathologists described "insulitis," a pathological feature seen in newly diagnosed type 1 diabetic patients in which the islets are infiltrated with mononuclear inflammatory cells. However, this finding was largely ignored because it was infrequently observed at the time of autopsy, by which time islet cell inflammation is already in its final stages and thus greatly diminished. Later evidence that cell-mediated immunity was involved in disease pathogenesis was provided in the 1960s by "leukocyte migration inhibition" tests, which demonstrated priming of patient T cells against islet cell antigens. The finding of delayed-type hypersensitivity reactivity to injected pig islet material in diabetic subjects further suggested a role for cell-mediated immunity against pancreatic antigens. The identification of islet cell antibodies in the 1970s provided the first evidence for humoral autoimmunity in type 1 diabetes. At the same time, two animal models helped to establish type 1 diabetes as an autoimmune disorder. Mice treated with subdiabetogenic doses of streptozotocin, a pancreatic toxin, were noted to have progressive islet cell destruction and insulin deficiency, even after the toxin was cleared from their systems. This process was therefore presumed to be immune-mediated. Also at that time, the first model of spontaneous type 1 diabetes was discovered, the BB rat. This animal, with classic insulin-deficient, ketosis-prone disease, had histological features of insulitis prior to disease onset. A second spontaneous diabetic animal model, the nonobese diabetic mouse, was later described in the 1980s. These rodent models have helped to further elucidate the pathogenesis of type 1 diabetes in humans, as outlined later in this article. Thus, the concept of type 1 diabetes as an autoimmune disorder first gained wide acceptance in the mid-1970s, and it is now recognized to be a process involving both the cellular and the humoral branches of the immune system.

It is now understood that the autoimmune process in type 1 diabetes results in the selective destruction of pancreatic beta cells. This process involves both cellular and humoral immunity, although it is thought to be mediated primarily by autoreactive T cells. This assumption is based on observations in both human subjects and animal models. In humans, lymphocytic islet cell infiltrates in newly diagnosed diabetics suggest that cytotoxic T-cell activity is the likely etiology of the islet cell destruction. In animal models, researchers have shown that transfer of diabetes from one animal to another requires both CD4$^+$ and CD8$^+$ T cells. The complete pathophysiological picture will likely involve a complex interaction between autoreactive T cells of both helper and cytotoxic subtypes, autoantibodies, and defective antigen-presenting cells. But what are the targets of these autoreactive lymphocytes? Ever since recognizing that type 1 diabetes is indeed an autoimmune disease, researchers have sought to define the targets of the immune response, as well as to understand why the immune system goes awry and begins to recognize these self-molecules as antigen. Answers to both of these questions will help to direct future interventions, defining what enemy to fight and what battle tactics to employ.

### A. Humoral Immunopathophysiology

Humoral immunopathophysiology in diabetic individuals has proven fairly straightforward to establish because of the relative ease in measuring antibody responses to a specific antigen. Islet cell antibodies (ICA) were first identified in the 1970s by indirect immunofluorescence techniques. Specific autoantigens were later immunoprecipitated from human islet cells, including glutamic acid decarboxylase 65 (GAD65), IA-2 (ICA-512), and IA-2β (phogrin). Insulin was also shown to be a target of the autoimmune response in newly diagnosed, previously untreated type 1 diabetic patients. Approximately 70–80% of children with new onset diabetes express GAD65 antibodies, compared with 1% of individuals in the general population and 8% of relatives of patients with type 1 diabetes. Similarly, IA-2 antibodies are seen in 60–70% of new onset diabetic patients, and insulin autoantibodies are seen in 50% of patients. Epidemiological studies have revealed several factors that seem to influence the expression of these autoantibodies, including age at diagnosis, gender, and HLA haplotype. Furthermore, prospective studies done in prediabetic individuals have shown that islet cell autoantibodies begin to appear between 9 months and 3 years of age, with insulin antibodies often being the first to appear, followed by GAD65 antibodies. Subjects may be positive for GAD65, IA-2, or insulin antibodies for many years without development of type 1 diabetes. However, the presence of two or more of these autoantibodies strongly predicts type 1 diabetes. Standardized antibody tests are now available and are beginning to be used to recruit participants to intervention trials. Although much has been learned about these autoantibodies as markers of disease, the role that they play in disease pathogenesis remains unclear.

### B. Cellular Immunopathophysiology

Because of the lack of reproducible cellular immunoassays, less is known about the cell-mediated autoimmune process in diabetes. The same autoantigens against which antibodies are produced (GAD65 and IA-2) have been shown to stimulate *in vitro* T-cell proliferation, using lymphocytes from diabetic subjects. Furthermore, immunodominant epitopes of GAD65 and IA-2 have been identified both from antigen-specific T-cell lines generated from diabetic patients and from transgenic mice expressing human “diabetes risk” HLA class II molecules. Although these studies have been informative, the methods are cumbersome, and they also potentially reflect a skewed sample, depending on which T cells are isolated. Investigations are currently underway using soluble major histocompatibility complex (MHC) tetramers to directly identify specific peptide/MHC-restricted T cells, with specificities both for known and unknown epitopes, in order to better understand the important targets of T-cell-mediated immunity.

Several hypotheses have been proposed for mechanisms of induction of a pathogenic T-cell response in type 1 diabetes. One explanation is that molecular mimicry, or sharing of common epitopes between islet antigens and foreign antigens (such as viruses), leads to loss of T-cell tolerance to these epitopes and progression to diabetes. Other possibilities include aberrant islet beta-cell expression of co-stimulatory molecules, adhesion molecules, or MHC. A third hypothesis suggests that the cytokine milieu is very important in disease progression. Studies have shown that the predominance of a Th1-mediated immune response, in which secretion of interferon-γ, interleukin-2 (IL-2), IL-12, and tumor necrosis factor α (TNFα) activates mainly cytotoxic T cells and macrophages, is pathogenic. On the other hand, a Th2-mediated response, in which secretion of IL-4, IL-5, IL-6, or IL-10 activates the humoral branch of the immune system, may be protective. The Th1 versus Th2 polarizing signals are primarily derived from antigen-presenting cells (APC), and one interpretation is that an APC defect is responsible for the shift from a benign Th2-dependent immune response to a destructive Th1-dependent immune response in diabetic individuals. This putative APC defect has not been well defined but is under active investigation.

## VI. CLINICAL COURSE

### A. Patient Presentation

The signs and symptoms of type 1 diabetes are directly related to the lack of insulin and consequent hyperglycemia. Classically, patients present with polyuria, polydypsia, and weight loss, despite polyphagia. Polyuria and polydypsia result from the osmotic diuresis that occurs when the serum glucose level becomes high enough (generally in the range 300–500 mg/dl) that sugar begins to spill into the kidney collecting system, carrying with it free water. Hyperglycemia and its consequent hyperosmolality lead to electrolyte imbalances; sodium, potassium, magnesium, chloride, and bicarbonate are lost in the urine, resulting in the depletion of these electrolytes. Blurred vision and central nervous system (CNS) dysfunction result from intracellular dehydration. The CNS dysfunction manifests as altered mental status ranging from mild drowsiness to profound lethargy.

A prolonged lack of insulin eventually leads to diabetic ketoacidosis (DKA). Because the peripheral tissues are unable to utilize glucose without insulin, protein catabolism and lipolysis are up-regulated, resulting in the production of ketones and a metabolic acidosis. The acidosis further contributes to electrolyte shifts and may cause life-threatening hyperkalemia (elevated serum potassium levels) resulting in cardiac arrhythmias, despite total body potassium depletion. Diabetic ketoacidosis may also be associated with abdominal pain, nausea, and vomiting. However, it is critical for the clinician to differentiate these symptoms of DKA from abdominal pain due to another process that may have played a role in triggering the disease onset, such as pyelonephritis, pancreatitis, or an acute abdomen of another etiology.

On physical examination, the patient generally has a thin body habitus, with signs of dehydration including decreased skin turgor, dry mucous membranes, tachycardia, and orthostatic hypotension. Patients often have rapid, deep breathing (Kussmaul's respirations), due to compensation for the metabolic acidosis with a respiratory alkalosis. Furthermore, the high plasma acetone level gives a distinctive fruity odor to the patient's breath.

## B. Natural History

The current understanding of the natural history of type 1 diabetes prior to the clinical onset of disease is limited. Many individuals positive for one or several islet cell antibodies have been examined prior to the clinical onset of overt hyperglycemia. In some of these so-called prediabetic subjects, it has been possible to demonstrate that the release of insulin in response to glucose stimulation has deteriorated over time. Other subjects have had a decreased ability to release insulin but have remained normoglycemic, presumably because their insulin sensitivity has remained high. The natural history of type 1 diabetes prior to clinical diagnosis therefore seems variable and further studies are needed to uncover factors that are important in the precipitation of hyperglycemia.

While once culminating in death within weeks to months, the short-term outcome for patients with a new diagnosis of type 1 diabetes is now very good, assuming that the diagnosis is made and insulin therapy is initiated in a timely manner. However, diabetes remains a disease associated with a high lifetime mortality risk. Rather than succumbing to the immediate consequences of hyperglycemia and its associated metabolic derangements, diabetic patients now die as a result of secondary effects of the diabetes, primarily heart disease. Chronic hyperglycemia results in damage to and dysfunction of many organs, including the heart (cardiomyopathy), blood vessels (vasculopathy), eyes (retinopathy), kidneys (nephropathy), and nerves (neuropathy). Medical care for diabetic individuals therefore is targeted at both maintaining tight glycemic control and careful monitoring for and treatment of the secondary sequelae of the diabetes.

## C. Diagnosis and Treatment

The diagnosis of diabetes mellitus is made based on a fasting blood glucose level greater than 126 mg/dl or a random blood glucose level greater than 200 mg/dl with associated signs and symptoms of diabetes, as outlined in Table 1. For type 1 patients, who generally present in DKA, the diagnosis is not subtle. In this case, the diagnosis is strongly suspected based on the clinical history and confirmed by the presence of hyperglycemia and acidosis. Initial treatment is directed at rehydrating the patient, correcting the electrolyte imbalances, and providing the deficient hormone, insulin, intravenously. Once clinically stable with blood sugars in the range 200–300 mg/dl, the patient is started on subcutaneous insulin injections, and measures to prevent the long-term consequences of diabetes are initiated.

The mainstay of treatment at this time for type 1 diabetics is subcutaneous insulin injections several times a day, with close monitoring of blood sugars. Glycemic control is monitored both by the patient on a day to day basis, with measured plasma glucose levels obtained by "finger sticks" four to seven times a day, and by the physician, who measures the hemoglobin A1C, a reflection of the mean blood glucose concentration over the previous 6 to 8 weeks. Target blood sugars are in the normal range (80 to 120 mg/dl), and target hemoglobin A1C values are between 6.5 and 7%. Newer modalities, such as insulin pumps, which control blood sugars minute to minute, peakless basal insulin, and aerosolized insulin (which is currently undergoing phase 3 clinical trials, expected to be available soon on the U. S. market), have contributed to improvements in diabetic management. Furthermore, the recent development of continuous glucose-monitoring systems and implantable insulin pumps is approaching the realization of an "artificial pancreas" concept, in which tight, hassle-free glycemic control may become a reality.

Prevention and treatment of the secondary effects of diabetes (target organ damage) include maintenance of tight blood pressure control, lowering LDL cholesterol, monitoring peripheral sensation with regular monofilament examinations of the feet, monitoring kidney function by measuring protein loss in the urine, screening for diabetic retinopathy with regular ophthalmological exams, and screening for cardiovascular disease with aggressive medical and/or surgical interventions as needed.

## D. Future Directions

During the past decade, pancreatic islet cell transplantation has also been under investigation as a treatment modality for type 1 diabetes. Initial results were disappointing, with success rates of only 8% (defined as patients still non-insulin-requiring at 1 year posttransplantation). However, with a better understanding of how to help these foreign grafts survive as well as how to alter the anti-islet cell autoimmune response in type 1 diabetics, success rates appear to be improving. Another area of active investigation is antigen-specific immunosuppression, in which prediabetic subjects with strong risk factors are vaccinated with targets of the autoimmune response, in an attempt to shift from pathogenic to protective immunity by inducing tolerance to the autoantigen. Studies in animal models have shown this to be an effective intervention, and phase 2 trials testing vaccination with the GAD65 antigen are currently underway. In addition, the ongoing Diabetes Prevention Trial 1 is examining whether low doses of injected or oral insulin can prevent progression to diabetes in susceptible individuals. Preliminary results suggest that injected insulin does *not* alter the natural history of the disease; results of the second arm of the study, using oral insulin, are not yet available. Furthermore, a host of immunosuppressive or immunomodulating agents are advancing to clinical trials.

At this time, a diagnosis of type 1 diabetes commits the individual to a lifetime of insulin injections. Studies seeking to elucidate why and how the immune system goes awry may provide clues about how to improve treatment options and even alter the course of this disease. Hopes for the future include immunomodulatory vaccines and islet cell transplantation.

## Glossary

**autoimmunity** Abnormal immune response in which B cells, T cells, or both are directed against self-antigens.

**cell-mediated immunity** Host defenses mediated by antigen-specific T cells, which protect against intracellular bacteria, viruses, and cancer.

**cytotoxic T-lymphocyte-associated antigen-4** Surface molecule on T cells that binds to the B7 molecule on antigen-presenting cells, resulting in delivery of a negative signal to the T cell and consequent downregulation of the immune response.

**diabetic ketoacidosis** Disease state characterized by hyperglycemia and acidosis, in which plasma ketones (β-hydroxybutyrate and acetoacetate) accumulate due to insulin deficiency and elevated glucagon levels.

**epitope** Site on an antigen that is recognized by a particular antibody or T-cell receptor.

**glutamic acid decarboxylase 65 (GAD65)** One of the major autoimmune targets in type 1 diabetes, GAD65 is an isoform of GAD, the enzyme that converts glutamic acid to γ-aminobutyric acid, an inhibitory neurotransmitter.

**human leukocyte antigen** Member of a family of cell surface heterodimers expressed on antigen-presenting cells that are responsible for presenting foreign antigens to T cells, via the T-cell receptor.

**humoral immunity** Host defenses, mediated by antibodies, which protect against extracellular bacteria and other foreign macromolecules.

**IA-2 (ICA-512)** One of the major autoimmune targets in type 1 diabetes, IA-2 is a protein tyrosine phosphatase-like molecule.

## See Also the Following Articles

**Diabetes Type 2 (Non-Insulin-Dependent Diabetes Mellitus) • Glucagon Secretion, Regulation of • Hypoglycemia in Diabetes • Insulin Actions • Insulin Gene Regulation • Insulin Processing • Insulin Secretion • Interleukin-10 • Interleukin-18**

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# Diabetes Type 2 (Non-Insulin-Dependent Diabetes Mellitus)

R. Paul Robertson
*University of Washington*

I. INTRODUCTION: DEFINITION OF TYPE 2 DIABETES MELLITUS
II. PHYSIOLOGY
III. PATHOPHYSIOLOGY
IV. DIAGNOSTIC TESTING
V. SECONDARY COMPLICATIONS
VI. TREATMENT

**Type 2 diabetes, also called non-insulin-dependent diabetes or adult-onset diabetes, refers to a pathologic condition that occurs commonly in humans and in some animals. This condition involves a chronic elevation of blood glucose and, over time, the development of secondary complications that adversely affect the function of many tissues and organs throughout the body.**

## I. INTRODUCTION: DEFINITION OF TYPE 2 DIABETES MELLITUS

The onset of type 2 diabetes, the hallmark of which is hyperglycemia, in most instances occurs in middle age and is associated with obesity. However, lean individuals can also develop type 2 diabetes and even children can develop this disease if they grow up under conditions of insufficient physical activity, improper diet, and excessive weight gain. It has been estimated that 7–9% of individuals in the United States will eventually develop type 2 diabetes. It is likely that type 2 diabetes is not a single disease and it is more accurately referred to as a syndrome that can have many causes. Clinical expression of the syndrome probably involves participation of more than one gene in a setting of adverse environmental influences favoring expression of the abnormal genes. For full expression, the syndrome of type 2 diabetes requires both an abnormality in pancreatic islet beta cell function and an abnormal resistance to the action of insulin in the cells throughout the body.

## II. PHYSIOLOGY

### A. Anatomy

The pancreas is located within the abdomen next to the stomach, intestines, and spleen. The pancreas is made up primarily (97%) of nonislet tissue, referred to as exocrine tissue. This tissue is drained by a pancreatic duct that runs the length of the pancreas and empties directly into the small intestine, where it delivers the digestive enzymes made by the exocrine pancreas. Scattered throughout the exocrine pancreas and comprising the remaining 3% of the pancreatic mass are the islets of Langerhans. The islets contain four major cell types, namely, alpha, beta, delta, and PP cells. These cells secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively.

### B. Regulators of Islet Function

The primary stimulus for insulin secretion is glucose, but beta cells can also be stimulated by amino acids, other hormones, and drugs (Table 1). Insulin secretion automatically turns off when blood glucose levels become low, thus preventing the development of hypoglycemia. The primary regulators for alpha cell stimulation to release glucagon function in just

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TABLE 1 Regulators of Pancreatic Islet Cell Secretion

| Cell type | Product | Stimulators | Inhibitors |
|---|---|---|---|
| Alpha | Glucagon | Hypoglycemia, arginine | Hyperglycemia, somatostatin |
| Beta | Insulin | Hyperglycemia, arginine, acetylcholine, glucagon | Hypoglycemia, somatostatin, prostaglandin E2, galanin |
| **Delta** | Somatostatin | Glucose, arginine | α-Adrenergic agonists, dopamine |
| PP | Pancreatic polypeptide | Hypoglycemia, secretin | Atropine |

the opposite way, i.e., hypoglycemia stimulates glucagon secretion and hyperglycemia inhibits the secretion of this hormone. Somatostatin secretion is stimulated by glucose and amino acids. Pancreatic polypeptide secretion is stimulated by hypoglycemia and another hormone called secretin. The interplay of these regulators of hormone secretion by the pancreatic islet provides a highly important and sophisticated means by which the products of the cells within the islet regulate overall body metabolism.

In general, the hormone insulin is essential for normal growth and for storage of fuels in body tissues. It is the primary regulator of blood glucose and is responsible for maintaining glucose within physiologic levels. Failure of pancreatic beta cells to secrete insulin inevitably is associated with very high levels of glucose and the development of diabetes. Beta cells sense the level of blood glucose and either release or stop releasing insulin, regulating blood glucose levels within a narrowly defined range both in the fasting and fed states. Hypoglycemia, however, can result from exogenous insulin, as in injected doses used to treat diabetes; overdosing can cause blood glucose to drop to abnormally low levels. In this scenario, glucagon secretion from alpha cells is then stimulated and recovery from hypoglycemia begins. Alpha cells sense when the blood glucose level becomes dangerously low (<56 mg/dl) and release the hormone glucagon, the main function of which is to prevent prolonged periods of hypoglycemia. As soon as the blood glucose level returns to normal, glucagon secretion is inhibited. Somatostatin is a local regulator of both insulin and glucagon secretion by virtue of its capacity to inhibit secretions from beta and alpha cells. The function of pancreatic polypeptide in humans is unknown.

## C. Insulin and Glucagon Action

Insulin and glucagon have opposite actions on many important bodily processes (Table 2). Insulin inhibits and glucagon stimulates lipolysis, a process by which stored triglyceride within fat cells is released into the bloodstream in the form of fatty acids and glycerol. Insulin inhibits and glucagon stimulates gluconeogenesis, a biochemical process by which amino acids are used to synthesize glucose. Insulin inhibits and glucagon stimulates glycogenolysis, a process by which stored glycogen is broken down into glucose for release into the bloodstream. Insulin inhibits and glucagon stimulates ketogenesis, a biochemical process in the liver through which free fatty acids form ketones in the liver for release into the bloodstream. As mentioned previously, insulin decreases and glucagon increases blood glucose levels. The major function of these two partners is to finely regulate blood glucose levels so that neither prolonged hypoglycemia nor hyperglycemia is allowed to develop. The major sites for the regulatory actions of insulin and glucagon are the liver, fat tissue, and muscle. The liver is the major site for glycogenesis, glycogenolysis, gluconeogenesis, and ketogenesis. The fat cell is the major site for lipolysis. Insulin-induced removal of glucose from the blood occurs in most tissues of the body, with neural tissue being one important exception. The biochemical mechanism that insulin uses to exert its effects is a matter of debate. It is known that there are insulin binding sites, termed insulin receptors, on target tissues and that insulin phosphorylates many proteins subsequent to its binding. The ultimate phosphorylation step that is required for insulin action has not yet been identified and may differ from tissue to tissue. The biochemical mechanism of glucagon also involves specific binding sites and postreceptor effects involving

TABLE 2 Metabolic Actions of Insulin and Glucagon

| Process | Insulin | Glucagon |
|---|---|---|
| Gluconeogenesis | Decrease | Increase |
| Glycemia | Decrease | Increase |
| Glycogenesis | Increase | Decrease |
| Glycogenolysis | Decrease | Increase |
| Lipolysis | Decrease | Increase |
| Ketogenesis | Decrease | Increase |

phosphorylation of proteins, a common consequence of which is the formation of cyclic adenosine monophosphate (cAMP).

## III. PATHOPHYSIOLOGY

### A. Syndrome of Type 2 Diabetes

As previously discussed, type 2 diabetes is more appropriately referred to as a syndrome than a single disease entity. This condition has a polygenic cause and there is usually a strong family history of the disease in immediate family members. The onset of type 2 diabetes is usually insidious, with the patient noticing gradual weight loss and the development of thirst, increased appetite, and copious urination. Because the onset is so insidious, there are long periods of time when the patient can have diabetes without knowing it unless the blood glucose level is measured. The average age of onset of the disease is usually around 50 years, although young persons can also develop a form of the disease, referred to as maturity-onset diabetes of the young (MODY). It is not yet known which genes cause common type 2 diabetes mellitus, although much is known about genes contributing to MODY. The concordance rate for the development of type 2 diabetes is very high in identical twins.

### B. Type 2 vs Type 1 Diabetes: Comparisons and Contrasts

Type 1 and type 2 diabetes mellitus are entirely different diseases. Many differences and a few similarities distinguish these diseases (Table 3). Middle age is the typical age of onset of type 2 diabetes, whereas onset is usually before adulthood in type 1 diabetes. Excess development of ketone bodies because of insulin deficiency is common in type 1 but not type 2 diabetes. Obesity is common in type 2 diabetes but not type 1. Type 2 diabetes is at least 20 times more prevalent than type 1 diabetes and in certain populations occurrence is much higher. There is no evidence for autoimmune causation of type 2 diabetes whereas an autoimmune process definitely contributes to type 1 diabetes. The concordance rate in identical twins is roughly 90% in type 2 diabetes but is only 50% in type 1 diabetes. Insulin therapy is absolutely required for type 1 diabetes but it is typically an option for type 2 diabetes. The only major similarity between these two diseases is that the secondary complications are frequent in both forms of the disease and are indistinguishable pathologically, reflecting the role of chronic elevations of high glucose as the cause of the complications.

### C. Other Causes of Type 2 Diabetes

Other than the common syndrome of type 2 diabetes, the exact genetic causes of which are poorly understood, many other conditions are able to impact negatively on pancreatic islet function and/or insulin action on its target tissues, thereby causing hyperglycemia (Table 4). These adverse situations involve pancreatic disease, such as pancreatitis, trauma, tumors, cystic fibrosis, and hemochromatosis; endocrinopathies, such as excess secretion of steroids (Cushing syndrome), growth hormone (acromegaly), and glucagon (glucagonoma); drugs and chemicals, such as glucocorticoids and certain diuretics; abnormalities in the insulin receptor; and several genetic syndromes, such as diabetes insipidus/diabetes mellitus/optic atrophy/deafness (DIDMOAD) and myotonic dystrophy. In these instances, the development of chronic hyperglycemia very closely resembles that of classic type 2 diabetes, and the disease will be treated similarly.

**TABLE 3 Clinical Manifestations of Type 1 vs Type 2 Diabetes Mellitus**

| Measure | Type 1 | Type 2 |
|---|---|---|
| Age of onset | <30 years | >40 years |
| Body weight | Lean | Obese |
| Prevalence | 0.5–1.0% | 7–8% |
| HLA-positive | Yes | No |
| Concordance in identical twins | 50% | 95% |
| Islet cell antibodies | 85% | <10% |
| Insulin treatment | Required | Optional |
| Secondary complications | Frequent | Frequent |

**TABLE 4 Causes of Type 2 Diabetes Mellitus**

- **Primary**
  - Polygenic (insulin independent, maturity onset)
    - Obese
    - Nonobese
    - Maturity-onset diabetes of the young (MODY)
- **Secondary**
  - Pancreatic disease
  - Nonpancreatic hormonal abnormalities
  - Drugs, chemicals
  - Insulin receptor abnormalities
  - Genetic syndromes

## D. Gestational Diabetes

The condition known as gestational diabetes refers to a temporary elevation in blood glucose level during pregnancy. It is an important condition because hyperglycemia can have adverse effects on a fetus. Consequentially, great care is taken by obstetricians to monitor patients for development of diabetes during the course of a pregnancy. Commonly, oral glucose tolerance tests are given; if abnormalities are observed, patients are advised to alter their diet and in some cases insulin treatment is required to maintain glucose levels in the normal range. Typically, after delivery, the diabetic state in the mother remits. The exact cause of gestational diabetes is not known but it is felt that a contributing factor is the complex array of hormonal changes that occur during pregnancy, coupled with an intrinsic borderline function of the maternal pancreatic islets. A greater percentage of women who develop gestational diabetes will later develop type 2 diabetes mellitus compared to women who are able to sustain pregnancies without developing hyperglycemia.

## E. Maturity-Onset Diabetes of the Young

Onset of the MODY form of type 2 diabetes usually occurs before the age of 25 and frequently occurs in childhood. This disease involves a primary defect in beta cell function and can result from mutations in one of at least six different genes (Table 5). The genes that can be involved in specific types of MODY encode specific proteins, hepatic nuclear factors (HNFs), insulin promoter factors (IPFs), and transcription factors (NeuroD1/BETA2) as follows: HNF-4α (MODY 1), glucokinase (MODY 2), HNF-1α (MODY 3), IPF-1 (MODY 4), HNF-1β (MODY 5), and NeuroD1/BETA2 (MODY 6). All of the conditions that are the consequences of these genetic defects have similarities and differences, including abnormal regulation of insulin gene transcription, sensitivity of beta cells to glucose, defective storage of glucose as liver glycogen, and abnormal regulation of beta cell development and function. Treatment of the different forms of MODY can vary from control of diet and exercise to use of oral hypoglycemic agents or insulin. It has been estimated that MODY may account for 1–5% of all cases of diabetes in the United States and other western countries. The chief clinical characteristics distinguishing patients with MODY from those with conventional type 2 diabetes include a prominent family history of diabetes in three or more generations, absence of obesity, and the young age of presentation.

## F. Relative Roles of Insulin Secretory Abnormalities and Insulin Resistance

In most instances, type 2 diabetes mellitus does not develop unless there is both a defect in beta cell function and a defect in insulin action at the level of its target tissues. This multiplicative effect has been referred to as the multiplier hypothesis (Fig. 1).

**TABLE 5 Classification of Maturity-Onset Diabetes of the Young**

| MODY type | Protein encoded by abnormal gene[a] | Molecular defect | Usual treatment |
|---|---|---|---|
| 1 | HNF-4α | Abnormal beta cell gene transcription, causing defective signaling of beta cell mass and secretion | Oral hypoglycemic drugs |
| 2 | Glucokinase | Defective beta cell sensitivity to glucose; defective liver glycogen storage | Diet, exercise |
| 3 | HNF-1α | Abnormal beta cell gene transcription, causing defective signaling of beta cell mass and secretion | Oral hypoglycemic drugs |
| 4 | IPF-1 | Abnormal beta cell gene transcription and function | Oral hypoglycemic drugs, insulin |
| 5 | HNF-1β | Abnormal beta cell gene transcription, causing defective signaling of beta cell mass and secretion | Insulin |
| 6 | NeuroD1 or BETA2 | Abnormal beta cell gene transcription and function | Insulin |

[a] HNF, Hepatic nuclear factor; IPF, insulin promoter factor.

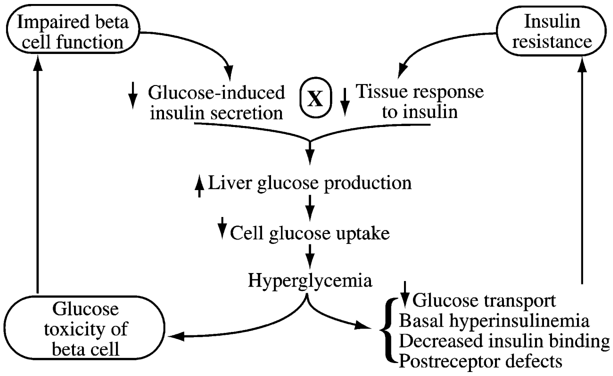
Impaired beta cell function
Insulin resistance
↓ Glucose-induced insulin secretion
X
↓ Tissue response to insulin
↑ Liver glucose production
↓ Cell glucose uptake
Hyperglycemia
Glucose toxicity of beta cell
↓ Glucose transport
Basal hyperinsulinemia
Decreased insulin binding
Postreceptor defects

FIGURE 1 Multiplier hypothesis. The pathogenesis of type 2 diabetes mellitus involves a double defect of abnormal beta cell function coupled with resistance to the actions of insulin on its target tissues. This combination leads to elevated glucose levels and worsening of insulin resistance as well as glucotoxic effects on many tissues throughout the body, including the beta cell. Modified from Robertson, R. P. (1995), Diabetes and insulin resistance: Philosophy, science, and the multiplier hypothesis, *J. Lab. Clin. Med.* **125**, 560–564, with permission.

The overall hypothesis is that patients with type 2 diabetes mellitus are born with one of several possible genetic defects in beta cell mass or function and also have genetic or environmentally induced insulin resistance. The combination of these defects cause hyperglycemia in the diabetic patient, which in turn leads to worsening insulin resistance as well as worsening beta cell function and adverse effects of chronic hyperglycemia on tissues throughout the body. It is unlikely that insulin resistance alone can cause type 2 diabetes because virtually all obese individuals as well as individuals treated with exogenous glucocorticoids are insulin resistant, and yet most of these individuals never develop hypoglycemia. Similarly, certain disease states, such as cystic fibrosis, cause abnormalities in insulin secretion, yet these patients usually are lean and insulin sensitive and usually do not develop hyperglycemia. This multifactorial etiology of type 2 diabetes presents the opportunity for multiple modes of treatment to control the hyperglycemia. Treatment can be directed both toward improving beta cell function as well as decreasing insulin resistance.

## G. Glucose Toxicity

Whatever the cause of hyperglycemia, the chronic elevation of glucose levels can have adverse metabolic effects in many tissues. Whereas glucose in normal concentrations is an important fuel for tissues, glucose in excess can be shunted away from its normal metabolic pathway into other pathways that produce compounds toxic to cell function. Some of these products are sorbitol, glucosamine, and highly reactive radicals involving either oxygen or nitrogen atoms. For example, glucose can generate reactive oxygen species (ROS), such as superoxide ions and hydroxyl radicals. The hydroxyl radical, in particular, is able to cross cell membranes rapidly and enter the nucleus, where, if in high enough concentrations, it can damage nuclear DNA. DNA mutations, in turn, can lead to abnormalities in protein synthesis and cell function.

## IV. DIAGNOSTIC TESTING

The chief means by which diabetes is diagnosed is the blood glucose level. Current criteria use a fasting plasma glucose level of greater than 126 mg/dl as the cutoff point for diagnosing diabetes. Oral glucose tolerance testing is also utilized. The oral glucose test involves ingesting a set amount of glucose (usually 75 g) and measuring blood glucose levels over the ensuing 2 h. Current diagnostic criteria indicate impaired glucose tolerance if the 2-h level is greater than 140 mg/dl and frank diabetes if the 2-h level is greater than 200 mg/dl. Beyond these clinical criteria, research data indicate that a fasting plasma glucose level greater than 100 mg/dl may be abnormal because individuals with a fasting glucose level less than 100 mg/dl have twice the magnitude of insulin secretion in response to an intravenous glucose challenge.

## V. SECONDARY COMPLICATIONS

The major secondary complications of hyperglycemia develop in the kidney, nerves, retinas, and walls of small and large arteries. Kidney disease, referred to as diabetic nephropathy, is a leading cause of death and disability in patients with diabetes. Roughly half the cases of end-stage renal disease in the United States are due to diabetic nephropathy. The prevalence of this complication varies from 15 to 60% of type 2 diabetes patients, depending on ethnic background. Typically, hyperglycemia over a period of 10–15 years is required before diabetic nephropathy is expressed at a clinical level. Abnormal consequences include the appearance of protein in the urine and excessive levels of creatinine in the blood, reflecting a decline in the ability of the kidney to filter proteins. Ultimately, diabetic nephropathy can lead to renal failure, which requires renal dialysis or kidney transplantation to prevent death. The adverse effect of diabetes on nerves, referred to as diabetic neuropathy, takes many forms. The most common manifestations are sensory and motor neuropathies. Sensor neuropathy is expressed clinically by symptoms such as numbness, pain, and extreme sensitivity to touch. Motor neuropathies are expressed clinically by muscle weakness and incoordination. Treatment of diabetic neuropathy is largely ineffective, which causes sustained and significant stress for the diabetic patient. Involvement of the optic retinae is referred to as diabetic retinopathy. Diabetic retinopathy is the leading cause of blindness in the United States. Even though many patients develop the retinopathic changes, most diabetic patients do not become blind. Typically, retinopathy involves the formation of microaneurysms, dilated veins, proteinaceous exudates, retinal detachment, and vitreal hemorrhage. Retinopathy is usually not apparent in the first 5 years of diabetes but eventually greater than 50% of patients develop some form of this complication. The treatment for diabetic retinopathy is photocoagulation, which is performed to decrease the incidence of hemorrhaging and scarring and to prevent an advanced stage of retinopathy referred to as proliferative retinopathy. Both small and large arteries are adversely affected by chronic hyperglycemia, outcomes that are worsened by an often associated elevated level of lipoproteins. Oxidized low-density lipoproteins favor the formation of atherosclerotic lesions and this process appears to be accelerated in diabetes. Complications that follow the development of atherosclerosis in arteries include coronary heart disease and stroke as well as peripheral vascular disease, which in combination with peripheral neuropathy, predisposes diabetic patients to poor healing of skin lesions in the feet. The latter complication, if not properly attended to, can cause gangrene and the need for amputation to varying degrees.

This range of secondary complications of chronic hyperglycemia provides important incentives for patients and physicians to treat diabetes optimally. A very important clinical trial, the Diabetes Control and Complications Trial (DCCT), has established that the rate of development of secondary complications of diabetes involving eyes, nerves, and kidneys is inversely related to mean circulating glucose levels (Fig. 2). In this study, the major marker of hyperglycemia was hemoglobin A1c, a fraction of hemoglobin that is glycosylated by glucose in direct proportion to the circulating glucose level. A single hemoglobin A1c measurement reflects the average daily mean blood glucose level for the prior 3 months. Ready access to this measurement, in conjunction with the ability of patients to monitor their own glucose levels at home with portable glucose meters, has greatly assisted patients in devising the proper therapeutic regimens to maintain their glucose levels as close to the normal range as possible, with the major goal of greatly diminishing the probability that they will develop secondary complications of chronic hyperglycemia.

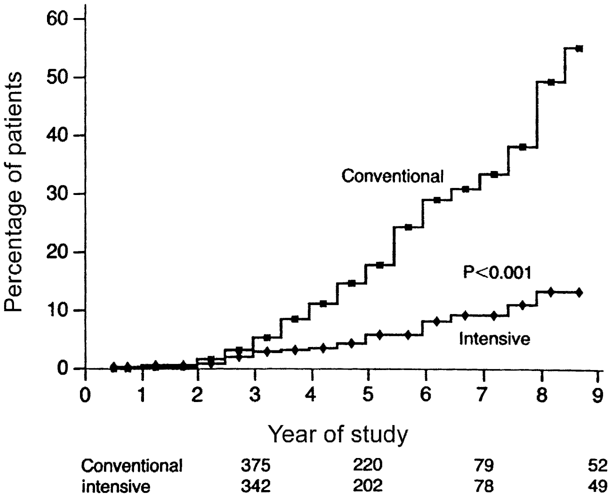

FIGURE 2 Cumulative incidence of a change in retinopathy in patients with type 1 diabetes receiving intensive or conventional treatment in the Diabetes Control and Complications Trial. Reprinted from The Diabetes Control and Complications Trial Research Group (1993), The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus, *N. Engl. J. Med.* **329**, 977–986, with permission.

## VI. TREATMENT

Medical treatment for type 2 diabetes involves a wide spectrum of options involving exercise, dietary changes, and the use of oral hypoglycemic agents and exogenous insulin (Table 6). There is no single ideal diet for diabetic patients. Rather, each individual's diet should be tailored to suit their metabolic

**TABLE 6 Treatment of Type 2 Diabetes Mellitus**

- Diet
- Exercise
- Oral hypoglycemic agents
  - Sulfonylureas
  - Insulin sensitizers (metformin, thiazolidinediones)
- Insulin

needs, considering blood glucose, hemoglobin A1c, lipid levels, blood pressure, weight, and quality of life. Approaches to manage obesity include changes in the caloric content of the diet. In this case, restriction of fat is usually beneficial. If hypercholesterolemia is a problem, reduced cholesterol intake is recommended. The approach to carbohydrate intake is highly complicated and varies with the individual, with attention given to the type and fiber content of any carbohydrate under consideration. Dietary therapy is greatly facilitated by consultation with a nutritionist, who can create an individualized diet for the patient that meets individual metabolic needs and yet is reasonable enough that the patient is not discouraged from following it. All patients with diabetes benefit from exercise because physical exertion facilitates insulin action and the removal of glucose from blood. Oral hyperglycemic agents that can be used include sulfonylureas, biguanides, and thiazolidinediones. Sulfonylureas facilitate insulin release from the pancreatic beta cell when it is stimulated by glucose. Biguanides and thiazolidinediones are insulin sensitizers that facilitate the action of insulin at its major metabolic sites, mainly the liver, fat cells, and muscle. Commonly, combinations of sulfonylureas and insulin sensitizers are used to maintain the hemoglobin A1c level in the desired range. Exogenous insulin can also be used for type 2 diabetic patients in the same manner that it is used for type 1 diabetic patients. Typically, a dose of long-acting insulin given at bedtime will greatly facilitate controlling glucose levels in type 2 diabetic patients. However, these patients may also elect to use a shorter acting insulin prior to eating their evening meal to facilitate reduction of postprandial hyperglycemia. A point of common confusion that should be avoided is that the use of insulin by a type 2 diabetic patient does not mean that the patient has become a type 1 diabetic patient. Rather, it simply means that the type 2 diabetic patient has elected to use insulin to facilitate management of hyperglycemia. The difference between a type 1 and type 2 diabetic patient is that the former requires insulin for survival and oral hyperglycemic agents cannot manage the disease alone, whereas the latter elects to use insulin as an additional help to maintain glucose levels to as close to normal as possible.

## Glossary

**glucose toxicity** Adverse biochemical effects in many tissues within the body resulting from prolonged elevations of glucose.

**hyperglycemia** Elevated blood glucose level.

**insulin** Major hormone released by the beta cells within pancreatic islets.

**oral hyperglycemic agents** Drugs that can be taken by mouth to treat hyperglycemia.

**pancreatic islet** Cluster of cells contained within the pancreas. The human pancreas contains approximately 1 million of these clusters, each of which contains four major cell types that release hormones directly into the bloodstream for distribution to all parts of the body.

**secondary complications** Adverse effects on many structures and organs in the body resulting from chronic diabetes.

## See Also the Following Articles

**Diabetes Type 1 (Insulin-Dependent Diabetes Mellitus) • Glucagon Processing • Glucagon Secretion, Regulation of • Hypoglycemia in Diabetes • Insulin Actions • Insulin Gene Regulation • Insulin Processing • Insulin Secretion • Leptin**

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# Dihydrotestosterone, Active Androgen Metabolites and Related Pathology

GUIDO VERHOEVEN
*Catholic University of Leuven, Belgium*

needs, considering blood glucose, hemoglobin A1c, lipid levels, blood pressure, weight, and quality of life. Approaches to manage obesity include changes in the caloric content of the diet. In this case, restriction of fat is usually beneficial. If hypercholesterolemia is a problem, reduced cholesterol intake is recommended. The approach to carbohydrate intake is highly complicated and varies with the individual, with attention given to the type and fiber content of any carbohydrate under consideration. Dietary therapy is greatly facilitated by consultation with a nutritionist, who can create an individualized diet for the patient that meets individual metabolic needs and yet is reasonable enough that the patient is not discouraged from following it. All patients with diabetes benefit from exercise because physical exertion facilitates insulin action and the removal of glucose from blood. Oral hyperglycemic agents that can be used include sulfonylureas, biguanides, and thiazolidinediones. Sulfonylureas facilitate insulin release from the pancreatic beta cell when it is stimulated by glucose. Biguanides and thiazolidinediones are insulin sensitizers that facilitate the action of insulin at its major metabolic sites, mainly the liver, fat cells, and muscle. Commonly, combinations of sulfonylureas and insulin sensitizers are used to maintain the hemoglobin A1c level in the desired range. Exogenous insulin can also be used for type 2 diabetic patients in the same manner that it is used for type 1 diabetic patients. Typically, a dose of long-acting insulin given at bedtime will greatly facilitate controlling glucose levels in type 2 diabetic patients. However, these patients may also elect to use a shorter acting insulin prior to eating their evening meal to facilitate reduction of postprandial hyperglycemia. A point of common confusion that should be avoided is that the use of insulin by a type 2 diabetic patient does not mean that the patient has become a type 1 diabetic patient. Rather, it simply means that the type 2 diabetic patient has elected to use insulin to facilitate management of hyperglycemia. The difference between a type 1 and type 2 diabetic patient is that the former requires insulin for survival and oral hyperglycemic agents cannot manage the disease alone, whereas the latter elects to use insulin as an additional help to maintain glucose levels to as close to normal as possible.

## Glossary

**glucose toxicity** Adverse biochemical effects in many tissues within the body resulting from prolonged elevations of glucose.

**hyperglycemia** Elevated blood glucose level.

**insulin** Major hormone released by the beta cells within pancreatic islets.

**oral hyperglycemic agents** Drugs that can be taken by mouth to treat hyperglycemia.

**pancreatic islet** Cluster of cells contained within the pancreas. The human pancreas contains approximately 1 million of these clusters, each of which contains four major cell types that release hormones directly into the bloodstream for distribution to all parts of the body.

**secondary complications** Adverse effects on many structures and organs in the body resulting from chronic diabetes.

## See Also the Following Articles

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# Dihydrotestosterone, Active Androgen Metabolites and Related Pathology

Guido Verhoeven
*Catholic University of Leuven, Belgium*

I. INTRODUCTION
II. DHT AND THE CONCEPT OF ACTIVE METABOLITES
III. STEROID 5α-REDUCTASE: TWO GENES/TWO ENZYMES
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V. BENIGN PROSTATIC HYPERPLASIA AND THE DEVELOPMENT OF 5α-REDUCTASE INHIBITORS
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VII. REMAINING QUESTIONS

**Testosterone is the major steroid hormone produced by the testis and the major circulating androgen in men. Many effects of androgens, however, are not mediated by testosterone itself, but rather by so-called "active metabolites." In several androgen target tissues, a steroid 5α-reductase converts testosterone into the more potent androgen 5α-dihydrotestosterone (DHT). This conversion is absolutely required for the virilization of the external genitalia during embryonic development and DHT remains the main androgen acting in the prostate during adulthood as well. Aromatization of testosterone may result in a second active metabolite, 17β-estradiol, which plays an important role in some of the effects of androgens on bone and on the brain. The concept of active metabolites has had important repercussions on our understanding of the physiology and pathology of androgen action.**

## I. INTRODUCTION

This article focuses on 5α-dihydrotestosterone (DHT) (Fig. 1) and the enzymes responsible for its formation. The role of DHT in normal sexual differentiation as well as a form of male pseudo-hermaphroditism caused by the absence of 5α-reductase activity is discussed. Furthermore, the role of DHT in the prostate and in benign prostatic hyperplasia and the therapeutic potential of 5α-reductase inhibitors are examined.

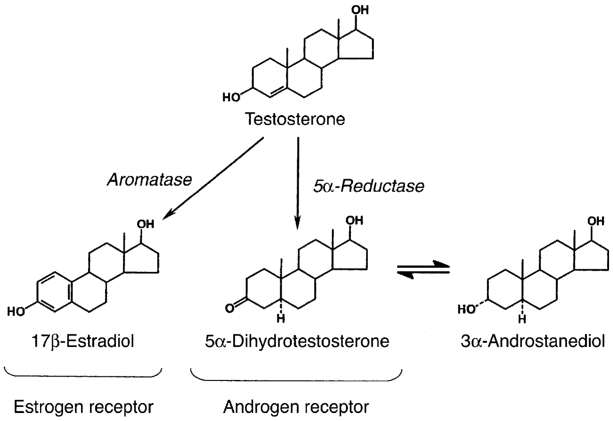

FIGURE 1 Production of active metabolites from testosterone. Five-α-reduction of the main circulating androgen, testosterone, results in the production of 5α-dihydrotestosterone, a more potent androgen that acts via the same receptor as testosterone (the androgen receptor). Aromatization of testosterone produces 17β-estradiol, which acts via the estrogen receptor. Some tissues may use 3α-androstanediol as a source of 5α-dihydrotestosterone.

## II. DHT AND THE CONCEPT OF ACTIVE METABOLITES

In 1968, two independent studies focused attention on DHT as a critical intracellular mediator of androgen action. Bruchovsky and Wilson analyzed the fate of [$^3$H]testosterone in castrated, eviscerated, and functionally hepatectomized rats and came to the surprising conclusion that DHT rather than testosterone was the predominant radiolabeled hormone accumulating in nuclei from the rat ventral prostate. Similar observations were made by Anderson and Liao after incubation of prostate slices with [$^3$H]testosterone. These initial findings were followed by the demonstration that several androgen target tissues actively convert testosterone into DHT and that this conversion is related to a 5α-reductase associated with the cell nucleus. The idea rapidly gained prominence that some and maybe all effects of androgens might require conversion of circulating testosterone into its active metabolite, DHT. This hypothesis received further support from bioassay data showing that—at least in some test systems—DHT is a more potent androgen than testosterone and from studies on the androgen receptor that revealed that this receptor has a higher affinity for DHT than for testosterone.

The full implications of these findings became evident after a detailed analysis of the embryologic development of the 5α-reductase in the genital tract. Androgens are essential for the virilization of the male genital tract during embryogenesis, and accordingly, the question was asked as to whether these effects were mediated by testosterone or whether they required conversion into the active metabolite DHT. The answer to this question proved complex. Comparison of 5α-reductase activity in urogenital tracts of various species including human revealed that, in the anlage of the prostate and external genitalia, the capacity to form DHT was present prior to the onset of virilization and precedes testicular androgen secretion, whereas in the Wolffian duct, which gives rise to the epididymis, vas deferens, and seminal vesicle, the capacity to form DHT was not acquired until after the onset of androgen secretion and after male differentiation. Final proof for the contention that some androgen effects require DHT formation (virilization of the external genitalia) whereas others may be induced by testosterone itself (virilization of the genital ducts) came from a hereditary disorder of sexual differentiation shown to be due to 5α-reductase deficiency. Before discussing this disease, however, it may be good to take a closer look at the 5α-reductase enzyme.

## III. STEROID 5α-REDUCTASE: TWO GENES/TWO ENZYMES

The steroid 5α-reductase was described in the 1950s and 1960s as an enzyme that irreversibly catalyzes the catabolism of various Δ4 steroids into their 5α-reduced metabolites using NADPH as a co-factor. The observation that the enzyme could also contribute to an anabolic reaction, converting testosterone into a more active metabolite, strongly stimulated attempts to purify the protein(s) involved. All efforts to solubilize or purify the protein remained unsuccessful, however, and the impasse was broken only after the introduction of expression cloning techniques. The first cDNA thus isolated encoded a rat liver 5α-reductase. Cross-hybridization screening of a human prostate cDNA library using this rat cDNA resulted in the identification of a human 5α-reductase. Surprisingly, the corresponding enzyme (5α-reductase 1) displayed an alkaline pH optimum (in contrast with the acidic pH optimum observed in prostate tissue and in genital skin fibroblasts). Moreover, it was only weakly inhibited by a 5α-reductase inhibitor (finasteride) known to be active in the above-mentioned androgen target tissues, and no mutations were found in the corresponding gene in patients with 5α-reductase deficiency. These findings suggested the existence of a second 5α-reductase. A new round of expression cloning indeed allowed the isolation of the relevant cDNA. The corresponding enzyme (5α-reductase 2) displayed the expected acidic pH optimum and proved sensitive to finasteride. In addition, the gene encoding this enzyme was mutated in patients with 5α-reductase deficiency. Some of the main characteristics of the type 1 and type 2 5α-reductases and the corresponding genes are summarized in Table 1. The genes encoding both human 5α-reductases show extensive structural homology, suggesting a common ancestor. Both have five exons separated by four intervening sequences. They are localized on different chromosomes and expression varies considerably in different tissues. Type 2 is predominantly expressed in the male urogenital tract and in genital skin, whereas type 1 is expressed in nongenital skin and particularly in the sebaceous glands. Both isozymes are expressed in the human liver. Some polymorphisms that may be useful for inheritance studies in the gene encoding type 2 have been described.

TABLE 1 **Comparison of Human Steroid 5α-Reductase Isozymes**

| | Type 1 | Type 2 |
|---|---|---|
| pH optimum | 6.5–9 | 5.5 |
| Apparent $K_m$ (μM) | 1–5 | 0.1–1 |
| $M_r$ | 29,462 | 28,398 |
| Chromosomal localization | 5p15 | 2p23 |
| Gene | SRD5A1 | SRD5A2 |
| Tissue distribution | | |
| Prostate | − | ++ |
| Genital skin | − | ++ |
| Liver | + | + |
| Nongenital skin | ++ | − |

## IV. STEROID 5α-REDUCTASE TYPE 2 DEFICIENCY

The link between the above-described fundamental observations on androgen action and a candidate disease due to 5α-reductase deficiency was established in 1974 by the study of two families, one in Dallas, Texas, and the other in the Dominican Republic. Both families presented a phenotype compatible with an autosomally recessive form of male pseudo-hermaphroditism previously known as pseudo-vaginal perineoscrotal hypospadias or incomplete male pseudo-hermaphroditism type 2. Patients were 46, XY males presenting at birth with an external female phenotype, bilateral testes, and normally virilized Wolffian structures terminating in the vagina (Fig. 2). In the Dallas family, 5α-reductase deficiency was demonstrated by measurements of the enzyme in slices and cell cultures of genital tissue and skin. In the large pedigree of the Dominican Republic, the diagnosis was made based upon the low levels of 5α-reduced metabolites in plasma and urine.

The clinical phenotype of 5α-reductase deficiency shows some variability. Classically, the external genitalia are not masculinized at birth, and affected

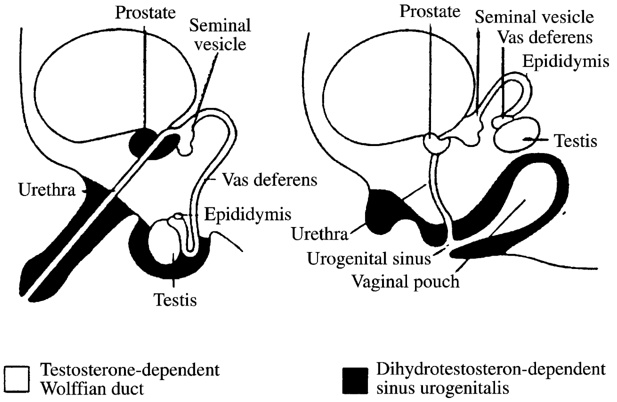

FIGURE 2 External and internal genitalia in the normal male (left) and in a patient with 5α-reductase type 2 deficiency (right). Reprinted with permission from Bartsch *et al.* (2000). Dihydrotestosterone and the concept of 5α-reductase inhibition in human benign prostatic hyperplasia. *Eur. Urol.* 37, 367–380. Copyright Karger AG, Basel.

patients exhibit a blind-ending vagina or a urogenital sinus opening in a single perineal orifice. The phallus is hypospadic, resembling a clitoris, and the scrotum is bifid and labia-like. Most of the patients are therefore described as girls at birth. In some cases, however, the phallus may be so large that affected infants are identified as males with hypospadias. The internal genitalia—vas deferens, epididymis, and seminal vesicles—are fully formed and the Müllerian structures are lost The prostate is absent or severely hypoplastic. The testes are in the inguinal canal, labia, or scrotum.

At puberty, marked virilization takes place. The muscle mass gives the body a masculine shape, the voice deepens, the penis enlarges to 4–8 cm, and the testes enlarge and descend into the labioscrotal folds. There is no gynecomastia. These predictable changes in phenotype have led to descriptions of affected individuals in the Dominican Republic as "guevedoces" or "penis at 12." The prostate remains tiny (approximately 10% of the volume observed in matched controls) and the pattern of body hair is female with terminal hair in the axilla and pubic triangle only. Male pattern baldness has not been described and acne is very rare. In the absence of medical interventions or sociocultural factors interrupting the natural sequence of events, psychosexual orientation is male. Even when unambiguously raised as girls, the vast majority of subjects change to male gender identity and a male gender role during or after puberty. This suggests that testosterone itself is active in the brain or that it acts via another active metabolite (e.g., estrogens) to produce changes in psychosexual behavior.

The main endocrine features of 5α-reductase deficiency are normal male to high levels of plasma testosterone and low levels of DHT. The ratio of testosterone to DHT is elevated in adulthood and after stimulation with human chorionic gonadotropin in childhood. In urine, the ratios of 5β/5α androgen and C21 metabolites are increased. Gonadotropins are at normal or slightly elevated levels.

Early studies suggested that affected patients had a defect in the activity of a 5α-reductase with a pH optimum of approximately 5.5. In some patients, activity was absent, and in others, activity remained measurable but was kinetically abnormal. The cloning of the two 5α-reductase enzymes allowed unequivocal localization of the defect in the 5α-reductase 2 enzyme. This enzyme not only exhibited the expected acidic pH optimum, it also displayed preferential expression in genital tissues and sensitivity to a 5α-reductase inhibitor known to interfere with androgen action in the prostate. Approximately 30 different mutations in the type 2 gene have now been identified and characterized at the clinical, genetic, and biochemical levels. Mutations that affect the affinity for testosterone map to the two ends of the type 2 isozyme, and mutations that affect NADPH binding map throughout the carboxy-terminal half of the enzyme. The described form of male pseudohermaphroditism is now universally referred to as steroid 5α-reductase 2 deficiency.

## V. BENIGN PROSTATIC HYPERPLASIA AND THE DEVELOPMENT OF 5α-REDUCTASE INHIBITORS

There is an overwhelming amount of evidence indicating that DHT is the major androgen acting in the human prostate. As already mentioned, the prostate remains vestigial in the absence of the 5α-reductase 2. Moreover, the level of DHT in the prostate is nearly 10 times higher than the level observed in the circulation, whereas the opposite is found for testosterone. These high intraprostatic levels of DHT are maintained with age, despite decreasing circulating levels of testosterone, and part of the intraprostatic DHT is derived from adrenal androgens. Finally, prolonged administration of DHT or 3α-androstanediol to dogs results in prostatic hyperplasia, an effect that can be markedly enhanced by simultaneous treatment with 17β-estradiol. The predominant 5α-reductase in the prostate is the 5α-reductase 2 isozyme and this enzyme is located mainly in the stroma and in the basal cells, suggesting that DHT produced in the prostate may act as an autocrine agent in the stroma and as a paracrine factor in the lumenal cells.

There is little doubt that androgens and in particular DHT contribute to the pathogenesis and progression of benign prostatic hyperplasia (BPH) but the exact role of androgens in the pathophysiology of the disease remains unclear. BPH is the most common nonmalignant proliferative abnormality observed in any internal organ in men. Autopsy studies reveal that microscopic disease may be present as early as 25–30 years of age. With increasing age, the prevalence of microscopic BPH rapidly increases to reach approximately 80% in the eighth decade. Approximately 35% of men 60 years or older display one or more clinical symptoms of prostatism (hesitancy, straining, weak stream, and intermittency). Androgens play at least a permissive role in the pathogenesis of BPH. BPH does not occur in men castrated before puberty and is extremely rare in men castrated before the age of 40. Moreover, androgen ablation therapy reduces prostatic volume in patients with BPH. Nonetheless, other factors may be involved. In fact, in its initial stages, BPH is not a *diffuse* disease—as would be expected if an endocrine imbalance were the primary pathogenic factor—but a *focal* disease situated in the transition zone and the periurethral region of the prostate gland. Disturbed stromal–epithelial communications and a reawakening of embryonic inductive interactions between these tissue components have been suggested as important pathogenic events.

The reliance of the prostate on androgens and particularly on the active metabolite DHT stimulated the search for specific 5α-reductase inhibitors. A whole series of steroidal and nonsteroidal inhibitors have now been developed but the best studied remains the 4-azasteroid finasteride (17β-(*N*,t,-butyl) carbamoyl-4-aza-5α-androst-1-en-3-one). At low concentrations, finasteride acts as a time-dependent inactivator of the type 2 isozyme. Much higher concentrations are required to also block the type 1 enzyme. Finasteride reduces circulating levels of DHT by approximately 70% and increases testosterone only slightly, suggesting that the feedback of luteinizing hormone (LH) depends directly on testosterone or on other active metabolites such as estradiol. Intraprostatic levels of DHT drop to less than 15% of their original value but intraprostatic testosterone increases some sevenfold. In a U.S. trial, prostatic volume was reduced by 19%, the maximum urinary flow rate improved by 1.6 ml/s, and the total symptom score decreased by 2.6 after 12 months of therapy. Some further improvement was observed after longer treatment periods. Treatment proved more effective in men with large prostates. Complications such as acute urinary retention and BPH-related surgery were decreased by more than 50% after a 4-year treatment period. Very few side effects were noted, with a decrease in libido or impotence reported in approximately 5% of the patients, suggesting again that the latter effects may not be dependent on DHT but on testosterone and/or estradiol.

The remaining levels of circulating DHT in finasteride-treated patients are most likely due to residual activity of the type 1 5α-reductase. In fact, in recent studies, a more effective dual inhibitor of the type 1 and type 2 enzymes (GI 198745) decreased circulating DHT by 98%. Whether this decrease will result in further therapeutic benefit remains to be proven. In fact, it is conceivable that the residual and increasing intraprostatic testosterone levels may limit the therapeutic potential of 5α-reductase inhibitors.

## VI. OTHER APPLICATIONS OF 5α-REDUCTASE INHIBITORS

Prostate cancer is obviously another common disease in which androgens play an important role. Taking into account that even low levels of androgens may be sufficient to permit continued growth of prostate cancer cells, it is unlikely that monotherapy with 5α-reductase inhibitors will have a future in the therapy of this disease. Reduction of prostate-specific antigen secretion has been observed in prostate cancer patients treated with finasteride, however, and combined with the anti-androgen flutamide, finasteride has been shown to be an effective form of maximal androgen blockade. Finasteride is being evaluated in a large primary prostate cancer prevention trial involving more than 18,800 healthy men at low to average risk. This landmark trial underscores the feasibility of chemoprevention but the size of the expected treatment is limited (25% reduction in the period prevalence of prostate cancer over 7 years of treatment), and there remains some concern that this form of prevention may select for androgen-resistant tumors.

Another important androgen target organ is the skin. Since patients with 5α-reductase deficiency have sparse facial and body hair and since they do not develop male pattern baldness, it might be expected that DHT could contribute to conditions such as acne, hirsutism, and baldness. Acne is probably not a good indication. In fact, sebum production is not affected in patients treated with finasteride and is comparable in patients with 5α-reductase deficiency and in normal controls. Finasteride has proven to be effective, however, in the treatment of androgenic alopecia in men. After 2 years of treatment, approximately two-thirds of men show improved scalp coverage and approximately one-third have the same amount of hair as they did at the onset. With prolonged treatment, the clinical impression is that scalp coverage continues to increase. Increased 5α-reductase activity is considered a pathogenic mechanism of idiopathic hirsutism and higher levels of 5α-reductase activity have been reported in dermal papilla cells from follicles derived from beard and balding scalp than in comparable cells derived from nonbalding scalp. Accordingly, hirsutism might be another indication for the use of 5α-reductase inhibitors. Such inhibitors should not be used, however, in women who are or may become pregnant because of the potential interference with the development of the genital tract in male fetuses. Nonetheless, some studies suggest that finasteride is as effective for the treatment of hirsutism as the anti-androgens flutamide and spironolactone. Moreover, the 5α-reductase inhibitor seems to improve the results of contraceptives containing the anti-androgenic compound cyproterone acetate.

## VII. REMAINING QUESTIONS

There remain several intriguing questions with respect to the physiological role of DHT. It remains unclear why some androgen-regulated processes (development of internal genital tract, feedback of LH, and spermatogenesis, etc.) rely on testosterone, whereas others require the active metabolite DHT. Is DHT simply a more active ligand or are there differences in the spectrum of genes activated by the DHT–receptor complex and the spectrum controlled by the testosterone–receptor complex? What is the role of circulating DHT in the response of some target organs (such as muscle) with low 5α-reductase activity? What is the role of the 5α-reductase 1 in health and disease? What is the role of genetic and dietary factors in differences in 5α-reductase activity reported in different populations? It is likely that many of these questions will be answered in the near future.

## Glossary

**hirsutism** Excess facial and body hair in women in a pattern similar to that found in men.

**male pseudo-hermaphroditism** Condition in which the gonads are testes but the genital ducts and/or external genitalia are incompletely masculinized. Most often there is a normal XY karyotype.

**steroid 5α-reductase** NADPH-dependent 3-oxo-5α-steroid Δ4 dehydrogenase (EC 1.3.99.5). Enzyme that irreversibly catalyzes the conversion of various Δ4 steroids into their 5α-reduced metabolites using NADPH as a co-factor.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptors and Prostate Cancer • Androgen Receptor-Related Pathology • Sexual Differentiation, Molecular and Hormone Dependent Events in • Spermatogenesis, Hormonal Control of • Testis Descent, Hormonal Control of**

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# Diuretic Hormones and the Regulation of Water Balance in Insects

JEFFREY H. SPRING* AND
S. RENEE HAZELTON-ROBICHAUX†
*University of Louisiana, Lafayette ● †Louisiana State University, Eunice

I. EXCRETORY ORGANS
II. SOURCE OF HORMONES
III. STRUCTURE
IV. TARGETS AND TRANSDUCERS
V. SUMMARY OF EFFECTS

**Because of their small size, insects have a most unfavorable surface area-to-volume ratio, which makes them susceptible to rapid desiccation. They compensate for this by having a waxy, waterproof surface coating to limit evaporative water losses and by carefully regulating their internal water balance. There are a number of neuropeptides that stimulate fluid secretion (diuretic peptides; DPs) and fluid reabsorption (antidiuretic peptides; ADPs). Although there is some overlap, the DPs and the ADPs generally target different regions of the excretory system.**

## I. EXCRETORY ORGANS

Insects have an open circulatory system and the blind-ended Malpighian tubules extend throughout most areas of the hemocoel, or blood space. The tubules are a monolayer epithelium and are normally regionally segmented with up to four discrete segments per tubule. Most insects appear to have a single cell type within each region; however, the Diptera have two cell types, primary and stellate, which are quite different in both structure and function. Blood pressure is very low, precluding filtration for urine formation, and so the primary urine is generated by secretion. Vacuolar-type proton pumps (H-ATPases, also known as V-ATPases) located on the microvilli establish a pH gradient across the luminal membrane. Antiporters on the same membrane exchange $H^+$ for $K^+$ or $Na^+$, generating the observed cation gradient into the lumen. Counterions, primarily $Cl^-$ or to a lesser extent $HCO_3^-$, follow passively. A very slight osmotic gradient ($\leq 1\%$) causes water and other hemolymph components to flow into the tubule lumen, often very rapidly. In most insects, this primary urine is rich in KCl and would rapidly alter the ionic composition of the hemolymph were it not for the action of the hindgut (ileum/rectum), where salts and water are reabsorbed. In most insects, there are four to six rectal pads with columnar transporting cells surrounded by layers of muscle and connective tissue. The hindgut is lined with cuticle, which provides mechanical and chemical protection from the gut contents. Here, the actively transported ion is $Cl^-$, with $Na^+$ and $K^+$ transported in various quantities as counterions. Both the Malpighian tubules and the rectum have specialized transport proteins to eliminate or recover specific organic substances (Fig. 1).

## II. SOURCE OF HORMONES

The primary source of diuretic peptides (DPs) and antidiuretic peptides (ADPs) is a complex containing the median neurosecretory cells/corpora cardiacum (MNC/CC). The MNC are located near the anterior dorsal surface of the brain, in the central region known as the pars intercerebralis. In those insects where the lobes are separate and not fused, the MNC axons terminate in the storage lobes of the CC, much like the vertebrate hypothalamus/posterior pituitary. The glandular lobes of the CC are analogous to the anterior pituitary and contain neurosecretory cells that synthesize and secrete their own intrinsic hormones (Fig. 2). Some DPs

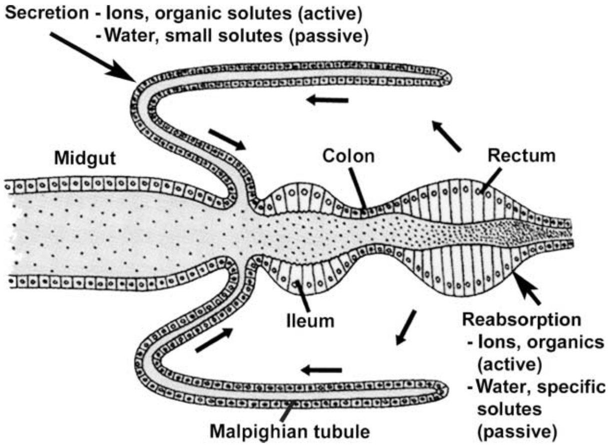
Secretion - Ions, organic solutes (active)
- Water, small solutes (passive)
Midgut
Colon
Rectum
Ileum
Reabsorption
- Ions, organics (active)
- Water, specific solutes (passive)
Malpighian tubule

FIGURE 1 Diagram of the general structure of the insect excretory system, showing the anatomical relationships between the Malpighian tubules and the regions of the gut and the functions of each region.

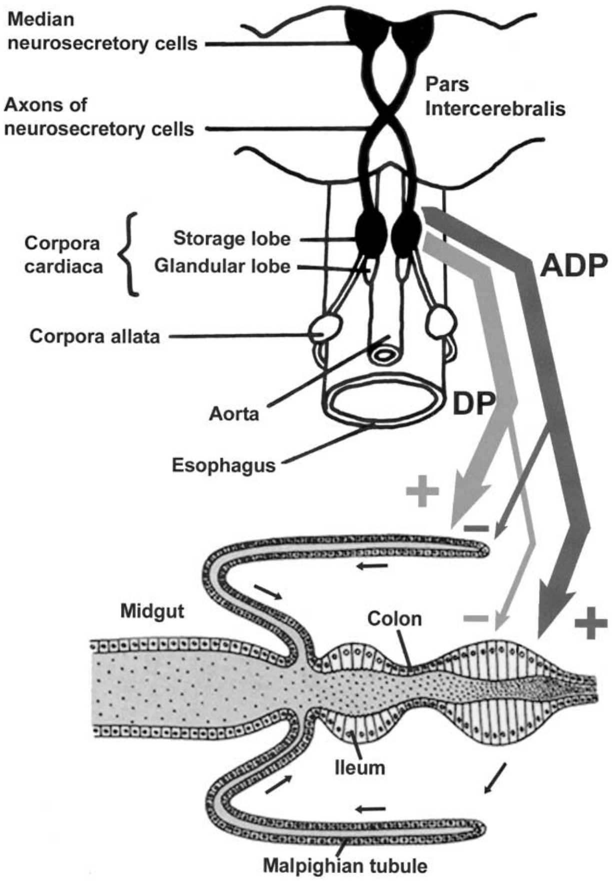

FIGURE 2 Diagram of the typical arrangement of the neurohemal organs (MNC/CC) and the primary and secondary targets of DPs and ADPs released from the CC. For further explanation, see text.

have been isolated from the ventral ganglia, and immunolocalization studies suggest that each ganglion contains a handful of neurosecretory cells that contain DPs and are presumed to act locally, affecting individual Malpighian tubules or segments of the ileum and rectum.

## III. STRUCTURE

In general, most of the DPs fall into two groups: those with varying degrees of homology to the vertebrate corticotropin-releasing factor/urotensin/sauvagine family of peptides and the smaller kinins. There are, of course, exceptions. For example, a DP related to the arginine vasopressinpeptide family has been isolated from the subesophageal and thoracic ganglia of *Locusta migratoria*. The cardioacceleratory peptide, $CAP_{2b}$, apparently acts as a DP in *Drosophila melanogaster*, and the biogenic amine, serotonin (5-hydroxytryptamine), in addition to its known pharmacological effects on tubule secretion, may act as a true diuretic factor in the blood-sucking bug, *Rhodnius prolixus*.

There are approximately 10 known corticotropin-releasing factor-like diuretic peptides (CRF-DPs) (some have only been partially sequenced) found in several orders of insects. They range in size from 30 to 46 amino acids, and sequence homology is relatively low, approximately 50% in most cases, although it has been suggested that nucleotide homology may be much higher, perhaps 70–80%. In all cases, gaps are required in the sequences to show maximal homology with the other members of the family. With a single exception, only one CRF-DP per species has been noted.

Members of the second major family of diuretic peptides were initially isolated on the basis of their myotropic activity on the hindgut of *Leucophaea maderae* and were named the insect kinins. There are currently two dozen known kinins, and there are usually several different kinins (five to eight) within a single species. These peptides are small, 6–13 residues, and have a highly conserved C-terminal pentapeptide sequence.

Serotonin, of course, is a single amino acid derivative, and $CAP_{2b}$ is an octapeptide. The situation with ADPs is much less clear-cut. The neuroparsins, a family of three larger neuropeptides (78, 81, and 83 residues), isolated from *L. migratoria* stimulate water reabsorption by the rectum. Two peptides isolated from *Schistocerca gregaria*, ion-transport peptide (72 residues) and chloride-transport stimulating hormone (structure unknown), act on the hindgut to stimulate ion and water reabsorption. Antidiuretic peptides (structure unknown) isolated from *Acheta domesticus* and *Formica polyctena* act to inhibit secretion by the Malpighian tubules.

## IV. TARGETS AND TRANSDUCERS

All of the known DPs and ADPs are peptides and so require a second-messenger system to exert their effects at the cellular level. The mode of action of the CRF-DPs appears to be relatively uniform across species, and there are varying degrees of interspecific bioactivity. In most cases, interspecific activity is relatively low, so, for example, even at high doses, Mas-DP1 (the primary CRF-DP from *Manduca sexta*) produces only 60% of the maximal response in *A. domesticus* Malpighian tubules, as might be expected given the relatively low sequence homology between peptides. The CRF-DPs activate an adenylate cyclase in the primary cells of the Malpighian tubules, elevating cAMP levels in these cells. The action of cAMP in the primary cells is to increase $Na^+$ conductance across the basolateral membrane, presenting the apical $Na^+/H^+$ antiporter with a higher concentration of substrate, thereby increasing cation transport, with water and other solutes following passively. In the tsetse fly, cAMP appears transiently to increase Malpighian tubule cell volume by increasing basolateral permeability to both $Na^+$ and $Cl^-$, with cell volume returning to normal as the transcellular movement of fluid increases. There is also electrophysiological evidence that in *D. melanogaster*, cAMP can act to stimulate the H-ATPase directly.

The effect of the kinins on Malpighian tubule secretion is both independent of and additive to the action of the CRF-DPs. In those systems where it has been studied, the kinins appear to act by increasing $[Ca^{2+}]_i$, which in turn increases the anion permeability of the tubules. It appears that by increasing the permeability of the tubules to anions, the driving force for cation transport is reduced, enhancing overall fluid transport. In the Diptera, a low-resistance $Cl^-$ pathway transforms the Malpighian tubule from an electrically "tight" to a "leaky" epithelium. The change in $Cl^-$ conductivity does not occur within the primary cells and has been shown to occur in the stellate cells characteristic of the Diptera. In *D. melanogaster* the kinins also stimulate fluid transport, mediated by $[Ca^{2+}]_i$, and activate a similar shunt pathway. In other insects, the kinins also open a low-resistance $Cl^-$ pathway, although the tubules appear to be composed of a single cell type. It has been believed that the two systems (CRF-DPs and kinins) are independently regulated; however, recent evidence suggests that in mosquitoes there is a dose-dependent interaction, in which low concentrations of CRF-DP activate the shunt pathway, whereas higher concentrations activate both the shunt and the cAMP-mediated response. This is consistent with the observation that the 8-bromo analogue of cAMP activates both pathways in *D. melanogaster*.

The biogenic amine, serotonin, has long been known to be a potent *in vitro* stimulant of Malpighian tubule secretion, particularly in the blood-sucking bug, *R. prolixus*. Initially considered to be a pharmacological agent, recent evidence suggests that serotonin acts as a second DP in *R. prolixus*, promoting diuresis by elevating intracellular levels of cAMP in the Malpighian tubules. Serotonin acts synergistically with the presumptive CRF-DP found in the mesothoracic ganglionic mass, and blocking the release of serotonin with the neurotoxin 5, 7-dihydroxytryptamine delays or completely blocks the normal postprandial diuresis of fifth-instar *R. prolixus*.

## V. SUMMARY OF EFFECTS

Most bioassays for diuretic activity involve an *in vitro* preparation of the Malpighian tubules or hindgut, which may or may not reflect actual diuretic activity in the whole animal. The Malpighian tubule assays are further complicated by the fact that many insects have tubules with as many as four segments, some of which are secretory and some of which are absorptive. For the sake of simplicity, in this article, we considered any factor that increases tubule secretion, inhibits hindgut reabsorption, or increases whole-animal water loss to be a DP and any factor that inhibits tubule function, promotes hindgut reabsorption, or inhibits whole animal water loss to be an ADP.

The effect of DPs on the Malpighian tubules is dramatic. In blood-feeding insects, such as *R. prolixus*, endocrine stimulation can increase the rate of fluid secretion more than 1000-fold and all insects show at least a doubling of the rate of primary urine formation. All insect species studied thus far, even the phytophagous ones whose diet is rich in $K^+$, show a shift in the $Na^+/K^+$ ratios, so that the urine becomes relatively more $Na^+$ rich.

Even with sophisticated chromatographic and immunological methods, detecting these peptides in the hemolymph is problematic. A few studies have linked increased levels of DP in the hemolymph with feeding, but there appear to be relatively high levels of DP in the hemolymph of normally hydrated animals. It has been suggested that many diuretics may in fact simply act to increase the filtration rate of the hemolymph to rid the animal of unwanted solutes. As long as increased secretion by the Malpighian tubules is matched by increased reabsorption by the rectum, there will be no net effect on water balance, but unwanted or toxic solutes will be more rapidly removed from the hemocoel. Thus, many of the DPs may better be termed "clearance factors" than diuretics.

Water balance in insects is crucial to their ability to exploit marginal habitats. There appear to be a suite of neuropeptides that regulate ionic balance, osmotic balance, hemolymph clearance, and the overall water budget of the animal. A great many aspects of this regulation, particularly the interactions among the DPs and ADPs, remain to be explored.

## Glossary

**corpora cardiaca (CC)** The paired CC form part of the wall of the dorsal aorta and are analogous to the pituitary, having both intrinsic secretory cells and axon endings from the median neurosecretory cells.

**corticotropin-releasing factor-like diuretic peptides (CRF-DP)** The primary diuretic factors are the CRF-DP, which show a strong structural homology to the vertebrate corticotropin-releasing factor.

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**median neurosecretory cells (MNC)** Functionally analogous to the vertebrate hypothalamus, the MNC have axon endings in the corpora cardiaca.

**rectum** Lined with cuticle, the ileum and rectum are the primary sites for water and salt reabsorption.

## See Also the Following Articles

***Drosophila* Neuropeptides • Insect Endocrine System • Insect Gut as an Endocrine Organ**

## Further Reading

Audsley, N., Goldsworthy, G. J., and Coast, G. M. (1997). Circulating levels of *Locusta* diuretic hormone: The effect of feeding. *Peptides* **18**, 59–65.

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# *Drosophila* Neuropeptides

Paul H. Taghert
*Washington University Medical School, St. Louis*

I. INTRODUCTION
II. THE *DROSOPHILA* GENOME
III. BEHAVIORAL NEUROPEPTIDES
IV. DEVELOPMENTAL NEUROPEPTIDES
V. HOMEOSTATIC NEUROPEPTIDES
VI. NEUROPEPTIDE BIOSYNTHESIS
VII. NEUROPEPTIDE RECEPTORS
VIII. SUMMARY

**In this article, *Drosophila* neuropeptide genes are divided into three categories: behavioral, developmental, and homeostatic. These categories are neither comprehensive nor mutually exclusive; they simply offer a template by which to provide an overview of the identified fly neuropeptide genes, to illustrate some methods of analysis common in *Drosophila* studies of neuropeptide signaling, and to convey some lessons learned. In addition, this article summarizes information concerning *Drosophila* genes that encode neuropeptide biosynthetic enzymes. This field is very small compared to parallel studies in vertebrates. However, given the strengths of *Drosophila* genetics, it has great potential regarding the manipulation and elucidation of neuropeptide functions *in vivo*. Finally, information concerning sequences encoding known or potential neuropeptide receptors is categorized according to their relatedness to vertebrate orthologues.**

## I. INTRODUCTION

Studies of insect neuropeptides have been particularly informative for two technical reasons: a high degree of cellular resolution and the power of genetic analysis. Concerning cellular resolution, in *Drosophila* and in other insects, such studies can be pursued at the level of single identifiable peptidergic neurons and single

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Studies of insect neuropeptides have been particularly informative for two technical reasons: a high degree of cellular resolution and the power of genetic analysis. Concerning cellular resolution, in *Drosophila* and in other insects, such studies can be pursued at the level of single identifiable peptidergic neurons and single identifiable target cells. This simplicity greatly facilitates the analysis and interpretation of peptidergic signaling. Likewise, fly genetics presents an ever-increasing array of sophisticated tools by which to examine specific gene function regardless of the complexity of the gene's expression pattern or the pleiotropic effects that the gene may have. For each category, examples are provided to illustrate the type of progress that has been achieved. As befits a complex and timely subject, several other, contemporaneous articles have recently covered, in part or in whole, similar information.

## II. THE *DROSOPHILA* GENOME

### A. Numbers of Genes

In the earliest annotations, the number of genes in the fly genome was estimated to approach 14,000. More recent estimates that include results from subsequent expressed sequence tag analyses have suggested that the final number may be higher, perhaps by a few thousand genes. The projected upward revision stems primarily from two technical issues. First, the genome sequence is largely, but not completely, finished. Second, the current estimate derives in large part from prediction programs that are not 100% accurate; these programs are still being optimized, by comparing results to those derived from other methods. In the near future, completion of genomic sequences from a related *Drosophila* species (work that is on-going and that will be very beneficial for several reasons) will help provide more precise estimates of the total gene number.

### B. Identifying Neuropeptide Genes

Recent estimates place the number of neuropeptide-encoding genes in *Drosophila* at approximately 20; in this article, 32 such genes are described. That number is likely to be an underestimate. It is based largely on the identification of sequences related to those defined by functional studies in other insects. In some cases, the endogenous peptides have been purified directly from *Drosophila*, but this is the case for only a minority of predicted peptides. In general, peptide-encoding genes have certain features by which the majority can be recognized (including a signal sequence, specific motifs that promote posttranslational processing, and a repetitive structure). However, not all neuropeptide genes share all these features; additionally, certain nonneuropeptide genes include one or more such features and yet have different functions. The final number (if higher) is likely to be no more than twice the current estimate (i.e., a total of ~40); that prediction is based on the parallel estimates of the number of peptide receptor genes (~44; see below). However, the reader will recognize this as a circular argument and should remain skeptical until more direct observations are recorded.

### C. Identifying Neuropeptide Biosynthetic Enzyme Genes

Fifteen genes encoding neuropeptide biosynthetic enzymes are reviewed here, based primarily on their sequence relatedness to known genes in vertebrates. For some of these genes, there is useful evidence based on direct observations of their expression patterns or of the enzymatic activities of the fly enzymes. For a single gene in this group, *PHM*, there is *in vivo* genetic evidence consistent with the hypothesis that it is required for normal neuropeptide biosynthesis. Hence, much work remains to be done to capitalize on the completed genomic sequences to identify and characterize all relevant neuropeptide biosynthetic enzyme genes in flies.

### D. Identifying Neuropeptide Receptor Genes

Unlike neuropeptide-encoding genes, gene sequences for neuropeptide receptors can be more easily identified because most peptide receptors are seven-transmembrane, G-protein-coupled receptors (GPCRs). This large class of receptor proteins has well-defined and highly conserved structural features that make it possible to search among novel genomic sequences and efficiently identify most or all such genes. Current estimates for the peptide GPCR complement in *Drosophila* stand at 44, and these genes are briefly described in this article.

## III. BEHAVIORAL NEUROPEPTIDES

In insects, neuropeptides play important roles in the modulation and triggering of specific behaviors or switching between behavioral states. At least 15 *Drosophila* neuropeptide genes can be grouped in the behavioral category based on behavioral observations and based on *in vitro* and *in vivo* muscle responses (Table 1). For example, many members of the "RFamide" neuropeptide family produce somatic,

TABLE I *Drosophila* Behavioral Neuropeptide Genes

| Gene | Neuropeptide | CG No. | Location | Peptide(s) purified? | Alleles? |
|---|---|---|---|---|---|
| *eh* | Eclosion hormone | CG6400 | 90B1 | No | No |
| *eth* | Ecdysis-triggering hormone | CG18105 | 60D15 | No | No |
| *ccap* | Crustacean cardioactive peptide | CG4910 | 94C4 | No | No |
| *pdf* | Pigment-dispersing factor | CG6496 | 97B2 | No | Yes |
| *fmrfa* | dFMRFamide | CG2346 | 46C5 | Yes, some | No |
| *dms* | Dromyosuppressin | CG6440 | 95F6–9 | Yes | No |
| *dsk* | Drosulfakinin | CG18090 | 82E1 | Yes | No |
| *npf* | Neuropeptide F-like | CG10342 | 89D5–6 | Yes | No |
| *sNPF* | Head peptides | CG13968 | 38B4 | Yes[a], one | No |
| *hug* | Pyrokinin gene 2 | CG6371 | 87C1 | No | No |
| *IFamide* | IFamide | CG4681 | 60D5–7 | Yes[a] | No |
| *tk* | Tachykinins | CG14734 | 87A8–9 | No | No |
| — | Proctolin (putative) | CG7105 | 28D3–4 | No | No |
| *amn* | Amnesiac | CG11937 | 19A1 | No | Yes |
| *crz* | Corazonin | CG3302 | 87B7–8 | No | No |

*Note*. The involvement of these pro-hormones in behavioral regulation has been directly implicated or inferred on the basis of *in vitro* bioassays.

[a] Unpublished data (J. A. Veenstra, personal communication).

visceral, and/or cardiac muscle responses, and in flies, they derive from each of several genes (*dFMRFa*, *dms*, *dsk*, *npf*, and *sNPF*). Also, the tachykinin-like peptides, a proctolin-like peptide, certain *hugin*-derived peptides, and IFamide all affect (or are likely to affect) hindgut or oviduct motility, among other biological activities. Finally, corazonin is cardioactive, CCAP regulates a central motor pattern generator, and peptides predicted to be derived from the *amnesiac* gene product affect several central nervous system functions, including the consolidation of long-term memory. Three additional, behaviorally active neuropeptides are then described in more detail: ecdysis-triggering hormone and eclosion hormone, which together control ecydsis behavior, and pigment-dispersing factor, which controls daily circadian locomotion.

## A. Ecdysial Behavior: Ecdysis-Triggering Hormone and Eclosion Hormone

Two distinct peptides contribute to the regulation of insect ecdysis/eclosion behavior and positively and directly regulate each other's release. One peptide is called the ecdysis-triggering hormone (ETH), and in *Drosophila*, it is produced by as many as 18 identified peripheral endocrine cells that release ETH in concert, rapidly, and in large amounts. A small deficiency of the ETH gene region produces larval lethality consistent with a critical role for ETH in coordinating ecdyses. The other peptide is called the eclosion hormone (EH), and in *Drosophila*, it is produced by only two identified neuroendocrine neurons of the brain. Genetic ablation of the EH-producing neurons has indicated the precise contribution of EH to the regulation of this complex behavior: A majority of animals display clear deficits in the temporal organization of adult eclosion, but a significant percentage of animals still succeed in completing the escape behavior. Thus, sophisticated genetic tools and the two very small cellular complements in *Drosophila* have made it possible to efficiently define the times of ETH and EH cellular activity, define the nature of their cross-regulatory activities, and begin to define the precise contributions of each peptidergic system to the control of movements that together generate the elaborate ecdysial behavior.

## B. Circadian Locomotion: Pigment-Dispersing Factor

The neuropeptide pigment-dispersing factor (PDF) is the primary output of the circadian clock system; it does not appear to trigger behavior as much as shape its temporal organization. PDF is a *Drosophila* peptide as predicted by a gene encoding its pro-hormone (CG6496 at 97B2). Neuropeptides related to PDF were first studied in crustacea based on their hormonal activity in a pigment dispersion assay:

α- and β-PDHs (pigment-dispersing hormones) contribute to the dispersion and diurnal movements of pigment granules in the eye and in epithelial chromatophores. In larger insects, β-PDH has pharmacological properties consistent with a circadian role, and in *Drosophila*, a subset of the very limited number of β-PDH-positive neurons in the brain are also positive for *period* gene expression. The last two observations suggest that PDF-related peptides might be candidate transmitters for circadian pacemaker neurons.

*pdf* mutant animals (apparent nulls) are viable and can entrain to light:dark cycles with rhythmic daily locomotion, but the majority of flies are unable to sustain such rhythms under prolonged, constant conditions. The PDF neurons survive in the peptide mutant background, and the behavioral deficits can be largely attributed to lack of *pdf* expression. This phenotype is closely mimicked by selective ablation of PDF neurons following their expression of the *hid* and *reaper* cell death genes (this matches the experimental design by which EH neurons were studied; see above). Misexpression of the *pdf* prohormone in several ectopic locations within the brain causes disruption of circadian locomotor behavior. These and other data support the hypothesis that *pdf* encodes the principal circadian transmitter in *Drosophila*. No PDF-like peptide has been identified in vertebrate species to date. However, given the relatedness of sequences that encode the majority of peptide receptor genes in vertebrates and *Drosophila* (see below), PDF signaling may nevertheless be phylogenetically conserved. Identification of ligands for the orphan peptide GPCRs in *Drosophila* will permit a better interpretation of which hormonal signaling pathways have been conserved and which are phylogenetically specific.

## IV. DEVELOPMENTAL NEUROPEPTIDES

The field of neuroendocrinology began nearly 100 years ago when Kopec first demonstrated a hormonal activity present in the silkmoth brain. Since that time, studies of insect endocrinology have focused on the numerous factors that regulate the precise embryonic and postembryonic developmental physiology of these animals. Numerous neuropeptides have been implicated in both positive and negative control of the steroid and steroid-like factors that trigger and modulate the molting cycle. In addition, other peptide factors regulate the timing of senescent states (diapause) and the maturation of the reproductive systems in adult insects. In the *Drosophila* genome, there is evidence for at least 11 genes encoding developmental neuropeptides (Table 2). A recently defined set of developmental neuropeptides in the insects are related to vertebrate insulins. This signaling system was initially studied in several insect species, not just *Drosophila*. However, sequencing of the *Drosophila* genome and renewed interest in fly insulins have brought a spate of new information in the past 2 years.

### A. Example: Insulin

In *Drosophila*, elements of the entire insulin signaling pathway—from receptor to phosphorylation targets—have been functionally conserved. A *Drosophila* gene encoding an insulin receptor, *InR* (CG18402 at 93E9), was identified in 1985 and subsequently shown to be essential for normal growth. Further conserved

TABLE 2 *Drosophila* Development Neuropeptide Genes

| Gene | Neuropeptide | CG No. | Location | Peptide purified? | Alleles? |
|---|---|---|---|---|---|
| *Dilp-1* | Insulin-like peptide 1 | CG14173 | 67C1–2 | No | No |
| *Dilp-2* | Insulin-like peptide 2 | CG8167 | 67C1–2 | No | No |
| *Dilp-3* | Insulin-like peptide 3 | CG14167 | 67C1–2 | No | No |
| *Dilp-4* | Insulin-like peptide 4 | CG6736 | 67C1–2 | No | No |
| *Dilp-5* | Insulin-like peptide 5 | AE003550.1 | 67C1–2 | No | No |
| *Dilp-6* | Insulin-like peptide 6 | CG14049 | 2F4 | No | No |
| *Dilp-7* | Insulin-like peptide 7 | CG13317 | 3F1–2 | No | No |
| — | PTTH (putative) | CG13687 | 21C6 | No | No |
| *Ast* | Allatostatin A/YXFGLamides | CG13633 | 96A22 | No | No |
| *MIP* | Allatostatin B/W(X6)Wamides | CG6456 | 74B1 | No | No |
| *Ast2* | Allatostatin C | CG14919 | 32D2-3 | No | No |

*Note*. Neuropeptide genes that contribute primarily (though not exclusively) to developmental physiology.

elements include *chico* (a *Drosophila* homologue of the vertebrate insulin receptor substrates), phosphatidylinositol 3′ kinsase and its downstream target (the Akt serine/threonine kinase), the PTEN tumor suppressor, and S6 kinase. Mutants in most of the *Drosophila* homologues display predictable effects on body size, cell size, and cell number. Overexpression of *Dinr* in eye precursor cells leads to an increase in the number of ommatidia and in ommatidial cell size. Current models suggest that, in *Drosophila*, insulin signals through the mitogen-activated protein kinase to induce cell division and through phosphatidylinositol 3′kinase to stimulate growth.

Remarkably, there appear to be seven different *Drosophila* insulin genes encoding seven different *Drosophila* insulin-like peptides. Five of the *Drosophila* insulin genes—*dilp1–4* (CG14173, CG8167, CG14167, and CG6736, all at 67C1–2) and *dilp-5* (AE003550.1: 259,432–259,854)—form a gene cluster, whereas *dilp-6* (CG14049 at 2F4) and *dilp-7* (CG13317 at 3F1–2) are found on a different chromosome. Their sequence similarity to insulin is limited: *dilp-2* is most closely related to human insulin, with 35% identity. Overexpression of *dilp-2* also leads to increases in cell size and numbers, and these effects are dependent on *dinr*.

Evidence to date suggests that many of the *Drosophila* insulin genes are normally expressed at different times and places, suggesting some functional diversity. The occurrence of multiple insulin-encoding genes in insects has precedent in the silkworm, *Bombyx mori*, in which more than 30 insulin-like (bombyxin) genes have been identified. There is as yet little understanding of how such molecular diversity translates into (potential) functional diversity. Some hints come from parallel studies in the nematode *Caenorhabditis elegans*. In the past few years, rapid progress has been made in the analysis of insulin signaling in the worm, and recent work indicates the possibility that, under conditions of overexpression, some insulin-like peptides can act as functional antagonists. *C. elegans* insulin regulates metabolism, development, and longevity by mechanisms that are similar to the endocrine regulation of metabolism and fertility by mammalian insulin. In addition, the genetic analysis in the worm has extended our understanding of insulin signaling by revealing novel molecular features that may be variant in diabetic pedigrees. It is clear that continued studies of insulin signaling in developmental physiology, in both flies and worms, will continue to contribute useful information that may inform all branches of work on insulin signaling.

## V. HOMEOSTATIC NEUROPEPTIDES

Several regulatory neuropeptide systems monitor the internal state of the insect to maintain electrolyte balance and energy reserves in the face of changing internal or environmental conditions. There are six *Drosophila* neuropeptide genes whose functions are thought primarily to involve such homeostatic functions (Table 3). At present, few to no physiological or genetic analyses of these genes have been reported. Most of what is thus far known reflects information from other insects or some initial expression data for RNAs and proteins. Below is offered a brief description of putative *Drosophila* hormones that are involved in water balance. The probable complexity argues for the use of *Drosophila* genetics to define redundant and compensatory roles of the various diuretic and anti-diuretic peptides. Such information will be a useful complement to the large amount of physiological data already accumulated.

### A. Water Balance and Diuretic Hormones

Water and ion metabolism in insects is regulated in large part by the Malpighian tubules and the hindgut. Insect neuropeptides, called diuretic or anti-diuretic

TABLE 3 *Drosophila* Homeostatic Neuropeptide Genes

| Gene | Neuropeptide | CG No. | Location | Peptides purified? | Alleles? |
|---|---|---|---|---|---|
| *Akh* | Adipokinetic hormone | CG1171 | 64A10 | Yes | No |
| *Dh* | CRF-like diuretic hormone | CG8348 | 85E4-5 | No | No |
| *Dh31* | Calcitonin-like diuretic hormone | CG13094 | 29D1 | No | No |
| *leukokinin* | Leukokinin | CG13480 | 70E3-4 | Yes | No |
| *capa* | Pyrokinin gene 1 | CG15520 | 98C8-D1 | No | No |
| *ITP* | Intestinal transport peptide-like | CG13586 | 60D5 | No | No |

hormones, stimulate fluid secretion by the Malpighian tubules and fluid reabsorption by the tubules or hindgut. A large number of insect neuropeptides are known to increase the rate of fluid secretion by the Malpighian tubules. They include the products of several distinct neuropeptide genes, including one related to corticotrophin-releasing factor (CRF *Dh*, CG8348 at 85E2) and another related to calcitonin (*Dh31*, CG13094 at 29D1). Two other neuropeptides also display diuretic effects—leukokinin and $Cap_{2b}$. In *Drosophila*, orthologous sequences are found in the genes *leukokinin* (CG13480 at 70E3–4) and *capa* (CG15520 at 99D1).

Malpighian tubules contain two main cell types, principal cells and stellate cells. Both cAMP and cGMP stimulate a vacuolar $H^+$-motive ATPase present in the apical membrane of the principal cells. Fluid secretion is achieved by the passive flow of water, following a net transport of $K^+$ in the principal cells and $Cl^-$ flow in the stellate cells. The calcitonin-like Dh31 stimulates intracellular cAMP and increases transepithelial voltage in the Malpighian tubules, indicating that it acts on the principal cells. Although CRF-like Dhs have not yet been tested in *Drosophila*, studies in larger insects suggest that Dh also acts on the principal cells. Like Dh and Dh31, the CAPA-1 and CAPA-2 peptides (predicted to derive from the *capa* gene) appear to act on the principal cells, and these promote actions via calcium, nitric oxide, and cGMP. Notably, CAPA-related peptides have anti-diuretic actions in at least one other insect. Finally, leukokinin-like peptides act on the stellate cells to increase $Cl^-$ conductance via an increase in intracellular calcium.

Of all the putative diuretic hormones, leukokinin appears 10 times more potent than the others in producing a significant increase in the rate of fluid secretion. Interestingly, synergistic effects have been described for leukokinin and the calcitonin-like diuretic hormone, as well as for Dh and leukokinin in locusts. Two other hormones have anti-diuretic effects in insects—neuroparsin and intestinal transport peptide (ITP); these stimulate fluid reabsorption by the hindgut. Neuroparsin-related sequences have not yet been identified in the *Drosophila* genome, but *Drosophila* prepro-ITP is encoded by CG13586 (at 60D5). Thus, the multiplicity of peptides, their alternative diuretic versus anti-diuretic actions, and their potential synergy of action all make for complex physiological processing. Clearly gain- and loss-of-function genetic analyses in *Drosophila* can help resolve important questions regarding the precise roles, mechanisms, and sites of action of peptides affecting homeostatic functions.

## VI. NEUROPEPTIDE BIOSYNTHESIS

Secretory peptides are derived from pro-hormone precursors that undergo endoproteolytic cleavage and further processing to generate one or more final, bioactive products. While the basic outline of secretory peptide biosynthesis has advanced from biochemical and molecular studies, genetics has contributed to the understanding of these events *in vivo*. Such work has provided evidence for functional roles played by specific endoproteases and peptidases and has also suggested novel, alternative processing mechanisms. Further genetic analysis in a model system like *Drosophila* could help overcome difficulties that arise from studying limited amounts of critical biosynthetic components. The *Drosophila* genome includes 15 genes that appear to play roles in neuropeptide biosynthesis (Table 4).

### A. Proteolytic Cleavage

Neural and endocrine peptide pro-hormones are cleaved by endoproteolytic enzymes that recognize definable *cis* amino acid motifs. In mammals, the best studied are the pro-hormone convertases (PCs), which are members of the subtilisin-like, serine

**TABLE 4** ***Drosophila* Genes That Contribute (or That May Contribute) to Neuropeptide Biosynthesis**

| Gene | Activity | CG No. | Location | Alleles |
|---|---|---|---|---|
| *dfurin1* | Cleavage | CG10772 | 96D1 | No |
| *dfurin2* | Cleavage | CG18734 | 14C1 | No |
| *dPC2 (amontillado)* | Cleavage | CG6438 | 97C3 | No |
| *ACER* | Cleavage | CG10593 | 29D1 | Yes |
| *ANCE* | Cleavage | CG10593 | 34D7 | Yes |
| *ANCE2* | Cleavage | CG16869 | 34D7 | No |
| *ANCE3* | Cleavage | CG17988 | 34D8 | No |
| *ANCE4* | Cleavage | CG8196 | 45A6 | No |
| *ANCE5* | Cleavage | CG10142 | 60E3 | No |
| *Prolyl endoprotease* | Cleavage | CG5355 | 31E | No |
| *silver* | Cleavage | CG4122 | 1B3 | Yes |
| *silver-related* | Cleavage | CG4678 | 15A2 | No |
| *dPHM* | Amidation | CG3832 | 60B1–2 | Yes |
| *dPAL1* | Amidation | CG12130 | 46C6–7 | No |
| *dPAL2* | Amidation | CG5472 | 59F4–6 | No |

*Note.* “Activities” refers to either known activity, or simply presumed based on sequence homologies to identified enzyme-encoding genes.

endopeptidase family of enzymes. Their substrates include polypeptide hormone precursors, growth factors, and viral envelope glycoproteins. For example, the PC1/3 and PC2 endopeptidases have well-defined roles in processing numerous neuropeptide pro-hormones in both neural and endocrine tissues, and both enzymes are packaged within secretory granules.

Sequence information supports the hypothesis that insect neuropeptide pro-hormones are also often cleaved at dibasic and monobasic residues. In *Drosophila*, there are three genes that are highly related to PCs, and at least some of these appear to be involved in neuropeptide biosynthesis. The *dfurin 1* (CG10772 at 96D1) and *dfurin 2* (CG18734 at 14C1) genes were identified by sequence homology to mammalian furin. When tested with specific substrates *in vitro*, both have enzymatic activities similar to that of mammalian furin and different from that of mammalian PC2. *dfurin1* is expressed in a small number of identified peptidergic neurons. No phenotypic analysis has been reported for either *dfurin* gene. The third *Drosophila* PC-like gene, called *amontillado* (*amon*, CG6438 at 97C3), is highly related to mammalian PC2. Reported alleles of *amon* include one with a deficiency in its genomic region and another that may also have a P-element in close proximity. Deletion analysis indicated that animals homozygous for *amon* deficiencies are morphologically normal but die trying to hatch from the egg. It is presumed that *amon* is required for the production of one or more secretory peptides needed to display the normal hatching behavior. AMON enzymatic activity has not been examined nor have its effects on neuropeptide biosynthesis been reported. However, the recombinant protein does display predicted interactions with both *Drosophila* and mammalian 7B2′, the protein normally associated with its maturation in mammals.

Angiotensin-converting enzyme (ACE) is a $Zn^{2+}$ peptidyl-dipeptidase that plays an important role in blood pressure homeostasis in mammals. There are six ACE-related gene sequences in the *Drosophila* genome, of which only two, ACER (CG10593 at 29D1) and ANCE (CG10593 at 34D7), are predicted to be active enzymes. The active sites of ANCE and ACER have structural features that are (respectively) highly similar to those of the two adjacent, active domains of mammalian somatic ACE. In other insects, ACE protein distribution is consistent with a role in neuropeptide processing; it is especially enriched in neurons producing peptides of the FXPRLamide family. Insect ACE can process neuropeptide pro-hormone intermediates to remove C-terminal dibasic residues. In *Drosophila*, *ACER* is represented by a single recessive lethal and *ANCE* is represented by two recessive lethals. The possible consequences of these mutations on neuropeptide biosynthesis have not yet been evaluated.

After pro-hormone cleavage by the pro-protein convertases, C-terminal basic residues are removed by a separate carboxypeptidase (CP) activity. Of the >15 characterized mammalian metallocarboxypeptidase enzymes, only CPE/H and CPD are thought to participate in neuropeptide biosynthesis. In *Drosophila*, *silver* (CG4122 at 1B3) and a second gene (CG4678 at 15A2) are the two closest orthologues to the mammalian CP genes associated with neural and endocrine peptide biosynthesis. *silver* (*svr*) is a complex locus encoding large proteins that contain as many as four predicted enzymatic domains. It is represented by 40 null and hypomorphic alleles; some hypomorphic alleles display alterations in wing development and in melanization and sclerotization of the cuticle (the latter phenotypes generate the gene name). The degree to which neuropeptide biosynthesis is altered in *svr* mutant animals has not been determined. No alleles of CG4678 have yet been recorded.

## B. Amidation

C-terminal peptide alpha-amidation is a late event in the biosynthesis of secretory peptides and likely to be the rate-limiting step in many instances. This modification is widespread: In vertebrates, more than half the known secretory peptides are amidated, whereas in insects, greater than 90% are amidated or predicted by DNA sequence to be amidated. Peptide amidation is functionally significant: Its absence often disrupts the activity or receptor-binding properties of peptide ligands. Finally, secretory peptides are the principal substrates for amidation—the enzymes catalyzing this reaction are exclusively associated with a lumenal intracellular compartment. Amidation results from the sequential actions of two enzymes: PHM (peptidylglycine α-hydroxylating mono-oxygenase) and PAL (peptidyl α-hydroxyglycine α-amidating lyase).

In vertebrates, the PHM and PAL enzymes are co-synthesized: The enzymes occupy adjacent domains of a bifunctional protein called PAM and, like the convertases, PAM is incorporated into nascent

TABLE 5 *Drosophila* Neuropeptide Receptor Genes

| Gene | Synonym | Location | Identified ligand? | ESTs |
|---|---|---|---|---|
| CG1147 | *NPFR1* | 83E1 | NPF-like peptides | 1 |
| CG2114 | | 63A1 | | 0 |
| CG2872 | *AlstR*, *DAR-1* | 3E2 | Allatostatin peptides | 0 |
| CG4187 | | 11D1 | | 0 |
| CG4395 | | 11D1 | | 0 |
| CG5042 | | 96E9 | | 1 |
| CG5811 | *NepYr* | 97D14 | RFamide-like peptides | 1 |
| CG5911 | | 93D4 | | 0 |
| CG5936 | | 17A2 | | 0 |
| CG6111 | | 96F7 | | 0 |
| CG6515 | *NKD* | 86C1 | Tachykinin-like peptides | 0 |
| CG6857 | | 17D3 | Cholecystokinin-like peptides (likely) | 1 |
| CG6881 | | 17D3 | Cholecystokinin-like peptides (likely) | 0 |
| CG6986 | | 4C8 | | 1 |
| CG7285 | | 75D1 | | 0 |
| CG7395 | *NPFR76F* | 76F1 | NPF-like peptides | 0 |
| CG7665 | *Fsh*, *DLGR-1* | 90C1 | | 1 |
| CG7887 | *DTKR* | 99C1 | Tachykinin-like peptides | 1 |
| CG8422 | | 50F6 | DH peptides likely | 1 |
| CG8784 | | 87E7 | | 0 |
| CG8795 | | 87E6 | | 0 |
| CG8930 | *rk*, *DLGR-2* | 34E1 | | 1 |
| CG8985 | | 62C4 | | 1 |
| CG9918 | | 88A7 | | 0 |
| CG10001 | *DAR-2* | 98E2 | Allatostatin peptides | 0 |
| CG10626 | | 64E1 | Leukokinin peptides (likely) | 1 |
| CG10698 | | 69A4 | | 1 |
| CG10823 | | 93D1 | | 1 |
| CG11325 | *GRHR* | 26D7 | Adipokinetic hormone | 2 |
| CG12370 | | 48F5 | DH peptides likely | 0 |
| CG12610 | | 45F4 | | 0 |
| CG13229 | | 47C6 | | 1 |
| CG13575 | | 60B4 | | 2 |
| CG13702 | | 75C4 | | 0 |
| CG13758 | | 3A7 | | 6 |
| CG13803 | | 62C4 | | 0 |
| CG13995 | | 26A8 | | 0 |
| CG14003 | | 25F4 | | 0 |
| CG14484 | | 54D6 | | 0 |
| CG14575 | | 78C7 | | 0 |
| CG14593 | | 41F10 | | 0 |
| CG16726 | | 67B5 | | 0 |
| CG17415 | | 49F2 | | 2 |

*Note*. The expression and functionality of these putative receptors for neuropeptide ligands have been determined for only a subset. This table is adapted and updated from Table 1 of Hewes and Targhert (2001). ESTs are listed based on a BLASTP search conducted in March 2002 of BDGP using sequences listed at http://thalamus.wustl.edu/flyGPCR/sequences.html.

secretory granules. In *Drosophila*, both the PHM and the PAL enzyme activities are present but are physically and genetically distinct. The *Drosophila* genome predicts one unlinked *PHM* gene (CG3832 at 60B1–2) and two unlinked *PAL* genes (CG12130 at 46C6–7, and CG5472 at 59F4–6). The *Drosophila* PHM protein is found in most tissues, but within them, it is expressed in a cell type-specific fashion. *Drosophila* PHM enzyme activity mimics the properties of its mammalian counterpart. The *PHM* gene mutates to give a lethal phenotype that is at least superficially similar of that of dPC2 (*amon*), a late embryo/early larval lethal phase, with no indication of gross morphological abnormality. *PHM* nulls and severe hypomorphs lack detectable PHM enzymatic activity; they display severely altered neuropeptide biosynthesis at both larval and adult stages. *PHM* nulls often die during early larval molts, whereas synthetic hypomorphs die attempting to complete later molts or pupal head eversion. These results indicate that PHM is required for the processing of one or more neuropeptides required for completion of different molt cycles.

## VII. NEUROPEPTIDE RECEPTORS

Neuropeptides and peptide hormones signal primarily via GPCRs. These receptors define a very large gene family that provides sensitivity to a variety of environmental, developmental, and physiological signals. GPCRs display a uniform topology with seven-transmembrane domains, whose conserved features permit the use of sequence analysis to identify and classify those devoted to (for example) peptide hormones and neuropeptide ligands ("peptide GPCRs"). The *Drosophila* genome contains ~100 genes encoding GPCRs, including 21 receptors for classical neurotransmitters and neuromodulators (biogenic amines, related compounds, and purines) and ~44 peptide GPCRs (Table 5; see also http://thalamus.wustl.edu/flyGPCR/peptideGPCR.html). Peptide GPCRs are classified within Family A (rhodopsin-like) and Family B (secretin-like) of the GPCRs. Interestingly, the majority of *Drosophila* peptide GPCRs bear strong sequence similarities to different vertebrate peptide GPCRs, as described below. Thus, the set of 44 known and candidate *Drosophila* peptide GPCRs contains up to 39 representatives of Family A and 5 of Family B.

### A. *Drosophila* Peptide GPCRs Are Closely Related to Those of Vertebrate Peptide GPCRs

*Drosophila* peptide GPCRs contain representatives related to at least 15 monophyletic vertebrate GPCR subgroups. There are at least 19 *Drosophila* peptide GPCRs belonging to Family A/Group III-B. Two of these bear strong resemblance to vertebrate gastrin/cholecystokinin receptors (CG6881 and CG6857, both at 17D3). Four fly GPCRs bear similarity to the Neuropeptide Y receptor subgroup (CG1147 at 83D2; CG7395 at 76F1; CG12610 at 17B1; and CG13995 at 26A8). There are six GPCRs related to vertebrate neurokinin receptors [CG6515 (*NKD*) at 86D; CG7887 (*DTKR*) at 99D; CG5811 (*NepYR*) at 97D14; CG10626 at 67B5; CG10823 at 93D1; and BACR48G21.1 at 45F4]. At least 6 *Drosophila* GPCRs are related to neurotensin and neurotensin-related receptors (CG8784 and CG8795, both at 87E6; CG14575 at 78C7; CG9918 at 88A7; CG5911 at 93D1; and CG14003 at 25F4). The bombesin-/gastrin-releasing peptide receptor group contains two *Drosophila* representatives (CG14494 and CG14593 at 41F10).

There are 11 representatives of Family A/Group V among *Drosophila* peptide GPCRs. Four genes are related to the vertebrate glycoprotein hormone-like receptors [CG4187 at 11D1; CG5042 at 96E9; CG7665 (*Fsh*) at 90C1; and CG8930 (*rk*) at 34E1]. The galanin- and somatostatin/opioid-like receptors include four loci [CG2872 (*AlstR*) at 3F1; CG7285 at 75C4; CG10001 (*DAR-2*) at 98E2; and CG13702 at 75C4], whereas the gonadotropin-releasing hormone- and oxytocin-/vasopressin-like receptors include three loci [CG6111 at 96F7; CG10698 at 69A4; and CG11325 (*DRHR*) at 26D7]. Among the 5 *Drosophila* peptide GPCRs that belong to Family B, 4 are clearly related to one or two vertebrate GPCR subgroups: the calcitonin-like (CG4395 at 11D1; and CG17415 at 49F2) and corticotropin-releasing factor-like receptors [CG8422 (*DHR1*) at 50F6 and CG12370 (*DHR2*) at 48F5]. The fifth (CG13578 at 3A7) may be distantly related to the corticotropin-releasing factor-like receptor clade. Thus, a large majority of the *Drosophila* and vertebrate neuropeptide GPCR signaling pathways appear to share common evolutionary origins. Functional and genetic analyses of individual receptor genes will be needed to determine whether the functions of these signals have been similarly conserved.

### B. *Drosophila* Peptide GPCRs Are Distantly Related to Those of Vertebrate Peptide GPCRs

An additional seven genes (CG 2114 at 63A1; CG5936 at 17A2; CG6986 at 4C8; CG8985 at 62C4; CG13229 at 47C6; CG13803 at 62C4; and CG16727 at 67B5) likely encode peptide GPCRs and may also be members of the neurotensin-related receptor subgroup. In fact, all seven appear most closely related to a large set of orphan receptors that were previously identified in *C. elegans* (C. Bargmann, personal communication).

## VIII. SUMMARY

A new era in the study of neuropeptide signaling has begun with the publication of whole genomes for the worm, for the fly, and for various mammals. The utility of the *Drosophila* model lies in the ease of manipulating gene expression to ask specific questions regarding the regulation or function of a particular neuropeptide signaling pathway in an organism that displays complex physiology and behavior. As most of the genes relevant to neuropeptide signaling continue to be identified, and as the DNA constructs and mutant stocks are made available, the opportunities for interesting experiments in the fly will continue to expand. However, as indicated in this article, our numerical estimates for any group of relevant molecules—whether neuropeptide genes, biosynthetic enzyme genes, or receptor genes—are all currently provisional and await experimental analysis. The point of providing a provisional overview such as this is to gauge the landscape and to estimate the measure of the work and the possibilities ahead. In this regard, the future looks busy and promising.

## Glossary

**deficiency** A cytogenetic term referring to a deletion of specific genetic material that ranges in size from 100 s to 1000 s of basepairs and that may remove one or several genetic loci.

**ecdysis** A specific behavior that represents the final step in the molt of an insect—the shedding of the old cuticle—that differs among different insects and that may include distinct subbehaviors.

**eclosion** The specific ecdyses of embryonic and adult stage insects.

**G-protein-coupled receptor** Receptor that contains the characteristic seven-transmembrane motif representative of the large family of proteins that confers sensitivity to a variety of environmental and internal signals. A subset of these receptors confer sensitivity to most peptide hormones.

**P-element** A widely used transposable genetic element in *Drosophila* that is used to create transgenic animals and/or to mutate specific genes.

**pro-hormone** A precursor to an active peptide hormone or peptide transmitter; a pro-hormone is a direct gene product and is produced in an obligate biosynthetic pathway.

## See Also the Following Articles

**Diuretic Hormones and Regulation of Water Balance in Insects • Ecdysone Action in Insect Development • Ecdysone Action in Insect Reproduction • GPCR (G-Protein-Coupled Receptor) Structure**

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# Eating Disorders

P. SÖDERSTEN AND C. BERGH
*Karolinska Institute, Sweden*

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**There are three classifications of eating disorders, anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified, as defined by the Diagnostic and Statistical Manual of Mental Disorders. AN can be further divided into two subtypes, restricting and binge-eating/purging types, and BN can be divided into two subtypes as well, purging and nonpurging types. The regulation of body weight is a main public health concern today because of the great increase in obesity, and it is now estimated that the medical consequences of too much food are as devastating as those of too little food worldwide. The focus here, however, is on the eating disorders listed above and their prevalence, incidence, prognosis, psychopathology, endocrine and physical effects, treatment, and causes.**

## I. INTRODUCTION

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), eating disorders are classified broadly into three types: anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified (EDNOS). There are two subtypes of AN, restricting and binge-eating/purging types, and two subtypes of BN, purging and nonpurging types. The main symptom of AN is the refusal to maintain a normal body weight, so that the body mass index (BMI) is reduced to $\leq 17.5$ (kg/m$^2$) from a normal of 19–24 for women and 20–25 for men. Because of their restricted food intake and their low body weight, anorexics do not menstruate and amenorrhea is a diagnostic criterion of AN. According to the DSM-IV, anorexics fear gaining weight and perceive their body as unrealistically large. Bulimic patients are of normal weight; their main behavioral disorder is that they eat large amounts of food in a brief period of time and subsequently display compensatory behavior to maintain their body weight. These behaviors include vomiting, the use of laxatives, and excessive exercise. Patients suffering from an EDNOS have some of the symptoms characteristic of AN and/or BN but not all these symptoms.

Other types of disordered eating behavior or eating behaviors that cause medical problems, e.g., obesity, are not included in the DSM-IV as eating disorders because they may not be associated with a psychological or behavioral syndrome. However, it is estimated that at least 20% of obese patients display a pattern of eating behavior that is similar to that of patients with BN, although they do not compensate for the increased intake of energy and hence become obese. Binge-eating disorder, i.e., intake of large amounts of food in a short period of time but no other signs of BN, may be viewed as an EDNOS.

In addition to the symptoms mentioned above, anorexic patients are often overactive physically and always hypothermic. These symptoms are obvious in most clinical settings and are likely to be included among the diagnostic criteria in future editions of the DSM. Likewise, patients with bulimia can display physical hyperactivity and deficiencies in thermoregulation.

## II. PREVALENCE, INCIDENCE, AND PROGNOSIS

The specific condition of AN has been recognized for several hundred years, although it is debated whether the different types of emaciation that have been described during the course of medical history are equivalent to the present DSM-IV description of the condition. It is not surprising that AN has been observed for a long time in medical practice, because of its conspicuous physical marker, i.e., the low BMI. By contrast, the specific diagnosis of BN is only 20 years old. Most likely the condition was brought to medical attention only recently because bulimic patients are of normal weight and display no obvious external markers.

The prevalence of AN has remained constant, at approximately 1%, for as long as it has been documented. The patients are typically 14–19 years old at the onset of the disorder and approximately

95% are female. The prevalence of BN is approximately 1–1.5%, the patients are 20–25 years old at the onset of the disorder, and the majority are females and have a history of AN. However, the probability that a young woman may develop an eating disorder symptom, such as binge-eating or vomiting, during a restricted period of her life is as high as 5–25%. On the other hand, there is some evidence that the incidence of both AN and BN has increased somewhat during the past 40 years. The incidence of EDNOS, however, has most likely increased markedly.

The prognosis of patients with eating disorders is poor. Anorexics typically have less than a 50% chance of recovery in 10 years and 25% of the cases become chronically ill. Sadly, the mortality rate is high; as many as 6–15% may die within 10 to 20 years after the onset of the disorder. A low BMI is regarded a poor prognostic factor; with a BMI $\leq 13$, the risk of becoming chronically ill approaches 100%, and with a BMI $\leq 10$, the risk of dying approaches 50%. Bulimics have been reported to have a better prognosis, but at least one-third of the patients continue to binge-eat and purge 10 years after the onset of the disorder.

## III. PSYCHOPATHOLOGY

Patients with AN suffer from concomitant psychopathology. This is not surprising, however, because animal studies have shown that food deprivation alters the concentration, synthesis, and turnover of the neurotransmitters that are involved in reward, mood, and emotion, e.g., dopamine. It is commonly recognized that humans subjected to experimental starvation develop the psychopathological symptoms found in patients with AN. These symptoms include depression, anxiety, and obsessive thoughts and actions. Patients with BN show a similar psychopathology, and periods of self-induced starvation are common among bulimics. Also, 10–20% of patients with AN show bulimic behavior and are, therefore, classified as a subgroup of AN, binge-eating/purging type. Although it has been argued that AN and BN are separable disorders and that EDNOS constitute several different disorders, the similarities between these disorders with regard to behavior and psychopathology are more conspicuous than are the differences. Moreover, there is a general consensus that the psychopathology of eating disorders is enhanced by food restriction. The most parsimonious interpretation, therefore, is that psychopathology emerges from the physical condition of eating-disorder patients. The alternative hypothesis, i.e., disordered eating behavior is an epiphenomenon of an underlying psychopathology, is not supported by any existing evidence. This hypothesis has been proposed in many versions, the most recent being that AN develops because of an underlying obsessive–compulsive disorder. Contradicting this view is the observation that obsessive-compulsive behaviors in AN patients are markedly reduced when the patient's body weight increases. Also, it is unclear how an obsessive personality trait translates into a disordered pattern of eating behavior. Most patients with eating disorders have been examined in a state of illness and, therefore, making a distinction between the potential effects of trait and those of state is not possible. Although patients show reductions in most psychopathological symptoms by the time of clinical discharge, there are no reports of patients who are free of all of their psychopathological symptoms. Consequently, these remaining symptoms may be an effect of a partially effective method of treatment rather than a personality trait of the patient.

## IV. ENDOCRINE AND PHYSICAL EFFECTS

### A. Reproduction

Reduction of food intake and a subsequent loss of body weight cause cessation of menstruation and amenorrhea in AN. Many patients do not experience menarche and therefore suffer from primary amenorrhea. The physical hyperactivity of anorexia may also contribute to their amenorrhea. Thus, amenorrhea is common among normal-weight elite female athletes; in fact, as many as 65% of those engaging in long-distance running are affected. Physical hyperactivity and reduction of food intake, therefore, both contribute to cessation of menstruation. An associated decrease in the level of pituitary gonadotropins and an increase in prolactin and pro-opiomelanocortin-derived hormones are also secondary to the reduced food intake and low body weight. Neither induction of menstrual cyclicity nor restoration of any of these hormonal imbalances has thus far been found useful in restoring the eating behavior of anorexics.

Approximately 50% of bulimics have menstrual disturbances. It is doubtful, however, that menstrual irregularities, including polycystic ovaries, predispose a woman for an eating disorder. Animal experiments suggest instead that the ovarian dysfunction in patients with eating disorders is caused by their disordered eating behavior.

Dieting and/or physical hyperactivity can cause functional hypothalamic amenorrhea, i.e., reduced pulsatile secretion of gonadotropin-releasing hormone (GnRH), and can therefore disrupt the menstrual cycle in women as well as testicular function in men. Consequently, the secretion of gonadal hormones (estradiol and progesterone in women and testosterone in men) is impaired. Puberty, therefore, is delayed.

Thus, the disturbances in reproductive neuroendocrine function in eating-disorder patients are secondary to reduced or altered intake of food and physical hyperactivity. Reproductive quiescence or disturbance induced in this way is reversible.

### B. Adipose Tissue

Leptin is a 15 kDa protein secreted by adipocytes that was first thought to inhibit food intake and play a role in preventing excessive weight gain. However, it has become apparent that reduced leptin facilitates the adaptation to starvation by entraining a complex set of behavioral and neuroendocrine responses that favor survival during periods of limited energy availability. The concentration of leptin is directly proportional to the body fat mass. In anorexia, fat mass is gradually decreased, and leptin levels therefore decrease in parallel. The increase in physical activity that also occurs in anorexia causes a further decrease in fat mass and therefore leptin. It has also been suggested that physical hyperactivity per se might influence leptin levels independent of its effect on fat mass. In addition, experiments on animals suggest that leptin reduces physical activity. The inhibition of pulsatile GnRH secretion and menstrual cyclicity that is caused by both food restriction and excessive physical activity can be reversed by the administration of leptin. Changes in peripheral leptin levels may therefore provide a signal to the brain essential for the adaptations that occur during periods of food shortage and/or abundance.

In view of the fact that many studies have shown that leptin inhibits the food intake of animals, the low levels of leptin and low intake of food in anorexic patients are paradoxical. However, the role of leptin in food intake in humans has been examined only in the obese and has been found to have a minor inhibitory effect. The eating behavior and body weight of anorexics are, of course, radically different from those of the obese, including those who show a bulimic pattern of eating behavior. Yet it has been recently reported that both anorexics and bulimics have low levels of leptin. These findings argue against a direct role of leptin in the eating behavior of patients with eating disorders.

### C. Other Hormonal and Physical Effects

Most likely, all of the other endocrine and physical changes that occur in eating-disorder patients are also the result of periods of dieting or continuous dieting; that is to say, the hormonal changes in starvation are the same independent of the way in which starvation is induced. Thus, hyperprolactinemia, hypercortisolism, bradycardia, hypotension, and hypothermia follow starvation in anorexia just as they do in starvation induced by enforced shortage of food. Other indirect effects of a restricted availability of food are reduced gastrointestinal motility and gastric emptying, constipation, and early satiation. Although most of the endocrine and physical effects in eating-disorder patients are reversible, some are not. Thus, reduced bone mass follows long periods of starvation and estrogen depletion. Physical activity is necessary for the development of bone mass, and the high physical activity in AN may counteract the effect of low food intake and low levels of circulating estrogens. However, normal-weight women with a history of AN will have a permanent reduction in bone mass, and osteoporosis may be a consequence in women with frequent periods of relapse. In BN, permanent dental damage may occur due to recurrent episodes of vomiting.

Interestingly, patients with AN rarely catch infections. This phenomenon is well known but has yet to be explained.

Patients suffering from type 2 diabetes have an increased prevalence of BN. The intriguing possibility that BN may be a risk factor for the development of diabetes deserves investigation.

## V. NEUROTRANSMITTERS

The neural mechanisms controlling food intake are currently the subject of intensive investigation. Although pharmacological studies have shown that many neurotransmitters affect food intake in laboratory animals, these results have not yet allowed the formulation of a model for the development of either AN or BN.

### A. Peptides

Work in animals has failed to demonstrate a factor that initiates feeding under physiological conditions. By contrast, gastrointestinal secretions during a meal are known to cause a physiologically relevant inhibition of food intake. The mechanisms of inhibitory control are localized mainly in the caudal brainstem, and neural systems related to initiation of behavior, including ingestive behavior, are localized in the forebrain, particularly in the ventral striatum. As animals, including humans, eat meals, forebrain and brainstem control mechanisms must interact in the onset and termination of ingestion, but there is no information on how this occurs. The octapeptide cholecystokinin (CCK-8) is in all probability a physiological satiety signal. Inhibition of food intake by CCK-8 is mediated by the vagal nerve and occurs as a result of interplay among CCK, dopamine, and glutamate receptors in the brainstem satiety relay system in the nucleus of the solitary tract. Little is known about how these brainstem mechanisms interact with the neural networks in the forebrain, which are activated by food deprivation before an animal starts ingesting food.

The arcuate nucleus of the hypothalamus lies outside of the blood–brain barrier and senses the leptin signal from adipose tissue. High leptin levels down-regulate the synthesis and release of Neuropeptide Y (NPY) in the arcuate nucleus. Numerous studies over the past 15 years have demonstrated that an infusion of NPY into the brain of experimental animals stimulates food intake, carbohydrate intake in particular. Closer inspection of the behavioral effect of NPY shows, however, that NPY also causes an increase in physical activity and an increase in the intake of water. The effect on water intake is at least as marked as the effect on food intake. Thus, contrary to the common notion that NPY is a potent stimulator of food intake, i.e., an orexigen, its behavioral effect is far from specific.

The hypothalamic circuit activated by hypoleptinemia following fat depletion, and, conversely, the circuits activated by hyperleptinemia following fat repletion have been outlined in impressive detail in animal studies. The theoretical framework of these studies remains much the same as in the early days of regulatory physiology, i.e., that body weight is controlled by precise negative feedback loops. The increase in the incidence of eating disorders and particularly the marked increase in obesity suggest, however, that negative feedback of food intake in humans is easily disrupted. In addition, the rather small effect of most weight-reducing pharmacological treatments and the almost immediate relapse that occurs after the termination of such treatments suggest that food intake in humans has redundant controls.

Animal studies, therefore, have thus far had limited bearing on the understanding on human eating disorders. For example, there is no unequivocal evidence that CCK-8 has any relation to the enhanced feeling of satiety that anorexic patients experience during meals. And the low level of leptin and the associated increase in brain levels of NPY that occur in anorexia, as in starving animals, are not followed by an enhanced intake of food. Interestingly, however, NPY has a much smaller effect on food intake in food-deprived animals than in nondeprived animals. In view of the effects of leptin and NPY on behaviors other than food intake, e.g., water intake and physical activity, the absence of a stimulatory effect on food intake in anorexics is not surprising. In an anorexic state, NPY may cause enhancement of physical activity to facilitate the search for food rather than stimulating the actual intake of food. Food deprivation has long been known to stimulate physical activity, not only in mammals, but in other animal species as well.

## B. The Hypothalamus–Pituitary–Adrenal Axis and Dopamine and Noradrenalin

Food deprivation activates the secretion of corticotropin-releasing hormone (CRH) by the hypothalamus and so acts as a stimulus for the activation of the hypothalamus–pituitary–adrenal (HPA) axis. As a consequence, patients with eating disorders have elevated plasma levels of cortisol. CRH is a well-known appetite suppressant per se, but the activity of the HPA axis can also influence food intake indirectly. Corticosterone, the rat adrenocortical equivalent to cortisol, stimulates the release of dopamine in the ventral striatum. This is the brain's reward pathway, which consequently is sensitized by food deprivation and also by enhanced physical activity. Adrencortical activation of the mesolimbic dopaminergic pathway provides an underlying substrate for the experience of reward, enjoyment, and pleasure that is typical in the beginning of an episode of self-imposed dieting or enhanced physical activity in humans. If dieting runs out of control, as occurs in AN, patients often say they must diet or run excessively, not for an experience of reward or enjoyment, but because they must get away from their feelings of anxiety and depression. Apparently, the neural substrate of reward is activated during the course of anorexia in a way not unlike the way the system is activated during the development of biologically based addictions.

Forebrain dopamine has long been thought of as an inhibitor of food intake, but recent work suggests that its main role is to initiate the behavioral sequences necessary to gain access to various rewards, while not affecting the consumption of those rewards. In addition, dopamine interacts with other transmitter systems, e.g., opioid peptides and benzodiazepines, to assign hedonic qualities to the ingestion of food.

Food restriction also activates the noradrenergic cell group in the locus coeruleus in the brainstem, the neural substrate for selective attention. CRH may mediate this effect as well.

### C. Serotonin

Serotonin is one of the best-known anorexic neurotransmitters. Thus, it was demonstrated 40 years ago that peripheral administration of serotonin inhibits food intake by activating a stretch receptor in the muscular wall of the stomach, thereby stimulating a vagally mediated satiety message to the brain. Subsequently, it has been found that intracerebral administration of serotonin also inhibits food intake. These observations provide the basis for the use of serotonergic agonists in the treatment of obesity.

Given this background, it is surprising that selective serotonin reuptake inhibitors (SSRIs), which facilitate the activity at serotonergic synapses, have been used to treat AN. It is somewhat less surprising that SSRIs are used in bulimia but the risk that the patients might develop anorexia is obvious. Other well-known effects of serotonin, e.g., inhibition of pubertal development, gonadotropin secretion, sexual behavior, and induction of hypothermia, are also incompatible with the use of SSRIs in anorexia.

## VI. TREATMENT

### A. Evaluation of Treatment

There are few if any reports of effective treatments for patients with eating disorders. Over the past 20 years, it has been reported many times that fewer than half the anorexic patients who have been treated recover and that recovery occurs over a prolonged period of time and only after repeated episodes of treatment. There is some evidence that treatment of patients who are younger than 17 years, who have had the disorder for less than a year, and who have not been treated extensively before may recover after treatment with a family-based method. However, similar modestly ill patients have also been shown to recover spontaneously with time.

There is no evidence that older patients with AN benefit from currently available treatments. In fact, the possibility that they may has not been properly examined. Thus, treatment interventions have not been evaluated against a no-treatment or minimal intervention control group in randomized controlled trials (RCTs). It is commonly argued that such comparisons are difficult for ethical reasons, but unless such studies are performed we will never know the effect of such a treatment. Of the few RCTs performed to date, none have shown a major effect of treatment. Furthermore, it has been reported recently that treatment by specialist units is not a predictor of outcome at follow-up 5 years later. This latter situation should encourage investigators to examine the effect of current methods of treatment using RCT methodology.

It is generally thought that the situation for patients with BN is better because cognitive behavioral therapy is effective in this group of patients. This type of therapy aims at changing the way the patient thinks about his or her condition. However, only half of the patients respond to such treatment and the evidence suggests that other types of individualized treatment are equally effective.

Pharmacological treatment has no beneficial effect in AN but treatment with anti-depressants offers some help in BN. Although SSRIs are thought to be useful in BN, their effect is not as great as that of cognitive behavioral therapy and is therefore rather minor.

Follow-up studies have shown that relapse is a significant problem in weight-restored anorexics. Thus, 30–50% of the patients have been reported to relapse within a year after their weight has been restored. There are no reliable figures for relapse of bulimic patients. The existing studies are difficult to interpret because many of the patients were given additional treatment during the follow-up period.

In most studies, the patients are only in partial remission at clinical discharge. Thus, many of them display eating-disorder symptoms or psychopathological symptoms; i.e., they may suffer from an EDNOS. The problem, therefore, remains as to whether these residual symptoms are signs of trait-dependent patient characteristics or simply the effect of a less than optimal method of treatment. This issue is of considerable importance because most of the currently available diagnostic tools do not allow a clear distinction to be made between state- and trait-dependent psychopathological symptoms.

## B. Treatment with Serotonin Agonists

The recent discussion on the possible role of serotonergic mechanisms in BN offers an example of the problem of distinguishing trait from state. Thus, it has been suggested that elevated levels of 5-hydroxyindole acetic acid in the spinal fluid and reduced 5-$HT_{2A}$ receptor binding in the frontal cortex of bulimic patients in remission are signs of trait-dependent patient characteristics. Although these patients did not fulfill the DSM-IV criteria of BN, none of them were free of eating-disorder symptoms or other psychopathological symptoms. Therefore, most of these patients were merely in partial remission and suffered from an EDNOS. Experimental reduction of 5-HT levels by giving patients a diet free of the 5-HT precursor tryptophan has offered better support for the possible role of 5-HT in bulimia. Thus, patients in complete remission with a history of bulimia have been reported to develop symptoms such as feeling fat and fear of losing control over eating immediately after intake of such a diet.

Surprisingly, because of the difference in eating behavior, it has been suggested that the 5-$HT_{2A}$ receptor may also be altered in patients with a history of AN or at risk of developing AN. The results thus far have been inconsistent. The evidence for the use of serotonergic agonists in the treatment of eating disorders is, therefore, weak. Not surprisingly, such drugs have minor effects.

In addition, the hypothesis has been advanced that treatment with SSRIs may prevent relapse in weight-restored anorexics. Some relapse prevention has been reported, but the main result in these studies has been a rapid relapse in both SSRI- and placebo-treated groups or a marked dropout rate. Thus, although it is well known that 5-HT has a role in the control of eating behavior in experimental animals, its possible involvement in eating disorders in humans remains unclear.

## VII. CAUSES

It is commonly stated that eating disorders have multifactorial causes or unknown etiologies. This may reflect the fact that there are only partially effective methods of treatment available and that these methods may target the wrong symptoms.

Obviously, a reduced intake of food is necessary for a loss of body weight and therefore for the development of AN. Also, as mentioned above, reduced availability of food causes an increase in physical activity and it is known that those who are physically active, i.e., elite athletes and ballet dancers, are at risk of developing AN or an EDNOS. Thus, we know that dieting and physical activity are risk factors for the induction of self-starvation, a subsequent loss of the control of food intake, and, eventually, therefore, AN.

It has been demonstrated that animals will run in running wheels if offered the opportunity and that they will run progressively more if the supply of food is restricted. As food availability is further restricted in this situation, the animals will eventually lose control over body weight. This situation, activity-based anorexia (ABA), is virtually identical to the development of AN in humans with the exception that if the animals are allowed *ad libitum* access to food, they stop running, eat, gain weight, and return to a normal physiological state. By contrast, AN develops despite the continuous presence of food. Yet, animals that have developed ABA are conspicuously similar to patients with AN. For example, hypothermia is obvious in both conditions and it has been recently reported that when animals in a state of ABA are treated with external heat they run less, eat more, and gain weight. Similarly, it was noticed in the first description of AN that heat treatments enhance recovery. Treatment with heat, therefore, is likely to become part of a future effective treatment perhaps not only of AN but of other eating disorders as well.

In the first descriptions of AN it was noted that patients were restless. This physical hyperactivity was considered paradoxical because it was argued that the patients would be better off if they were less active and conserved energy. Also, the physical hyperactivity appeared "agreeable" to the patient, i.e., rewarding. In later clinical descriptions, it was noted that losing weight is "enjoyable, pleasurable, and rewarding" to a patient. These clinical observations are compatible with the research on the neural pathways of reward discussed briefly above. Thus, reduced food intake and(or) enhanced physical activity activate the mesolimbic dopaminergic reward pathway in the brain. Interestingly, both dieting and physical activity also activate the locus coeruleus noradrenergic pathway of selective attention. During conditions of reduced food intake and enhanced physical activity, the substrates for reward and attention are activated, thus providing an optimal situation for learning. Therefore, it can be hypothesized that AN develops because it is rewarding to reduce one's food intake and that it is maintained by conditioning to the stimuli that provided the reward.

This hypothesis offers an explanation for the development and maintenance of AN that is compatible with established neurophysiological principles. Because of the symptomatic similarities between anorexics and bulimics and because most bulimics have a history of anorexia, it is possible that AN and BN develop in similar ways. However, it must be added that the marked sex difference in eating disorders await an explanation. Based on the present hypothesis, an attempt to set up an effective treatment for eating disorders should include a method for teaching patients how to eat and how to reduce their physical activity. Although there are no pharmacological methods for reduction of physical activity, it is possible that a supply of external heat might be used.

It seem likely that most of the current methods of treating patients with eating disorders fail because most of them do not rely on sound physiologic hypotheses or are incompatible with basic neurophysiology, e.g., the frequent use of SSRIs. The hypothesis outlined above is offered as a starting point but is likely to be replaced by a better hypothesis as soon as our understanding of the neurobiology of ingestive behavior, physical activity, and temperature regulation has increased beyond its present state.

## VIII. SUMMARY

A considerable amount of information has accumulated about the hypothalamic control of food intake and body weight through negative feedback signals from gastrointestinal and adipose tissues. Thus far, this information has had little influence on the management of patients with eating disorders. These patients still have a poor prognosis and there are few if any effective methods of treatment. Most pharmacological treatments have failed. Also, there are only a few investigations of the effect of treatment interventions that meet the criteria of random assignment and proper control. The effect of most of the methods that are currently used is, therefore, unknown. This situation is most likely caused by the absence of realistic explanations of why eating disorders develop and how they are maintained. Future attempts should take into consideration the possibility that eating disorders might be disorders of physical activity as much as they are disorders of eating behavior or mental disorders. Most endocrine and physical changes observed in patients with eating disorders are consequences rather than causes of self-imposed starvation that is maintained by conditioning to cues in the patient's environment.

## Glossary

**activity-based anorexia (ABA)** Animals run excessively and lose control over body weight if very little food is provided and they enter a state of ABA, which is conspicuously similar to self-starvation in humans.

**anorexia nervosa** An eating disorder with severe, self-imposed starvation and commonly associated with a high level of physical activity and hypothermia.

**body mass index** An indicator used to define nutritional status and derived from the formula: weight divided by the square of height (kg/m$^2$). The acceptable range is 19–24 in women.

**bulimia nervosa** An eating disorder with episodes of excessive food intake, followed by compensatory methods to maintain a normal body weight.

**eating disorder not otherwise specified** An eating disorder that does not fulfill the diagnostic criteria of anorexia nervosa or bulimia nervosa.

**5-hydroxytryptamine** A neurotransmitter long known to inhibit food intake; it is the focus of many treatment interventions aimed at normalizing disordered eating behavior. Also known as 5-HT or serotonin.

**neuropeptide Y** A neuropeptide thought to increase food intake.

**orexigen** A compound that stimulates or increases food intake.

**randomized controlled trial** The standard method of random assignment to a treatment and a control condition necessary for the proper evaluation of medical interventions.

**selective serotonin reuptake inhibitors** Widely used antidepressants that enhance serotonin availability at synaptic sites thought to be altered in patients with eating disorders.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Cholecystokinin (CCK) • Gastrointestinal Hormone-Releasing Peptides • Ghrelin • Leptin • Neuropeptide Y (NPY)**

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# Ecdysone Action in Insect Development

MICHAEL BENDER
*University of Georgia*

I. INTRODUCTION
II. BIOLOGICAL RESPONSES TO ECDYSONE
III. THE ECDYSONE RECEPTOR
IV. GENETIC REGULATION OF THE ECDYSONE RESPONSE
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VI. ECDYSONE AND NEURONAL REMODELING
VII. SPECIFICITY OF ECDYSONE SIGNALING
VIII. SUMMARY

**During metamorphosis, a process in which many larval tissues are replaced by adult tissues, the insect steroid hormone ecdysone triggers distinct stage- and tissue-specific responses in its many target tissues. These responses are very diverse, including cell proliferation and differentiation in future adult tissues, programmed cell death in larval tissues, and remodeling of neuronal cell types during metamorphosis. Different forms of the ecdysone receptor may contribute to the specificity of ecdysone signaling.**

## I. INTRODUCTION

The insect steroid hormone 20-hydroxy-ecdysone is a key regulator that triggers transitions between developmental stages including larval molting and the metamorphosis from larva to adult. Ecdysone also regulates female reproduction and coordinates simple behaviors. During metamorphosis of the insect nervous system, ecdysone governs the remodeling of sets of neurons that function during both larval and adult stages.

Like the vertebrate steroid hormones and other small lipophilic hormones such as retinoic acid and thyroid hormone, ecdysone acts through a heterodimeric nuclear receptor that functions as a hormone-regulated transcription factor to activate or repress target genes. The ecdysone receptor is composed of the EcR and USP nuclear receptor proteins. Molecular mechanisms of steroid hormone signaling show remarkable evolutionary conservation in organisms as diverse as insects and human. This fact, coupled with the advantages for experimental endocrinology exhibited by insect species such as the lepidopterans *Bombyx mori*, *Manduca sexta*, and *Hyalophora cecropia* and the mosquito *Aedes aegypti* as well as the superb genetic and molecular tools available in the fruit fly *Drosophila melanogaster*, has made the study of ecdysone signaling an important model for understanding steroid hormone signaling during development.

The response to ecdysone is controlled by a genetic hierarchy in which a small number of primary response genes are directly induced by ecdysone and a larger set of secondary response genes execute specific biological responses to the hormone. Recent studies employing DNA microarray analysis of gene expression during metamorphosis have begun to identify the entire genomic complement of ecdysone-responsive genes active at this time. These experiments, and others that examine gene expression in a particular tissue, hold out the prospect of identifying batteries of ecdysone-regulated genes required for particular tissue-specific responses to ecdysone. Conventional molecular genetic and

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# Ecdysone Action in Insect Development

Michael Bender
*University of Georgia*

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**During metamorphosis, a process in which many larval tissues are replaced by adult tissues, the insect steroid hormone ecdysone triggers distinct stage- and tissue-specific responses in its many target tissues. These responses are very diverse, including cell proliferation and differentiation in future adult tissues, programmed cell death in larval tissues, and remodeling of neuronal cell types during metamorphosis. Different forms of the ecdysone receptor may contribute to the specificity of ecdysone signaling.**

## I. INTRODUCTION

The insect steroid hormone 20-hydroxy-ecdysone is a key regulator that triggers transitions between developmental stages including larval molting and the metamorphosis from larva to adult. Ecdysone also regulates female reproduction and coordinates simple behaviors. During metamorphosis of the insect nervous system, ecdysone governs the remodeling of sets of neurons that function during both larval and adult stages.

Like the vertebrate steroid hormones and other small lipophilic hormones such as retinoic acid and thyroid hormone, ecdysone acts through a heterodimeric nuclear receptor that functions as a hormone-regulated transcription factor to activate or repress target genes. The ecdysone receptor is composed of the EcR and USP nuclear receptor proteins. Molecular mechanisms of steroid hormone signaling show remarkable evolutionary conservation in organisms as diverse as insects and human. This fact, coupled with the advantages for experimental endocrinology exhibited by insect species such as the lepidopterans *Bombyx mori*, *Manduca sexta*, and *Hyalophora cecropia* and the mosquito *Aedes aegypti* as well as the superb genetic and molecular tools available in the fruit fly *Drosophila melanogaster*, has made the study of ecdysone signaling an important model for understanding steroid hormone signaling during development.

The response to ecdysone is controlled by a genetic hierarchy in which a small number of primary response genes are directly induced by ecdysone and a larger set of secondary response genes execute specific biological responses to the hormone. Recent studies employing DNA microarray analysis of gene expression during metamorphosis have begun to identify the entire genomic complement of ecdysone-responsive genes active at this time. These experiments, and others that examine gene expression in a particular tissue, hold out the prospect of identifying batteries of ecdysone-regulated genes required for particular tissue-specific responses to ecdysone. Conventional molecular genetic and physiological analyses have revealed much about the genetic regulation of ecdysone response in individual tissues or cell types. Two examples are discussed here: the regulation of programmed cell death in larval tissues fated to die during metamorphosis and the control of neuronal remodeling during metamorphosis.

A general question in hormonal signaling is how tissue- and cell-specific responses are generated from a global signal. Expression of EcR isoforms in distinct temporal and spatial patterns suggests that distinct EcR isoforms may mediate different responses to ecdysone. This view is supported by *in vivo* rescue experiments carried out in cells of the larval salivary gland and in neurons undergoing remodeling during metamorphosis that show that EcR isoforms are not functionally identical in these cell types.

## II. BIOLOGICAL RESPONSES TO ECDYSONE

During insect development, pulses of ecdysone act as temporal signals to coordinate the transitions between developmental stages. Careful measurements of ecdysteroid titers have been made throughout development for several insect species, including the tobacco hornworm *M. sexta* and the fruit fly *D. melanogaster.* A composite ecdysteroid titer profile for *Drosophila* is shown in Fig. 1. For simplicity, the description below pertains to ecdysone action during *Drosophila* development. Ecdysone carries out similar functions in other insects that undergo complete metamorphosis, although these insects vary in the number of larval molts and in the style of puparium formation. In *Drosophila*, ecdysone pulses during first- and second-instar larval development trigger molts to the next larval instar as the animal increases in size. Larval molting involves the separation of the larval cuticle from the underlying epidermis (apolysis), the formation of the new cuticle, and finally, the shedding of the old larval cuticle (ecdysis).

A series of three ecdysone pulses drives the metamorphic transitions between larval, prepupal, pupal, and adult stages (Fig. 1). A prominent pulse at the end of third-instar development triggers formation of the puparium, the hardened structure within which metamorphosis will take place. Puparium formation involves shortening of the larva to form a barrel-like structure, eversion of the anterior

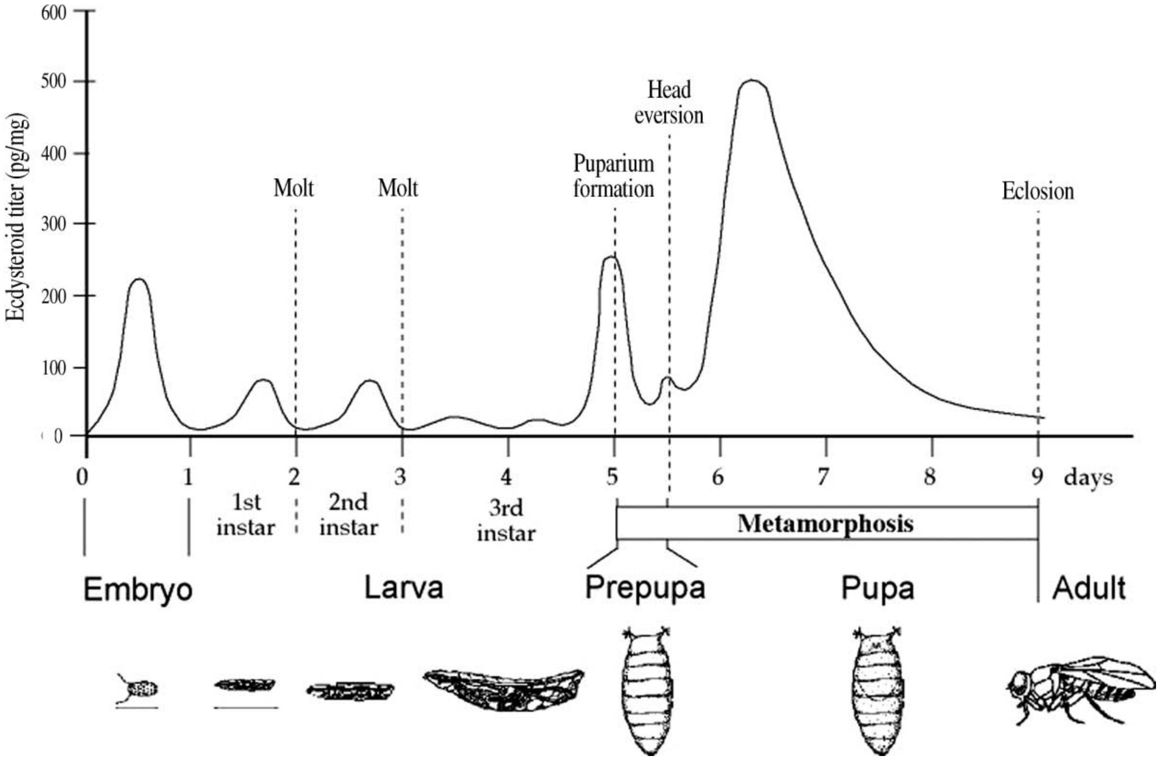

**FIGURE 1** Ecdysone controls developmental transitions in *Drosophila*. Ecdysteroid titer is indicated on the y axis and developmental stage is indicated on the *x* axis. Reprinted from Thummel (2001), with permission from Elsevier Science.

spiracles, and formation of the operculum at the anterior end of the puparium, a structure through which the adult fly will ultimately emerge. Twelve hours after the pulse that triggers puparium formation, a smaller pulse of ecdysone drives head eversion, an event that marks the end of the prepupal period. Another large pulse occurs during the pupal period and is responsible for development of adult structures.

Two other ecdysone pulses can be detected in *Drosophila*. The function of the midembryonic pulse is not yet definitively established, although genetic studies suggest that it may play a role in morphogenesis and cuticle deposition. Finally, a small ecdysone pulse detected soon after the midpoint of the third instar may trigger larval wandering, a behavioral change characterized by the larva's departure from the food site and search for a suitable site for puparium formation.

During metamorphosis, the larva, which is specialized for feeding and growth, is completely reorganized into the reproductively competent adult. In this process, many larval tissues degenerate and are replaced by adult tissues. The timing of larval cell death varies between tissues. For example, the cells of the larval midgut initiate cell death in response to the ecdysone pulse that triggers puparium formation, and salivary gland cell death is triggered by the prepupal ecdysone pulse.

The cells that will give rise to adult structures, the imaginal cells, are set aside during embryogenesis and can be divided into two types, imaginal disc cells and imaginal histoblast cells. Imaginal disc cells, which will form the adult head, thorax, and genitalia, invaginate from the embryonic epidermis to form small sac-like structures and proliferate throughout larval development, finally forming discs containing thousands of cells at the end of the larval period. At the time of puparium formation, the imaginal discs respond to ecdysone by evaginating and elongating to form the rudiments of the adult appendages. The prepupal ecdysone pulse triggers head eversion, in which head structures assume their normal adult position, as well as further elongation of legs and wings as they assume their adult form. Differentiation of imaginal disc cells into adult cell types takes place in response to the pupal ecdysone pulse.

The imaginal histoblast cells are also set aside during embryogenesis but, in contrast to imaginal disc cells, do not proliferate during larval development, instead remaining in small clusters or rings until metamorphosis. The adult abdomen and internal tissues, including the adult foregut, midgut, hindgut, and salivary glands, arise from histoblast cells. The response of the histoblast imaginal cells to ecdysone is characterized by rapid cell proliferation, followed in some cases by cell migration to form the appropriate adult structures.

## III. THE ECDYSONE RECEPTOR

In the early 1990s, the *Drosophila ecdysone receptor* (*EcR*) gene was cloned by homology to another *Drosophila* nuclear receptor, E75. The finding that the EcR protein bound a labeled ecdysone analogue, bound a previously identified ecdysone-response element, and rescued ecdysone responsiveness when transfected into a mutant cell line lacking EcR provided evidence that EcR was a necessary component of the *Drosophila* ecdysone receptor. The fact that an additional protein was necessary for activity when EcR was expressed in a heterologous system indicated that a second protein was required for ecdysone receptor activity and this protein was subsequently identified as the product of the *ultraspiracle* (*usp*) gene. Both the EcR subunit and the USP subunit of the ecdysone receptor are members of the nuclear receptor protein family. USP is the insect homologue of the vertebrate retinoic acid X receptor (RXR) protein that serves as a heterodimeric partner of a family of nuclear receptor proteins including the retinoic acid receptor, the thyroid hormone receptor, and the vitamin D receptor. EcR is most closely related to the farnesol X receptor, which functions in bile acid homeostasis in vertebrates. The recent purification of EcR and USP from *Drosophila* showed that a complex of molecular chaperones is required to activate the ecdysone receptor for DNA binding and that the EcR subunit is the primary target of the chaperone complex.

For certain nuclear receptors, binding of hormone leads to dissociation of co-repressors and their replacement by co-activators, leading to transcriptional activation of hormone target genes. Co-repressors and co-activators may act by recruitment of chromatin remodeling complexes that modify chromatin via histone deacetylation or histone acetylation, respectively. The *Drosophila* co-repressor SMRTER, a functional homologue of the vertebrate co-repressors SMRT and N-Cor, has been shown to associate with the ecdysone receptor. SMRTER mediates repression by interacting with the SIN3 adapter protein, a component of a histone deacetylase complex. These results suggest that gene regulation by the ecdysone receptor may involve chromatin remodeling mediated by histone modification.

The EcR and USP subunits of the ecdysone receptor exist in several different forms. In *Drosophila*, multiple promoters and alternative splicing within the *EcR* gene result in a family of three EcR isoforms (EcR-A, EcR-B1, and EcR-B2). The isoforms share a common carboxy-terminal region containing DNA- and hormone-binding domains and differ in their amino-termini. Only one *Drosophila* USP protein is known based on cDNA sequence, although two proteins with different molecular weights can be distinguished, suggesting the possibility of an alternate translational start site. EcR and USP have been identified from a large number of insect species and from the Atlantic sand fiddler crab (*Uca pugilator*). Several insect species, including *M. sexta*, *B. mori*, the mealworm *Tenebrio molitor*, and the spruce budworm *Choristoneura fumiferana*, encode both EcR-A and EcR-B forms. Multiple USP isoforms are also common among insect species and two USP forms are encoded by separate genes in *M. sexta*, *B. mori*, and *A. aegypti*.

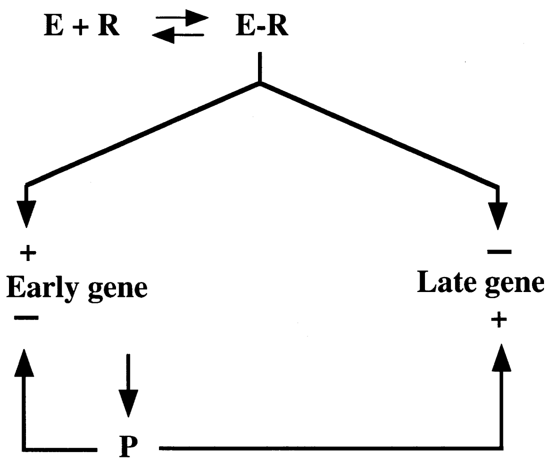

FIGURE 2 A regulatory model for ecdysone response. Ecdysone (E) binds to its receptor (R) and activates early gene expression while repressing late gene expression. An early gene product(s) (P) activates the late genes, overcoming the repressive effects of the receptor, and in addition acts negatively to limit early gene expression. Redrawn from Ashburner *et al.* (1974), with permission from Cold Spring Harbor Laboratory Press.

## IV. GENETIC REGULATION OF THE ECDYSONE RESPONSE

The first global examination of genetic response to a steroid hormone was carried out in the 1960s in experiments examining ecdysone response in the midge *Chironomus tentans* and in *Drosophila*. These experiments took advantage of the giant polytene chromosomes of these species in which changes in gene activity could be directly visualized in the form of transcriptional puffs and provided evidence that ecdysone acted through a cascade of gene activation. Subsequent experimentation by Michael Ashburner and his colleagues in the 1970s, in which *Drosophila* polytene chromosome puffing responses were examined *in vitro* in the presence of ecdysone or in the presence of ecdysone plus protein synthesis inhibitors, established a regulatory model for ecdysone response (Fig. 2). At the time of metamorphosis, a temporal progression of polytene chromosome puffing can be seen in which a small set of early gene loci puff and then regress, followed by puffing and regression of a larger set of late gene loci. The puffing sequence was shown to be controlled by ecdysone *in vitro*, and a series of experiments demonstrated that late puff induction and early puff regression were sensitive to protein synthesis inhibitors but early puff induction was not. In addition, late puffs could be prematurely induced following ecdysone addition and withdrawal. In the model shown in Fig. 2, ecdysone acts via a receptor protein to directly activate the early response genes and to repress the late genes. The products of the early genes in turn activate the late response genes and repress their own expression.

Cloning of the early genes showed that a number of these genes encode transcriptional regulatory proteins, supporting the proposed regulatory model. Each of the *E74*, *E75*, and *BR-C* early genes encodes a family of transcription factors (of the ets [proto-oncogene], nuclear receptor, and zinc-finger types, respectively) through the use of multiple promoters and alternative splicing. Biochemical and mutational analyses have shown that each of these genes acts as an ecdysone-induced transcription factor and is required for normal ecdysone response early in metamorphosis. In contrast to these three early genes, the *E23* and *E63* early genes do not encode transcription factors, indicating that the primary genetic response to ecdysone is not limited to proteins that function directly in gene transcription. The *E63* gene encodes a calmodulin-related protein, and *E23* encodes a homologue of an ABC transporter protein. Because ABC proteins can function to transport steroids in other systems, this latter identity has led to the intriguing suggestion that *E23* may function to regulate ecdysone response by controlling intracellular ecdysone levels in target cells. Among the late genes cloned, *L71* encodes a family of antimicrobial peptides, *L82* encodes a protein of unknown function, and *L63* encodes a protein with homology to cyclin-dependent protein kinases.

The recent sequencing of the *Drosophila* genome and the advent of DNA microarray technology have allowed the examination of ecdysone-induced gene expression on a genome-wide scale. A recent study by Kevin White and his colleagues examined the expression of genes in a temporal window encompassing the ecdysone pulse that triggers puparium formation and the subsequent pulse that triggers head eversion (see Fig. 1). In these experiments, RNA populations were isolated from wild-type animals at seven time points starting at 18 h prior to puparium formation and ending 30 h later at the time of the prepupal ecdysone pulse. Labeled probes prepared from each time point were hybridized along with a reference probe to DNA microarrays containing 4500 unique cDNA elements, representing approximately one-third of all genes in the *Drosophila* genome. Genes that increase or decrease in expression level relative to the time of pupariation were selected and grouped by similarities in expression patterns. Four hundred sixty-five genes, or approximately 10% of the genes tested, that reproducibly increase or decrease using the selection criteria chosen (a minimum of a threefold change in expression) were identified. In addition to known ecdysone-responsive genes, whose expression profiles from microarray analysis matched those determined earlier by RNA Northern analysis, a large number of candidate genes that may be induced or repressed by ecdysone at this time were identified in these experiments. Each of these newly identified genes potentially provides a new entry point for experiments designed to elucidate the molecular and cellular mechanisms underlying ecdysone response during early metamorphosis. Challenges that remain include distinguishing ecdysone-regulated genes within this set from those genes that are regulated by other developmental signals at this time, separation of primary from secondary ecdysone-responsive genes, and refinement of the technique to allow examination of gene expression within individual tissue types. The first two tasks should be aided by the availability of mutants that lack key ecdysone regulatory proteins or that are defective in ecdysone synthesis, as well as molecular genetic tools for overexpression of ecdysone regulatory proteins.

## V. ECDYSONE AND THE CONTROL OF CELL DEATH

During metamorphosis, many larval tissues undergo programmed cell death and do not contribute to adult structures. Extensive molecular, genetic, and histological analyses of larval salivary gland and midgut destruction in *Drosophila* have revealed much about the molecular and genetic mechanisms by which cell death is carried out in these tissues. The ecdysone-induced *E93* gene plays a key role in programmed cell death of the midgut and salivary gland as these tissues are replaced by adult cells early in metamorphosis. *E93* expression precedes cell death in the midgut and salivary gland, and genetic studies have shown that *E93* is required for cell death in each of these tissues. Furthermore, ectopic expression of *E93* in wing imaginal disc cells can trigger cell death, even though these cells do not normally undergo this process.

In the larval salivary gland, *E93* is expressed in a stage-specific fashion, responding to the prepupal ecdysone pulse but not to the earlier pulse that triggers puparium formation. Fig. 3 shows the current regulatory model for how stage-specific expression of *E93* is achieved in this tissue. The late third-instar ecdysone pulse acts through the early genes *BR-C* and *E74* to repress glue genes, whose products are used early in puparium formation, and to induce the *L71* late genes. Following a sharp drop in ecdysone titer after puparium formation, expression of the early genes also drops. The early genes are then re-induced by the prepupal ecdysone pulse that occurs 12 h after puparium formation and triggers destruction of the larval salivary glands. Normal expression of *E93* during the prepupal ecdysone pulse requires βFTZ-F1, a nuclear receptor protein whose expression is limited to the low-ecdysone-titer environment of the mid-prepupal period. The expression of β*FTZ-F1* is tightly regulated. The timing of β*FTZ-F1* gene induction is determined by interaction of the E75B repressor and the DHR3 activator, both ecdysone-induced nuclear receptor proteins. The extent of β*FTZ-F1* expression is then limited by repression by the EcR/USP heterodimer in response to the rising prepupal ecdysone titer as well as by autorepression by the βFTZ-F1 protein. Inactivation of β*FTZ-F1* by mutation leads to submaximal induction of *E93* and failure of salivary gland destruction, suggesting that βFTZ-F1 acts as a competence factor to ensure stage-specific expression of *E93*. β*FTZ-F1* is also required for maximal expression of *BR-C* and *E74*. In contrast to the situation in the salivary gland, E93 is expressed in the larval midgut at the time of puparium

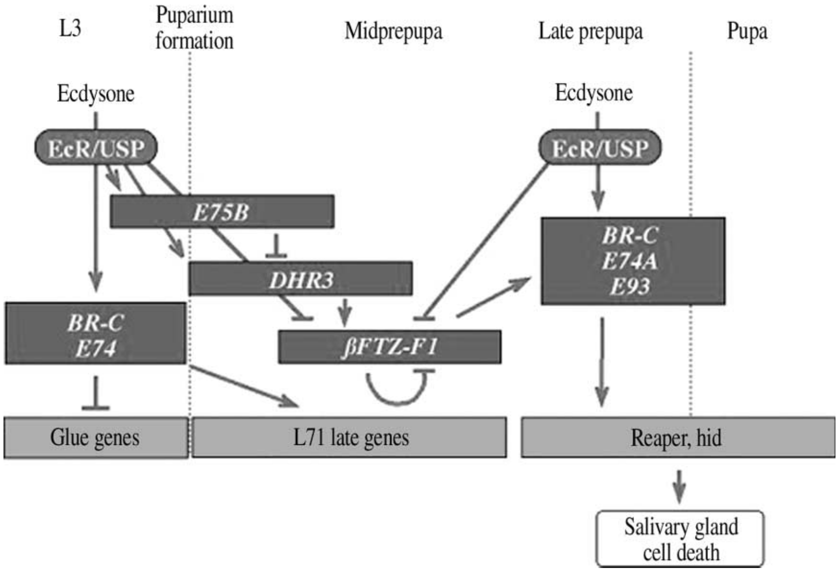

**FIGURE 3** Genetic control of salivary gland cell death. Shaded bars indicate temporal patterns of expression of ecdysone regulatory genes relative to the developmental stages indicated at the top. Arrows indicate gene activation and bars indicate gene repression. Reprinted from Thummel (2001), with permission from Elsevier Science.

formation when midgut cell death is initiated. Although little is currently known about *E93* regulation in the midgut at this earlier time, comparison of *E93* regulatory mechanisms in this tissue to those operating during salivary gland destruction should reveal how the ecdysone-regulated cell death pathway is deployed at distinct developmental times to control cell death in different tissues.

*E93* encodes a novel nuclear protein that has been shown to be associated with specific polytene chromosome sites, suggesting that *E93* may act through transcriptional activation of target genes. Transcription of genes involved in programmed cell death is defective in *E93* mutants. Thus, the *Drosophila* homologue of APAF-1/ced-4, the caspase DRONC, and the cell death genes *reaper* and *hid* require *E93* for normal induction at the time of the prepupal ecdysone pulse. *Croquemort*, a gene required for engulfment of apoptotic cell corpses during embryogenesis, is also expressed at the time of the prepupal ecdysone pulse and requires *E93* function for its expression at that time. In addition, *BR-C* function is required for *reaper* and *hid* expression and *E74* is required for maximal *hid* expression. These results suggest that *E93*, *BR-C*, and *E74* act to trigger a programmed cell death pathway involving known cell death genes in the larval salivary gland in response to the prepupal ecdysone pulse.

Recent work by Cheng-Yu Lee and Eric Baehrecke suggests that programmed cell death in the larval midgut and salivary gland proceeds by an autophagic pathway rather than by an apoptotic pathway. During autophagy, the cell is destroyed by its own lysosomal contents, whereas during apoptosis, remnants of dying cells are degraded by a second phagocytic cell. This observation suggests that the autophagic cell death pathway makes use of some of the same downstream components as apoptotic cell death pathways despite the morphological distinctions between the two cell death pathways. Lee and Baehrecke also show that some cellular aspects of cell death triggered by ectopic *E93* expression (including formation of cell corpses, engulfment by macrophages, and cell elimination) are independent of the cell death genes *reaper*, *grim*, and *hid* but other aspects of cell death (including DNA fragmentation and acridine orange staining) depend on these genes. These intriguing observations suggest the existence of genetically distinct pathways that regulate cellular changes during programmed cell death.

## VI. ECDYSONE AND NEURONAL REMODELING

During metamorphosis of the insect central nervous system, ecdysone triggers the cell death of certain neurons that function only during the larval stage or during the larval and pupal stages, and it stimulates adult differentiation of other neurons that arise from neuronal stem cells that have proliferated during the larval period. In contrast to these effects, which mirror ecdysone's influence on larval and imaginal tissues described earlier, ecdysone also controls the development of a third class of neurons that function during larval development and are then remodeled during metamorphosis to serve adult-specific functions. The study of remodeling neurons, which constitute a major fraction of the adult nervous system, was pioneered in *M. sexta*. The large size of *Manduca* allowed identification of individual neurons that could be marked and followed throughout the course of metamorphosis and allowed dye-filling experiments so that the extensive dendritic branching of these cells could be visualized. Fig. 4 shows the morphological changes during larval and pupal development in a *Manduca* abdominal motoneuron (MN-3) that innervates body wall muscles. During the final larval instar, this neuron innervates larval abdominal body wall muscles and has an extensive arbor of dendritic processes. Early in pupal development, however, MN-3 undergoes a severe pruning of these processes so that the extent of dendritic branching is much reduced soon after entry into the pupal stage. Regrowth of adult-specific processes takes place during pupal development, and the adult MN-3 cell that innervates the newly created adult abdominal muscles has extensive branching. These morphological changes are correlated with changes in ecdysteroid titer. Dendritic pruning is initiated after the late larval ecdysone pulse and regrowth takes place during the broad pupal ecdysone pulse (Fig. 4).

Hormone deprivation and restoration experiments in another *Manduca* cell type that undergoes dendritic loss prior to cell death (the PPR neurons) show that an ecdysone signal is required for dendritic pruning and that pruning can be rescued by restoration of ecdysone to ecdysone-deprived neurons. Ecdysone control of regrowth of dendritic processes was examined in the MN-1 abdominal motoneuron cells of *Manduca*. In these experiments, injection of ecdysone into diapausing pupae, a quiescent stage with low ecdysone levels, was sufficient to trigger the regrowth of adult processes in MN-1 cells. These experiments indicate that dendritic regression and outgrowth are controlled by ecdysone.

Cell-specific markers that identify individual neurons or sets of neurons have allowed the analysis of the *Drosophila* nervous system during metamorphosis, opening the door to genetic analysis of neuronal remodeling. Like *Manduca*, many *Drosophila* neurons are remodeled during the metamorphosis from the larval stage to the adult stage. One particularly well-characterized example of neuronal remodeling takes place in a set of neurosecretory cells that react with an antibody to a secreted peptide, SCP-B. SCP-B reactive cells undergo extensive pruning of dendritic and axonal processes after puparium formation and regrowth of adult-specific processes

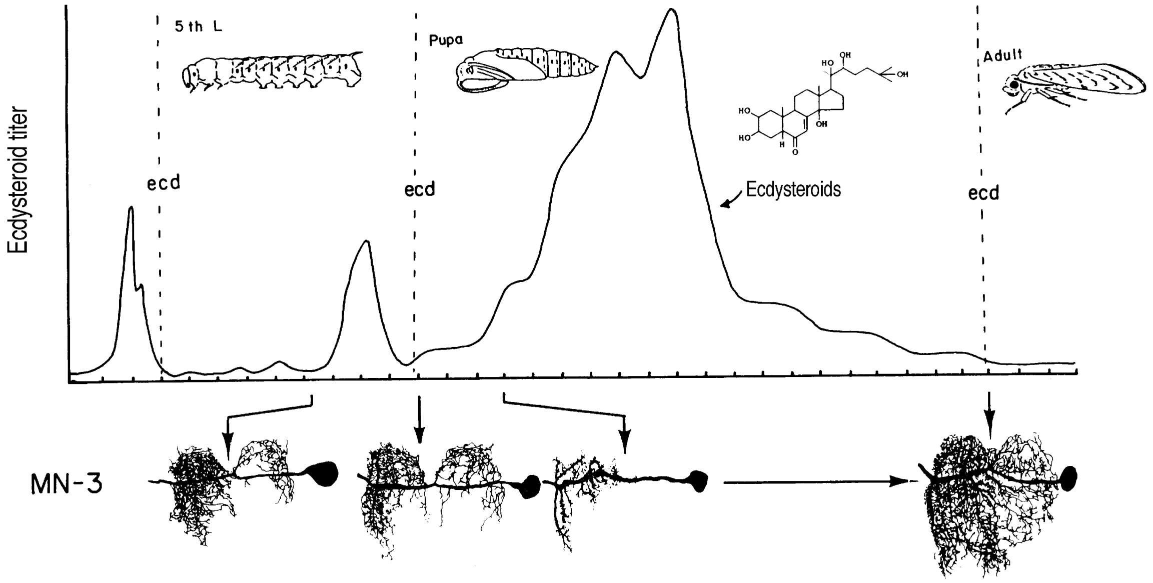

FIGURE 4 Neuronal remodeling during metamorphosis. The drawings at the bottom show the appearance of the *Manduca* MN-3 neuron at different stages during metamorphosis. The graph at the top shows ecdysteroid titers during *Manduca* metamorphosis. Ecdysis (ecd) at the end of each stage is indicated by a dashed line. Modified from Truman (1996), with permission from Academic Press.

during pupal development. Examination of *EcR* expression in the SCP-B reactive cells showed that expression of high levels of the EcR-B1 isoform precedes pruning of larval processes and that EcR-A is the predominant isoform during the later process of adult outgrowth. These observations led to the hypothesis that distinct forms of the ecdysone receptor might govern distinct responses in remodeling neurons, with an EcR-B1 form responsible for larval pruning and an EcR-A form responsible for adult outgrowth and maturation. This model is supported by analysis of neuronal remodeling in *EcR* mutants. Mutants lacking both EcR-B1 and EcR-B2 fail to undergo pruning, indicating that EcR-B functions are required for this aspect of neuronal remodeling. The unanticipated finding that pruning can proceed in an *EcR-B1* mutant suggests that either the EcR-B1 or the EcR-B2 receptor form alone is sufficient to trigger the pruning response. These experiments also suggest that neuronal remodeling in *Drosophila*, as in *Manduca*, is regulated by ecdysone.

Examination of another *Drosophila* neuronal cell type that undergoes remodeling, the mushroom body (MB) gamma neurons, showed that both USP and EcR-B1 components of the ecdysone receptor are required for axonal pruning in this cell type. Interestingly, the downstream ecdysone-response genes *BR-C*, *E75*, and *E74* were not required for remodeling of the MB gamma neurons. These results suggest that remodeling of these neurons proceeds via a novel pathway of ecdysone-induced genes.

## VII. SPECIFICITY OF ECDYSONE SIGNALING

The sheer diversity of cellular responses to ecdysone prompts the question of how a global signal such as ecdysone can specify diverse cell- and tissue-specific responses. One model proposes that distinct responses to ecdysone would require particular combinations of ecdysone receptor isoforms. This model grew out of the finding that expression of specific EcR isoforms could be correlated with patterns of ecdysone response characteristic of particular cell or tissue types. Mutational analysis of the *EcR* gene is thus far consistent with this model. Mutations that inactivate EcR-B1 lead to a non-pupariating phenotype in which tissues that predominantly express EcR-B1 are blocked in their normal response to ecdysone, whereas EcR-A predominant tissues initiate their normal ecdysone response. Mutants that lack both EcR-B1 and EcR-B2 show defects in larval molting and, as described above, are blocked in an early stage of neuronal remodeling that is correlated with high levels of EcR-B1 expression. It will be interesting to examine *EcR-A* mutant phenotypes to determine whether these phenotypes are distinct from those of *EcR-B* mutants, thus indicating distinct developmental functions for EcR-A.

Several *in vivo* studies have addressed the question of whether the different EcR proteins are functionally interchangeable or functionally distinct by measuring the ability of an EcR isoform to rescue a defect caused by the loss of a different EcR isoform. In the first of these, the loss of transcriptional puffing in larval salivary gland cells of an *EcR-B1* mutant could be completely rescued by heat-shock-driven expression of EcR-B1 and partially rescued by EcR-B2 expression. In contrast, EcR-A expression was incapable of significant rescue. These experiments indicate that the EcR isoforms are functionally distinct in this cell type during early metamorphosis.

Another study made use of the EcR-B1-expressing MB gamma neurons, described above, that are remodeled during pupal development and require both EcR-B1 and USP to complete this process. Targeted expression of EcR to the MB gamma neurons using the GAL4-UAS system was used to rescue defective remodeling of these neurons in an *EcR-B1* mutant. In these experiments, the expression of EcR-B1 or EcR-B2, but not EcR-A, was sufficient to rescue remodeling defects. In a third study, a similar strategy was used to express EcR in SCP-B$^+$ neurons in an *EcR-B* mutant background. As described above, these cells express high levels of EcR-B1 prior to undergoing pruning of larval-specific processes and larval pruning is blocked in *EcR-B* mutants. In these experiments, EcR-B2 and EcR-B1 were most efficient at rescuing defective larval pruning and EcR-A rescued less well. In this study, the phenotypic consequences of expressing the different EcR isoforms in a wild-type background were also found to be distinct. Together, these experiments suggest that the EcR isoforms are not functionally interchangeable in the cellular contexts examined. In all cases, the functional distinction is greatest between EcR-A and the EcR-B isoforms, with the greatest degree of overlap between EcR-B1 and EcR-B2 function.

The various EcR isoforms differ in their amino-terminal A/B domains, domains that in vertebrate nuclear receptors encode a transcriptional activation function. Several studies have examined the transcriptional activation functions of EcR in cell culture and shown that the various EcR isoforms differ in their abilities to activate test promoters.

These experiments suggest that distinct EcR AF-1 domains may function to differentially control gene transcription of ecdysone target genes.

## VIII. SUMMARY

Ecdysone acts through the ecdysone receptor, a heterodimer of the EcR and USP nuclear receptors, to regulate reproduction, larval molting, and metamorphosis in insects. The sets of genes regulated by ecdysone include early genes that are direct targets of the receptor and late genes that constitute a downstream response and execute cell- and tissue-specific hormone responses. Cellular responses to ecdysone are very diverse, including development and differentiation of adult tissues, programmed cell death of larval tissues fated to die, and remodeling of neuronal cell types during metamorphosis. Multiple isoforms of EcR and/or USP exist in most insect species examined. In *Drosophila*, expression of different EcR isoforms is correlated with distinct cell and tissue responses to ecdysone, suggesting that different ecdysone receptor forms contribute to the specificity of ecdysone signaling. *In vivo* rescue experiments that show that the EcR isoforms are generally incapable of complete functional substitution support this model.

## Glossary

**ecdysone receptor** Insect specific nuclear receptor that mediates the action of the steroid hormone 20-hydroxy-ecdysone.

**ecdysone-response element** A specific DNA regulatory sequence found near the transcription start site of ecdysone-regulated genes that is bound by the ecdysone receptor heterodimer.

**ecdysones** Natural compounds (e.g., α-ecdysone and 20-hydroxy-ecdysone) that control molting and metamorphosis in insects. As used here, the term refers to biological activity and is analogous to "estrogens" and "androgens."

**EcR** A nuclear receptor that is the protein product of the *ecdysone receptor* gene.

**metamorphosis** A hormone-induced process in which the larval form of an organism is transformed into the adult form.

**USP** A nuclear receptor that is the protein product of the *ultraspiracle* gene.

## See Also the Following Articles

**Ecdysone Secretion in Lepidopteran Insects • Ecdysteroid Action in Insect Reproduction • Ecdysteroidogenic Pathway • Ecdysteroid Receptors (EcR/USP) • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development**

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# Ecdysone Secretion in Lepidopteran Insects

HIROSHI KATAOKA
*University of Tokyo*

I. INTRODUCTION
II. PURIFICATION AND PRIMARY STRUCTURE OF *Bombyx* PTTH
III. cDNA STRUCTURES OF *Bombyx* PTTH
IV. DIMERIC STRUCTURE AND DISULFIDE BOND LOCATION OF *Bombyx* PTTH
V. STRUCTURE OF THE CARBOHYDRATE CHAIN OF *Bombyx* PTTH
VI. MOLECULAR CLONING OF THE PTTH cDNAs FROM OTHER LEPIDOPTERAN INSECTS
VII. PHYSIOLOGY OF *Bombyx* PTTH AND OTHER FACTORS INVOLVED IN ECDYSONE SECRETION OR SIGNAL TRANSDUCTION CASCADES IN THE PGs

**Ecdysones are poly-hydroxylated steroid hormones that control arthropod development and reproduction. This article focuses on the actions of a brain peptide prothoraciotropic hormone (PTTH) to promote the secretion of ecdysone by the prothoracic gland.**

## I. INTRODUCTION

Insects are characterized by molting and metamorphosis during postembryonic development. Approximately 80 years ago, the mechanisms of these physiologically important events were first investigated by a Polish scientist, Kopec.

During the following 40 years, the roles of the brain, the corpora allata (CA), and the prothoracic glands (PGs), along with their functional relationships, were clarified. Molting and metamorphosis are controlled by three hormones: prothoracicotropic hormone (PTTH), produced in the brain; juvenile hormone, produced in the CA; and ecdysone, produced in the PGs. PTTH activates the PGs to secrete ecdysone, thereby playing a central role in the endocrine control of insect molting and metamorphosis. PTTH was first isolated from heads of the adult silkworm, *Bombyx mori*, and subsequently, the cDNA coding for *Bombyx* PTTH and its cDNA homologues from several kinds of other lepidopteran insects were cloned. This article presents a history of the studies of *Bombyx* PTTH, the current status of lepidopteran PTTH research, and a description of other factors that regulate ecdysone secretion from the PGs.

## II. PURIFICATION AND PRIMARY STRUCTURE OF *Bombyx* PTTH

During the purification of *Bombyx* PTTH, debrained dormant pupae were used for bioassay. When the brain is extirpated from *Bombyx* pupae shortly after pupation, brainless pupae remain as pupae for several months without undergoing adult development. Injection of material containing PTTH into debrained dormant pupae brings about the resumption of adult development, whereas material without PTTH caused no development. In 1986, *Bombyx* PTTH was first isolated from *Bombyx* adult heads and as little as approximately 0.1 ng of pure PTTH induced adult development in a *Bombyx* brainless pupa. The amino acid sequence of *Bombyx* PTTH up to the 104th residue from the amino-terminus was determined from the sequencing data of fragment peptides obtained by enzyme digestion of the sample from $3 \times 10^6$ heads and purified by the same purification scheme. The 41st residue was undetermined since no PTH amino acid was detected at this cycle of Edman degradation.

## III. CDNA STRUCTURES OF *Bombyx* PTTH

A cDNA coding for *Bombyx* PTTH was cloned from a cDNA library by an expression cloning strategy using an antibody against a synthetic peptide corresponding to the first 15 amino acids at the amino-terminal. As shown schematically in Fig. 1, the *Bombyx* PTTH cDNA encodes, in the following order starting from the 5′-end, a signal peptide (29 amino acids), a 2 kDa peptide (21 amino acids), a 6 kDa peptide (57 amino acids), and PTTH (109 amino acids). The first 104 deduced amino acids exactly matched the amino acid sequence obtained from peptide sequencing of the purified PTTH. The 41st residue, which remained unidentified by peptide analysis, was eventually shown to be asparagine, indicating that a carbohydrate moiety is attached to this site. Thus, the entire amino acid sequence of *Bombyx* PTTH has been elucidated.

## IV. DIMERIC STRUCTURE AND DISULFIDE BOND LOCATION OF *Bombyx* PTTH

By sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis under nonreducing

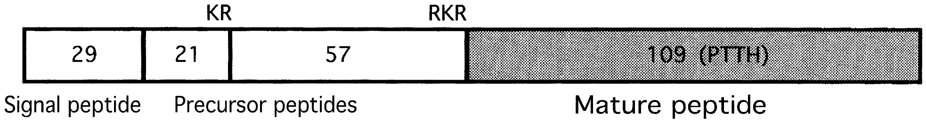

**FIGURE 1** Schematic structure of *Bombyx* prepro-PTTH. The numbers in the boxes indicate the number of amino acid residues. K and R indicate the lysine and arginine residues in the amino acid sequence of PTTH. KR and RKR are the possible proteolytic processing sites in the *Bombyx* prepro-PTTH.

and reducing conditions, a broad band corresponding to 30 kDa and two bands of 17 and 16 kDa, respectively, were detected. When the purified PTTH was reduced, alkylated, and sequenced, all peaks obtained gave the same sequence as the amino-terminal sequence of the intact PTTH. Therefore, it was concluded that *Bombyx* PTTH is a homodimeric protein, the subunits of which are linked together by a disulfide bond(s).

When a portion of the cDNA designed to express only the PTTH subunit peptide was introduced into *Escherichia coli*, the cell lysate contained PTTH activity that was indistinguishable from that of natural PTTH, proving that the cloned cDNA encoded *Bombyx* PTTH. The purified recombinant PTTH possessed specific activity comparable with that of natural PTTH. Furthermore, SDS–PAGE analysis showed that the recombinant PTTH also had a dimeric structure linked by a disulfide bond(s). Determination of the disulfide bond location in this recombinant *Bombyx* PTTH has shown their positions at Cys-15–Cys-15′ as an interchain disulfide linkage and Cys-17–Cys-54, Cys-40–Cys-96, and Cys-48–Cys-98 as intrachain disulfide linkages (Fig. 2).

Interestingly, when PTTH was partially reduced with all intrachain disulfide bonds remaining intact, PTTH retained half of its biological activity compared with the intact dimeric PTTH. However, the fully reduced and S-alkylated PTTH monomer had no PTTH activity. This clearly indicates that the intrachain disulfide bonds are essential for attaining the peptide conformation required for manifesting the biological activity. The interchain disulfide bond has a clear additive effect by maintaining the dimeric structure of native PTTH. Although any significant sequence homology between PTTH and other peptide hormones or protein factors has not been discovered by database searching, the sequential arrangement of intrachain disulfide bonds indicates a striking similarity to that of several growth factors with a cysteine-knot motif, such as nerve growth factor and transforming growth factor-β (TGF-β).

## V. STRUCTURE OF THE CARBOHYDRATE CHAIN OF *Bombyx* PTTH

The structure of the carbohydrate chain of recombinant *Bombyx* PTTH produced by the baculovirus system was determined to be Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc–. The saccharide chain of the native hormone has been determined to be identical to that of the recombinant hormone. The biological activities of the native hormone and the recombinant hormones, produced in *E. coli* and in the baculovirus expression system, were compared in the bioassay using brainless *Bombyx* pupae. The native hormone and the baculovirus-generated recombinant hormone showed identical dose responses, whereas the *E. coli*-produced recombinant hormone showed only slightly weaker activity. This finding suggests that the glycosidic side chain in PTTH is likely not essential for its biological activity; however, it might stabilize the hormone molecule in the hemolymph.

## VI. MOLECULAR CLONING OF THE PTTH cDNAs FROM OTHER LEPIDOPTERAN INSECTS

Immunohistochemical studies using an antibody against *Bombyx* PTTH revealed that molecules homologous to *Bombyx* PTTH exist in the brains of other lepidopteran insects. Thus far, cDNAs for *Bombyx* PTTH homologues have been isolated and sequenced from three species of lepidopteran insects, *Samia cynthia ricini*, *Antheraea pernyi*, and *Manduca sexta*. The structure of each precursor molecule is similar to that of *Bombyx* PTTH, and each of these PTTHs contains seven cysteine residues and an *N*-glycosylation site in the mature domain, although the glycosylation site is slightly different. The positions of the seven cysteine residues are conserved, indicating that these hormones are all structurally similar to those of the TGF-β family. Amino acid sequences of the mature PTTH regions are shown in Fig. 3. The deduced amino acid sequence of *Bombyx* PTTH and the sequences of *Samia*, *Antheraea*, and *Manduca*

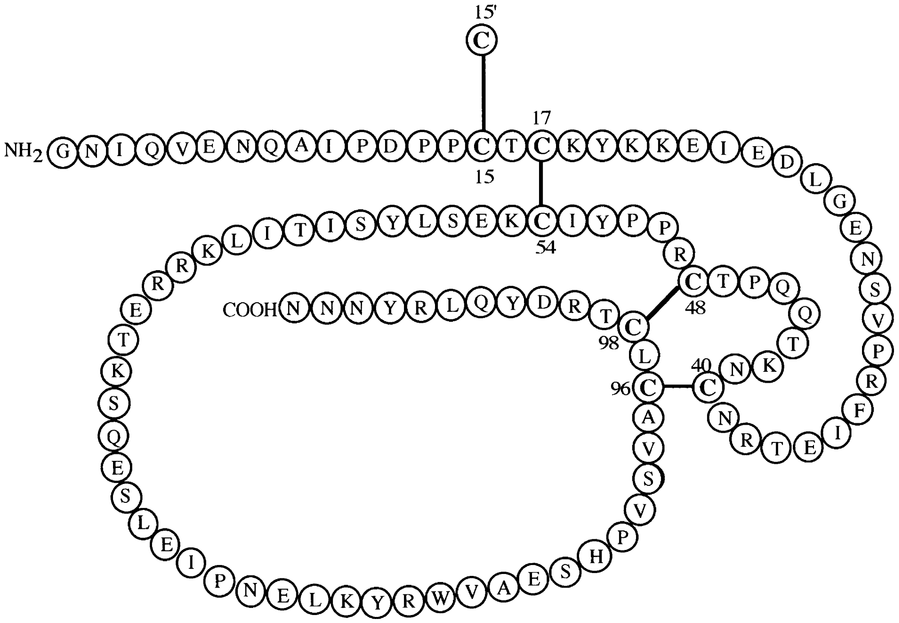
15'
C
17
NH2 G N I Q V E N Q A I P D P P C T C K Y K K E I E D L G E N S V P R F I E T R N C N K T Q Q P T C R P P Y I C K E S L Y S I T I L K R R E T K S Q E S L E I P N E L K Y R W V A E S H P V S V A C L C T R D Y Q L R Y N N N COOH
15
54
48
40
98
96

FIGURE 2 The amino acid sequence and the disulfide bond location of *Bombyx* PTTH. Circles represent amino acids. $NH_2$ and COOH indicate the N-terminus and C-terminus, respectively, of *Bombyx* PTTH. Two identical units are held together by the disulfide bond between Cys-15 and Cys-15′.

PTTHs show 51, 52, and 60% identity, respectively. The C-terminal portion following the last cysteine residue of these PTTHs was variable in length.

*Samia* PTTH and *Manduca* PTTH were expressed in *E. coli* and the biological activities of *Bombyx*, *Samia*, and *Manduca* PTTH were determined by debrained pupal assay systems. The recombinant *Samia* or *Manduca* PTTHs were capable of inducing adult development at a dose of approximately 0.3 ng/pupa when injected into brainless *Samia* or *Manduca* pupae, respectively. This activity is about the same as that of the recombinant *Bombyx* PTTH in debrained *Bombyx* pupae. Despite the relatively high level of sequence similarity, the biological activities of PTTHs were species specific. For example, *Samia* PTTH showed PTTH activity in *Samia* but not in *Bombyx* or *Manduca*. Conversely, *Manduca* PTTH showed PTTH activity in *Manduca* but not in *Samia*

```
                                20                  40                  60
                       .         .         .         .         .         .
Bombyx:     GNIQVE--NQAIPDPPℂTℂKYKKEIEDLGENSVPRFIETRNℂℕKTQQPTℂRPPYIℂKESLYS
Samia:      *DLRR*KH****Q***ℂSℂG*TQTLL*F*K*AF**HVV**ℕℂSD-**QSℂLF**Vℂ**T**D
Antheraea:  ***KRQ--*--*****ℂSℂE*TN*TV*F***AF**HV*S*ℕℂSELR*SSℂLF**Vℂ**T**D
Manduca:    ***K**EY********ℂSℂE***GFIN****VF*SN***Iℕ ℂSTN**QSℂP****ℂ***IYE

                              80                 100                 120
                     .         .         .         .         .         .
Bombyx:     ITILKRRETKSQESLEIPNELKYRWVAESHPVSVAℂLℂTRDYQLRYNNN
Samia:      VN*******ST*I*E*V*R***F**IG*KWQI**GℂMℂ****RNSTEDYQPRLLTKIIQQRDLS
Antheraea:  *SV****QSTT*P*EKV*****F**I**KWQI**GℂVℂ****RDTI*QD
Manduca:    *K**RK*KSMAEK**AR*TD*EIG*****L*I**GℂIℂ****VI
```

FIGURE 3 The amino acid sequences of lepidopteran PTTHs. Each amino acid sequence was deduced from cDNA sequences obtained from *Bombyx mori*, *Samia cynthia ricini*, *Antheraea pernyi*, and *Manduca sexta*. Amino acid residues identical with *Bombyx* PTTH are indicated by asterisks. C, cysteine residues; N, potentially glycosylated asparagine residues.

or *Bombyx*, and *Bombyx* PTTH showed PTTH activity in *Bombyx* but not in *Samia* or *Manduca*.

## VII. PHYSIOLOGY OF *Bombyx* PTTH AND OTHER FACTORS INVOLVED IN ECDYSONE SECRETION OR SIGNAL TRANSDUCTION CASCADES IN THE PGs

Immunohistochemistry with an antibody against *Bombyx* PTTH demonstrated that PTTH is produced by two pairs of dorsolateral neurosecretory cells of the brain and is transported to the CA by axons running through the contralateral hemisphere of the brain. The PTTH titer in the hemolymph was quantified using a very sensitive time-resolved fluoroimmunoassay, and it changed dramatically during *Bombyx* development, with a small peak in the middle of the fourth instar, medium-sized peaks at the wandering and prepupal stages in the fifth instar, and a large prolonged peak during early pupal-adult development. The changes were, overall, closely correlated with those in the hemolymph ecdysteroid titer.

Recently, a peptide inhibiting ecdysone secretion from the PGs, prothoracicostatic peptide (PTSP), was isolated from *Bombyx* larval brains and determined to have the amino acid sequence H-Ala-Trp-Gln-Asp-Leu-Asn-Ser-Ala-Trp-$NH_2$. This peptide inhibited PTTH-stimulated ecdysone secretion in the PGs at both the spinning and the feeding stages, indicating that PTSP interferes with PTTH-stimulated ecdysone secretion. In addition, two factors other than PTTH and PTSP involved in regulating signal cascades of ecdysteroidogenesis in the PGs were discovered in *Bombyx*. One of them is a factor that can elevate glandular cyclic AMP levels in the PGs in the early stages of the fifth instar, and the other is a factor that can elevate intracellular inositol 1,4,5-trisphospate levels in the PGs throughout the fifth instar and during the first day of the pupal stage. It is hoped that these two factors will be identified chemically and that the molecular mechanisms of endocrine control in ecdysteroidogenesis will be further clarified.

## Glossary

**prothoracic glands** Glands in lepidopteran insects that synthesize and secrete the molting hormone ecdysone.

**prothoracicostatic peptide** A peptide hormone that acts on the prothoracic glands and inhibits ecdysone secretion.

**prothoracicotropic hormone** A neuropeptide hormone that is secreted in the insect brain and stimulates the prothoracic glands to secrete ecdysone.

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysteroid Action in Insect Reproduction • Ecdysteroidogenic Pathway • Ecdysteroid Receptors (EcR/USP) • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development**

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# Ecdysteroid Action in Insect Reproduction

Alexander S. Raikhel*, Leeanne McGurk†, and Mary Bownes†

*University of California, Riverside • †University of Edinburgh*

I. INTRODUCTION
II. MOLECULAR ENDOCRINOLOGY OF MOSQUITO VITELLOGENESIS
III. MOLECULAR ENDOCRINOLOGY OF FRUIT FLY VITELLOGENESIS

**This article focuses on two insects for which the role of 20-hydroxyecdysone (20E) in reproduction is best understood at the molecular level. In the yellow fever mosquito, *Aedes aegypti*, the ecdysteroid-regulatory hierarchy governs transcription of yolk protein precursor (*YPP*) genes. Following blood feeding, the ecdysone receptor (EcR)-ultraspiracle (USP) heterodimer activates the early genes, *E74* and *E75*, as well as directly acting one the YPP gene vitellogenin, allowing its expression in the fat body. In turn, the products of *E74* and *E75* genes act as powerful activators of the gene transcription. Prior to blood feeding, such activation is prevented by USP heterodimerization with an orphan receptor AHR38, which is a repressor of the EcR-USP transcriptional activation. In the fruit fly, *Drosophila melanogaster*, *YPP* genes are expressed in two tissues, the fat body and the ovary. The regulation of *YPP* gene expression is quite different in these two sites of synthesis. In the fat body, ecdysteroids stimulate YPP synthesis. In the ovary, however, JH and 20E seem to be antagonistic, with JH promoting YPP synthesis and oocyte progression and ecdysone leading to apoptosis. The molecular control of *YPP* gene transcription in *Drosophila* is well understood in terms of sex-specific regulation. A great deal has been elucidated concerning the mechanism leading to expression of these genes in the female and not in the male. Mutations in genes controlling sex-specific expression of *YPP* genes lead to sexual transformations of males to females to males, as well as to intersex flies.**

## I. INTRODUCTION

As in vertebrates, some insects utilize a steroid hormone to govern key events in their reproduction. It was first established in *Aedes aegypti* that the female ovaries produce a precursor form of a steroid hormone, ecdysone (E), which is transformed by target tissues into its active form, 20-hydroxyecdysone (20E). The parallel with oviparous vertebrates is even more striking considering that E is produced by the follicular epithelium in the ovary in both instances. Moreover, in both vertebrates and insects, steroid hormones activate the expression of yolk protein genes in the liver and the fat body, respectively. Remarkably, only some groups of insects, mainly Diptera, represented by mosquitoes and flies, have 20E as the key regulator of reproduction. In contrast, in the majority of insects, it is juvenile hormone (JH) that plays this role in reproduction. The reason for such dramatic differences in the hormonal control of reproduction among insects remains an enigma.

## II. MOLECULAR ENDOCRINOLOGY OF MOSQUITO VITELLOGENESIS

The yellow fever mosquito, *A. aegypti*, in addition to its importance as a pathogen vector, represents an outstanding model system for arthropod vector research due to the exceptional knowledge base amassed on its physiology, biochemistry, and development. The great wealth of information that has accumulated over the past two decades concerning vitellogenesis in *A. aegypti* has made it one of the best studied insects. The vitellogenic cycle of *A. aegypti* can be divided into three distinct periods. First, in the previtellogenic period, the mosquito undergoes a preparatory phase in which the fat body becomes capable of intense synthesis of yolk protein precursors (YPPs). This process is thought to be under the control of JH III. It is followed by a state of arrest that is maintained until a blood meal is taken, after which the mosquito enters the synthetic phase of the vitellogenic period. The fat body then produces YPPs to be accumulated and stored in the yolk bodies of the oocytes. 20-Hydroxyecdysone is involved in the control of the vitellogenic period. Finally, in the termination period, YPP production is halted in the fat body (Fig. 1).

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## II. MOLECULAR ENDOCRINOLOGY OF MOSQUITO VITELLOGENESIS

The yellow fever mosquito, *A. aegypti*, in addition to its importance as a pathogen vector, represents an outstanding model system for arthropod vector research due to the exceptional knowledge base amassed on its physiology, biochemistry, and development. The great wealth of information that has accumulated over the past two decades concerning vitellogenesis in *A. aegypti* has made it one of the best studied insects. The vitellogenic cycle of *A. aegypti* can be divided into three distinct periods. First, in the previtellogenic period, the mosquito undergoes a preparatory phase in which the fat body becomes capable of intense synthesis of yolk protein precursors (YPPs). This process is thought to be under the control of JH III. It is followed by a state of arrest that is maintained until a blood meal is taken, after which the mosquito enters the synthetic phase of the vitellogenic period. The fat body then produces YPPs to be accumulated and stored in the yolk bodies of the oocytes. 20-Hydroxyecdysone is involved in the control of the vitellogenic period. Finally, in the termination period, YPP production is halted in the fat body (Fig. 1).

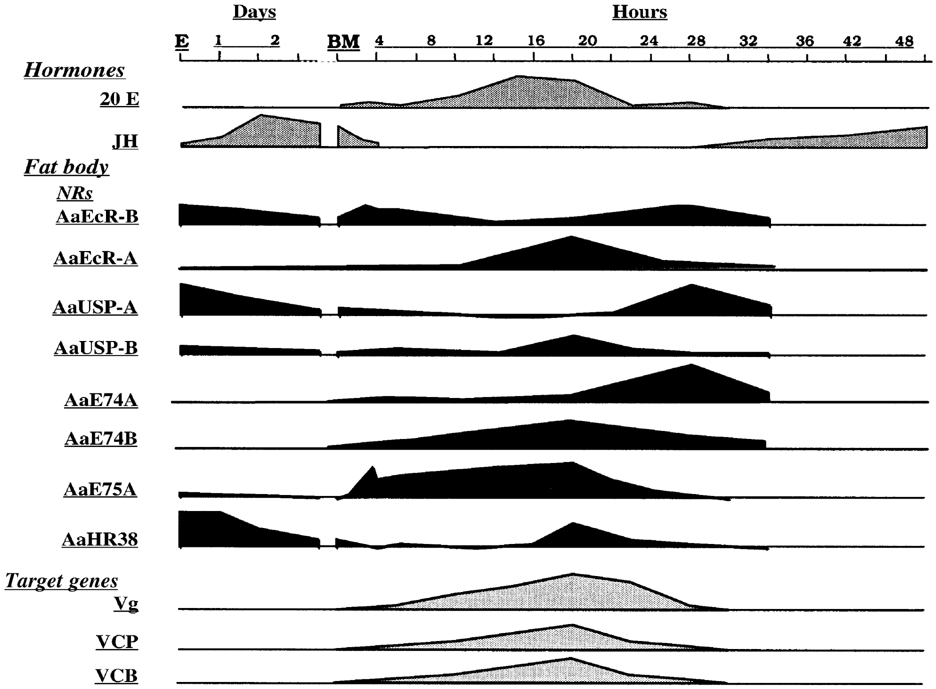

FIGURE 1 Transcript profiles of major genes of the ecdysteroid-regulatory hierarchy during the first cycle of egg maturation in the mosquito, *Aedes aegypti*. The previtellogenic period begins at eclosion (E) of the adult female. During the first 3 days of posteclosion life of the female, both the fat body and the ovary become competent for subsequent vitellogenesis. The female then enters a state of arrest; yolk protein precursors are not synthesized during the previtellogenic period. Only when the female mosquito ingests blood (BM) is vitellogenesis initiated. Hormones: hormonal titers of juvenile hormone (JH) and ecdysteroids (20E) in *A. aegypti* females. Fat body: relative levels of RNAs for nuclear receptors (NRs) determined by reverse transcription-polymerase chain reaction. AaEcRA, ecdysone receptor isoform A; AaEcRB, ecdysone receptor isoform B; AaUSPA, Ultraspiracle isoform A; AaUSPB, Ultraspiracle isoform B; AaE74B, E74 isoform B; AaE74A, E74 isoform A; AaE75A, E75 isoform A; AaHR38, mosquito homologue of *Drosophila* HR38; Vg, vitellogenin; VCP, vitellogenic carboxypeptidase; VCB, cathepsin B-like protease. Modified from Raikhel *et al.* (2002), Vitellogenesis of Disease Vectors, from Cell Biology to Genes. *Insect Biochemistry and Molecular Biology* 32 (10), with permission from Elsevier.

The gene encoding vitellogenin (Vg), a major YPP in most oviparous animals, is expressed in extraovarian tissues in a sex-, tissue-, and stage-specific manner. *A. aegypti* Vg is a member of large family of evolutionally conserved proteins serving as major yolk proteins in organisms ranging from nematodes to birds. In vitellogenic female insects, the fat body, a powerful metabolic and secretory organ, is engaged in massive production of YPPs for developing oocytes. The fat body's role in vitellogenesis in the female mosquito *A. aegypti* is not limited to the production of Vg. Together with Vg, the vitellogenic fat body of *Aedes* females produces two other YPPs, which are proenzymes deposited in developing oocytes and activated during embryogenesis: 53 kDa vitellogenic carboxypeptidase (VCP) and 44 kDa cathepsin B-like protease (VCB). Moreover, the regulation of these YPPs appears to be similar: they are synthesized exclusively by the fat body in response to a blood meal and are maximally expressed at 24 h post-blood meal (PBM). Lipophorin (Lp), the insect lipid transport molecule, also plays a role as a YPP in *A. aegypti*.

The hemolymph titers of ecdysteroids in female mosquitoes are correlated with the rate of YPP synthesis in the fat body (Fig. 1). The ecdysteroid titers are only slightly elevated at 4 h PBM; however, they rise sharply at 6–8 h PBM and reach their maximum level at 18–20 h PBM. Many studies have established that the ecdysteroid control of vitellogenesis is a central event in the blood meal-activated regulatory cascade leading to successful egg maturation.

## A. Molecular Basis of Competence to the 20E Response

In *A. aegypti*, a preparatory, previtellogenic, developmental period is required for the mosquito fat body to attain competence for 20E responsiveness as well as for the adult female to attain competence for blood feeding. βFTZ-F1, the orphan nuclear factor implicated as a competence factor for stage-specific responses to ecdysteroid during *Drosophila* metamorphosis, serves a similar function during mosquito vitellogenesis. The transcript of the *Drosophila* *βFTZ-F1* homologue is expressed at high levels in the mosquito fat body during pre- and postvitellogenic periods when ecdysteroid titers are low. However, there is a delay in the appearance of active *AaFTZ-F1* factor that coincides with the onset of competence for 20E response (Fig. 2). In addition, a homologue of *Drosophila HR3* is expressed in the vitellogenic tissues of the female mosquito. The expression of the mosquito homologue, *AHR3*, correlates with the titer of 20E, peaking in late pupae and adult vitellogenic females at 24 h PBM and preceding *AaFTZ-F1* expression peaks. The orphan nuclear receptor *DHR3*, an early-late gene, is one of the key genes in the ecdysone-mediated genetic regulatory network. Recent studies have revealed that during insect metamorphosis *DHR3* has a dual role in repressing the early genes while activating *βFTZ-F1*. Thus, the regulation and function of *FTZ-F1* during mosquito vitellogenesis closely resemble those shown at the onset of *Drosophila* metamorphosis, and *FTZ-F1* is therefore part of a conserved and broadly utilized molecular mechanism controlling the stage specificity of the ecdysteroid response.

## B. Ecdysteroid-Regulatory Hierarchy in Mosquito Vitellogenesis

Analysis of ecdysone's effects on polytene chromosome puffing patterns in the late larval and prepupal salivary gland of *Drosophila* have suggested that the initial activation of a small number of early ecdysone-inducible genes leads to the subsequent induction of a large number of late target genes. Elucidation of this genetic hierarchy at the molecular level has led to the identification of the ecdysone receptor as a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), the insect homologue of the vertebrate retinoid X receptor. Furthermore, these studies have shown that the action of the ecdysone–receptor complex is indeed mediated by early genes such as the *BR-C*, *E74*, and *E75* genes encoding transcription factors involved in the regulation of late gene expression. The ecdysone-mediated regulatory network is further refined by the presence of genes that are involved in setting up the timing and stage-specificity of gene activation by this genetic hierarchy.

Consistent with the proposed role of 20E in activating mosquito vitellogenesis, experiments using an *in vitro* fat body culture have shown that physiological doses of 20E ($10^{-7}$ to $10^{-6}$ M) activate two *YPP* genes, *Vg* and *VCP*. Transcripts of two different isoforms of EcR, EcR-A and EcR-B, are present in previtellogenic and vitellogenic ovaries and fat bodies. Transcripts encoding two different USP

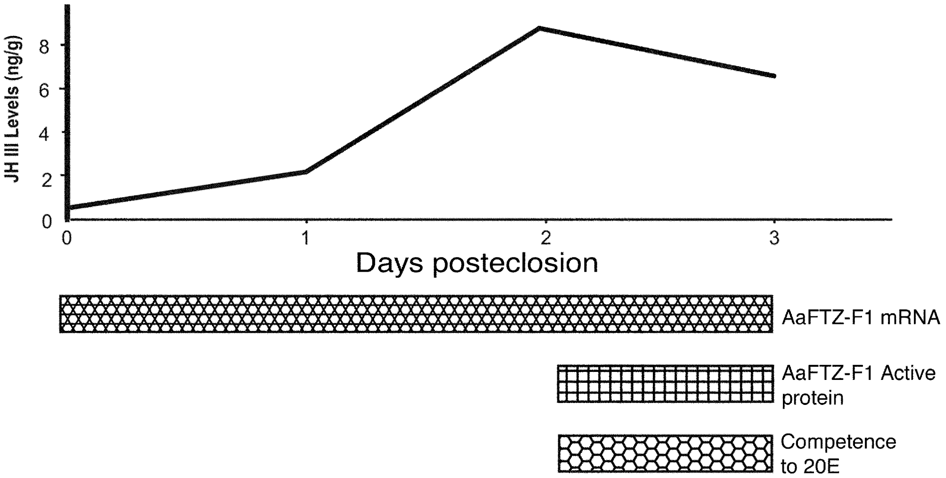

**FIGURE 2** βFTZ-F1, the orphan nuclear factor, is implicated as a competence factor for stage-specific response to ecdysteroid in the mosquito fat body. The *AaFTZ-F1* mRNA is present at the late pupal and previtellogenic stages of newly eclosed females; however, the appearance of active *AaFTZ-F1* factor coincides with the onset of competence for 20E response. Based on Li *et al.* (2000); reprinted from Raikhel *et al.* (2002), with permission.

isoforms are differentially expressed in these tissues as well (Fig. 1). The mosquito EcR–USP heterodimer has been shown to bind to various ecdysteroid-response elements (EcREs) to modulate ecdysteroid regulation of target genes. EcREs are present in the *Vg* and *VCP* genes.

Several genes of the ecdysteroid-regulatory hierarchy are conserved between vitellogenesis in mosquitoes and metamorphosis in *Drosophila*. The *A. aegypti* homologue of the *Drosophila E75* gene, a representative of the next level in the ecdysteroid response hierarchy, is expressed in the ovary and fat body following a blood meal. Similar to *Drosophila*, there are three E75 isoforms in *A. aegypti* that are inducible by 20E. Interestingly, in the mosquito fat body, E75 transcripts show two peaks, with a small peak coinciding with the first peak of 20E. The correlation between midvitellogenic expression of the *E75* and *Vg* genes suggests that the *YPP* genes are direct targets of E75 (Fig. 1). Indeed, analysis of the *Vg* gene regulatory regions has identified an E75-binding site within a region required for high-level *Vg* expression. These findings suggest that *AaE75* mediates fat body ecdysteroid responses and that the ecdysteroid-triggered regulatory hierarchies, such as those implicated in the initiation of metamorphosis, are reiteratively utilized in the control of the reproductive ecdysteroid response.

Two isoforms of the homologue to the *Drosophila* transcription factor E74, which share a common C-terminal Ets DNA-binding domain, yet have unique N-terminal sequences, are present in the mosquito *A. aegypti*. They exhibit a high level of identity to DmE74 isoforms A and B and show structural features typical for members of the Ets transcriptional factor superfamily. Furthermore, both mosquito E74 isoforms bind to a *Drosophila* E74-binding site with the consensus motif C/AGGAA. The AaE74B transcript is induced by a blood meal-activated hormonal cascade in fat bodies and peaks at 24 h PBM, the peak of vitellogenesis (Fig. 1). In contrast, AaE74A is activated at the termination of vitellogenesis, exhibiting a peak at 36 h PBM in the fat body and at 48 h PBM in the ovary. AaE74A and AaE74B isoforms likely play different roles in the regulation of vitellogenesis in mosquitoes, as an activator and a repressor of *YPP* gene expression, respectively. The *Vg* gene regulatory portion containing an E74-binding site is required for the high level of *Vg* expression.

Analysis of the 5′-upstream regulatory region of the mosquito *Vg* gene has revealed the presence of putative binding sites for EcR–USP along with those to the early genes, E74 and E75. This suggests a complex system of regulation of this gene through a combination of direct and indirect hierarchies. The *Vg* gene contains a functionally active EcRE that binds the heterodimer EcR–USP. A direct repeat with a 1 bp spacer (DR-1) with the sequence AGGCCAaTGGTCG is the major part of the EcRE in the *Vg* gene. Thus, the *A. aegypti Vg* gene is the target of a direct and indirect regulation by 20E (Fig. 3).

## C. Molecular Nature of Previtellogenic Arrest

Anautogeny is a fundamental phenomenon underlying the vectorial capacity of mosquitoes. It involves numerous adaptations, from host-seeking behavior to a complete repression of egg maturation prior to a blood meal. An important adaptation for anautogeny is the establishment of previtellogenic developmental arrest (the state of arrest) preventing the activation of *YPP* genes in previtellogenic competent females prior to blood feeding. In *A. aegypti*, both AaEcR and AaUSP proteins are abundant in the nuclei of the previtellogenic female fat body at the state of arrest; however, the EcR–USP heterodimer capable of binding to the specific EcREs is barely detectable in these nuclei. Studies have shown that the ecdysteroid

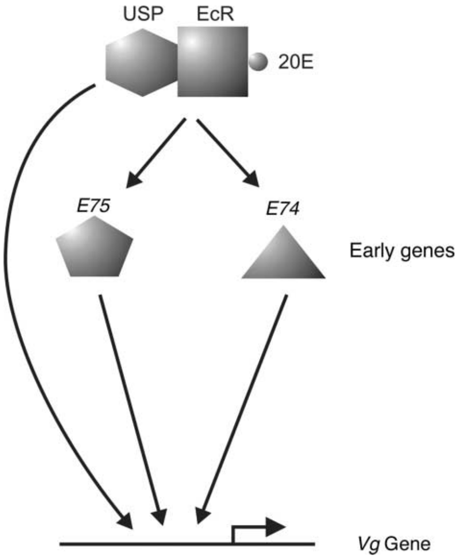

**FIGURE 3** Direct and indirect regulation of the yolk protein precursor gene *Vg* by 20-hydroxyecdysone in the mosquito fat body. After binding 20E, the EcR–USP heterodimer activates the early genes *E74* and *E75* and directly acts on the *Vg* gene, allowing its expression. In turn, the products of the *E74* and *E75* genes act as powerful activators of *Vg* gene transcription. Based on Kokoza *et al.* (2001) and Martin *et al.* (2001); reprinted from Raikhel *et al.* (2002), with permission.

receptor is a primary target of the 20E signaling modulation in mosquito target tissues at the state of arrest. A possible mechanism through which the formation of ecdysteroid receptor activity can be regulated is the competitive binding of other factors to either EcR or USP. Indeed, at this stage, AaUSP exists as a heterodimer with the orphan nuclear receptor AHR38. AHR38, the mosquito homologue of *Drosophila* DHR38 and vertebrate NGFI-B (nerve growth factor-induced protein B) orphan receptors, is a repressor that disrupts the specific DNA binding of the ecdysteroid receptor and interacts strongly with AaUSP. However, in the presence of $10^{-6}$ M 20E, EcR can efficiently displace AHR38 and form an active heterodimer with USP, as occurs after a blood meal (Fig. 4).

## D. Structure and Function of Regulatory Regions of Mosquito Yolk Protein Genes

Transcriptional activation of hormonally controlled, tissue-specific genes involves interactions of sequence-specific transcription factors with enhancer/promoter elements of these genes. Synergistic involvement of a number of regulatory factors governs the fat body-specific expression of the *Aedes Vg* gene. The 5′-regulatory region of the gene consists of three modules required for its blood meal activation and high-level expression. *Drosophila* and *Aedes* transformation and DNA-binding assays have been used to identify *cis*-regulatory sites in the *Vg* gene regulatory region, responsible for stage- and fat body-specific activation of this gene via a blood meal-triggered cascade. These analyses revealed three regulatory regions in the 2.1 kb upstream portion of the *Vg* gene that are sufficient to bring about the characteristic pattern of *Vg* gene expression (Fig. 5). The proximal region, adjacent to the basal transcription start site, contains binding sites to several transcription factors: EcR–USP, GATA (GATA-binding transcription factor), C/EBP (CAAT-binding protein), and HNF3/fkh (hepatocyte nuclear factor 3/forkhead transcription factor). This region is required for correct tissue- and stage-specific expression. It appears that a combinatorial action of these transcription factors is essential to bring about fat body-specific expression. EcR–USP acts as a timer, allowing the gene to be turned on. However, the level of expression driven by this regulatory region is low. Analysis of the mosquito *Vg* gene has revealed that the *Vg* 5′-regulatory region contains a functional ecdysteroid-responsive element (VgEcRE) that is necessary to confer responsiveness to 20E. VgEcRE is directly bound by EcR–USP produced *in vitro* or from extracts of vitellogenic fat body nuclei. The binding intensity of the EcR–USP–EcRE complex from nuclear extracts corresponds to the levels of ecdysteroids and of the Vg transcript during the vitellogenic cycle. However, given the modest level of 20E-dependent activation, it is likely that the EcR–USP receptor acts synergistically with other transcription factors to bring about the high level of *Vg* gene expression. The median region carries the sites for early gene factors E74 and E75. It is responsible for a stage-specific hormonal enhancement of *Vg* expression. The addition of this region increases the expression of the gene in a hormonally controlled manner. Finally, the distal region of the 2.1 kb upstream portion of the *Vg* gene is characterized by multiple response elements for the transcription factor GATA. In transgenic experiments utilizing both *Aedes* and *Drosophila*, this GATA-rich region has been found to be required for extremely high expression levels characteristic of the *Vg* gene (Fig. 5).

In the mosquito, VCP is the second most abundant YPP. The *VCP* gene is expressed in synchrony with the *Vg* gene: both their transcripts appear within 1 h PBM, reach maximal levels at 24 h

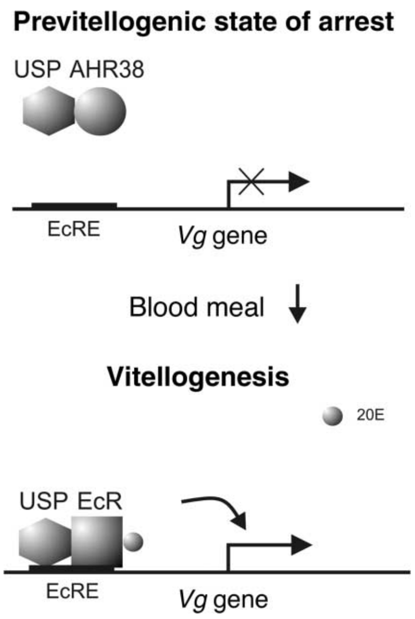

**FIGURE 4** The ecdysteroid receptor is a primary target of the 20E signaling modulation in mosquito target tissues at the state of arrest. At the previtellogenic state of arrest, AaUSP exists as a heterodimer with the repressor AHR38, which prevents the formation of the functional ecdysteroid receptor. After a blood meal, EcR displaces AHR38 and forms a functional heterodimer with USP that is capable of activating ecdysteroid-regulated genes. Based on Zhu *et al.* (2000); reprinted from Raikhel *et al.* (2002), with permission.

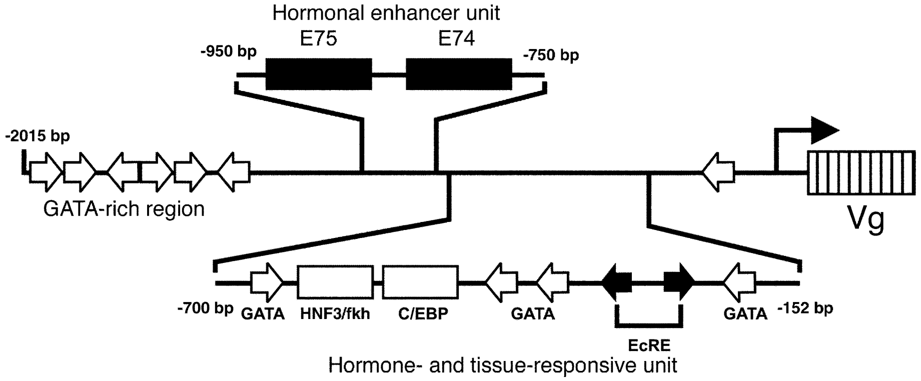

**FIGURE 5** The regulatory regions of the *Aedes aegypti Vg* gene. Numbers refer to nucleotide positions relative to the transcription start site. C/EBP, response element of C/EBP transcription factor; EcRE, ecdysteroid-response element; E74 and E75, response elements for respective early gene products of the ecdysone hierarchy; GATA, response element for GATA transcription factor; HNF3/fkh, response element for HNF3/forkhead factor; Vg, coding region of the *Vg* gene. Reprinted from Raikhel *et al.* (2002), with permission.

PBM, and rapidly decline to background levels by 36 h PMB (Fig. 1). 20-Hydroxyecdysone activates both these genes in fat body organ culture and in *in vitro* cell transfection assays. Furthermore, the same concentration of 20E ($10^{-6}$ M) is required for maximal activation of both these genes. In both genes, most of the putative regulatory elements are located in the 2 kb upstream region of the transcription initiation site. Comparative analyses have revealed conservation of putative binding sites for transcription factors responsible for tissue- and stage-specific expression in the putative regulatory regions of both genes. Both genes contained sites for the EcRE and for the early ecdysone-regulatory gene E74. There are also similar tissue-specific binding sites including those for GATA, C/EBP, and HNF3/fkh.

Another level in the regulation of *YPP* gene expression in the mosquito fat body is provided by the control of transcription factors themselves. Levels of transcripts and active factors critical for transcription of *YPP* genes such as EcR, USP, E74, E75, and GATA are greatly enhanced during the vitellogenic cycle and are themselves targets of the blood meal-mediated regulatory cascade. This additional control provides yet another mechanism for the amplification of *YPP* gene expression levels.

## III. MOLECULAR ENDOCRINOLOGY OF FRUIT FLY VITELLOGENESIS

The control of vitellogenesis in higher dipteran flies has been extensively studied in several species. The precise hormonal regulatory mechanisms depend upon whether the species lays eggs continuously or in batches. In the former case, as exemplified by the extensive studies on the model organism, the fruit fly *D. melanogaster*, each individual egg chamber differentiates and its progress through vitellogenesis is modulated by hormonal signals, thus linking its development to environmental factors. This has been studied using molecular, genetic, developmental, and physiological approaches. In species laying eggs in batches, the maturation of a synchronous group of oocytes is controlled by changes in hormone levels. Probably the best studied species in this category is the housefly, *Musca domestica*. Many aspects of the regulation of vitellogenesis in the housefly are quite similar to those described for the mosquito, *A. aegypti*, in the previous section. JH acts at an early stage to prime the fat body for yolk protein synthesis and ensure that the oocytes are arrested in a previtellogenic stage. Only after a protein feed does the fat body start to synthesize yolk, and vitellogenesis commences in the ovary as endocytosis of yolk begins. This phase is controlled by the circulatory levels of ecdysone in the hemolymph, which is produced by the egg chambers, and some time afterward the fat body shuts down yolk protein synthesis and the eggs are completed, ready to be laid.

In the fruit fly, the fat body matures and differentiates at eclosion, again under the control of JH, but it immediately begins to synthesize yolk proteins and secrete them into the hemolymph. Oocytes progress into vitellogenesis at once and begin to produce mature eggs, which are fertilized and laid continuously. However, if the fly does not mate or does not have available food, these "ready to lay" eggs are retained and oocytes arrest at a previtellogenic stage and do not enter vitellogenesis. The fat body will gradually produce less yolk proteins, though it rarely ceases production completely, as in the housefly and mosquito. Whether an oocyte progresses into vitellogenesis or arrests is controlled by the balance of JH and ecdysone. Thus, the fruit fly does relate egg production to feeding but the mechanism is not by producing batches of eggs after a feed but rather by feeding and laying eggs continuously unless there is a reason not to do so, such as lack of sperm, lack of food, or crowded conditions. There must therefore be subtle mechanisms that relate egg production rates to food availability in *Drosophila*.

There are several *yolk protein* genes that encode a small family of related yolk proteins in most fly species. The genes have been cloned in *D. melanogaster*, *M. domestica, Calliphora erythrocephala*, *Calliphora*, *Ceratitus capitata*, and other species and are well conserved throughout evolution. These genes have functions that are similar to those of the vitellogenins of mosquitoes, although they are not closely related genes in evolutionary terms. Yolk proteins are made in the fat body, as in the mosquito, but there is a second site of synthesis in the follicle cells, which are associated with the oocyte in developing egg chambers. As far as is known, the gene products, the yolk proteins (YPs), are the same for each tissue. The proteins are approximately 45 kDa in size and are produced with a small leader sequence that is cleaved prior to secretion. Those made in the follicle cells are secreted unidirectionally toward the oocyte. Those made in the fat body travel to the ovary via the hemolymph.

The regulation of *yp* gene expression seems to be quite different in the two sites of synthesis. In the fat body, ecdysteroids can stimulate YP synthesis in males and up-regulate it in females and JH also increases YP synthesis in the fat body. However, these effects are rather modest in *Drosophila* compared to the dramatic effects in the mosquito. In the ovary, however, JH and 20E seem to be antagonistic, with JH promoting YP synthesis and oocyte progression and ecdysone tending to lead to apoptosis if it is present at high levels.

Many of the response genes that have been shown to be crucial in the mosquito and that are homologues of *Drosophila* genes used to regulate the response of the organism to ecdysone during metamorphosis have not been studied in *Drosophila* with respect to yolk protein synthesis in the fat body. This is largely because in normal, mated, well-fed females the genes are constitutively active rather than regulated in response to a blood meal; thus, the normal control seems secondary to their sex-specific activation in females. However, the same set of genes including *BR-C*, *E74*, *E75*, and the *Ecdysone receptor EcR* have all been shown to be crucial for oocyte development and the progression of vitellogenesis that leads to a mature egg. They are needed in the egg chambers themselves to decide how many eggs to develop in relation to environmental conditions (Fig. 6).

When flies are provided with a starvation diet rather than normal food, egg chambers stop developing prior to the vitellogenic period. Those already in the stages of active yolk accumulation are triggered to undergo apoptosis. This effect can be mimicked by injection of 20E into females or can be rescued by treatment with JH. When nutritionally challenged, the spatial and temporal expression patterns of *BR-C*, *E74*, *E75*, and *yp* mRNAs are altered, suggesting that the decision to undergo vitellogenesis or apoptosis is controlled by the same set of genes that regulate expression of the *yp* genes in the fat body of mosquitoes and perhaps *Drosophila*.

Although sites where ecdysone exerts its effects on *yp* gene expression in the fat body have been mapped, it is not clear how much of this effect is a direct action via binding of the EcR and how much is due to downstream genes such as *BR-C*, *E74*, and *E75*.

In the mosquito, three other yolk protein precursors are important in vitellogenesis, namely, Lp, VCP, and VCB. Lipophorin activity has been purified from *D. melanogaster*, but a gene was not identified. Searches of the *Drosophila* genome show that there are two genes that encode low-density lipoprotein receptors that could potentially be lipophorin equivalents and transport lipids (genes *CG4861* and *CG13257*) but they have not been studied in the context of fruit fly vitellogenesis. The VCP of *A. aegypti* has two related genes in *D. melanogaster*; these are a serine carboxypeptidase (gene *BG:DS00365.3*) and carboxypeptidase C (gene *CG4572*). *Drosophila* also has a cathepsin B-encoding gene (gene *CG10992*) but it is not clear whether this encodes the homologue of VCB. None of these genes have been studied during fruit fly oogenesis.

## A. Structure and Function of Regulatory Regions of Fruit Fly Yolk Protein Genes

Rather little is known about the *cis*-acting regions of the *yp* genes needed for hormonal regulation in any

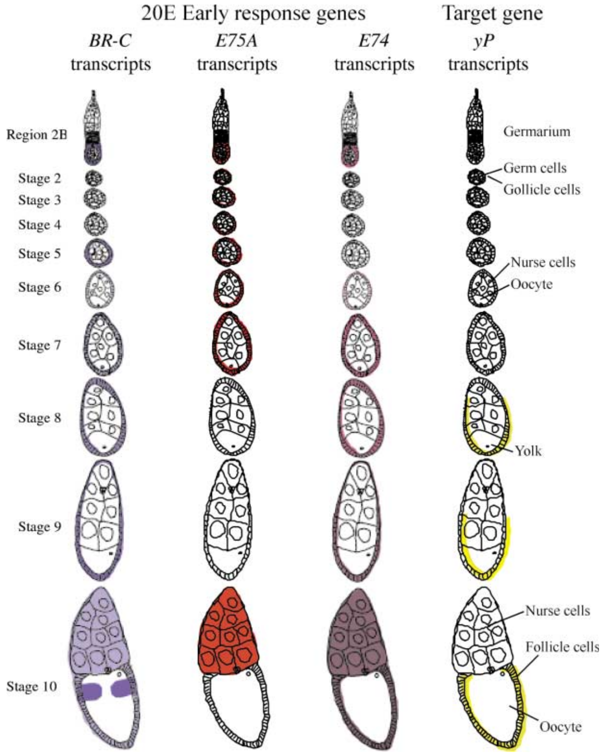

**FIGURE 6** Ovariole of *Drosophila* indicating *BR-C*, *E75A*, *E74*, and *yp* expression patterns during oocyte development. The expression pattern of several key ecdysone-response genes during oogenesis is shown. Stem cells divide in the germarium to produce a cluster of 16 germ cells. One becomes the oocyte and the other 15 become the polyploid nurse cells that produce macromolecules needed for oogenesis. Oogenesis is divided into 14 morphologically distinct stages as the oocyte gradually matures. Yolk is produced in the follicle cells from stage 8 to stage 11. It is produced mostly in the columnar follicle cells that migrate to surround the oocyte. *yp* gene expression is shown. The ecdysone-response genes *BR-C*, *E75A*, and *E74* are also expressed in follicle cells at certain stages of oogenesis. The regulation of *yp* genes is certainly not their only function in the ovary and *yp* genes do not always respond to their expression since *BR-C*, *E75*, and *E74* are also expressed in the nurse cells late in oogenesis when *yp* genes are not expressed. They are also expressed much earlier in follicle cells than the *yp* genes. It is likely then that the genes interact with other tissue-specific factors in the follicle cells to modulate the progress of oogenesis, including the regulation of the *yp* genes. From Jun Terashima and Mary Bownes, unpublished work, with permission.

fly species. The most information pertains to *D. melanogaster*, in which several DNA sequences can confer ecdysone-inducible *yp* gene expression in males in response to injection of 20E. These are located 5′, 3′, and within the coding sequences of the *yp* genes. These regions do contain the putative EcRE sequences that have been shown to bind and respond to the EcRE–USP heterodimer and lead to the transcription of other genes in metamorphosis and of the vitellogenin genes in the mosquito. JH also up-regulates *yp* gene expression in the fat body, but those sites could not be mapped to flanking sequences and JH may in fact achieve its effect by affecting the stability of *yp* RNA.

Although in *Drosophila* the study of the molecular control of *yolk protein* gene transcription is less well understood in terms of hormonal regulation, a great deal is known about how it is that the genes are expressed only in the female and not in the male. A pathway of sex determination genes controls the male and female sexual characteristics, including the expression of the *yolk protein* genes. Mutations in these genes lead to sexual transformations of males to females and vice versa and to intersex flies. The most important gene for *yp* control is *dsx*. The *dsx* gene encodes different proteins in male and female adults as a result of alternate transcript splicing. The two proteins generated have similar DNA-binding domains but different carboxy-terminal extensions. Although the male and female proteins (DSXF and DSXM) bind to the same DNA sequences *in vitro*; *in vivo* DSXF and DSXM are essential for maintaining *yp* transcription in the female fat body and repressing it in males. YP expression in the ovarian follicle cells is not dependent on the sex determination pathway in the adult but depends on ovarian tissue-specific factors, which at the moment are poorly understood. It is likely that 20E is a key to their correct expression in the ovarian follicle cells.

A number of factors that interact with *cis*-acting sequences close to the *yp* genes have been identified by *in vitro* binding studies. Some of these *cis*-acting sites have been shown to be crucial for correct tissue-, sex-, and hormone-regulated gene expression by creating transgenic flies with normal or mutated response elements fused to reporter genes that can be monitored *in vivo*. There are also other genes that modify sexual differentiation in *Drosophila* that have been shown to be crucial for normal yolk protein gene expression.

There is overlap in the binding sites between DSX and some of these tissue-specific regulatory proteins, which suggests that it is a complex interaction between DNA-binding sites and multiple proteins that leads to the production of yolk being a female fat body-specific trait. The *dsx* gene is well conserved in different flies such as the housefly and Mediterranean fruit fly. It is alternately spliced in males and females in these species; thus, this mechanism of regulation is likely to be well conserved throughout evolution.

Somehow, the hormonal regulation is superimposed on this mechanism and can in fact overrule it in *Drosophila*, since the injection of 20E into males transiently overrides the repression by DSXM and leads to the expression of *yp* genes in the male fat body for a few hours. Somewhat surprisingly, however, the *cis*-acting sequences 5′ of the *yp* genes of *Calliphora* and *Musca* are not regulated in a sex-specific manner when they are transformed into *Drosophila*, despite having DNA-binding sites to which the *Drosophila* DSX proteins bind *in vitro*. It seems that in those species that lay their eggs in batches, the hormonal regulation is more important in the adult, and the *Drosophila* hormones present in the hemolymph in combination with the *Drosophila* DSX proteins lead to expression in both sexes. More detailed studies on the hormonal control of *yp* gene expression at the molecular level are needed in *D. melanogaster*, *M. domestica*, and *C. capitata* in order to understand how vitellogenesis is controlled in flies.

## Glossary

**ecdysone** A precursor form of the steroid hormone 20-hydroxyecdysone.

**ecdysone receptor** Insect-specific nuclear receptor that mediates the action of the steroid hormone 20-hydroxyecdysone.

**fat body** Insect metabolic tissue analogous to the vertebrate liver and the adipose tissue combined.

**20-hydroxyecdysone** Active form of the steroid hormone found in insects and crustaceans.

**juvenile hormone** Insect-specific hormone of a sesquiterpenoid nature.

**lipophorin** Lipid carrier protein.

**ultraspiracle** The insect homologue of vertebrate retinoid X receptor, an obligatory partner of the ecdysone receptor.

**vitellin** A storage form of the major yolk protein.

**vitellogenesis** A major stage during egg maturation in egg-laying animals during which yolk protein precursors are synthesized and accumulate in developing oocytes.

**vitellogenin** A precursor form of the major yolk protein.

**yolk protein** Major yolk protein in flies.

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysone Secretion in Lepidopteran Insects • Ecdysteroidogenic Pathway • Ecdysteroid Receptors (EcR/USP) • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormone Biosynthesis**

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**prothoracic glands** Paired organs in the prothorax (region between the head and thorax) of insects, which are the major site of ecdysteroid synthesis in insect larvae.

**sterols** Compounds having three 6-sided carbon rings, one 5-sided carbon ring, and a side chain, e.g., cholesterol.

## See Also the Following Articles

**Ecdysteroid Action in Insect Reproduction • Ecdysteroid Receptors (EcR/USP) • Ecdysteroids, Overview • Insect Endocrine System**

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# Ecdysteroid Receptors (EcR/USP)

Margarethe Spindler-Barth and
Klaus-Dieter Spindler
*University of Ulm, Germany*

I. MOLECULAR CHARACTERIZATION OF EcR
II. MOLECULAR CHARACTERIZATION OF USP
III. GENERAL COMMENTS
IV. APPLICATION AND PERSPECTIVES

**The heterodimer of the ligand-dependent transcription factors ecdysteroid receptor (EcR) and USP is considered as functional ecdysteroid receptor that coordinates arthropod development and metabolism and modifies the expression of a multitude of different genes in a tissue- and time-specific manner. As is typical for steroid hormone receptors in general, the different functions of this receptor are associated with defined molecular domains, which are modified by intra- and intermolecular interactions. Both EcR and USP can dimerize with several other transcription factors, but homodimerization has been described for both partners as well. Isoforms of EcR and USP are detected in various species and are engaged in different functional roles. Species-specific variations demonstrate that different solutions have evolved within arthropods. The ecdysteroid receptor is an important target for insecticides and is also used as a ligand-controlled switch in heterologous systems for gene expression.**

## I. MOLECULAR CHARACTERIZATION OF EcR

### A. General Features

Thus far, the ecdysteroid receptor (EcR) has been sequenced from 12 insects, a crustacean, and a tick. The common features of EcRs include a highly conserved two-zinc-finger DNA-binding domain, which shares a considerable level of sequence identity with vertebrate steroid hormone receptors (Table 1), an A/B domain, which in most instances is responsible for transactivation, and a D–F domain, which, in addition to binding ligand, mediates various other functions (Fig. 1). The degree of sequence identity that EcRs share with vertebrate steroid receptors is considerably lower than that within arthropods.

**TABLE 1** Sequence Identities of EcRs and Comparison with Human Nuclear Receptors

| | A/B domain | DNA-binding domain | D domain | E domain |
|---|---|---|---|---|
| Diptera EcR | 21[a], 44–49 | 95–96 | 50[a], 64–82 | 74[a], 87–95 |
| Lepidoptera EcR | 31–48 | 93–95 | 55–62 | 70–72 |
| EcRs of other arthropods | 25–44 | 86–89 | 52–62 | 58–70 |
| Human nuclear receptors | | | | |
| PPAR α | 15 | 56 | 14 | 19 |
| PPAR β | 19 | 55 | 16 | 23 |
| PPAR γ | 5 | 53 | 15 | 20 |
| TR α | 17 | 59 | 18 | 23 |
| TR β | 4 | 59 | 22 | 27 |
| VDR | 21 | 57 | 20 | 27 |

*Note*. The *Drosophila melanogaster* EcR isoform B is set at 100%. Analysis was performed using ClustalW. PPAR, peroxisome proliferation-activated receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor.

[a] Values for Chironomus tentans.

The EcR of *Chironomus tentans* shows pronounced differences compared to the EcRs of all other dipterans. As is also typical for vertebrate nuclear receptors, the hinge region D and the A/B domains are highly diverse.

## B. Regulation of Expression

Expression and isoform patterns vary in a time- and tissue-specific manner and are regulated by ecdysteroids and juvenile hormone. This was investigated thoroughly in *Manduca sexta* but was also confirmed for other species. The highly complex gene-specific modulation of expression by EcR and USP is the basis for the hierarchic hormone-dependent gene cascades during development.

## C. Nuclear Transport, Nuclear Localization, and DNA Binding

Using biochemical as well as immunocytochemical techniques, localization of EcR in the cytoplasm and nucleus has been demonstrated, but the underlying mechanisms of transport and nuclear localization signals have not yet been identified. Hormone response elements (HREs; receptor-binding motifs in the promoter region) have been characterized. Both naturally occurring and synthetic HREs are either palindromes or direct repeats with the consensus sequence AGGTCA, as tested by gel mobility shift assays, determination of affinity constants, and transactivation assays. Half-sites are not functional and do not bind EcR or EcR/USP, although their presence in the promoter region of ecdysteroid-dependent genes has been demonstrated. Not every ecdysteroid response element is active *in vivo* but the cellular context seems to determine the functionality of a certain response element.

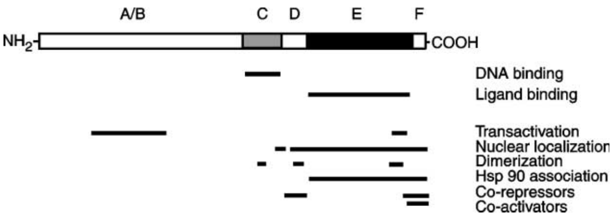

**FIGURE 1** Generalized scheme of nuclear receptors and their functional domains.

## D. Ligand Binding

The X-ray structure of the ligand-binding pocket of EcR has not been available until now. However, computer modeling of the two- and three-dimensional structures reveals the same general architecture as that determined for vertebrate steroid hormone receptors, which consist of 11–12 α-helices and a β-sheet arranged as anti-parallel sandwich. The dimerization partner has a profound influence and either enables or enhances ligand binding considerably. Ligand binding changes the interaction with co-modulators, enhances DNA binding, and promotes dimerization. As was determined for vertebrate nuclear receptors, it was concluded that these effects were due to a conformational change in the ligand-binding pocket, especially in helix 12.

## E. Dimerization

Three different dimerization interfaces have been described for EcR. As in vertebrate nuclear receptors, a conserved region is present in the zinc-finger domain, which mediates DNA-dependent dimerization. This was confirmed for vertebrate receptors by mutational analysis. Due to the high level of sequence identity, the same function also seems likely for EcR. An additional dimerization site in the hinge region was identified in *Choristoneura fumiferana* EcR by analysis of truncated versions of the receptor. A highly conserved region is present in arthropod receptors adjacent to the C-terminal end of the C domain. The T-box, which mediates dimerization, is located at the same position in vertebrate receptors. It remains to be clarified whether or not the same region is also involved in dimerization in arthropod receptors.

As shown for vertebrate nuclear receptors, ligand-controlled dimerization in the E domain is mediated primarily by helix 10. This has also been confirmed for EcR by mutational analysis using the ligand-binding pocket of *Drosophila melanogaster* EcR expressed in yeast cells. Heterodimerization between EcR and USP seems to be most common; however, interaction with other partners, such as seven-up (a homologue of vertebrate COUP-TF), can contribute to the diversity of ecdysteroid-regulated processes.

Whether or not all three dimerization sites are involved simultaneously is still unknown, and the question of dimerization partners remains unanswered. For example, it is reasonable to assume that binding to direct repeats or palindromic HREs has a profound influence on the type of dimerization, as has been demonstrated for vertebrate nuclear receptors.

### F. Transactivation, Receptor Isoforms, and Co-modulators

The A/B domain, which harbors the ligand-independent transactivation domain (AF1), varies considerably between different arthropods, but also varies between isoforms within the same species. An indication of specific functions associated with a certain isoform is given by the different expression profiles in various tissues during development. Mutational analysis of EcR from *Drosophila* revealed that receptor isoforms cannot replace one another.

Depending on the species, the transactivation domain can be either activating (e.g., *Drosophila*) or repressing (e.g., *Chironomus*), as has been demonstrated using chimeric receptors.

For *Drosophila* imaginal discs, it has been shown that the ecdysteroid receptor exerts repressing functions in the absence of USP and activates gene expression in its presence. Ligand-dependent transactivation is mediated mainly by helix 12 of the E domain. The change in position of helix 12 following hormone binding seems to be a prerequisite for ligand-dependent activation and allows the release of co-repressors and the binding of co-activators. Some co-modulators, such as *alien*, are quite conserved between vertebrates and invertebrates, whereas others, such as *Smrter*, are insect specific. *Alien* and *Smrter* bind in transactivation domain AF2 (helix 12) in the signature motif between helix 3 and helix 4 of the ligand-binding domain.

## II. MOLECULAR CHARACTERIZATION OF USP

### A. Expression

The pattern of USP expression does not always follow in parallel with that of EcR, which suggests a possible separate functional role in addition to heterodimerization with EcR. This is further substantiated by specific effects of USP mutants during development. The discrepancies in specific mRNA titer and USP protein concentration are indicative of additional posttranscriptional control. As with EcR, different USP isoforms have been described for several insect species. The only exception is *Drosophila*, for which only one isoform has been described thus far.

### B. Nuclear Transport and DNA Binding

USP is transported into the nucleus independent of the presence of EcR. *In vivo* and *in vitro* experiments have clearly demonstrated that USP also binds to specific HREs in the absence of EcR. In contrast to the heterodimer, direct repeats are preferred over palindromic structures.

### C. Ligand Binding

USP is considered an orphan receptor since a specific ligand has not yet been identified. However, phospholipids are bound in the unusually large and hydrophobic ligand-binding pocket, as determined by X-ray studies. The lipid occupies only part of the ligand-binding pocket and leaves additional space for another presumably more specifically bound ligand. Juvenile hormone was proposed as a possible ligand but no definite proof has been made available yet.

The high level of sequence identity shared with human retinoic acid X receptor (RXR) (Table 2) suggests that USP can also be replaced functionally by RXR. This is in fact possible, when EcR is expressed in vertebrate cells. The ligand for RXR, retinoic acid, can also replace juvenile hormone functionally in crustaceans.

TABLE 2 **Sequence Identities of USPs and Comparison with Human Nuclear Receptors**

| | A/B domain | DNA-binding domain | D domain | E domain |
|---|---|---|---|---|
| Diptera USP | 26–50 | 90–92 | 46–70 | 49–72 |
| Lepidoptera USP | 20–21 | 95–96 | 65–70 | 44–45 |
| USPs of other arthropods | 23–30 | 90–93 | 57–70 | 38–48 |
| Human nuclear receptors | | | | |
| RXR α | 6 | 86 | 50 | 51 |
| RXR β | 16 | 83 | 55 | 57 |
| RXR γ | 7 | 83 | 38 | 47 |
| ER α | 11 | 53 | 23 | 23 |

*Note*. The *Drosophila melanogaster* USP is set at 100%. Analysis was performed using ClustalW. RXR, retinoic X receptor; ER, estradiol receptor.

The sequence identity of the ligand-binding pocket is rather low not only compared to vertebrate receptors, but also within arthropods and even within Diptera or Lepidoptera. This may indicate either that no ligand, or no highly specific ligand, is necessary for USP function or that different ligands may be used in different species.

X-ray analysis revealed that the ligand-binding pocket of USP has a unique architecture. Helix 12 is fixed by hydrophobic interactions in a groove between helix 1 and helix 3 of USP even in the absence of a specific ligand. In vertebrate receptors, this configuration is observed only after binding of an antagonist. Interestingly, the three-dimensional structure of the ligand-binding domain of USP is more similar to estrogen receptor (ER) than to RXR, although the sequence identity to ER is considerably lower (Table 2). This finding demonstrates that conclusions based on alignments of different receptor sequences should be considered cautiously and that the two- or three-dimensional architecture must be taken into account.

## D. Transactivation and Isoforms

In most arthropods investigated thus far, with the exception of *Drosophila*, isoforms with different A/B domains have been found. For *Drosophila*, it was shown that DNA binding of USP is necessary for repression of transcription, whereas for activation only the ligand-binding domain is required.

For *Choristoneura* USPs, it was shown that the A/B domain of USP is essential for basal transactivation of the EcR/USP complex in yeast cells. This confirms that dimerization of EcR/USP occurs in the absence of ligand as shown previously by electrophoretic mobility shift assay experiments with EcR/USP. These data also demonstrate that the A/B domains of both nuclear receptors are necessary for correct transcriptional regulation.

# III. GENERAL COMMENTS

## A. Posttranslational Modifications

Like nuclear receptors in general, EcR and USP occur in several isotypes with different degrees of phosphorylation. Although the specific phosphorylated amino acids have not yet been identified, phosphorylation is of functional importance, e.g., for the regulation of ecdysteroid synthesis. Numerous putative phosphorylation sites are present in the A/B and E domains of both nuclear receptors. In the DNA-binding domain, phosphorylation sites are present only in USP.

## B. Interdomain Signaling

As outlined earlier for vertebrate nuclear receptors, the function of individual receptor domains is modulated by interdomain signaling. The most prominent example is the stimulation of DNA binding of EcR/USP by ecdysteroids. The degree to which different HREs are influenced by hormones varies and is more pronounced for palindromic HREs than for direct repeats. Another example is the inhibition of ligand-dependent activation, which is mediated by helix 12 in the E domain and by the A/B region of EcR.

## C. Species-Specific Differences

Individual receptor domains have presumably evolved separately within arthropods.

The hinge region of EcR is quite conserved within Lepidoptera and within Diptera, but there are pronounced differences between the taxa, which might contribute to their different sensitivities to hormone mimics, e.g., diacylhydrazines, since the C-terminal segment of the hinge region seems to be important for ligand binding.

In contrast to EcR from other species, e.g., *Chironomus*, EcR from *Drosophila* is capable of binding ligand in the absence of its heterodimerization partner USP. This offers an additional pathway for hormonal regulation by EcR alone. However, heterodimerization also enhances ligand binding to EcR considerably in *Drosophila*.

There are glycine stretches in the ligand-binding domain of *Drosophila* EcR that are absent in EcRs from other species.

The hydrophobic F domain, which is especially extended in *Drosophila*, is highly variable among insects and its contribution to receptor function is unclear.

Although helix 12 of USP is of functional importance for ligand binding to EcR in *Drosophila*, the amino acid sequences vary considerably among insects. Calculation of the two-dimensional structure revealed that even in *Bombyx mori* there is a helix 12 despite the low level of sequence identity. Only one leucine out of nine amino acids is conserved.

## IV. APPLICATION AND PERSPECTIVES

The EcR ligand-binding domain of the ecdysteroid receptor has been characterized extensively, since molting hormone mimics are used successfully as pesticides with low vertebrate toxicity. Comparative aspects of ligand binding not only are of interest for target specificity of potential insecticides, but also provide new insight into the evolution of hormonal signal transduction pathways.

The ecdysteroid receptor is used as gene switch for ligand-dependent regulation of heterologous protein expression. The efficiency of the ecdysteroid-inducible expression system can be enhanced considerably using receptor chimeras of different arthropod species or combinations of insect and vertebrate receptor domains. Stable ecdysone-inducible systems, such as that described recently for mammalian colon cancer cells, are an important step toward the possible utilization of such systems in gene therapy.

## Glossary

| | |
|---|---|
| ***Bombyx mori*** | Silkworm. |
| ***Chironomus tentans*** | Nonbiting midge. |
| ***Choristoneura fumiferana*** | Spruce budworm. |
| **co-modulator** | Substance that interacts with a nuclear receptor and modifies transcriptional activity. |
| ***Drosophila melanogaster*** | Fruit fly. |
| **ecdysteroid receptor** | Nuclear receptor for ecdysteroids. |
| **ecdysteroids** | Generic name of a class of steroid hormones that are mandatory for molting. |
| **HRE** | Hormone response element that specifically binds nuclear receptor. |
| ***Manduca sexta*** | Tobacco hornworm; member of the Lepidoptera. |
| **receptor isoforms** | Variants of a receptor. |
| **USP** | Ultraspiracle; heterodimerization partner of ecdysteroid receptor. Insect orthologue of vertebrate retinoic acid X receptor. |

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysone Secretion in Lepidopteran Insects • Ecdysteroid Action in Insect Reproduction • Ecdysteroidogenic Pathway • Ecdysteroids, Overview • Insect Endocrine System**

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# Ecdysteroidogenic Pathway

Huw H. Rees
*University of Liverpool*

I. INTRODUCTION
II. SIDE CHAIN DEALKYLATION OF PLANT STEROLS
III. ECDYSTEROID BIOSYNTHESIS
IV. ECDYSONE 20-MONOOXYGENASE
V. SUMMARY

**In common with many other classes of arthropods, insects cannot synthesize sterols *de novo* from small molecules and must obtain them from the diet. Although carnivorous species obtain cholesterol directly from the diet, many, but not all, investigated phytophagous species, as well as omnivorous species, can dealkylate $C_{28}$ and $C_{29}$ phytosterols to yield cholesterol. In immature stages of insects, the prothoracic glands are the major site of ecdysteroid synthesis, whereas in adults, synthesis of the hormones occurs in the ovarian follicle cells and in testes. Although 7-dehydrocholesterol is an early intermediate in ecdysone biosynthesis, subsequent early steps of the pathway are not clear. Nonetheless, there is good evidence for the intermediacy of 5β-ketodiol (2,22,25-trideoxyecdysone) or its 3-dehydro derivative. The subsequent cytochrome P450-dependent hydroxylations (at C-25, C-22, and C-2) to yield ecdysone are somewhat better understood. Ecdysone then undergoes 20-monooxygenase-catalyzed hydroxylation to yield the major active hormone, 20-hydroxyecdysone, in several peripheral tissues. A major mechanism of regulation of ecdysone biosynthesis occurs via the neuropeptide, prothoracicotropic hormone.**

## I. INTRODUCTION

Elucidation of the pathway of biosynthesis of ecdysone from cholesterol has proven difficult, because ecdysteroids, being hormones, occur in low concentrations and because of the small size of insects and of the ecdysiosynthetic tissues investigated, primarily the prothoracic glands and, to a lesser extent, the follicle cells. Consequently, intermediates in the biosynthetic pathway do not generally accumulate in sufficient quantities for identification. Early studies have depended almost entirely on incorporation of synthetic, radioactive putative intermediates. More recently, the availability of the *Drosophila melanogaster* genome sequence has provided an impetus for studies in this area, coupled with detailed studies on various *Drosophila* embryonic cuticle mutants.

## II. SIDE CHAIN DEALKYLATION OF PLANT STEROLS

Although insects, in common with other arthropods, lack the ability to synthesize sterols *de novo* from small molecules, the majority of insect species investigated require cholesterol (12), or a sterol that is convertible into cholesterol, for satisfactory growth, development, and reproduction. Whereas carnivorous insects obtain cholesterol directly from their diet, many but not all examined phytophagous species, together with omnivorous species, can obtain cholesterol or a related $C_{27}$ sterol by dealkylation in the gut of dietary plant 24-alkyl sterols such as sitosterol (1), campestrol (2), or stigmasterol (3) (Fig. 1). There is much evidence that dealkylation of the latter three predominant phytosterols occurs via the analogous pathways shown in Fig. 1 in several insect species. In each case, the initial step involves oxidation to yield a $\Delta^{24(28)}$ bond, followed by an epoxidation reaction before dealkylation of the $C_1$ or $C_2$ fragment to yield a $\Delta^{24}$ bond. In the case of stigmasterol dealkylation, an extra step is involved, reduction of the $\Delta^{22}$ bond of 22*E*-cholesta-5,22,24-trien-3β-ol (11), to furnish the common terminal intermediate, desmosterol (10). There is very little information on the enzymes involved in the dealkylation pathways (Fig. 1), but it would be envisaged that cytochrome P450 is involved at more than one step.

Some phytophagous and omnivorous insect species, e.g., certain Hymenoptera, Hemiptera, and Diptera, as well as a coleopteran, cannot dealkylate the sterol side chain. Many such species use campesterol (2) as a precursor for the $C_{28}$ ecdysteroid, makisterone A (13) (see Fig. 2).

Furthermore, identification of the $C_{29}$ ecdysteroid makisterone C (14) as a major embryonic ecdysteroid in *Dysdercus fasciatus* (Hemiptera) suggests that a $C_{29}$ steroid can serve as a molting hormone.

## III. ECDYSTEROID BIOSYNTHESIS

### A. Sites of Synthesis

The prothoracic glands are the major physiologically important source of ecdysteroid during insect

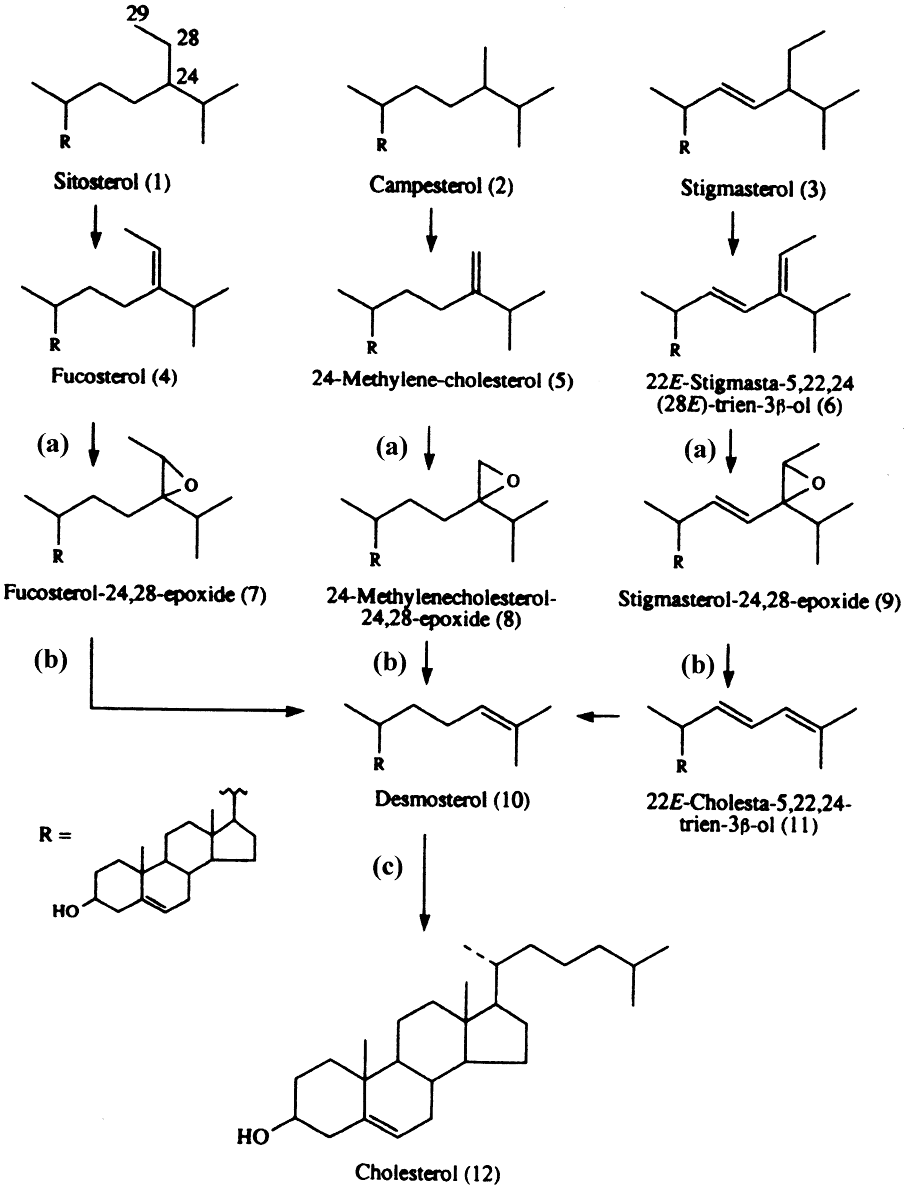
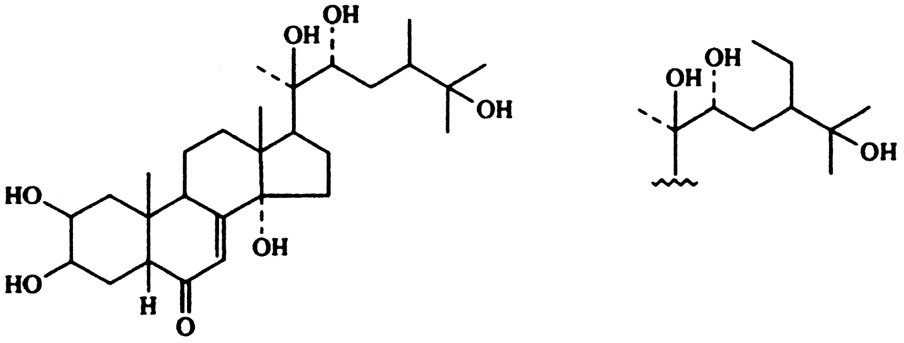

29
28
24
R
Sitosterol (1)
R
Campesterol (2)
R
Stigmasterol (3)
R
Fucosterol (4)
R
24-Methylene-cholesterol (5)
R
22E-Stigmasta-5,22,24 (28E)-trien-3β-ol (6)
(a)
O
R
Fucosterol-24,28-epoxide (7)
(a)
O
R
24-Methylenecholesterol-24,28-epoxide (8)
(a)
O
R
Stigmasterol-24,28-epoxide (9)
(b)
(b)
(b)
R
Desmosterol (10)
R
22E-Cholesta-5,22,24-trien-3β-ol (11)
R =
HO
(c)
HO
Cholesterol (12)

FIGURE 1 Pathways of side chain dealkylation of phytosterols. Dealkylation at C-24 of the major plant sterols occurs via analogous pathways. Initial oxidation produces a $\Delta^{24(28)}$ bond, followed by an epoxidation reaction, before dealkylation of the $C_1$ or $C_2$ fragment forming a $\Delta^{24}$ bond. In the case of stigmasterol dealkylation, an extra step is required to reduce the $\Delta^{22}$ bond. Finally, the common terminal intermediate, desmosterol (10), is reduced to cholesterol (12). Enzymes involved include (a) epoxidase; (b) epoxide lyase; and (c) desmosterol 24,25-reductase. Reprinted from Rees (1995), with permission.

postembryonic development. However, physiological ecdysteroid production has been demonstrated in the abdomens of some species, with epidermal synthesis being observed in certain cases. During pupal–adult development, the prothoracic glands completely (or nearly completely) disappear by programmed cell death. In adult life, not only are ecdysteroids produced by the epidermis, but by the ovarian follicle cell epithelium in females and by the testes sheath in males.

In the Crustacea, another class of arthropod that has been extensively studied, the Y-organs have been established as an ecdysiosynthetic tissue.

Whereas the product of the prothoracic glands in most insect species is ecdysone (24), in many lepidopteran species, 3-dehydroecdysone (20),



**Makisterone A**
**(13)**

**Makisterone C**
**(14)**

FIGURE 2 Formulae of makisterone A (13) and makisterone C (14). Reprinted from Rees (1995), with permission.

accompanied by varying amounts of ecdysone, is a major product of the prothoracic glands, being reduced to ecdysone by a hemolymph reductase enzyme. In a somewhat similar manner, in Crustacea, ecdysone, 3-dehydroecdysone, and 25-deoxyecdysone have been detected as products of Y-organs in various combinations, although reduction of 3-dehydroecdysone has not been detected in these cases.

## B. Early Stages

It is now clear that during transformation of cholesterol (12) into ecdysteroids, there is extensive modification of the nucleus before side chain hydroxylation, with appreciable evidence for the intermediacy of 2,22,25-trideoxyecdysone [5β-ketodiol (21); Fig. 3].

The intermediacy of 7-dehydrocholesterol (15) in ecdysteroid biosynthesis has been firmly established. Introduction of the $\Delta^7$ bond into cholesterol involves removal of the 7β- and 8β-hydrogens and is a rapid, basically irreversible reaction, catalyzed by a microsomal cytochrome P450-dependent activity. This raises the possibility of the intermediacy of 7β-hydroxycholesterol. Interestingly, a *D. melanogaster* low-ecdysteroid mutant (*woc*; *without children*) lacks the 7(8)-dehydrogenase and can be partially rescued by feeding 7-dehydrocholesterol. However, the *woc* gene codes for a novel zinc-finger protein that may function as a transcription factor, rather than the expected cytochrome P450.

Indirect evidence has suggested that transformation of 7-dehydrocholesterol (15) to trideoxyecdysone [5β-ketodiol (21)] occurs in the mitochondria. It has been postulated that the rate-limiting step in prothoracicotropic hormone-stimulated ecdysteroidogenesis in prothoracic glands is the movement of 7-dehydrocholesterol (poorly water soluble) from the endoplasmic reticulum, where it is formed, to the mitochondria, where it is oxidized to ecdysteroid. This process may well have some analogy with the adrenocorticotropic hormone-mediated control of cholesterol movement within the mammalian adrenal mitochondria and may involve uncharacterized regulatory proteins.

Our lack of understanding of the initial part of the pathway following 7-dehydrocholesterol led to its designation as the "black box." Formation of the A/B *cis*-ring junction of ecdysteroids apparently occurs early in the biosynthetic pathway. Analogous transformation of a $\Delta^5$ bond to yield a 5β-hydrogen during formation of certain steroid hormone metabolites and bile acids in vertebrates involves a 3-oxo-$\Delta^4$-steroid intermediate. Similarly, demonstration of specific elimination of the 3α- and 4β-hydrogens during ecdysteroid formation was interpreted by postulating the intermediacy of an analogous 3-oxo-$\Delta^4$-steroid intermediate in the formation of the A/B *cis*-ring junction. More recently, evidence suggests that $\Delta^4$-diketol (cholesta-4,7-diene-3,6-dione-14α-ol; 16) may be an intermediate. The demonstration that the 14α-hydroxy function could not be introduced after formation of the 6-keto-7-ene system and that ecdysteroid analogues having a 6-hydroxyl group could not be oxidized to 6-keto compounds led to the notion that the 3-dehydro-6-oxo-7-en-14α-ol functionality, which characterizes ecdysteroids, must be formed in a concerted manner, i.e., simultaneously. They might perhaps be mediated by a single cytochrome P450. Although it is not clear when oxidation of the 3β-hydroxyl of 7-dehydrocholesterol occurs, it may well be an integral part of the presumed concerted overall mitochondrial oxidations leading to the $\Delta^4$-diketol (16) formation.

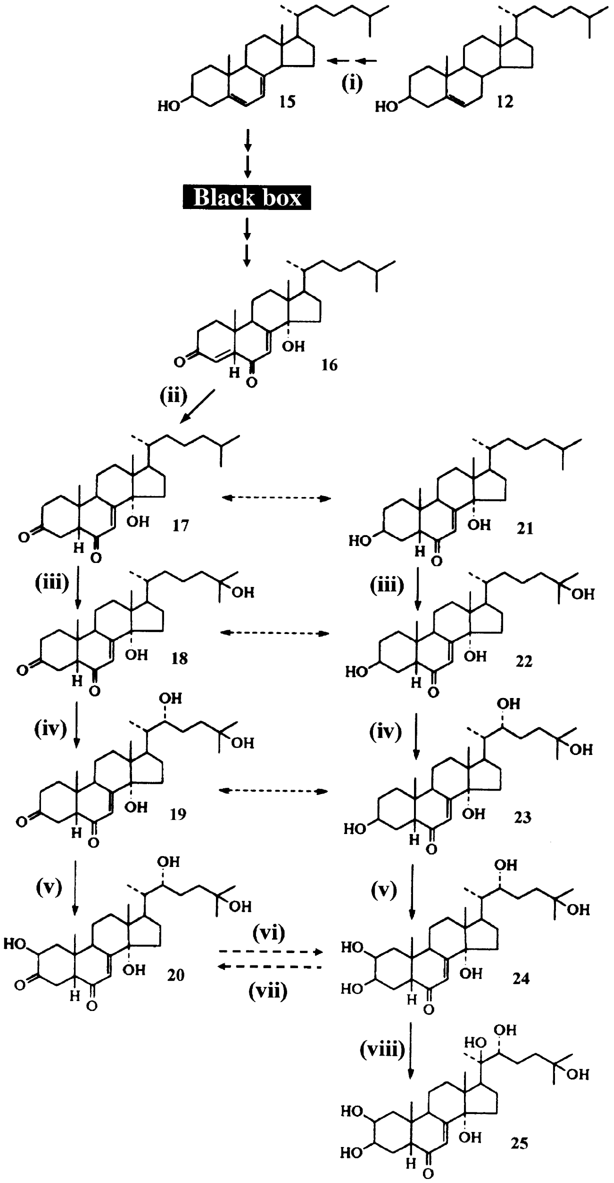


FIGURE 3 Composite scheme of ecdysteroid biosynthesis. Cytochrome P450-dependent formation of 7-dehydrocholesterol occurs in the microsomal fraction. Subsequent reactions (the "black box"; mitochondrial) yield the postulated intermediate, 5β-diketol (16), which then undergoes A-ring reduction to yield 3-dehydro-2,22,25-deoxy-ecdysone (17), and this may undergo further reduction to 2,22,25-deoxyecdysone (21; 5β-ketodiol). The latter two compounds may then undergo successive cytochrome P450-dependent hydroxylations at C-25 (microsomal) and C-22 and C-2 (both mitochondrial) to yield 3-dehydroecdysone (20) and ecdysone (24). Ecdysone undergoes 20-hydroxylation in certain peripheral tissues to yield the major active hormone, 20-hydroxyecdysone (25). Enzymes involved include (i) cholesterol 7,8-dehydrogenase; (ii) $\Delta^4$-diketol-5β-reductase; (iii) 25-hydroxylase; (iv) 22-hydroxylase; (v) 2-hydroxylase; (vi) 3-dehydroecdysone 3β-reductase; (vii) ecdysone oxidase; and (viii) ecdysone 20-monooxygenase.

This $\Delta^4$-diketol (16) intermediate can be reduced to diketol (17), thus, introducing the A/B *cis*-ring junction, characteristic of ecdysteroids. Such 5β-reductase activity has been demonstrated in crustacean tissues, being most active in the Y-organs. This NADPH-dependent enzymatic activity, which seems specific for 3-oxo-$\Delta^4$ substrates, was characterized in the cytosolic fraction but is also present in the crude mitochondrial and microsomal fractions. Such characteristics appear to be similar to those of vertebrate 3-oxo-$\Delta^4$-steroid 5β-reductases involved in steroid hormone catabolism and bile acid synthesis.

## C. Terminal Hydroxylations

The terminal hydroxylation reactions leading to ecdysone (24) formation have been primarily investigated, until recently, using various high-specific-radioactivity $^3$H-labeled substrates, including 5β-ketodiol (21) and 5β-diketol (17). As alluded to earlier, the demonstration that lepidopteran prothoracic glands produce 3-dehydroecdysone (20) in addition to ecdysone (24) raises the question as to the exact biosynthetic stage at which the 3-dehydro grouping arises. Evidence that a 3-oxo-$\Delta^4$ moiety is likely involved in the formation of the A/B *cis*-ring junction was presented earlier. Whether such a putative 3-dehydro feature is preserved throughout the biosynthetic pathway to yield 3-dehydroecdysone (20) or whether the 3-dehydro moiety is (re)introduced independently at a subsequent stage(s) and the stage(s) at which reduction of any obligatory 3-dehydro moiety occurs to yield ecdysone (24) as the final product are all pertinent considerations. These questions will be considered again later and the various possibilities are exemplified in Fig. 3.

The C-2, C-22, and C-25 hydroxylases are all cytochrome P450-dependent enzymes, being $O_2$- and NADPH-dependent and inhibited by the classical cytochrome P450 inhibitors fenarimol, metyrapone, and piperonyl butoxide. However, surprisingly, unlike the 22- and 25-hydroxylases, the 2-hydroxylase activity is not inhibited by carbon monoxide. Hydroxylation at both C-2 and C-22 occurs with retention of configuration. As expected, these three hydroxylase activities occur in prothoracic glands, follicle cells, and crustacean Y-organs of all species examined, but surprisingly, several other tissues, in addition to these major accepted ecdysiosynthetic organs, can effect at least some of these hydroxylations, albeit at a much lower level. The physiological significance of this finding is an enigma, unless such tissues can synthesize *de novo* or effect uptake from the hemolymph of a relevant precursor. Alternatively, such hydroxylations could conceivably be explained by the lack of substrate specificity of other unrelated cytochrome P450s.

Evidence in various species indicates that a major sequence of terminal hydroxylations involves hydroxylation at C-25, followed by C-22 and then C-2, without eliminating the possible operation of other sequences. Obviously, it is the combination of rates of individual steps that dictates the overall rate of a pathway and its dominance over others. The individual rates of the cytochrome P450-catalyzed reactions are reflected in the substrate and positional specificities of the P450s. In this respect, it is relevant that vertebrate steroidogenic P450s do not show absolute substrate or positional specificity. Clearly, the three terminal hydroxylases in ecdysone formation are distinct, since the two mitochondrial P450s (C-2 and C-22) not only differ in their CO sensitivity, but are encoded by different genes (see below), whereas the C-25 P450 is microsomal. Thus, it is also evident that extensive shuttling of intermediates between the endoplasmic reticulum and the mitochondria occurs during various stages of ecdysteroid biosynthesis, as occurs in vertebrate steroidogenesis.

Very recently, it has been shown that the wild-type genes corresponding to two members of the Halloween family of embryonic lethal mutations, *disembodied (dib)* and *shadow (sad)*, code for the mitochondrial cytochrome P450s that catalyze the 22- and 2-hydroxylations, respectively. Conceptual translation showed that both proteins possessed characteristic N-terminal mitochondrial import sequences. Hydroxylation activities of the expressed proteins were established by transfection of the *sad* (CYP315A1) and *dib* (CYP302A1) genes into *Drosophila* S2 cells, prior to incubation with appropriate [$^3$H]ecdysteroid precursors. Interestingly, *in situ* hybridization analyses have revealed that *sad* and *dib* expression is concentrated within the individual segments of the developing epidermis when there is a surge of ecdysteroid midway through embryogenesis, before the ring gland has developed, and suggests that the embryonic epidermis is a site of ecdysteroid biosynthesis. This expression then diminishes, and during late embryogenesis, expression of both genes is concentrated in the prothoracic gland cells of the developing ring gland. Furthermore, expression of both genes continues to be localized in these endocrine cells during larval development, being maximal at approximately the time of the premolt peaks in the ecdysteroid titer in late second- and third-instar larvae. As expected, expression was also observed in the follicle cells of the adult ovary.

Current evidence suggests parallel operation of both 3-dehydro and 3β-hydroxy routes to ecdysone (Fig. 3) but the exact points of interaction between them are uncertain. However, this may not apply to all systems, since the products of [$^3$H]5β-diketol (17) and [$^3$H]5β-ketodiol (21) metabolism may not always reflect the normal situation. The proportions of different ecdysteroid products may be explained by postulating that different steps are rate-limiting in various systems. For example, in lepidopteran prothoracic glands producing a preponderance of 3-dehydroecdysone, it can be assumed that a 3-dehydrosteroid 3β-reductase becomes limiting, thus, increasing the ratio of 3-dehydroecdysone/ecdysone product. There is no information on the enzymes catalyzing interconversion of the 3-dehydro and 3β-hydroxy moieties of the various intermediates in ecdysone biosynthesis in the prothoracic glands or follicle cells. Ecdysone oxidase, an enzyme that catalyzes oxidation of 3β-hydroxy ecdysteroids to the 3-dehydro derivatives, has been characterized in peripheral tissues and conceivably could be involved in oxidation of later intermediates in the pathway in prothoracic glands. As alluded to earlier, 3-dehydroecdysone (20), with lesser amounts of ecdysone (24), is a major product of the prothoracic glands in many lepidopteran species. The 3-dehydroecdysone (20) is reduced in the hemolymph by an NAD(P)H-dependent 3β-reductase enzyme, which is a member of the aldo-keto reductase superfamily. The 3-dehydroecdysone 3β-reductase mRNA transcripts in the cotton leafworm, *Spodoptera littoralis*, are widely distributed in various tissues, with a developmental profile that accounts for the peak in enzymatic activity just preceding the ecdysteroid peak during the instar.

## IV. ECDYSONE 20-MONOOXYGENASE

In immature stages of insects, ecdysone (24) produced in the prothoracic glands is transported to certain peripheral tissues, where it undergoes ecdysone 20-monooxygenase-catalyzed hydroxylation to yield 20-hydroxyecdysone (25). This 20-hydroxylation reaction is important, since 20-hydroxyecdysone is generally far more active than ecdysone in most assays and is believed to generally represent the major active ecdysteroid, although other compounds, including ecdysone, may have activity per se in certain biological systems. The 20-monooxygenase system occurs in several peripheral tissues, primarily in the fat body, midgut, and Malpighian tubules. Depending on species and tissue, it may occur in either mitochondrial or microsomal subcellular fractions or in both. The enzyme system requires NADPH and $O_2$ and is a typical cytochrome P450-dependent monooxygenase. The enzyme exhibits competitive inhibition by its product, 20-hydroxyecdysone.

It has been demonstrated in the final larval instar of several insect species that the 20-monooxygenase activity undergoes developmental changes, generally exhibiting a distinct peak. In some species, this peak coincides with the peak hemolymph ecdysteroid titer, whereas in others, the peaks are out of phase. The exact physiological significance of the latter situation is somewhat of an enigma. The molecular mechanisms involved in the regulation of the ecdysone 20-monooxygenase activity during development are not known. In the fat body of the cotton leafworm, *S. littoralis*, the ecdysone 20-monooxygenase is largely mitochondrial. When such extracts were subjected to immunoblot analyses using antibodies raised against components of vertebrate mitochondrial steroidogenic systems, anti-cytochrome $P450_{SCC}$, anti-cytochrome $P450_{11\beta}$, anti-adrenodoxin, and anti-adrenodoxin reductase antibodies revealed the presence of specific immunoreactive polypeptides. Furthermore, these antibodies effectively inhibited the mitochondrial ecdysone 20-monooxygenase activity, suggesting that the insect hydroxylating system may contain polypeptide components analogous to those in vertebrates. Correlation between developmental changes in mitochondrial 20-monooxygenase activity and the abundance of polypeptides recognized by anti-cytochrome $P450_{11\beta}$ antibody and a polypeptide recognized by the anti-adrenodoxin reductase antibody was observed, suggesting that developmental changes in the abundance of components of the monooxygenase system may be important in developmental regulation of enzyme expression. Furthermore, in these *S. littoralis* fat body mitochondrial (and microsomal) ecdysone 20-monooxygenase systems, indirect evidence that the enzyme may exist in an active phosphorylated state and an inactive dephosphorylated state has been furnished. This tenet awaits more direct confirmation, but such covalent modification potentially provides short-term, rapid modulation of the enzymatic activity in response to unidentified effectors. It is noteworthy that the adrenodoxin reductase gene (*dare*) has been identified in *Drosophila*, together with a sequence in the genome putatively encoding adrenodoxin.

## V. SUMMARY

Insects cannot synthesize sterols *de novo* and thus must obtain cholesterol for ecdysteroid synthesis from the diet, either directly or following phytosterol dealkylation. The principal sites of ecdysteroid synthesis in immature stages of insects are the prothoracic glands, whereas in adults synthesis occurs either in the ovarian follicle cells or in the testes. 7-Dehydrocholesterol is an early intermediate in the pathway and although subsequent early steps are unclear, there is good evidence for the intermediacy of 2,22,25-trideoxy-ecdysone (5β-ketodiol) or its 3-dehydro derivative. Subsequent cytochrome P450-dependent hydroxylations at C-25, C-22, and C-2 yield ecdysone, which then undergoes 20-monooxygenase-catalyzed hydroxylation in several peripheral tissues to yield the major active hormone, 20-hydroxyecdysone.

## Glossary

**arthropods** Animals possessing a hard, jointed exoskeleton and constituting the largest group (phylum) in the animal kingdom in terms of numbers of species, e.g., insects, crabs, spiders, and centipedes.

**cytochrome P450** A family of heme-containing proteins that reduce molecular oxygen, one atom of which is incorporated into the substrate as a hydroxyl group. When these proteins use steroids as substrates, they are frequently called steroid hydroxylases. Cytochrome P450s derive their name from their characteristic absorption of light at 450 nm when carbon monoxide is bound.

***Drosophila*** The fruit fly, a commonly used model organism.

**ecdysteroids** A unique class of steroids characteristic of arthropods, possessing the full cholesterol side chain (unlike most vertebrate steroids) and several hydroxyl groups. They act as hormones (regulators) controlling molting and aspects of reproduction.

**microsomes** Closed vesicles formed during fragmentation of the endoplasmic reticulum membrane system in eukaryotic cells, i.e., cells having the nucleus separated from the cytoplasm by a nuclear membrane and the genetic material borne on a number of chromosomes.

**mitochondria** Oval-shaped subcellular organelles in eukaryotic organisms that are responsible for producing ATP, the immediately available energy store.

**monooxygenases** A class of enzyme (catalyst) also called "mixed-function oxygenases" because one atom of oxygen appears in the reaction product and the other in water. The majority of hydroxylases, including the steroid hydroxylases, belong to this class of enzymes.

**prothoracic glands** Paired organs in the prothorax (region between the head and thorax) of insects, which are the major site of ecdysteroid synthesis in insect larvae.

**sterols** Compounds having three 6-sided carbon rings, one 5-sided carbon ring, and a side chain, e.g., cholesterol.

## See Also the Following Articles

**Ecdysteroid Action in Insect Reproduction • Ecdysteroid Receptors (EcR/USP) • Ecdysteroids, Overview • Insect Endocrine System**

## Further Reading

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# Ecdysteroid Receptors (EcR/USP)

Margarethe Spindler-Barth and
Klaus-Dieter Spindler
*University of Ulm, Germany*

I. MOLECULAR CHARACTERIZATION OF EcR
II. MOLECULAR CHARACTERIZATION OF USP
III. GENERAL COMMENTS
IV. APPLICATION AND PERSPECTIVES

**The heterodimer of the ligand-dependent transcription factors ecdysteroid receptor (EcR) and USP is considered as functional ecdysteroid receptor that coordinates arthropod development and metabolism and modifies the expression of a multitude of different genes in a tissue- and time-specific manner. As is typical for steroid hormone receptors in general, the different functions of this receptor are associated with defined molecular domains, which are modified by intra- and intermolecular interactions. Both EcR and USP can dimerize with several other transcription factors, but homodimerization has been described for both partners as well. Isoforms of EcR and USP are detected in various species and are engaged in different functional roles. Species-specific variations demonstrate that different solutions have evolved within arthropods. The ecdysteroid receptor is an important target for insecticides and is also used as a ligand-controlled switch in heterologous systems for gene expression.**

## I. MOLECULAR CHARACTERIZATION OF EcR

### A. General Features

Thus far, the ecdysteroid receptor (EcR) has been sequenced from 12 insects, a crustacean, and a tick. The common features of EcRs include a highly conserved two-zinc-finger DNA-binding domain, which shares a considerable level of sequence identity with vertebrate steroid hormone receptors (Table 1), an A/B domain, which in most instances is responsible for transactivation, and a D–F domain, which, in addition to binding ligand, mediates various other functions (Fig. 1). The degree of sequence identity that EcRs share with vertebrate steroid receptors is considerably lower than that within arthropods.

# Ecdysteroids, Overview

René Lafont, Chantal Dauphin-Villemant, and Catherine Blais
*Université Pierre et Marie Curie, Paris*

I. INTRODUCTION
II. CHEMICAL DIVERSITY (IN ARTHROPODS)
III. DISTRIBUTION–EVOLUTION

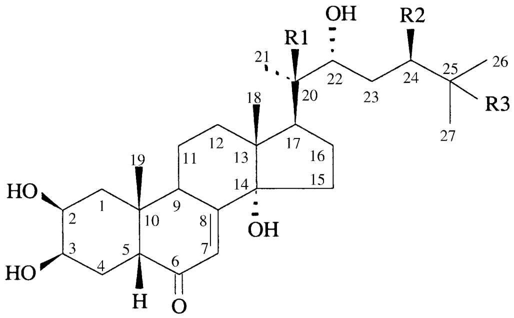
**Ecdysteroids are polyhydroxylated steroid hormones that control arthropod development and reproduction. Whether they also fulfill endocrine functions in lower invertebrate phyla is still conjectural. Their wide distribution in the plant kingdom as secondary metabolites results in their frequent presence in animals' diets; hence, when they are found in a given animal species, a demonstration of their endogenous origin is required. Different arthropod species may contain different ecdysteroids, and the variations concern in particular the number of carbon atoms (which is related to the sterol precursors used) and the number of hydroxyl groups. In addition, a single insect species may contain several molecules, but whether those have specific receptors (and thus possibly different physiological roles) or simply bind with various affinities to a single type of receptor remains an unanswered question.**

## I. INTRODUCTION

Evidence that molting and metamorphosis of insects are under hormonal control was provided by Kopec's pioneering experiments as early as 1917. Kopec demonstrated that a blood-borne factor originating in the brain was necessary for caterpillar molting, and this was in fact the first demonstration that the nervous system was able to secrete hormones (i.e., the first experimental demonstration of neurosecretion). Evidence that this brain hormone was relayed by a molting hormone produced by (pro)thoracic glands was obtained much later by Fukuda in 1941.

Ligation experiments by Fraenkel in 1935 provided the basis for a molting hormone bioassay; posterior portions (i.e., lacking the molting glands) of ligated *Calliphora erythrocephala* larvae could pupate when injected with a solution containing molting hormone, and this bioassay was used for the isolation of the hormone from silkworm pupae. Starting from 500 kg of pupae and using a combination of several chromatographic procedures, in 1954 Butenandt and Karlson obtained 25 mg of pure crystalline ecdysone, a quantity that did not allow determination of its structure by the physicochemical techniques available at that time (by comparison, modern nuclear magnetic resonance techniques would have allowed this to be completed within a single day using a few milligrams of ecdysone). The structure of ecdysone was elucidated 11 years later thanks to X-ray analysis.

The identification of ecdysone as a steroid was a surprise, as this molecule is very polar due to a large number of hydroxyl groups (Fig. 1). Ecdysone (E) was at first termed α-ecdysone, and a minor compound simultaneously isolated from *Bombyx mori* pupae was termed β-ecdysone. The latter was also isolated from a crustacean and proved to be in fact the major hormone in most arthropod species; it is now called 20-hydroxyecdysone (20E). Related compounds were then found in plants and this discovery stimulated extensive research. The generic name "ecdysteroid" was proposed by Goodwin *et al.* in 1978 for all the compounds "structurally related to ecdysone." The discovery of large amounts of ecdysteroids in plants made them available to many laboratories and was decisive for the development of research in insect endocrinology.



$R_1 = R_2 = R_3 = H$ : 25-Deoxyecdysone
$R_1 = R_2 = H, R_3 = OH$ : Ecdysone
$R_1 = H, R_2 = Me, R_3 = OH$ : 24-Methyl-ecdysone
$R_1 = OH, R_2 = R_3 = H$ : Ponasterone A
$R_1 = OH, R_2 = H, R_3 = OH$ : 20-Hydroxyecdysone
$R_1 = OH, R_2 = Methyl, R_3 = OH$ : Makisterone A
$R_1 = OH, R_2 = Ethyl, R_3 = OH$ : Makisterone C

FIGURE 1 Structures of major zooecdysteroids.

## II. CHEMICAL DIVERSITY (IN ARTHROPODS)

### A. Definition of Ecdysteroids

Whether ecdysteroids should be defined by using their chemical structure and/or their biological activity (as molting hormones) is a matter of debate, and a chemical definition is used in this article.

Their definition as compounds structurally related to ecdysone is indeed simple but it does not seem precise enough from a chemical point of view. In 1989, Lafont and Horn defined true ecdysteroids as "compounds where the steroid nucleus bears a *cis*-fused A/B ring junction, a 7-ene-6-one chromophore and a 14 α-OH" (Fig. 2), irrespective of being biologically active. Such a definition is still not fully satisfactory, as it leaves out very closely related compounds that may even co-occur with true ecdysteroids within the same animal or plant. Therefore, the limits of the ecdysteroid family are quite difficult to define, as will be exemplified below.

### B. Ecdysteroids and Their Parent Sterols

Arthropods are auxotroph for sterols, which are the precursors of ecdysteroids. The food of carnivorous or phytophagous insects contains either zoosterols, which are 27C molecules (in fact, mainly cholesterol), or phytosterols, which have either a 28C (e.g., campesterol, ergosterol) or a 29C (e.g., sitosterol, stigmasterol) skeleton. Most but not all phytophagous insects are able to dealkylate phytosterols and to convert them into cholesterol. Thus, according to their diet and their ability or inability to dealkylate phytosterols, insects contain 27C, 28C, and/or 29C sterols and they will accordingly produce ecdysteroids with different carbon skeletons (Table 1). The 28C and 29C ecdysteroids are found in phytophagous Hemiptera and Hymenoptera and also in *Drosophila melanogaster*. In *Drosophila*, as is also the case in some Hymenoptera, it has been shown that the proportions of the different ecdysteroids depend on the nature of the dietary sterols. This finding raises an interesting question concerning the substrate specificity of the ecdysteroid biosynthetic enzymes and also the ligand-binding properties of insect receptors. *Drosophila* can use both 27C and 28C molecules, whereas other species require specifically 27C or 28C molecules, as shown by the relative biological activities of exogenous 20E or makisterone A.

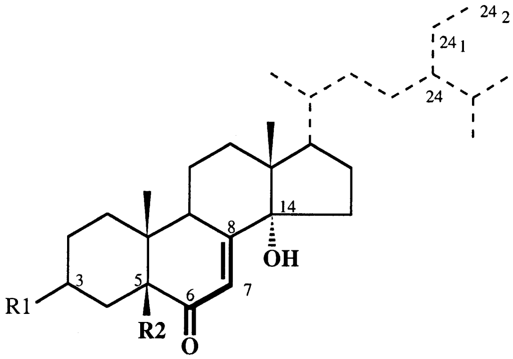


FIGURE 2 Common structural features of ecdysteroids (R1 = OH or O; R2 = H or OH).

TABLE 1 Relationship between Dietary Sterols and Ecdysteroid Structures

| Dietary sterols | Dealkylation | Major ecdysteroid |
|---|---|---|
| 27C | Indifferent | 20-Hydroxyecdysone (or ponasterone A) |
| 28C/29C | + | 20-Hydroxyecdysone |
| 28C | − | Makisterone A (or 24-epi-makisterone A) |
| 29C | − | Makisterone C |

### C. The Major Ecdysteroid Hormones

In all classes of arthropods, similar major ecdysteroid hormones are produced by steroidogenic organs. The major circulating hormone or "active ecdysteroid" of insects and crustaceans is most commonly 20E and makisterone A or makisterone C, respectively, for the phytophagous species that produce 28C or 29C ecdysteroids. Endocrine glands usually release a "pro-hormone," a precursor that is E, 3-dehydroecdysone (3DE), or a mixture of the two (or their 24-Me or 24-Et analogues in the above-mentioned phytophagous species). Conversion to the biologically active form always requires a hydroxylation at position 20 and eventually a reduction of the 3-ketone into the corresponding 3β alcohol. 20-Hydroxylation takes place mainly in fat body, gut, and Malpighian tubules and most probably occurs in all tissues, whereas 3β reduction takes place both in hemolymph (immediately following 3DE secretion) and in peripheral tissues. As a consequence, the half-life of molting gland secretory products is usually very short; thus, the isolation of E from *Bombyx* pupae resulted from the use of a very particular biological system in which this compound accumulates temporarily, which is not at all representative of the general situation. There are other exceptions to the rule; e.g., the molting glands of the beetle *Zophobas atratus* produce 2-deoxyecdysone (2dE) together with E. Given the small number of insect species analyzed thus far relative to the one million species known, it is possible that more variability exists.

The situation in Crustacea is very similar to that in Insecta, and the molting glands (Y-organs) of crustaceans usually secrete E and (or) 3DE, but in this case 3β-reductase seems only tissular. Several crab species contain another major ecdysteroid, ponasterone A (PonA), i.e., the 25-deoxy analogue of 20E. Their molting glands produce 25-deoxyecdysone, due to a low level of activity of the enzyme responsible for the 25-hydroxylation. These crabs contain a mixture of PonA and 20E, and it is not known whether these molecules have specific functions. PonA has also been found in *Thermobia*, a primitive insect. Amazingly, PonA is much more active than 20E in many bioassays using insect species (including *Drosophila*) although they do not produce this molecule.

The question of the biologically active form(s) of ecdysteroids in a given species is still open. Although several biological systems exist in which E and 20E elicit different responses, attempts to find specific receptors for a given ecdysteroid have thus far been unsuccessful. The *Drosophila* ecdysone (20E) receptors exist as three isoforms, but all of them share the same ligand-binding domain, a situation that does not support their specific binding to different ecdysteroids. The existence of a receptor that would specifically bind E cannot be ruled out, and this would make sense for the accumulation of E at specific developmental stages: E is the major ecdysteroid in young *Bombyx* pupae, and during pupal–adult development of *Pieris brassicae* and *Manduca sexta*, successive peaks of E and 20E are observed. E is also present in mid third-instar larvae of *Drosophila* and might activate a specific nuclear receptor. A role for 3-oxo ecdysteroids is more difficult to establish, due to their rapid interconversion with 3β-OH molecules.

### D. Other Biologically Active Ecdysteroids

Ecdysteroids are not only produced during larval development, they are also involved in the control of insect reproduction. The gonads of adult insects synthesize ecdysteroids. Both vitellogenic oocytes and newly laid eggs often contain high levels of ecdysteroids, which are produced by the follicular cells of the ovaries, are stored within the vitellus, and are probably used during early embryogenesis, before molting glands begin to differentiate.

In many insect species, these "maternal" ecdysteroids are E and 20E or analogues of E that either lack some –OH groups, mainly at positions 2 and/or 22 (precursors perhaps?), or bear additional –OH groups at unusual positions (positions 16, 23, or 26). They are essentially conjugated to polar or apolar moieties, and these conjugates are considered biologically inactive storage compounds. For example, large amounts of 2dE are stored in *Locusta migratoria* eggs, which are later activated by "steroidogenic enzymes" present in embryonic tissues. Such a conclusion is supported by the observation that normal development can take place within both anterior and posterior half-cut embryos and by recent expression studies on a steroidogenic cytochrome P450 enzyme (CYP302A1, the product of the *dib* gene) in early *Drosophila* embryos. Alternatively, less hydroxylated compounds could function as true "hormones"; thus, in *Locusta*, a role for 2dE in the control of meiotic reinitiation has been suggested.

*M. sexta* eggs accumulate huge amounts of another ecdysteroid, 26-hydroxyecdysone (26E). This ecdysteroid, which is considered a catabolite devoid of biological activity during larval and adult stages, appears to be a likely candidate hormone in the embryo, since the level of free 26E peaks during early embryonic development, when embryonic cuticle is secreted.

In *Bombyx* eggs, a wide array of ecdysteroids have been identified, including minor compounds that bear additional –OH groups at unusual positions (positions 16, 23, etc.). It is not clear whether this molecular diversity has any biological significance, whether it is unique to this stage, or whether it has been found here simply because the large amounts of available biological material and the extremely high ecdysteroid levels have allowed the identification of the minor ecdysteroids. Similar "cocktails" have also been found in several ecdysteroid-rich plant species in which extensive chemical investigations have been undertaken.

## III. DISTRIBUTION–EVOLUTION

### A. Which Animals Contain Ecdysteroids?

The ecdysteroid family comprises more than 300 members, i.e., ca. 70 molecules isolated from animals (zooecdysteroids) and 250 from plants (phytoecdysteroids), with some compounds being found in both phyla. These molecules are listed in the *Ecdysone Handbook* (see http://ecdybase.org) (see also Table 2).

**TABLE 2 Major Variations on the 20-Hydroxyecdysone Molecule (Zooecdysteroids Only)**

| Type | Positions on the molecule |
|---|---|
| Hydroxyl groups | |
| Additional –OH | 11α, 16β, 23, 26 |
| Missing –OH | 2, 14, 20, 22, 25 |
| Oxidation | |
| ($>CHOH \Rightarrow\ >C{=}O$) | 3 |
| ($-CH_2OH \Rightarrow -COOH$) | 26 |
| Epimerization | 3α/3β |
| Alkyl substitution | 24 (Me, Et) |
| Side-chain cleavage between C20 and C22 | Poststerone |
| Esterification | |
| Acetic acid | 2, 3, 22, 25 |
| Glycolic acid | 22 |
| Fatty acid | 22 |
| Phosphoric acid | 2, 22, 26 |
| Nucleotide | 22 |
| Etherification | |
| Glucosides | 22, 25, 26 |

*Note:* ME, methyl; ET, ethyl.

Ecdysteroids are not restricted to Arthropoda. They have also been isolated from more primitive invertebrates, including Annelida, Helmintha, Cnidaria, and even Porifera, but (with the exception of Cnidaria and Porifera) their concentrations are very low. An endogenous origin (i.e., the unambiguous demonstration of *de novo* biosynthesis from cholesterol) has thus far been obtained only in arthropods. There is in fact a discrepancy between the chemical nature of the compounds (E, 20E, and 20,26-dihydroxy-ecdysone) identified in helminths and annelids and the failure to detect several key hydroxylation steps that would be required to produce them (at least when using the precursors that are efficiently converted in insects). Thus, no 2-hydroxylation could be demonstrated in myriapods, annelids, or helminths, and 22-hydroxylation was observed only in arthropods.

In many instances, a dietary origin of these ecdysteroids cannot be ruled out (e.g., for sea anemones, which feed on planktonic Crustacea), but such an explanation does not apply so easily to all cases (e.g., for leeches).

## B. Ecdysteroid-Related Molecules and the Origin of Ecdysteroids

Ecdysteroids are multifunctional molecules and their biosynthesis requires probably at least 8–10 steps and thus 8–10 enzymes (including a large set of cytochrome P450 mono-oxygenases, a 3β-hydroxysteroid dehydrogenase, and a 5β-reductase). As is the case for vertebrate-type steroids, it seems unlikely that all these enzymes have appeared simultaneously, and we may therefore expect to find "protoecdysteroids" in primitive arthropods and also in nonarthropod invertebrates.

Although the lack of biosynthetic studies does not allow determination of whether these molecules have an endogenous origin, an impressive number of ecdysteroid-related molecules have been isolated from nonarthropod invertebrates. At present, such molecules do not have any established function, but the structures of some of these make them likely candidate protoecdysteroids. We may assume that classical ecdysteroids arose from the progressive appearance of CYP450-catalyzed hydroxylation reactions, and in this respect, the mitochondrial steps (2- and 22-hydroxylations) would probably have been the last to appear. This hypothesis is consistent with the isolation from invertebrates of cholesterol derivatives bearing hydroxyl groups at positions 20 and 25 and lacking hydroxyl groups at positions 2 and 22. For the B ring substituents, many oxysterols that have a 7,8-ene and a 6-one, a 5,6-diol, or a 5,7-diene-3,6-dione (Fig. 3) have been isolated.

The incomplete elucidation of the ecdysteroid biosynthetic pathway, concerning in particular the formation of the 7-ene-6-one-14αOH chromophore, does not allow a precise evolutionary scenario to be proposed as yet. Steroids of interest include compounds isolated from insects, which co-occur with ecdysteroids, e.g., bombycosterol in *Bombyx* ovaries and a steroid from exocrine (defensive) secretions of a Chrysomelid beetle (Fig. 3). It would be of great interest to determine whether bombycosterol is synthesized by follicle cells that produce ecdysteroids.

Oxysterols have recently become the subject of increasing interest, as they are natural ligands for formerly termed "orphan" nuclear receptors and they control a large set of cellular processes in vertebrates. It seems reasonable to expect that oxysterols play fundamental roles in invertebrates too and that among these molecules some have further evolved to give the currently known ecdysteroids.

Finally, it is worth mentioning that ecdysteroids do not always act as hormones. They can also be used as signaling substances by diverse sensory systems, which most probably involves their binding to membrane proteins. Very low concentrations of ecdysteroids evoke action potentials on lobster antennae, and it has been suggested that they might be used as sex pheromones. The huge amounts

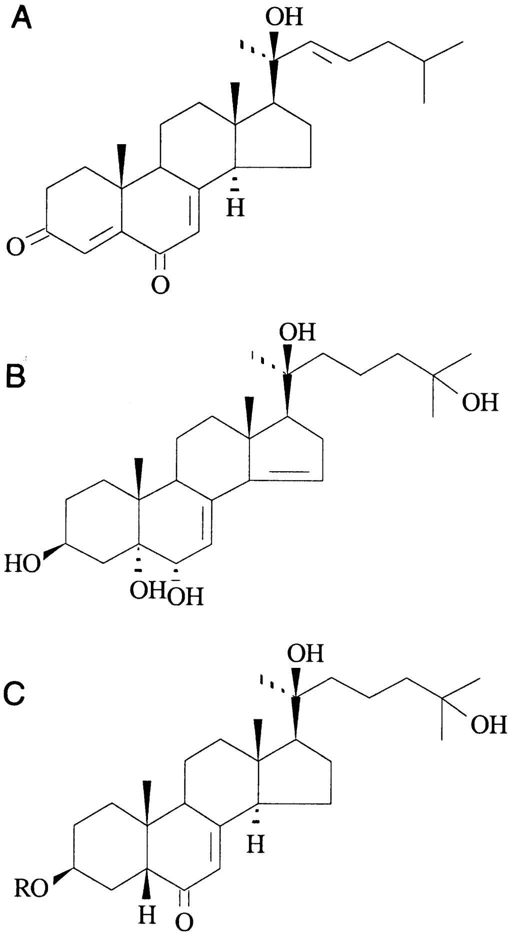


FIGURE 3 (A) An oxysterol from a sponge. (B) Structure of bombycosterol from silkworm ovaries/eggs. (C) A defensive steroid from a Chrysomelid beetle (R = sophorose).

of ecdysteroids that accumulate in the integument of pycnogonids can be rapidly released in water when the animal is attacked by crabs, and this reaction seems efficient enough to deter the crabs from eating them. These defensive effects are to some extent similar to those that phytoecdysteroids have on insects; when the phytoecdysteroids are detected by insect taste receptors, they exert an anti-feedant effect, and this could be the *raison d'être* for the presence of ecdysteroids in so many plant species.

## Glossary

**cytochrome P450** A heme–iron protein that uses molecular oxygen and NADPH to oxidize a substrate and produce a water molecule (this reaction is termed mono-oxygenation).

**ecdysone** 2β,3β,14α,22*R*,25-Pentahydroxy-5β-cholest-7-ene-6-one.

**20-hydroxyecdysone** 2β,3β,14α,20*S*,22*R*,25-Hexahydroxy-5β-cholest-7-ene-6-one.

**3β-hydroxysteroid dehydrogenase** A membrane-bound enzyme that catalyzes the reversible conversion of a 3β-OH into a 3-keto group.

**makisterone A** 2β,3β,14α,20*S*,22*R*,25-Hexahydroxy-5β-ergost-7-ene-6-one.

**makisterone C** 2β,3β,14α,20*S*,22*R*,25-Hexahydroxy-5β-stigmast-7-ene-6-one.

**ponasterone A** 2β,3β,14α,20*S*,22*R*-Penta-hydroxy-5β-cholest-7-ene-6-one.

**prohormone** The product of an endocrine gland that requires further modification (before or after its secretion) to become biologically active.

**5β-reductase** A cytosolic enzyme that reduces a 4,5 double bond with the formation of a *cis*-bond between cycles A and B.

## See Also the Following Articles

**Crustacean Endocrine Systems • Ecdysone Action in Insect Development • Ecdysone Secretion in Lepidopteran Insects • Ecdysteroid Action in Insect Reproduction • Ecdysteroidogenic Pathway • Ecdysteroid Receptors (EcR/UCP) • Insect Endocrine System**

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# Effectors

TAE H. JI, YONG SANG SONG, AND INHAE JI
*University of Kentucky*

I. INTRODUCTION
II. ADENYLYL CYCLASE
III. PHOSPHOLIPASE C
IV. ION CHANNELS
V. OTHER EFFECTORS

**Effectors are enzymes that can generate second messengers, which in turn trigger other molecules in hormone signaling pathways. Common effectors include adenylyl cyclase, phospholipase C, and $Ca^{2+}$ channels. Effectors are usually regulated by guanine nucleotide-binding proteins in response to hormones binding to receptors.**

## I. INTRODUCTION

Nearly 2000 seven-transmembrane domain (TM) receptors have been reported, and they share a general structure consisting of an N-terminal extracellular domain (exodomain), seven TMs, three extracellular loops (exoloops), three cytoplasmic loops (cytoloops), and a C-terminal cytoplasmic tail (cytotail) as shown in Fig. 1. These receptors associate with guanine nucleotide-binding protein (G-protein) and therefore are referred to as G-protein-coupled receptors (GPCRs). This receptor family is the major class of hormone receptor and represents more than 1% of the human genome. GPCRs are responsible for binding a wide spectrum of hormones from odorants, biogenic amines, nucleosides, eicosanoids, and lipid moieties (lysophosphatidic acid and sphingosine-1-phosphate) at the cell surface and trigger hormone actions. Hormones are considered first messengers that are produced by an originating tissue and sent to targets. At the target they bind to the exodomain, exoloops, and/or TM domain of the receptors, which is thought to modulate the receptor conformation and interaction with G-proteins. G-protein is a heterotrimeric protein consisting of the α-, β-, and γ-subunits. Gα has 17 isoforms, which are divided into four groups. The Gαs group has Gαs and Gαo; the Gαi group consists of Gαi1, Gαi2, Gαi3, GαoA, GαoB, Gαt1, and Gαt2; the Gαq group comprises Gαq, Gα11, Gα14, Gα15, and Gα16; and the Gα12 group consists of Gα12 and Gα13. Seven Gβ isoforms and 12 Gγ isoforms have been cloned. The combination of these isoforms of the three G-subunits produces a wide variety of the trimeric G-proteins specific for effectors and tissues.

Effectors are generally enzymes that are capable of generating second messengers, which in turn trigger other molecules along the hormone signal pathways. The common effectors are adenylyl cyclase, phospholipase C, and $Ca^{2+}$ channels. Generally, adenylyl cyclase is activated by Gαs to produce cAMP and is suppressed by Gαi to reduce the cAMP concentration. Phospholipase Cβ (PLCβ) is activated by Gαq to hydrolyze phospholipids and to produce diacylglycerol (DAG) and inositol phosphate (IP). $Ca^{2+}$ channels are activated by protein kinase C and suppressed by Gβγ. $Ca^{2+}$ channels also regulate adenylyl cyclases, PLCβ, ion channels, and phosphoinositide 3-kinases.

Although the majority of GPCRs mediate signal transduction via G-proteins, recent evidence indicates that some of these receptors are also capable of interacting with and sending signals to alternative signal molecules independent of G-proteins, e.g., Jak2 kinase, phospholipase Cγ, or protein kinase C. These alternative pathways are an indication of the functional diversity of the GPCR superfamily. Furthermore, there are putative seven-transmembrane molecules that do not appear to be coupled to a G-protein.

This article describes common effectors such as adenylyl cyclase, phospholipase Cβ, and $Ca^{2+}$ channels and briefly describes some other effectors as well.

## II. ADENYLYL CYCLASE

Adenylyl cyclase is an enzyme that produces cAMP by hydrolyzing ATP to produce cAMP, which in turn activates protein kinase A to phosphorylate target proteins and induces protein–protein interactions independent of phosphorylation. In response, target cells undergo physiological changes and gene expression. It is generally known that the enzyme is activated by Gαs and inactivated by Gαi. It has

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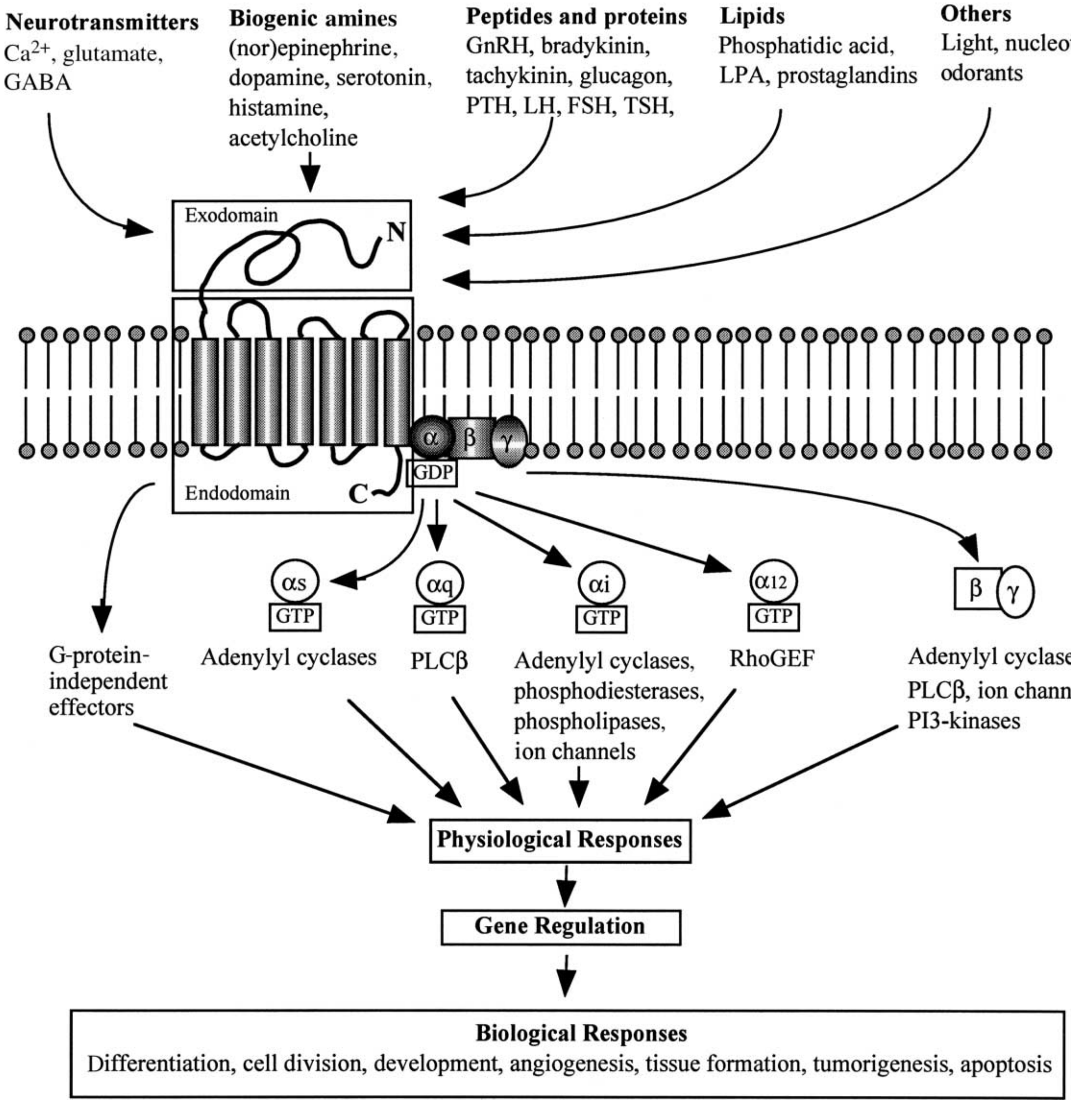
Neurotransmitters
Ca2+, glutamate, GABA
Biogenic amines
(nor)epinephrine, dopamine, serotonin, histamine, acetylcholine
Peptides and proteins
GnRH, bradykinin, tachykinin, glucagon, PTH, LH, FSH, TSH,
Lipids
Phosphatidic acid, LPA, prostaglandins
Others
Light, nucleotides, odorants
Exodomain
N
α
β
γ
GDP
Endodomain
C
αs
GTP
αq
GTP
αi
GTP
α12
GTP
β
γ
G-protein-independent effectors
Adenylyl cyclases
PLCβ
Adenylyl cyclases, phosphodiesterases, phospholipases, ion channels
RhoGEF
Adenylyl cyclases, PLCβ, ion channels, PI3-kinases
Physiological Responses
Gene Regulation
Biological Responses
Differentiation, cell division, development, angiogenesis, tissue formation, tumorigenesis, apoptosis

FIGURE 1 Network of G-protein-coupled receptors from hormones to effectors and their biological responses. A wide variety of hormones from small neurotransmitters, biogenic amines, peptides, proteins, lipids, and other molecules bind to the large number of G-protein-coupled receptors. The hormone binding activates diverse G-protein isoforms, which in turn regulate a wide spectrum of effectors. Other effectors that are not linked to G-proteins are also regulated by the receptors. These effectors elicit various physiological responses and regulation of various genes, leading to diverse biological responses.

a number of isoforms: nine membrane-bound isoforms and one soluble form have been cloned.

## A. Activity and Structure

All adenylyl cyclases are activated by both forskolin (a diterpene extracted from plants) or GTP-bound Gαs (GTP/Gαs). Adenylyl cyclase is inhibited by adenosine analogues termed P-site inhibitors such as 2′-deoxy-3′-cAMP. In addition, $Ca^{2+}$/calmodulin can activate some isoforms (Table 1). The membrane-bound adenylyl cyclases have molecular weights of approximately 120 kDa with nearly 1200 amino acids. Amino acid sequence analysis show a short amino-terminal extension, 12 TMs, suggesting a complex organization (Fig. 2). The 12 TMs indicate the presence of six exoloops, five cytoloops, the N-terminus, and the C-terminus. This orientation suggests that both the N- and the C-termini are exposed to the cytoplasm. This structure can roughly be divided into two sections, with the N-terminal section containing the first 6 TMs, three exoloops,

TABLE 1 Properties of Adenylyl Cyclase Isoforms

| Group | Isoform | Chromosome | Activators | Suppressors | Tissue distribution | Function |
|---|---|---|---|---|---|---|
| 1 | AC1 | 7p12–p13 | Gαs, $Ca^{2+}$/calmodulin | Gαi, Gβγ | Brain, adrenal gland | Learning, memory |
| 1 | AC3 | 2p22–p242 | Gαs, $Ca^{2+}$/calmodulin | Gαi | Brain, heart, lung, uterus | Odor stimulation |
| 1 | AC8 | 8q24.2 | Gαs, $Ca^{2+}$/calmodulin | Gαi | Brain, heart, lung, uterus | |
| 2 | AC2 | 5p15.3 | Gαs, Gβγ | | Brain, heart, lung muscle | |
| 2 | AC4 | 14q11.2 | Gαs, Gβγ | | Brain, heart, lung, uterus | |
| 2 | AC7 | 16q12–q13 | Gαs, Gβγ, PKC | | Brain, platelets, ubiquitous | |
| 3 | AC5 | 3q13.2–q21 | Gαs | Gαi, Gβ1γ2, $Ca^{2+}$, PKC, PKA | Brain, heart, lung, uterus | |
| 3 | AC6 | 12q12–q13 | Gαs | Gαi, Gβ1γ2, $Ca^{2+}$, PKC, PKA | Ubiquitous | Cell proliferation |
| 4 | AC9 | 16p13.3 | Gαs | | Brain, muscle, ubiquitous | |

and three cytoloops. The third cytoloop connecting TMs 6 and 7 is ~40 kDa with ~360 amino acids and is one of the catalytic domains (C1) of the enzyme. The C-terminal section comprises the next 6 TMs, three exoloops, two cytoloops, and the long C-terminal tail. This ~40 kDa (~350 amino acids) tail is the second catalytic domain (C2) of the enzyme. The upstream amino acid sequences of the C1 and C2 domains are conserved. When the synthetic polypeptides corresponding to these upstream sequences are expressed together, they show the adenylyl cyclase activity necessary to produce cAMP from ATP. This reaction is activated by GTP-bound Gαs or forskolin, a hallmark of adenylyl cyclase. However, neither of the two peptides is active alone, indicating that both peptides are required for the enzyme activity. These observations indicate that the C1 and C2 domains interact with each other and form an active catalytic site. This is proved to be correct according to the crystal structure. In fact, the two polypeptides form a forskolin-binding site, and forskolin binding promotes their interaction, leading to the active form. They also show a site for GTP-bound Gαs at the interface between the domains but primarily toward the C2 domain. It is thought that GTP-bound Gαs or forskolin stabilizes the dimer interaction, promotes a favorable structure, in particular, by bringing the Asp440 and 3′-hydroxyl group of ATP together, and thus, facilitates the enzyme activity. The inhibitory Gαi-subunit is also thought to bind to a similar site in the C1/C2 dimer as the stimulatory Gαs does, but has the opposite effect of inhibiting the enzyme activity, probably by inducing an unfavorable structure. However, the exact mechanism of this inhibition is unclear.

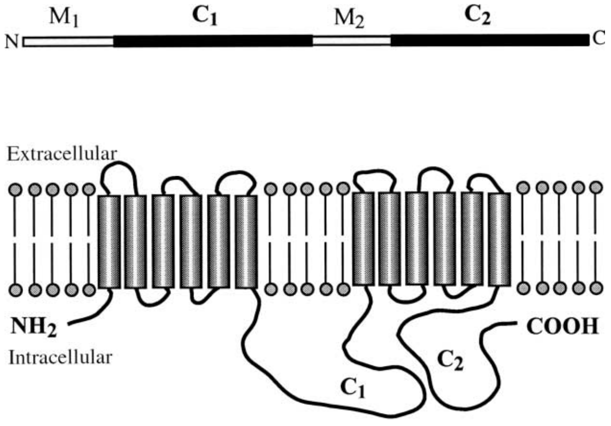

FIGURE 2 Domain structure and membrane topology of adenylyl cyclases. Adenylyl cyclases have two halves, each consisting of a membrane-associated domain (M) and a catalytic domain (C). The membrane domains (M1, M2) have six transmembrane domains. The two catalytic domains (C1, C2) form a catalytic site.

## B. Termination of the Adenylyl Cyclase Activity

It is essential to terminate the activity of an effector soon after a second messenger is produced, otherwise the overproduction of second messengers will poison a cell as clearly shown by cholera toxin, pertussis toxin, and forskolin. The activity of adenylyl cyclase can be terminated by dissociation of Gαs from the C1/C2 complex most likely when the bound GTP is hydrolyzed to GDP by the GTPase activity of the GTP-bound Gαs. It has been suggested that activated adenylyl cyclase may activate the GTPase activity of Gαs, thus producing the inactive form Gαs (GDP-bound Gαs) and terminating its own activity. If this is true, adenylyl cyclase is likely to activate the GTPase activity of Gαi, also.

### C. Isoforms, Tissue Distribution, and Functions

Nine different membrane-associated adenylyl cyclases, AC1–9, have been cloned, and two different isoforms are alternately spliced from AC8. These isoform genes are widely distributed among human chromosomes and the encoded enzymes share amino acid sequence homology (Table 1). Based on the sequence homology, the nine isoforms can be classified into four groups, 1–4 (Fig. 3). Group 1 comprises AC5 and AC6; group 2 consists of AC1, AC3, and AC8; group3 consists of AC2, AC4, and AC7; and group 4 consists of AC9.

The tissue distribution of the isoforms examined by mRNA hybridization is not ubiquitous, indicating that there may be some tissue-specific functions. Some isoforms are found in the same tissues and cells, but it is unclear whether their functional and regulatory specificities are the same or different. The enzymes have been extensively examined in the brain. AC1 and AC2 are highly expressed in certain brain regions that are associated with learning and memory, such as the hippocampus, cerebral cortex, and cerebellum. In AC1 knockout mice, the adenylyl cyclase activity of cerebellar cortex is reduced by 65% and cerebellar long-term potentiation is blocked. Mice with a double knockout of AC1 and AC3 show no long-term memory. Studies using AC3 knockout mice indicate an important role for AC3 in the sense of smell. In addition, AC8 appears to be involved in long-term memory. These studies taken together suggest crucial roles for AC1 and AC3 in brain function, particularly in long-term memory. In addition to the knockout mice study, transgenic mice have been generated to overexpress AC5 or AC6. Mice overexpressing AC5 show myocardial damage over their lifetime, and those with overexpressed AC6 exhibit increased cardiac responsiveness to adrenergic stimulation.

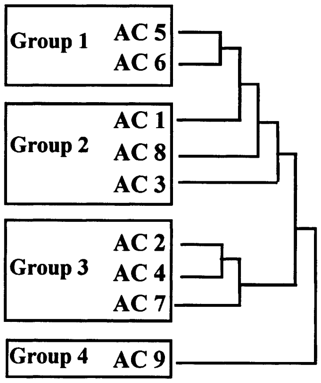

FIGURE 3 Classification of adenylyl cyclase isoforms. Nine isoforms of the enzyme have been cloned, and they can be classified into four distinct groups based on their sequence homology.

These isoforms are activated and suppressed differently by a number of distinct regulators. For example, Gαs can stimulate all AC isoforms but Gαi can suppress group 1 and group 3 adenylyl cyclases. Gβγ is capable of stimulating group 2 enzymes and it can suppress AC1. In addition to these G-proteins, $Ca^{2+}$/calmodulin can stimulate the group 1 enzymes, particularly AC1 and AC 8 in the brain. $Ca^{2+}$/calmodulin activates AC3 only in the presence of forskolin, and it is unclear whether AC3 is stimulated by $Ca^{2+}$/calmodulin *in vivo*. In contrast to the $Ca^{2+}$/calmodulin-sensitive group 1 enzymes, other groups are insensitive to $Ca^{2+}$/calmodulin. AC1 and the 1 and 3 enzymes are suppressed by Gβγ. AC5 and AC6 are also suppressed by protein kinases A and C, indicating the diversity in the regulatory mechanisms.

## III. PHOSPHOLIPASE C

Phospholipase C cleaves phospholipids to generate two products: one is DAG and the other can be inositol phosphate, phosphoethanol amine, phosphocholine, or phosphoserine. DAG and inositol phosphate, in particular, inositol 1,4,5-trisphosphate ($IP_3$), are recognized as important second messengers (Fig. 4). In addition, phosphoserine, phosphoethanol amine, and phosphocholine have been suggested to be second messengers. $IP_3$ is also generated by the interconversion of various inositol phosphates by a number of kinases that can phosphorylate and phosphatases that can dephosphorylate. These various inositol phosphates are also suggested to be second messengers. DAG activates protein kinase C, and $IP_3$ mobilizes $Ca^{2+}$ from intracellular stores such as the endoplasmic reticulum. This is accomplished by $IP_3$ binding to its receptor on the endoplasmic reticulum membrane, which in turn releases $Ca^{2+}$ from the endoplasmic reticulum lumen. Then, the released $Ca^{2+}$ regulates various molecules in the cell. $IP_3$ is also capable of modulating other molecules. For example, it interacts with actin-binding proteins and modulates actin polymerization; it is a co-factor for phosphatidylcholine-specific phospholipase D and a substrate for phosphoinositide 3-kinase, an important signal molecule.

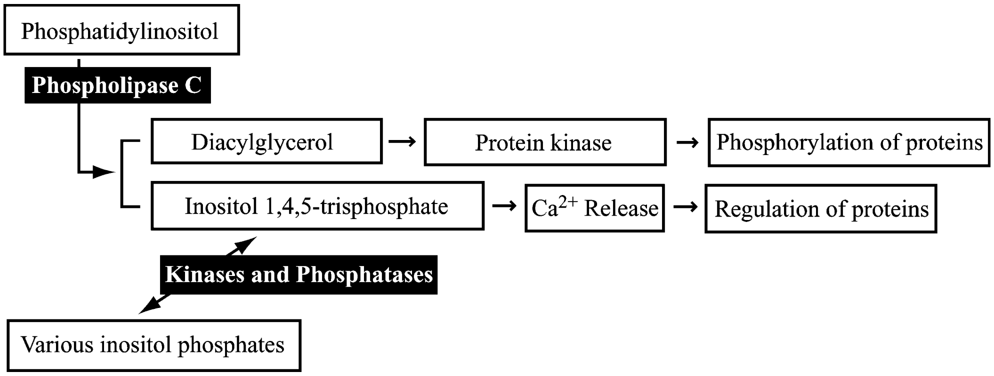
Phosphatidylinositol
Phospholipase C
Diacylglycerol
Protein kinase
Phosphorylation of proteins
Inositol 1,4,5-trisphosphate
Ca$^{2+}$ Release
Regulation of proteins
Kinases and Phosphatases
Various inositol phosphates

FIGURE 4 Phospholipase Cβ and production and conversion of inositol phosphates. Phosphatidyl-inositol is hydrolyzed by phospholipase Cβ to generate DAG and IP. DAG activates protein kinase C, which can phosphorylate various proteins and modulate them, whereas IP mobilizes Ca$^{2+}$ from intracellular stores, which regulates a variety of proteins. Various inositol phosphates are interconverted by kinases and phosphatases and are involved in signaling.

## A. Isoforms

There are 11 PLC isoforms, which are classified into four groups of different molecular weights, PLCβ with 4 isoforms (120–155 kDa), PLCγ with 2 isoforms (120–155 kDa), PLCδ with 4 isoforms (85 kDa), and PLCε with 1 isoform (230–260 kDa). These 4 PLC groups are found in higher eukaryotes, and lower eukaryotes such as yeast and slime molds have only PLCδ, suggesting the evolution of PLCβ, PLCγ, and PLCε from PLCδ.

PLC isoforms share a general domain structure (Fig. 5) although they have diverse amino acid sequences except for the conserved X and Y domains. The X and Y domains consist of 40–110 amino acids in PLCβ and PLCδ and 190 amino acids in PLCβε. The stretch of the X and Y domains in PLCγ spans ~400 amino acids and includes two Src homology 2 (SH2) domains of ~100 residues and one SH3 domain of ~50 residues, which recognize the sequences containing phospho-Tyr and multiple Pro residues, respectively. These isoforms also have a PH domain of ~100 amino acids that can bind inositol polyphosphates near the N-terminus of PLCβ, PLCγ, and PLCδ and at the center of PLCε. PLCγ has an additional PH domain that is split between the SH2 and the SH3 complexes. The PH domain is thought to interact with the inositol phosphate group of phosphatidylinositol of the cell membrane, thus anchoring it to the membrane, whereas the SH2 and SH3 domains mediate the interaction with other proteins. The isoforms also have the EF-hand domain and C2 domain, which is considered as an extension of the Y domain. PLCε is unique in possessing a domain similar to the Ras guanine nucleotide exchange factor (RasGEF) near the N-terminus and two Ras-binding (RA) domains near the C-terminus.

## B. Catalytic Site

The X and Y domains show ~60% amino acid similarity and form the core of the catalytic domain,

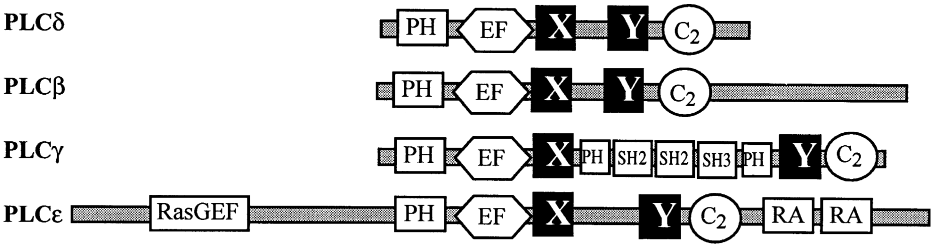
PLCδ
PH
EF
X
Y
C2
PLCβ
PLCγ
PH
SH2
SH2
SH3
PH
PLCε
RasGEF
RA
RA

FIGURE 5 Domain structure of phospholipase C isoforms. There are four groups of PLC isoforms, PLCδ, PLCβ, PLCγ, and PLCε. All of them possess X, Y, PH, EF, and C2 domains. The X and Y domains form the catalytic core, and the PH domain binds to the IP moiety of phosphatidylinositol.

TABLE 2 **Phospholipase Cβ Isoforms**

| Group | Isoform | Activators | Receptors | Tissue distribution | Function |
|---|---|---|---|---|---|
| PLCβ | 1 | Gαq ($K_d$ = 40–60 nM), Gα11, Gα14, Gα16, Gβγ | α, β, angiotensin II, bradykinin, bombasin | Hematopoietic cells | Learning, memory |
| | 2 | Gαq ($K_d$ = 400 nM), Gα11, Gα14, Gα16, Gβγ | Chemokines, histamine, GnRH, TSH, LH | Ubiquitous | Odor stimulation |
| | 3 | Gaq ($K_d$ = 40–60 nM), Gα11, Gα14, Gα16, Gβγ | Muscarinic acetylcholine, endothelin-1 | Ubiquitous | |
| | 4 | Gαq, Gα11, Gα14, Gα16 | Thromboxane, vasopressin, formyl | Retina, neuronal cells | |
| PLCγ | 1 | Gβγ, Gαs, Gαi | β2, angiotensin II, thrombin, acetylcholine | | |
| PLCδ | | Gαh (activator or suppressor) | α1 | | |
| PLCε | 2 | Gα12 | Thrombin, lysophosphatidic acid | | |

which is lined with eight ionic residues consisting of two Lys, one Arg, two His, two Glu, and one Asp, and one each of Asn, Ser, and Tyr according to the crystal structure of PLCδ. Their side chains bond with the phosphate and hydroxyl groups of polyphosphoinositol and Ca$^{2+}$, thus stabilizing them as well as the interaction between the two. The interaction explains why Ca$^{2+}$ is necessary for catalysis. The loss of the basic side chains impairs PLC's interaction with the substrate and catalysis. Therefore, the phosphoryl groups of the substrate lipid, phosphatidylinositol, are important for the affinity for the substrate binding and catalysis. In contrast, the products of the lipid hydrolysis, free inositolphosphates, are likely to suppress catalysis. The basic amino acids in the pleckstrin homology (PH) domain of PLC isoforms are likely to be involved in recruiting the enzyme to the cell membrane where the substrate is located. However, their sequences are not conserved and therefore, the interactions of the various PH domains with the phosphatidylinositol may not be the same. This further suggests that individual PLC isoforms may have differential affinity toward different inositol phosphate groups of the substrate lipid. In addition, the PH domains of PLCβ1 and PLCβ2 appear to be the contact sites for the Gβγ complex.

## C. Activation of PLCβ

All four PLCβ isoforms are directly activated by G-proteins linked to a vast number of G-protein-coupled receptors (Table 2), but their tissue distribution and activation mechanisms are diverse. All four isoforms of PLCβ can be activated by all four Gαq isoforms: Gαq, Gα11, Gα14, and Gα16 (Table 2). The Gβγ complex facilitates the activation of PLCβ1 and PLCβ2. In addition, there is evidence suggesting that Gβγ, Gαs, and Gαi are involved in the regulation of PLCγ. PLCδ may also be regulated by Gαh, whereas PLCε may be activated by Gα12.

Among the Gα-subunits, all of the Gαq family members are capable of activating all four isoforms of PLCβ, but their activation affinities vary widely. For example, Gαq and Gα11 activate the isoforms in the following order of activation affinity: PLCβ1 ≥ PLCβ3 ≥ PLCβ2. These activation affinities reflect the affinities of GTP-γ-S-bound Gαq for PLCβ1, PLCβ3, and PLCβ2. The equilibrium dissociation constants ($K_d$) of GTP-γ-S-bound Gαq isoforms are 40–60 nM for PLCβ1 and PLCβ3 and 400 nM for PLCβ2. Gα16 shares the least homology with the other Gαq family members; it is found to be expressed only in hematopoietic cells, but activates the PLCβ isoforms in a manner similar to other Gαq family members. Despite the Gαq family members' general affinities for the PLCβ isoforms, some receptors show a preference toward them. For example, the receptor for macrophage chemotactic protein-1 couples to Gα14 and Gα16, but not to Gαq or Gα11.

Before hydrolyzing membrane phospholipids, PLCβ must interact with the membrane. One mechanism to anchor it to the membrane is the interaction with the G-protein subunits. G-protein subunits are not hydrophobic but are anchored to the membrane. Gα is covalently linked to a fatty acid (myristic acid) near the N-terminus, and Gγ is either farnesylated or geranylated near the C-terminus.

Through these fatty chains, the two G-protein subunits are anchored to the cell membrane. Although Gβ does not have a fatty acyl chain, it is complexed with the Gγ-subunit, through which it is attached to the membrane. The C2 domain of the PLCβ isoforms binds to the activated GTP-bound Gαq with a $K_d$ value of 18 nM, showing a high affinity. When the activated GTP-bound Gαq is deactivated to become GDP-bound Gαq, the affinity between the GDP-bound Gαq and the C2 domain of the PLCβ drops significantly to $K_d$ values of 60–120 nM. This could provide a mechanism to release the enzyme from the membrane. Another mechanism of recruiting PLCβ to the membrane is the interaction of the PH domain of the enzyme with the inositol phosphate moiety of membrane-associated phosphatidylinositol. In fact, this interaction of the substrate might facilitate the enzyme catalysis.

Gβγ is also capable of activating PLCβ isoforms with varying affinities. Gβγ does not activate PLCβ4. Gβγ interacts with PLCβ2 with a high affinity, but the affinity for PLCβ1 and PLCβ3 is not high. PLCβ1 is the least sensitive to Gβγ. The PLCβ2 activation by Gβγ has been documented in a large number of receptors including those for luteinizing hormone (LH), β1, β2, formyl, V2 vasopressin, interleukin-8, and muscarinic acetylcholine. PLCβ2 is also activated by some of these receptors via Gαq. Although the Gβγ concentration necessary for activation is higher than that for Gαq, the maximum level of activation is similar. Therefore, both Gβγ and Gαq are likely to be involved in the activation of PLCβ but the existing evidence shows that Gαq is essential. Gαq and Gβγ interact with PLCβ at different sites: Gαq near the C-terminus and Gβγ at the PH domain. Although Gβγ and the inositol phosphates bind to the PH domain, their contact points are distinct. Interestingly, PLCβ and Gαq bind to Gβγ at the same site, and therefore, Gβγ can interact with one of them but not both. The 7 Gβ isoforms and 12 Gγ isoforms can produce a large number of combinations. Different Gβγ complexes show a specificity for PLCβ isoforms. For example, Gβ1γ can activate PLCβ1, PLCβ2, and PLCβ3, whereas Gβ5γ can activate PLCβ1 and PLCβ2, but not PLCβ3.

## D. Deactivation of PLCβ

PLCβ isoforms activated by the GTP-bound Gα-subunits can be turned off by the hydrolysis of the GTP bound to Gα-subunits. This hydrolysis is catalyzed by the GTPase activity of Gα-subunits, which is intrinsic to Gα-subunits but low in the absence of PLCβ. Activated PLCβ stimulates the GTPase activity by 6–60 fold, and therefore, PLCβ serves as a GTPase-activating protein (GAP) as is adenylyl cyclase. In the case of PLCβ1, the C-terminal fragment consisting of amino acid residues 903–1042 is involved in the activation.

The GTPase activity is also stimulated by regulators of G-protein signaling (RGS). Although they serve as GTPase activating proteins, their action mechanism differs from those of PLCβ and adenylyl cyclase. For example, RGS interact with receptors unlike the interaction of PLCβ and adenylyl cyclase with Gα. More than 20 isoforms of RGS have been cloned to date, and they are effective to Gαq and Gαi, but not for Gαs and Gα12. When the GTPase activity of Gα is activated and the GTP that is bound to Gα is hydrolyzed to GDP, the Gα disengages from PLCβ and adenylyl cyclase, leading to deactivation of the enzymes. The dissociated Gα now engages with the Gβγ complex, thus completing the activation and deactivation cycle of G-protein.

## E. Activation of PLCδ and PLCε

Gαh (high-molecular-weight G-protein) is co-immunoprecipitated with the thromboxane A2α receptor, but not the A2β, receptor. It has been shown that the receptors for α1 and oxytocin stimulate Gh to complex with PLCδ1 and activate the enzyme in the presence of GT-γ-S. These results suggest that the receptors interact with Gαh and activate it. Interestingly, these receptors are also capable of binding Gαq and activating PLCβ. Gα12 appears to activate PLCε. For example, a Gα12 mutant lacking the GTPase activity enhances the enzyme activity. This suggests the interesting possibility that Gα12 may activate PLCε, perhaps via a G-protein-coupled receptor.

# IV. ION CHANNELS

Nerve cells communicate one another and with other cells such as muscle and sensory cells through their contact points, referred to as synapses, which have specialized structures containing hormone receptors and ion ($Na^+$, $K^+$, $Ca^{2+}$, and $Cl^-$) channels. They communicate by two distinct signal mechanisms, electrical and chemical. Electrical signals are generated by differential ion concentrations and pass from presynaptic cells to postsynaptic cells through voltage-dependent ion channels. Chemical signals include neurotransmitters such as acetylcholine, glycine, glutamate, γ-amino butyric acid, serotonin, dopamine, epinephrine, and norepinephrine. They are released from presynaptic cells and bind to their cognate receptors on postsynaptic cells, which opens ion channels.

Voltage-dependent ion channels comprise several subunits, α (170–250 kDa) β (50–78 kDa), γ (36 kDa), and δ, and various ion channels have different subunit compositions. Among ion channels, $Ca^{2+}$ channels have been extensively investigated and play essential roles in neuronal activity. They modulate the release of neurotransmitters, activation of $Ca^{2+}$-dependent enzymes and regulators, and neuronal excitation. Defective $Ca^{2+}$ channels are associated with disorders including migraine, cerebella ataxia, night blindness, and epilepsy. There are several types (N, P/Q, L, R, and T) of $Ca^{2+}$ channels, which have specific α isoforms: α1A for P/Q, α1B for N, α1D for L, α1E for R, and α1G-I for R. The N-type channel is activated by protein kinase C, whereas the N- and P/Q-type channels are suppressed by G-protein-coupled receptors via the Gβγ-subunits of Gi and Go. This inhibition appears to occur by Gβγ-dependent tyrosine phosphorylation of the channel α-subunit. Involved in this regulation are the receptors for a wide variety of hormones including α2 and β2 adrenalines, dopamine D5, GABAa, a metabotropic glutamate, opioid, adenosine, and muscarinic acetylcholine. The β-subunit is primarily involved in the equimolar interaction with the channels. Gβ isoforms show a specificity toward the channels as Gβ1, but not Gβ2–4, is effective for the N-type channel. It appears to interact with the domain I and II linkers and C-terminal region of the α1 of the N-type channel. In addition, the channel β-subunits play a role in the interaction and the Gβ isoforms show specificity.

## V. OTHER EFFECTORS

Phosphodiesterases are activated by rhodopsin via Gαo, and phospholipase A2 is also activated by G-protein-coupled receptors for $Ca^{2+}$, α1 adrenaline, ATP, bradykinin, muscarinic acetylcholine, and endothelin-A. The $Ca^{2+}$ receptor activates phospholipase A2 via Gαq, and the endothelin-A receptor activates the enzyme through the Gαq–phospholipase Cβ–DAG–protein kinase C pathway. Gβγ interacts with phosphoinositide 3-kinase and activates it.

Receptor tyrosine kinases such as the epidermal growth factor receptor are stimulated by G-protein-coupled receptors such as the β adrenergic receptor. This appears to follow complex pathways involving Gβγ, phosphoinositide 3 kinase, Src, and receptor tyrosine kinase. Mitogen-activated protein kinase is one of the central components in the cell signaling pathways and is activated by Gαq-coupled receptors. Jun N-terminal kinase is also activated by a G-protein-coupled receptor but the pathway is unclear.

Arrestin is an adaptor molecule and binds to phosphorylated G-protein-coupled receptors, leading to endocytosis of the receptors. ADP ribosylation factor 6 is directly activated by liganded LH receptors, which in turn stimulate the desensitization and internalization of the receptors. In addition, there is evidence for the interaction of G-protein-coupled receptors with Jak2 kinase, phospholipase Cγ, and protein kinase C. Clearly, these observations predict a vast number of signal molecules to complex directly or indirectly with G-protein-coupled receptors and to elicit various hormonal responses.

## Glossary

**adenylyl cyclase** An extensively investigated effector that hydrolyzes ATP to produce cAMP, a second messenger.

**effectors** Usually enzymes that are regulated by G-proteins in response to hormones binding to receptors.

**G-protein-coupled receptor** Hormone receptors that are present on the cell surface and coupled to G-proteins in the cell. These receptors generally transfer hormone signals to G-proteins to trigger hormone actions from the cells.

**guanine nucleotide-binding protein (G-protein)** This protein binds the guanine nucleotides GDP or GTP and has three different subunits, the α-, β-, and γ-subunits. It interacts with G-protein-coupled receptors, receives hormone signals from the receptors, and modulates effectors in the cell.

**hormone receptor** Molecules on the cell surface that bind specific hormones. More than 1000 receptors have been cloned.

**hormones** Biological molecules, such as proteins, peptides, amino acid derivatives, and lipids, that are produced from an endocrine gland and spread to target tissues and cells, often through the circulatory system. They bind to specific receptors and trigger specific effects; therefore, they are considered first messengers.

**isoform** Variants of proteins and enzymes that share the same functions and similar structures.

**phospholipase C** An effector of G-protein-coupled receptors that is capable of hydrolyzing phospholipids to generate two second messengers, diacylglycerol and inositol phosphate.

## See Also the Following Articles

**GPCR (G-Protein-Coupled Receptor) Structure • Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Its Receptor • Protein Kinases • Receptor-Mediated Interlinked Systems, Mathematical Modeling of • Steroid Hormone Receptor Family: Mechanisms of Action • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# EGF

See ***Epidermal Growth Factor***

# Eicosanoids

Odette Laneuville
*University of Ottawa*

I. INTRODUCTION
II. NOMENCLATURE AND CHEMICAL STRUCTURES
III. SYNTHESIS
IV. EICOSANOIDS IN THE ENDOCRINE SYSTEM
V. PHARMACOLOGICAL IMPORTANCE
VI. SUMMARY

**The goals of this article are to describe the chemical structure of eicosanoids, their enzymatic synthesis, and the mechanisms by which they exert their actions on various physiological systems.**

## I. INTRODUCTION

Establishing the biological significance of eicosanoids was particularly laborious and still constitutes a very active area of research. The large number of eicosanoid family members, over 50, posed a difficulty in establishing their importance. The chemical structures of those eicosanoids and their metabolites have been determined, along with those of some recently added to the list. The general chemical structure of eicosanoids corresponds to that of oxygenated fatty acids formed from arachidonic acid by the addition of oxygen catalyzed enzymatically. There are three pathways for the formation of eicosanoids: the cyclo-oxygenase, lipoxygenase, and epoxygenase pathways. To add to the difficulty of establishing this ever-growing family of substances as hormones, they are, as a rule, synthesized and released on demand. In most cases, they do not circulate at considerably high levels and have a very short half-life. Moreover, eicosanoids are recognized as naturally occurring active substances produced by many organs and affecting multiple functions. The situation is much easier for hormones produced by specialized organs, where removal of the endocrine gland generally gives information about its biological significance. In the case of eicosanoids, their importance in the regulation of physiological functions and homeostasis was revealed by the availability of inhibitors of the three pathways for the formation of eicosanoids. The most important are the inhibitors of the cyclo-oxygenase enzymes extensively used in clinical settings for the treatment of inflammation in various contexts. Still, the most popular and lucrative representative of the nonsteroidal anti-inflammatory drugs is aspirin.

## II. NOMENCLATURE AND CHEMICAL STRUCTURES

Eicosanoid derives from the Greek “eicosa,” which means 20; eicosanoids constitute a broad group of compounds derived from 20-carbon polyunsaturated fatty acids. Arachidonic acid (AA) serves as the most common precursor and consists of a 20-carbon chain with a methyl (-$CH_3$) group at one end of the chain and a carboxyl (-COOH) group at the other end.

**phospholipase C** An effector of G-protein-coupled receptors that is capable of hydrolyzing phospholipids to generate two second messengers, diacylglycerol and inositol phosphate.

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The carbon chain forming arachidonate also includes 4 unsaturated double bonds and is designated 20:4 (20 carbons and 4 double bonds). The carbons are numbered 1 to 20 from the -COOH terminal; the COOH end is designated C1 and the $CH_3$ end is designated C20. According to this nomenclature, the COOH-terminal is termed the alpha-terminal and the double bonds in arachidonate are formed between carbons 5 and 6, 8 and 9, 11 and 12, and 14 and 15. This designation will be used in this article. The carbon chain can also be numbered from the $CH_3$-terminal and this is the omega (ω) or biological numeration. According to the ω nomenclature, the double bonds in arachidonate are formed between carbons 6 and 7, 9 and 10, 12 and 13, and 15 and 16. AA is also referred to as 20:4ω6 since the first double bond involves C6 in the chain. All the double bonds of AA are conjugated, which signifies they are interrupted by a methylene (-$CH_3$-) group and have a *cis* configuration.

All eicosanoids are biosynthesized by molecular oxygen fixation at well-determined sites on the AA molecules. There are three pathways for the formation of eicosanoids: the cyclo-oxygenase, lipoxygenase, and epoxygenase pathways (Fig. 1). The three pathways are collectively known as the arachidonate cascade. The cyclo-oxygenase pathway leads to the formation of prostanoids, including prostaglandins and thromboxanes. The lipoxygenase pathway leads to the formation of leukotrienes, hepoxilins, and lipoxins, and the P450 pathway leads to the formation of monohydroxylated fatty acids.

## A. Prostanoids

Prostanoids fall into several classes designated by letters (A to J) (Fig. 2). From the substrate AA, the unstable endoperoxide prostaglandin $G_2$ ($PGG_2$) is first produced. The hydroperoxy group (-OOH) on carbon 15 of $PGG_2$ is then reduced to a hydroxyl group (-OH) and the product formed is prostaglandin $H_2$ ($PGH_2$). All other prostanoids are derived from $PGH_2$ and are distinguished by substitutions on the cyclopentane ring of $PGH_2$. Prostaglandins of the E and D series are hydroxy ketones and prostaglandins $F_\alpha$ are 1,3-diols. Prostaglandin $I_2$, also termed prostacyclin, has a double-ring structure; in addition to a cyclopentane ring, a second ring is formed by an oxygen bridge between carbons 6 and 9. Thromboxanes contain a six-member oxane ring instead of the cyclopentane ring of the prostaglandins. Both prostacyclins and thromboxanes also result from the metabolism of $PGH_2$. Prostaglandin $J_2$ derives from $PGD_2$ and contains a keto substitution on the cyclopentane ring. Prostaglandins A, B, and C are unsaturated ketones that arise nonenzymatically from PGE during extraction procedures; there is no evidence that they occur biologically.

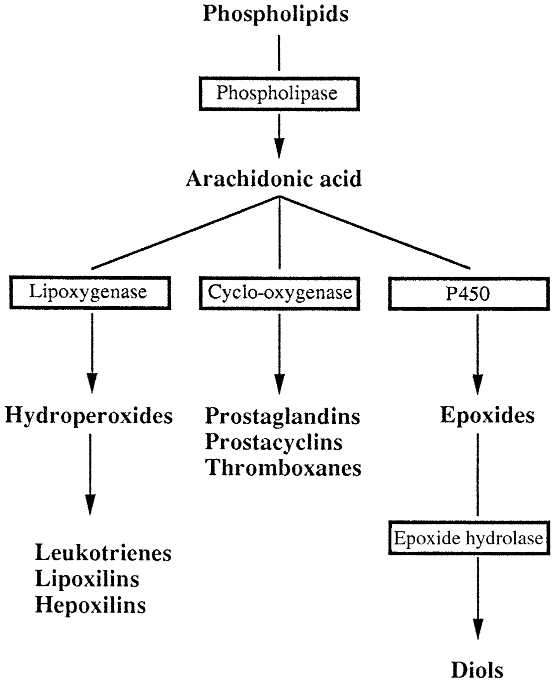

FIGURE 1 Arachidonic acid cascade. From the substrate arachidonic acid, three pathways lead to the formation of the various eicosanoids. The cyclo-oxygenase forms the prostanoids, which includes the prostaglandins and thromboxanes. The lipoxygenase pathway leads to the formation of HPETEs, HETEs, leukotrienes, hepoxilins, and lipoxins. The P450 pathway forms EETs and HETEs.

## B. Hydroperoxyeicosatetraenoic Acids, Hydroxyeicosatetraenoic Acids, Leukotrienes, Hepoxilins, and Lipoxins

The lipoxygenase pathway of AA leads to the formation of hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), leukotrienes, hepoxilins, and lipoxins. HPETE are named according to the location of the -OOH group; that is, 5-HPETE bears a hydroperoxide group at the carbon 5 position, and 8-HPETE, 9-HPETE, 11-HPETE, 12-HPETE, and 15-HPETE are designated in a similar manner.

HPETEs are short-lived compounds and are metabolized into more stable compounds belonging

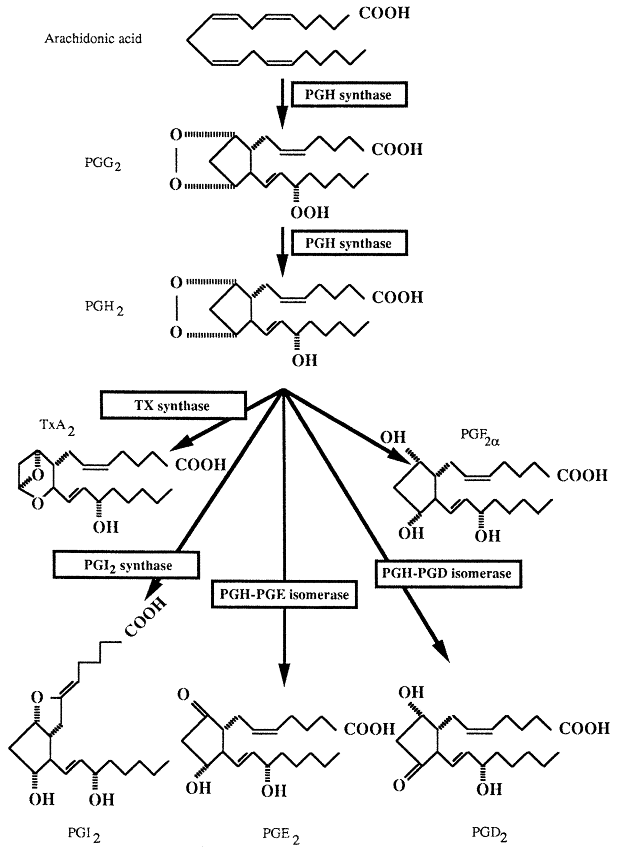
Arachidonic acid
COOH
PGH synthase
PGG2
COOH
OOH
PGH synthase
PGH2
COOH
OH
TX synthase
TxA2
COOH
OH
OH
PGF2α
COOH
OH
OH
PGI2 synthase
PGH-PGE isomerase
PGH-PGD isomerase
COOH
OH
OH
PGI2
COOH
OH
OH
PGE2
OH
COOH
OH
PGD2

FIGURE 2 The cyclo-oxygenase pathway of arachidonic acid. The transformation of arachidonic acid into prostanoids is initiated by the action of the enzyme cyclo-oxygenase that consecutively forms prostaglandin $G_2$ and prostaglandin $H_2$. The product $PGH_2$ then serves as a substrate for the various isomerases and synthases to produce $TXA_2$, $PGI_2$, $PGE_2$, $PGD_2$, and $PGD_2$.

to various families, of which the HETEs, leukotrienes, hepoxilins, and lipoxilins have been identified. The leukotriene family derives from 5(*S*)-HPETE, and the term leukotriene connotes the source from which the first substances in this family were isolated, the leukocytes, and the common essential structural feature, a conjugated triene. Leukotrienes (LTs) fall into classes designated by letters (A to F), in a manner consistent with the prostanoid nomenclature. Leukotriene $A_4$ ($LTA_4$) bears an epoxide group (-O-) on carbons 5 and 6 of the carbon chain, and leukotriene $B_4$ ($LTB_4$) has two hydroxyl groups, one on carbon 5 and one on carbon 12 (Fig. 3). Other leukotrienes derive from $LTA_4$.

Hepoxilins derive from 12(*S*)-HPETE and result from the rearrangement of the -OOH group. Two hepoxilins are formed, hepoxilin $A_3$ and $B_3$, and both compounds carry their epoxide group on carbon atoms 11 and 12. They are distinguished by the position of their hydroxyl group, on carbon atom 8 for hepoxilin $A_3$ and on carbon atom 10 for hepoxilin $B_3$. Lipoxins contain a conjugated tetraene structure, four conjugated double bonds, and three hydroxyl groups. The main compounds in this family were isolated from leukocytes and are named lipoxins $A_4$ and $B_4$.

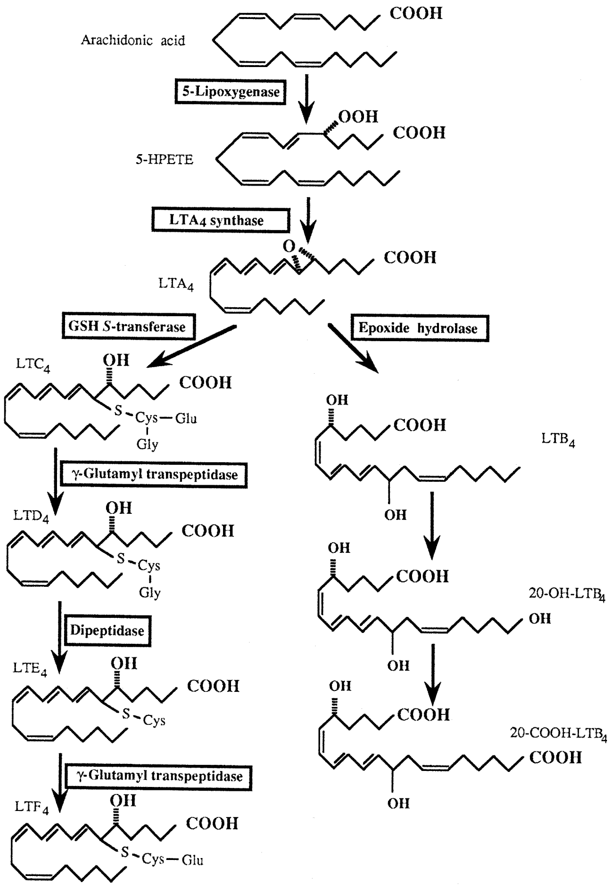
Arachidonic acid
COOH
5-Lipoxygenase
OOH
COOH
5-HPETE
LTA4 synthase
O
COOH
LTA4
GSH S-transferase
Epoxide hydrolase
LTC4
OH
COOH
S
Cys—Glu
Gly
γ-Glutamyl transpeptidase
LTD4
OH
COOH
S
Cys
Gly
Dipeptidase
LTE4
OH
COOH
S
Cys
γ-Glutamyl transpeptidase
LTF4
OH
COOH
S
Cys—Glu
OH
COOH
LTB4
OH
OH
COOH
20-OH-LTB4
OH
OH
OH
COOH
20-COOH-LTB4
COOH
OH

FIGURE 3 The lipoxygenase pathway of arachidonic acid. The specific incorporation of oxygen on carbon 5 of the arachidonic acid chain leads to the formation of 5-HPETE. This product becomes the substrate for $LTA_4$ synthase, which forms $LTC_4$. The product of the $LTA_4$ synthase reaction then becomes the substrate for both $LTB_4$ and $LTC_4$. The GSH moiety of $LTC_4$ is further metabolized to form $LTD_4$, $LTE_4$, and $LTF_4$.

## C. EETs and HETEs

The P450 or epoxygenase pathway leads to the formation of two groups of monohydroxylated fatty acids: the epoxyeicosatrienoic acids (EETs) and the monohydroxyeicosatetraenoic acids. All products of the P450 pathway derived directly from the substrate

AA, to which one atom of oxygen derived from molecular oxygen ($O_2$) is added to the carbon chain. In the case of EETs, the oxygen atom is inserted in the form of an epoxy group (-O-) across each of the double bonds of AA. The four possible epoxides formed are 5,6-EET, 8,9-EET, 11,12-EET, and 14,16-EET (Fig. 4). EETs are then converted by epoxide hydrolases to their corresponding vicinal diols, the dihydroxyeicosatrienoic acids (DiHETEs). For the HETEs, the oxygen atom is added in the form of a hydroxyl group (-OH) onto one of the carbons of AA, 5, 8, 9, 11, 12, or 15, and the HETEs formed are designated based on the position of the hydroxyl group: 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, and 15-HETE. As detailed in the previous section, HETEs are also produced from the lipoxygenase in which case the insertion of the oxygen atom is position- and orientation-specific. Oxidation of AA by hepatic microsomal cytochrome P450 produces HETEs that are not stereo-and region-specific.

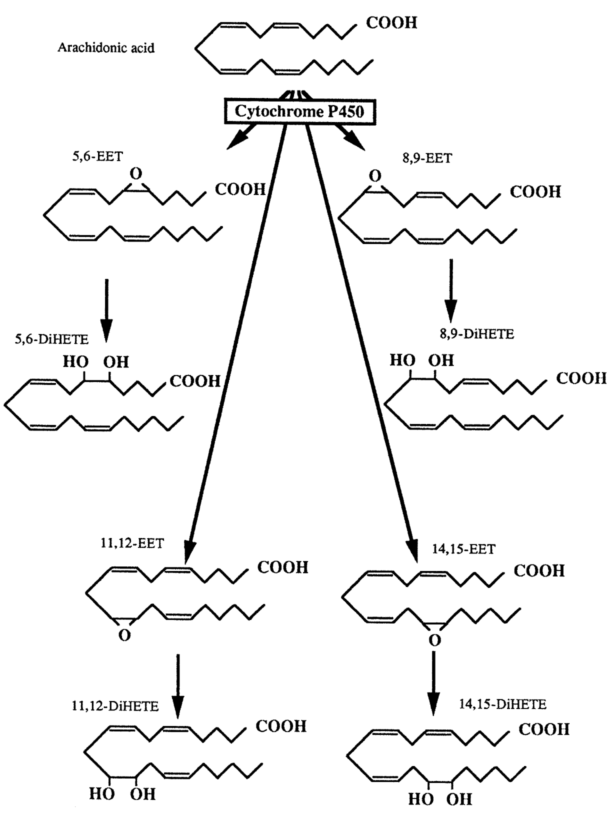

FIGURE 4 The cytochrome P450 pathway of arachidonic acid. P450 catalyzes the incorporation of oxygen to form all four possible EETs. The epoxide group is then subjected to hydrolysis catalyzed by epoxide hydrolases, and the products formed are diols.

## III. SYNTHESIS

Prostaglandins were discovered as products of the only organ where they are stored in large amounts: as the secretion from the vesicular gland. The occurrence of prostaglandins in the seminal fluid of some species is unique, in the sense that similar amounts are not found elsewhere in the organism. As a rule, there are no stores of eicosanoids and they are formed in most tissues, in response to various stimuli. The first step in the synthesis of eicosanoids is the release of the substrate arachidonic acid from membrane phospholipids. Various biosynthetic enzymes then catalyze the oxygenation of arachidonic acid, which leads to the formation of eicosanoids.

### A. Substrate Release

The most common and important substrate in the synthesis of eicosanoids is arachidonic acid. Two other polyunsaturated fatty acids can serve as substrates, homo-γ-linolenic acid (20:3) and eicosapentaenoic acid (20:5). Like arachidonic acid, they are composed of a chain of 20 carbons but differ in the number of double bonds that they possess: 3 for homo-γ-linolenic acid and 5 for eicosapentaenoic acid. The number of double bonds present in the fatty acid used for the synthesis of eicosanoids will dictate the number of double bonds present in the formed eicosanoids; homo-γ-linolenic acid, arachidonic acid, and eicosapentaenoic acid will be transformed in eicosanoids with 1, 2, and 3 double bonds, respectively. The number of double bonds is indicated in subscript; eicosanoids derived from arachidonic acid carry the subscript 2 and are the most abundant eicosanoid in mammals.

The substrates in the synthesis of eicosanoids are derived from two essential fatty acids, precursors that are provided by the diet. Linoleic acid (18:2), used to form homo-γ-linolenic and arachidonic acids, is particularly abundant in certain vegetable oil acids. α-Linolenic acid (18:3) is obtained mainly from marine animals and is used in the synthesis of eicosapentaenoic acid. In mammalian tissues, 18:2 and 18:3 are desaturated and elongated to produce the substrates in the synthesis of eicosanoids 20:3, 20:4, and 20:5, which are then stored in the membrane phospholipids. The cellular membrane is particularly rich in arachidonic acid and this is the most important substrate for the synthesis of eicosanoids.

## B. Cyclo-oxygenase Pathway

Cyclo-oxygenases catalyze the incorporation of two molecules of oxygen ($O_2$) on the AA chain. The details of the reaction of insertion of oxygen have been reported. Initially, the enzyme cyclo-oxygenase abstracts the hydrogen on carbon 13, the 13 pro *S* hydrogen, and inserts one molecule of $O_2$ on carbon 11. The insertion of oxygen occurs from the plane opposite that from which the hydrogen is abstracted; the insertion is said to be antarafacial. At this stage, a double bond is created between carbons 12 and 13 of the arachidonic chain, and one atom of oxygen from the added $O_2$ will form a link with C9, eliminating the C8–C9 double bond of AA. The relocalization of electrons will lead to formation of the cyclopentane ring within the AA chain. The second molecule of oxygen is inserted on carbon 15 of the AA chain. The oxygen added on C15 remains in the form of a hydroperoxide (-OOH) and the product formed is prostaglandin $G_2$. The cyclo-oxygenase will then reduce the hydroperoxide at position 15 to a hydroxyl group (-OH), and the product formed is prostaglandin $H_2$. This reduction is catalyzed by the second enzyme activity of the cyclo-oxygenase enzyme: the peroxidase activity. Both enzyme activities of cyclo-oxygenase have been mapped to different sites on the protein. Therefore, the designation cyclo-oxygenase for this enzyme refers to only one of the two activities the enzyme has: the cyclo-oxygenase activity. The term prostaglandin endoperoxide H synthase is also used and refers to the two activities the enzyme has: the cyclo-oxygenase and the peroxidase activities.

Cyclo-oxygenase enzyme exists as two isoforms encoded by two separate genes. The two cyclo-oxygenases, cox-1 and cox-2, catalyze the same reaction: the formation of $PGH_2$ from AA. Their patterns of expression and regulation differ significantly. The cox-1 enzyme is constitutively expressed in most cell types, where it plays a housekeeping role. Cox-2 has been termed the inducible cyclo-oxygenase because of its rapid up-regulation in response to various stimuli, including inflammatory cytokines.

The formation of the biologically active prostanoids, a term that includes prostaglandins and thromboxanes, from $PGH_2$ occurs through the action of a set of isomerases and synthases (Fig. 2). Prostanoid-forming enzymes include PGH–PGD isomerase, PGH–PGE isomerase, PGF synthase, PGI synthase, and TX synthase and form $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, and thromboxane $A_2$ ($TXA_2$). All prostanoid-forming cells produce $PGH_2$ through the action of cyclo-oxygenase, which is further metabolized into only one of these prostanoids because of the predominance of a single $PGH_2$ metabolizing enzyme. For instance, blood platelets contain mostly TX synthase and form mainly $TXA_2$.

## C. Lipoxygenase Pathway

The lipoxygenase enzymes catalyze the formation of HPETEs and are named according to the site of entry of the -OOH group on the carbon chain, e.g., 5-LOX, 8-LOX, 9-LOX, 11-LOX, 12-LOX, and 15-LOX. The positions of insertion are all part the double bonds of the AA chain. The addition of molecular oxygen to AA by lipoxygenases is not only position-specific but also stereo-specific; the added -OOH has an *S* configuration. A single molecule of oxygen is added to the AA chain by the lipoxygenase enzymes, whereas two $O_2$ molecules are added by the enzyme cyclo-oxygenase. Like the cyclo-oxygenases, lipoxygenases insert oxygen antarafacially to the AA chain. Similar to the cyclo-oxygenase pathway, the first product formed from AA is an unstable hydroperoxide termed HPETE.

HPETEs are subjected to three reactions depending on the biosynthetic enzymes present. Peroxidases reduce the HPETEs to their hydroxy analogues, HETEs. The second possible reaction is a dehydration and the formation of an epoxide group at positions 5 and 6 on the carbon chain; the product formed is $LTA_4$. The epoxide group of $LTA_4$ can be hydrolyzed into two hydroxyl groups, and the product formed is $LTB_4$. $LTA_4$ is also subjected to epoxide opening by the enzyme glutathione *S*-transferase with the addition of glutathione to form the sulfidopeptide series of leukotrienes. The initial product is leukotriene $C_4$ ($LTC_4$), which carries the glutathione peptide, Gly-Cys-Glu, at position 6 of the carbon ring as well as a hydroxyl group on carbon 5. The glutathione moiety is further metabolized by a γ-glutamyltranspepdidase, which cleaves the glutamic acid residue to form leukotriene $D_4$ ($LTD_4$) following digestion by a dipeptidase to form leukotriene $E_4$ ($LTE_4$). $LTD_4$ can also be converted to $LTF_4$ by the γ-glutamyltransferase. The leukotrienes $C_4$ and $D_4$ were originally known as slow-reacting substances of anaphylaxis with respect to their smooth muscle-contracting activity in allergic reactions.

The third possible transformation of the HPETE formed by the lipoxygenase pathway is the intramolecular isomerization of the hydroperoxide group to form the hepoxilins and the lipoxilins. Hepoxilins are hydroxy epoxide metabolites of AA formed through the 12-lipoxygenase pathway. The hydroperoxide group is rearranged into an epoxide group and a hydroxyl group to form $HxA_3$ and $HxB_3$. The formation of hepoxilins was initially described as a ferriheme-assisted reaction and more recently was shown to be catalyzed enzymatically. The term hepoxilin combines both the chemical structure and the function; hepoxi refers to the epoxide group of the molecule and lin refers to the first biological activity reported, the release of insulin. The name lipoxilins derives from lipoxygenase interaction products and these are formed as a result of an interaction between the 5- and the 15-lipoxygenases.

### D. Cytochrome P450 Pathway

The formation of EETs and of HETEs from AA is catalyzed by the cytochrome P450 enzymes and requires molecular oxygen as well as NADPH in a 1:1 stoichiometry. The insertion of oxygen by cytochrome P450 enzymes is characterized by the use of reducing equivalents to split the oxygen–oxygen bond in molecular oxygen and the transfer of one activated oxygen atom to the substrate AA while the other oxygen atom appears in water. The addition of oxygen in the form of an epoxide group leads to the formation of four possible epoxides: 5,6-, 8,9-, 11,12-, 14,15-epoxyeicosatrienoic acids (Fig. 4). These in turn can be converted by epoxide hydrolases to the corresponding vicinal diols, the dihydroxyeicosatrienoic acids. The cytochrome P450 can also perform allylic oxidations of AA to form HETEs. The distinction between P450 and lipoxygenase-derived HETEs is found in the stereoselectivity of the product formed. The lipoxygenase products are enantiospecific with an *S* configuration of the hydroxyl group. Hepatic P450 enzymes produce both *R* and *S* monohydroxylated fatty acids.

## IV. EICOSANOIDS IN THE ENDOCRINE SYSTEM

Prostanoids may serve both specific and general functions. All of the main systems contributing to homeostasis of mammals are under the influence of eicosanoids. In the circulatory system, the interaction between the circulating platelets and the vascular endothelial cells is of significant importance for the treatment and prevention of cardiovascular diseases. Under normal physiological conditions, eicosanoids produced by the cyclo-oxygenase pathway generally induce vasorelaxation and prevent platelet aggregation. In contrast, thromboxane $A_2$ produced by platelets will induce vasoconstriction and platelet aggregation. Inhibiting eicosanoid formation with aspirin will be more significant for platelets than for endothelial cells since platelets are anucleated cells and cannot replenish their pool of cyclo-oxygenase enzyme. Aspirin induces an irreversible inhibition of cyclo-oxygenase, which means that the enzyme is condemned and new enzyme must be resynthesized in order to produce prostanoids. Under aspirin exposure, platelets are irreversibly inhibited and will be less likely to aggregate and to form thrombus since their production of the pro-aggregatory thromboxane $A_2$ is inhibited. The production of the anti-aggregatory prostanoid $PGI_2$ by endothelial cells can be reestablished after aspirin exposure since endothelial cells are nucleated and active cyclo-oxygenase can be synthesized. The net result of aspirin inhibition is to favor the action of the anti-aggregatory substance $PGI_2$ and the net effect on the circulatory system is the reduction in cardiovascular diseases associated with hyperplatelet functions.

The digestive system is also under the influence of eicosanoids. Prostanoids regulate gastric acid secretion, intestinal mobility, and intestinal ion transport, prevent liver damage due to hepatotoxin, participate in liver regeneration, and regulate pancreatic secretions, to name a few functions. Most P450 enzymes are primarily expressed in the liver, where they oxidize, peroxidize, and reduce cholesterol, vitamins, steroids, and xenobiotics in an oxygen- and NADPH-dependent manner. Many P450 isozymes in the extrahepatic tissues are also capable of metabolizing AA to biologically active products such as EETs, which regulate vascular tone. The nervous system is influenced by the hormonal action of eicosanoids. $PGD_2$ regulates sleep episodes; $PGE_2$ regulates body temperature, cerebral vasospasm, and the transmission of pain signals to the central nervous system.

Prostaglandins were originally isolated in the seminal fluid where they accumulate and the name prostaglandins derives from the prostate. The reproductive system of females is largely influenced by eicosanoids. They seems to play a role in female fertility as demonstrated in cox-2 knockout mice, which have a significantly reduced fertility. The regression of the corpus luteum is influenced by $PGF_{2\alpha}$ produced by the uterus. $PGE_2$ is necessary for parturition and causes cervical dilation and myometrial contractions. There is substantial evidence that different prostaglandins are involved in the regulation of normal uterine contractility, dysmenorrhea, and menstruation. In the newborn, $PGE_2$ is responsible for the closure of the ductus arteriosus, a direct connection between the main pulmonary artery and the descending aorta that allows the circulation of the newborn to be directed to the left ventricule of the heart and then to the lungs. Several lines of evidence indicate that the fetal ductus arteriosus is relaxed and dilated by prostaglandin actions.

The four classical cardinal signs of inflammation, redness, swelling, heat, and pain, are all induced by different prostaglandins. This is due in part to their ability to dilate vessels, to increase vascular permeability, and to sensitize nerve terminals transmitting pain information. Experimental evidence also indicates that prostanoids are capable of potentiating the effects of other mediators of inflammation such as bradykinin and histamine. The most popular treatment for the symptoms of inflammation comprises the inhibitors of prostanoid synthesis, nonsteroidal anti-inflammatory drugs (NSAIDs), largely because of their anti-pyretic and analgesic properties.

The concept that leukotrienes play a pivotal role in airway disease such as asthma is derived mainly from evidence that several cell types that produce leukotrienes—mast cells, basophils, and eosinophils—are present in increased numbers in the lungs of patients with asthma. In addition, metabolites of leukotrienes detected in biological fluids from subjects with asthma further support this concept.

## A. Receptors

In agreement with the adopted theory of hormone action, eicosanoids attach to receptors on the cell surface or inside the cell before these initiate the process of response action. The receptors for prostanoids and leukotrienes have been extensively studied and all share a common topography, being composed of a single polypeptide with seven transmembrane-spanning domains. The receptor structure is linked to a G-protein responsible for initiating the cascade of reactions leading to the transduction of the message, the binding of the eicosanoid to its receptor inside the cell. The signal transduction system involves sequential phosphorylation reactions initiated by the hydrolysis of GTP by the G-protein.

Receptors for prostanoids and leukotrienes have been characterized pharmacologically in many bioassay systems using various prostanoids and leukotrienes and their stable analogues. Based on these studies, a pharmacological classification of the prostanoid and the leukotriene receptors is currently used. Receptors specific for PGD, PGE, PGF, PGI, and TX are named the DP, EP, FP, IP, and TP receptors, respectively. The EP receptors are further classified into four subtypes, $EP_1$, $EP_2$, $EP_3$, and $EP_4$, all of which respond to $PGE_2$ but differ in their actions and their responses to various analogues. Like the prostanoid receptors, the leukotriene receptors have seven transmembrane domains and are coupled to a G-protein. At least two distinct leukotriene receptors have been extensively studied and are termed $CysLT_1$ and $CysLT_2$. The prostanoid and leukotriene receptors have now been cloned and are currently used to screen and design chemical compounds that could either mimic or block the effect of endogenous eicosanoids with the goal of controlling conditions in which a lack of eicosanoid or an excess of a response to eicosanoids creates a pathological situation.

## B. Autocrine and Paracrine Hormones

Prostanoids are produced by a variety of cells in response to both physiological and pathological stimuli and are released outside the cells immediately after synthesis. They exert a variety of actions in the body, which are mediated via specific cell surface receptors. The evidence leading to the conclusion that eicosanoids are hormones is that they act as chemical messengers that possess receptors and generate a specific biological response.

The eicosanoids can be considered local hormones since, with few exceptions, they exert their effects principally in the tissues or organs in which they are synthesized. The very active catabolic system of eicosanoids at the site of their synthesis led to the widely accepted view that eicosanoids are local rather than circulating hormones acting at or near their site of synthesis. To support the notion of eicosanoids as local hormones, both the synthetic enzymes involved in their synthesis and the receptors conferring their activities have been co-localized in the same cell or system.

## C. Metabolism

Under basal conditions, prostanoids are considered to be formed and inactivated within the same cell prior to their release into the circulation as inactive catabolites. However, under conditions of stress when prostanoid synthesis is greatly activated, prostanoids accumulate in the circulation. The lungs contain a very active prostanoid catabolic system and the accumulation of prostanoids in the circulation is controlled through rapid degradation upon passage through the lungs. The low plasma concentrations of eicosanoids are a consequence of a combination of instability and/or active catabolism. In the case prostacyclin, the half-life at 37°C is less than 30 s. Prostanoid catabolism begins with the oxidation of the hydroxyl group on carbon 15 to yield 15-keto metabolites. These metabolites have 10- to 100- fold less activity than their parent compounds. The oxidation is catalyzed by 15-hydroxyprostaglandin dehydrogenases specific for the different prostanoids and concentrated in the lung, kidney, and placenta. Metabolites of eicosanoids are excreted in the urine mainly in the form of 16-carbon dicarboxylic acids, which result from the ω-oxidation and β-oxidation.

## V. PHARMACOLOGICAL IMPORTANCE

The importance of eicosanoids in the regulation of physiological functions and in the development of pathophysiological conditions was essentially demonstrated by inhibiting their synthesis. The major impetus for development of drugs to control the production of prostanoids and thromboxanes came from the work of Vane and co-workers in the early 1970s; these researchers showed that the inhibition of prostanoid production was a central feature of the mode of action of nonsteroidal anti-inflammatory drugs. Since then and based on that premise, drug companies have been actively developing drugs to control inflammation. In recent years, new inhibitors of prostanoid synthesis have been made available on the market, the cyclo-oxygenase-2-selective inhibitors with reduced side-effect profiles. The rationale for their development was the association of cox-1 with physiologically necessary prostanoids such as those involved in gastric cytoprotection and cox-2 with the production of prostanoids in inflammatory pathologic states such as rheumatoid arthritis. It was suggested, therefore, that the selective inhibition of cox-2 could be anti-inflammatory while avoiding the unwanted side effects of cox-1 inhibition.

It is now recognized that inhibition of prostanoid production could be beneficial in a wide variety of other physiopathologic conditions: prevention of thrombus formation, control of the menstrual cycle, correction of a patent ductus arteriosus in the newborn, or as an adjunct in the therapy of cancer. Considerable development is under way to produce selective inhibitors of the leukotrienes and their receptors for the treatment of asthma.

The complementary DNAs and genomic DNAs of the eicosanoid-forming enzymes and their receptors have all been cloned and sequenced. Their regulation of expression is being documented. The crystal structures of cyclo-oxygenases have revealed the molecular details in the formation of eicosanoids from the substrate AA as well as the mechanisms of inhibition of their formation by NSAIDs. Based on the information resulting from this work, new anti-inflammatory drugs are constantly being developed and added to the list of the most used medications in our society, NSAIDs.

## VI. SUMMARY

Although the cells of an organism all contain identical genetic material, they are physically different and perform different functions. This is in part because they switch different genes on and off depending on where the cells are located in the body and what information they receive. Information in the form of hormones takes various chemical forms and is delivered to the target cells via the bloodstream. Compared to other hormones, eicosanoids constitute a very unique form of information sent to cells. They are a family of lipid messengers produced on demand by a variety of different cells; they do not travel appreciably but instead affect the cells that are producing them or nearby cells. Eicosanoids act by affecting gene expression, cellular physiology, and overall organ function. Their importance was revealed by the use of inhibitors of their synthesis, the NSAIDs, which are used extensively for the treatment of pain and fever. The effects of eicosanoids on the various systems clearly place the eicosanoids in a conspicuous position among hormones.

## Glossary

**arachidonic acid** Polyunsaturated fatty acid that has 20 carbons and four double bonds.

**cyclo-oxygenase** Enzyme that catalyzes the formation of prostanoids from arachidonic acid.

**eicosanoids** Polyunsaturated fatty acids with 20 carbons.

**enzyme** A protein that catalyzes the transformation of a substrate into a product.

**epoxyeicosatrienoic acids** Eicosanoids that are the products of the P450 pathway.

**hepoxilins** Eicosanoids that are the products of the 12-lipoxygenase pathway.

**hydroxyeicosatetraenoic acids** Eicosanoids that are the products of the P450 and lipoxygenase pathways.

**hydroperoxyeicosatetraenoic acids** Eicosanoids that are the products of the lipoxygenase pathway.

**leukotrienes** Eicosanoids that are the products of the lipoxygenase pathway.

**lipoxins** Eicosanoids that are the products of the 5- and 15-lipoxygenase pathways.

**lipoxygenase** Enzyme that catalyzes the formation of leukotrienes, hepoxilins, and lipoxilins from arachidonic acid.

**nonsteroidal anti-inflammatory drugs (NSAIDs)** A class of drugs that inhibit the formation of prostanoids. NSAIDs are used in clinic mainly for the treatment of fever and pain.

**P450** Enzyme that catalyzes the formation of epoxyeicosatrienoic acids and of hydroxyeicosatetraenoic acids from arachidonic acid.

**prostanoids** Products of the cyclo-oxygenase pathway.

## See Also the Following Article

**Glucocorticoids and Asthma**

## Further Reading

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# Endocrine Rhythms: Generation, Regulation, and Integration

Fred J. Karsch[*] and Suzanne M. Moenter[†]
[*] *University of Michigan, Ann Arbor* • [†] *University of Virginia*

I. INTRODUCTION
II. BASIC CONCEPTS AND THEORETICAL FRAMEWORK
III. PULSATILE GONADOTROPIN-RELEASING HORMONE SECRETION—A HIGH-FREQUENCY NEUROENDOCRINE RHYTHM
IV. OVULATORY CYCLE—A LOWER FREQUENCY RHYTHM
V. SEASONAL REPRODUCTION—EXPRESSION OF A CIRCANNUAL RHYTHM
VI. CIRCANNUAL RHYTHM ENTRAINMENT—ROLE OF CIRCADIAN RHYTHMICITY
VII. SUMMARY AND FINAL CONSIDERATIONS

**Hormones are secreted in a periodic manner, and endocrine rhythms have frequencies that operate on various time scales. Various types of endocrine rhythms can interact, and rhythms on one time scale affect the generation and regulation of those on other time scales. The interplay among endocrine rhythms is important in the integration and timely expression of physiological and behavioral processes.**

## I. INTRODUCTION

Periodicity of hormone secretion is a characteristic of endocrine function. For example, a marked diurnal secretory pattern is evident for growth hormone, adrenocorticotropic hormone, and prolactin secretion from the anterior pituitary gland. These secretory patterns of pituitary hormones are the consequence of

**lipoxygenase** Enzyme that catalyzes the formation of leukotrienes, hepoxilins, and lipoxilins from arachidonic acid.

**nonsteroidal anti-inflammatory drugs (NSAIDs)** A class of drugs that inhibit the formation of prostanoids. NSAIDs are used in clinic mainly for the treatment of fever and pain.

**P450** Enzyme that catalyzes the formation of epoxyeicosatrienoic acids and of hydroxyeicosatetraenoic acids from arachidonic acid.

**prostanoids** Products of the cyclo-oxygenase pathway.

## See Also the Following Article

**Glucocorticoids and Asthma**

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## I. INTRODUCTION

Periodicity of hormone secretion is a characteristic of endocrine function. For example, a marked diurnal secretory pattern is evident for growth hormone, adrenocorticotropic hormone, and prolactin secretion from the anterior pituitary gland. These secretory patterns of pituitary hormones are the consequence of diurnal secretion of hypothalamic-releasing and/or -inhibiting hormones and, in the case of pituitary hormones that target another gland downstream in the endocrine system, there is a corresponding periodicity in target gland hormone secretion (e.g., glucocorticoid secretion from the adrenal cortex). Endocrine rhythms occur with different frequencies ranging from minutes, to hours, to days, to weeks, and even up to 1 year. Furthermore, a single hormone may exhibit cycles in more than one time domain. The goal of this article is to identify various types of endocrine rhythms, how they interact, and the importance of these interactions to the integration and timely expression of physiological and behavioral processes. In particular, the concept to be developed is that rhythms on one time scale are important to the generation and regulation of rhythms on other time scales.

## II. BASIC CONCEPTS AND THEORETICAL FRAMEWORK

At the outset, it is important to expand upon important terminology and to introduce a few basic concepts. In this article, a rhythm is considered to be a periodicity that is generated endogenously, from within the organism, rather than being driven by cyclical changes in the environment. Not all cyclical changes in endocrine function are due to rhythms in the sense used here. For example, the swings in insulin secretion that surround the periodic eating of meals are not due to a rhythm of pancreatic islet cell function. Rather, they are driven by external input, the periodic ingestion of food. In contrast, melatonin is secreted from the pineal gland on a 24 h basis in the complete absence of input from the external environment, due to an oscillatory process within the body. Thus, this is considered to be a rhythm. Establishing that cyclicity of hormone secretion is driven endogenously rather than by external input can be difficult, especially in the case of long-term (e.g., yearly) cycles, because it requires demonstration that they persist repeatedly in the absence of cyclical changes in relevant environmental variables.

The interval between repeated occurrences of the rhythm is its period, for example, 24 h in the case of the circadian rhythm of melatonin secretion. The period of many endocrine rhythms is regulated by variation in the internal and external environments. In the case of external cues, the period of the rhythm is adjusted to match that of a cyclical swing in ambient conditions (e.g., 24 h for circadian rhythms and 365 days for circannual rhythms). In addition, external cues set the rhythm phase, i.e., align rhythm stages appropriately to the environmental cycle. Such regulation of phase and period by the external environment is referred to as entrainment.

For illustrative purposes and to maintain focus, the remainder of this article describes how endocrine rhythms that have different periods interact to generate and regulate the ovulatory cycle of a seasonally breeding species. This specific example nicely illustrates how different types of rhythms are integrated to sustain a critically important biological function—reproduction. From an evolutionary perspective, seasonal reproduction is a common trait with significant selective advantage because it allows birth to occur at a time when the young stand the greatest chance of survival. From a practical perspective, seasonal reproduction is a natural process of reversible fertility. Knowledge of the underlying mechanisms should provide leads to novel approaches for manipulating and managing reproduction.

Figure 1 presents the conceptual framework: the seasonal expression of ovulatory cycles is the consequence of interactions among endocrine rhythms that operate in different time domains. Among these, the endocrine rhythm with the shortest period is the pulsatile rhythm of gonadotropin-releasing hormone (GnRH) secreted by neurons located in the hypothalamus (Rhythm No. 1 in Fig. 1). In its unregulated state, this rhythm has a period ranging from ~20 to 60 min, depending on species, and thus it has been classified as ultradian (period less than 1 day) or circhoral (period ~1 h). It is more commonly referred to as a pulsatile rhythm because GnRH is secreted in brief bursts (pulses) with

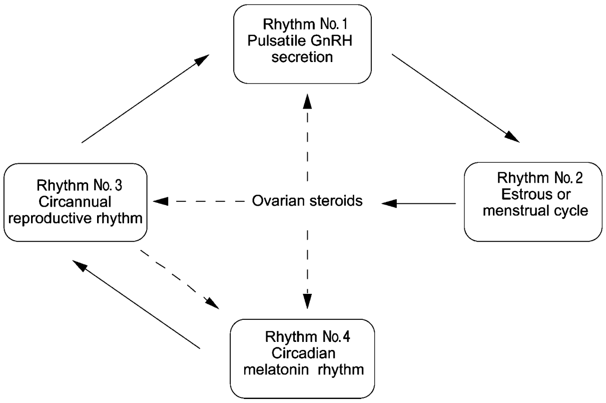


**FIGURE 1** Theoretical framework for interactions among endocrine rhythms that coordinate seasonal reproduction.

little or no intervening secretion. The period (i.e., frequency) of the rhythm is tightly regulated and this is critically important to fertility. When the period is appropriate, GnRH pulses drive the estrous or menstrual cycle; inappropriate GnRH pulse frequency leads to infertility.

The ovulatory cycle itself is a true rhythm as it is endogenously generated (Rhythm No. 2 in Fig. 1). There is great species variability in the period of this rhythm. Cycles last 4 or 5 days in rodents, approximately 2 weeks in sheep, 1 month in primates, and even longer in some other species. In a number of seasonal breeders, the pulsatile GnRH rhythm and thus the ovulatory cycle are influenced by a much slower rhythm, referred to as a circannual rhythm because its period is in the range of 1 year (Rhythm No. 3 in Fig. 1). This low-frequency rhythm drives the seasonal reproductive cycle. The circannual reproductive rhythm is, in turn, regulated by yet another rhythm, the circadian rhythm of melatonin secretion from the pineal gland (Rhythm No. 4 in Fig. 1). The circadian melatonin rhythm entrains the circannual reproductive rhythm so that the fertile phase occurs during the appropriate season.

One final generality, illustrated by the dashed lines in Fig. 1, is that reciprocal interactions regulate and integrate the various types of rhythms identified above. Among these, the best described are the feedback actions of gonadal steroid hormones on the pulsatile rhythm of GnRH secretion (other interactions considered at the end of the article). In the sections that follow, pertinent concepts related to each of the rhythms identified in Fig. 1 are developed. Of particular interest is how these different types of rhythms are integrated to regulate and coordinate the reproductive process.

## III. PULSATILE GONADOTROPIN-RELEASING HORMONE SECRETION—A HIGH-FREQUENCY NEUROENDOCRINE RHYTHM

### A. Description

GnRH is synthesized by a small population of neurons scattered throughout the preoptic area and medial ventral hypothalamus (800–2500 neurons in mammals) (Fig. 2a). Axons of these neurons project to the median eminence where GnRH is released in the vicinity of pituitary portal capillaries, carrying this and other hypothalamic hormones to the pituitary gland (Fig. 2b). The arrival of GnRH pulses at the pituitary drives a corresponding pulsatile secretion of the two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn regulate gonadal function (Fig. 2c).

The earliest evidence for a circhoral rhythm of GnRH secretion was obtained in the early 1970s from studies of primates. This led to the surprising discovery that the concentration of circulating LH oscillated. Values increased from a nadir to a peak within a matter of minutes and then declined more gradually at a rate reflecting the half-life of this gonadotropin. These early observations led to the concept that a hypothalamic oscillator or "pulse generator" drives episodic secretion of GnRH. This idea was substantiated and embellished by the following important observations: (1) LH pulses are eliminated by obliterating GnRH secretion. (2) Episodic administration of GnRH to individuals lacking endogenous GnRH restores LH pulsatility. (3) A GnRH pulse in pituitary portal blood accompanies each LH pulse secreted from the pituitary. (4) The pulsatile release of LH is coupled to a corresponding oscillation of hypothalamic electrical activity.

The phenomenon of pulsatile GnRH secretion has had a profound impact on our understanding of the brain mechanisms governing reproduction. In addition, it has driven investigations into the functional significance and mechanisms for generation and regulation of the pulsatile mode of release.

### B. Functional Significance

GnRH pulses are a prerequisite for fertility. The importance of the pulsatile pattern *per se* can be seen by testing pituitary responses to various patterns of GnRH delivery in animals lacking their own GnRH secretion. Pulsatile delivery profoundly stimulates gonadotropin synthesis and secretion, whereas continuous delivery does not. GnRH pulses vary in their amplitude, frequency, and shape. Among these characteristics, frequency modulation appears to be most important for driving changes in reproductive state. Shifts in GnRH pulse frequency are associated with progression of the ovulatory cycle and seasonal reproductive transitions, as discussed below. In addition, GnRH pulse frequency is an important determinant of differential secretion of the two gonadotropic hormones. Higher frequency favors LH release, whereas lower frequency favors FSH release. A change in the relative levels of the gonadotropins can be critical for ovarian follicular maturation.

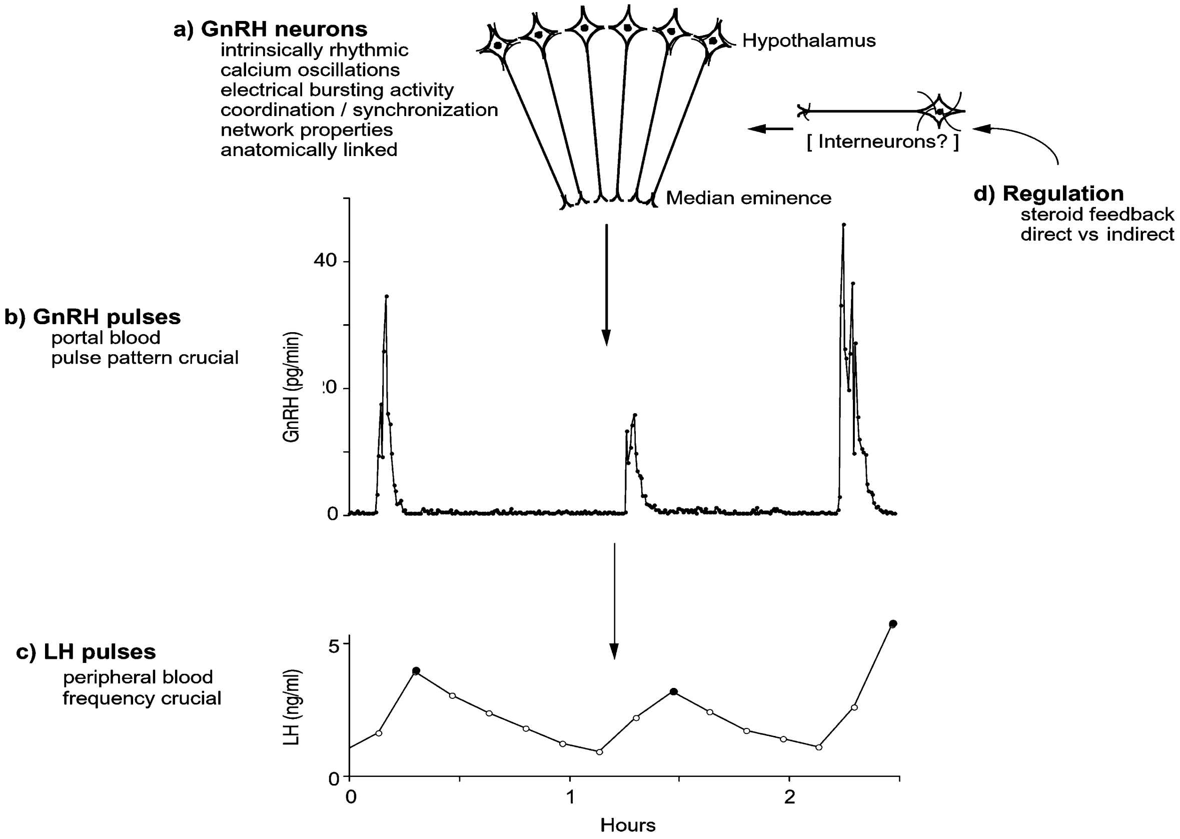

a) GnRH neurons
intrinsically rhythmic
calcium oscillations
electrical bursting activity
coordination / synchronization
network properties
anatomically linked
Hypothalamus
[ Interneurons? ]
Median eminence
d) Regulation
steroid feedback
direct vs indirect
b) GnRH pulses
portal blood
pulse pattern crucial
GnRH (pg/min)
40
0
c) LH pulses
peripheral blood
frequency crucial
LH (ng/ml)
5
0
0
1
2
Hours

FIGURE 2 Neuroendocrine processes involved in the generation and regulation of the pulsatile rhythm of GnRH and LH secretion (see text for details). GnRH in pituitary portal blood was determined at 30 s intervals and LH in jugular blood at 10 min intervals in ovariectomized sheep. Adapted from Moenter *et al.* (1992).

## C. Mechanism for GnRH Pulse Generation and Coordination

Perhaps the most fundamental question in the area of pulse generation is whether separate pacemaker cells drive the rhythm or whether GnRH neurons themselves are intrinsically rhythmic. Strong evidence that the oscillation arises within the GnRH neural network itself comes from studies using transformed GnRH neuronal cell lines or native GnRH neurons in culture. These cells exhibit spontaneous rhythmic electrical activity, rhythmic uptake of an exocytotic marker, and rhythmic secretion of GnRH. Whether or not single GnRH cells are rhythmic remains unknown. Emerging evidence favors the concept that, whereas individual cells are rhythmic, the overall pulsatile pattern of secretion emerges as a network property of multiple GnRH neurons. For example, individual GnRH neurons in culture exhibit oscillations of intracellular calcium. The oscillations, however, are generally not synchronized among cells and the oscillatory period is much shorter than that of GnRH pulses. Remarkably, calcium oscillations periodically synchronize at a frequency similar to that of pulsatile GnRH release *in vivo*, suggesting that the activity of multiple GnRH neurons is coordinated at the time of a pulse.

The foregoing observations suggest the following hypotheses, which are summarized in Fig. 2a: (1) individual GnRH neurons are intrinsically rhythmic; (2) firing patterns leading to the release of a GnRH pulse emerge from a more rapid oscillation (e.g., intracellular $Ca^{2+}$); (3) coordination of individual cells is required for secretion; and (4) hormone release emerges as a network property of GnRH neurons.

Another basic question concerning the mechanism of GnRH pulse generation is the molecular nature of the series of reactions within GnRH neurons that result in a pulse. Although this question has not been answered, pulse generation does not appear to be directly linked to GnRH synthesis. The GnRH gene is transcribed in a rhythmic manner, but the period of transcription (several hours) is much slower than that of GnRH release, and pulsatile release still occurs when mRNA or protein synthesis is blocked. Thus, rather than synthesis of new proteins, the molecular mechanism for pulsatile release more likely includes posttranslational events such as changes in the phosphorylation state of proteins, including ion channels and pumps that contribute to changes in membrane properties.

The above findings indicate the importance of coordination among multiple GnRH neurons in the generation of a pulse. Neuroanatomical observations suggest that coordination could occur at cell bodies, terminals, or both (Fig. 2a). Both axo-dendritic and axo-somatic synapses have been demonstrated between GnRH neurons. Furthermore, GnRH neurons form cytoplasmic bridges with one another, suggesting that cells may be linked into syncytia. Coordination of GnRH neuronal activity may also occur at the median eminence where terminals converge, as suggested by the finding that explants containing just the isolated median eminence retain some characteristics of pulsatile release.

Several types of chemical mediators coordinating GnRH neurons may be considered. GnRH cells have both large dense core vesicles filled with GnRH and small clear vesicles containing unidentified transmitter(s). Thus, either neurotransmitters in these vesicles or GnRH itself may serve to coordinate GnRH cells. Cultured GnRH cells contain GnRH receptors, although it remains to be determined whether GnRH receptor expression is induced in GnRH neurons *in vivo*. It is also possible that GnRH or transmitters could coordinate activity among neighboring GnRH cells by diffusion through gap junctions. GnRH cells express several connexins, proteins that form gap junctions, and dye transfer has been demonstrated between GnRH neuron cell bodies. Another proposed chemical mediator of coordinated GnRH neuronal activity is the gaseous transmitter nitric oxide, which alters GnRH release both *in vivo* and in cultured GnRH cells. Clearly, many questions remain as to how GnRH pulses are generated and coordinated at the cellular level. This is an area of intense current investigation using diverse model systems.

### D. Mechanisms for Regulation of GnRH Pulses by Gonadal Steroids

Feedback regulation of GnRH pulses by gonadal steroids is highly important to the generation of ovarian cyclicity and seasonality. At the cellular level, steroidal regulation of GnRH neurons has long been thought to be indirect, via steroid-sensitive afferents, as there was scant evidence for steroid receptor expression in GnRH neurons. Recent studies, however, indicate that GnRH neurons express estrogen receptor-β, a newly discovered form of the estradiol receptor. Progesterone receptors have also been identified in subpopulations of GnRH neurons in some species. Thus, it is likely that both direct and transsynaptic mechanisms cooperate in steroid feedback regulation of GnRH release (Fig. 2d).

Potential cellular mechanisms of steroid action include modulation of gene expression and nongenomic effects. Examples of gene expression include the regulated synthesis of GnRH or enzymes for neurotransmitters that regulate GnRH transsynaptically. Examples of nongenomic effects include activation of kinase signaling cascades and the opening of potassium channels on the membrane of GnRH cells. The latter actions may provide a means for rapid effects of steroids on GnRH pulsatility.

Regardless of whether steroids exert direct, indirect, genomic, or nongenomic actions, their effects are critically important to the ovulatory cycle. How their influence on the pulsatile GnRH rhythm leads to the generation of cyclicity is discussed in the next section.

## IV. OVULATORY CYCLE—A LOWER FREQUENCY RHYTHM

### A. Description

In its simplest sense, the ovulatory cycle (estrous or menstrual cycle) may be conceptualized as a circular series of causally related events in which each step leads to a subsequent step in the sequence. Because the series is circular, the sequence eventually loops back upon itself, forming a self-sustained cycle, i.e., a rhythm. From a regulatory perspective, the ovulatory cycle is the outcome of a well-orchestrated interplay of endocrine and neuroendocrine interactions. Ovulation is the most prominent single event, preceded by a follicular phase when estradiol rises, leading up to the preovulatory LH surge, and followed by a luteal phase when progesterone predominates.

Because this article emphasizes interactions among endocrine rhythms of different time domains ranging from circhoral (pulsatile) to circannual (seasonal), it is appropriate to consider the basis for ovarian cyclicity in a seasonal breeder. For this purpose, the sheep has been selected as a species of focus. Not only does the large blood volume of sheep facilitate detailed analysis of rhythmic hormone secretion, powerful techniques have been developed for direct measurement of the minute-to-minute secretory pattern of GnRH secretion in this species (example in Fig. 2b). Information from such studies enables us to piece together how interactions among the pulsatile rhythm of GnRH secretion and cycles of other reproductive hormones are integrated into a self-sustaining ovulatory cycle.

## B. Cycle Generation—Role of Pulsatile GnRH Rhythm

In its unregulated state, the period of the pulsatile GnRH rhythm in the ewe is 30 to 60 min and the amplitude of GnRH pulses is relatively large. Each of the ovarian steroids, progesterone and estradiol, exerts negative feedback on the pulsatile pattern, but they do so by very different mechanisms. The action of progesterone is relatively simple; it inhibits the frequency of the GnRH oscillation. Estradiol, in contrast, affects the dynamics of individual GnRH pulses, decreasing their amplitude, duration, and rate of increase, each of which reduces the mass of hormone released during a pulse. Moreover, estradiol enhances basal GnRH release between pulses. All of these parameters alter the response of pituitary gonadotropes and thus have physiological relevance to the reproductive state. Of importance, estradiol does not inhibit GnRH pulse frequency during the cycle and, if anything, this steroid increases frequency during the follicular phase. Because GnRH pulses drive LH pulses, these ovarian steroids similarly affect the episodic secretion of LH from the pituitary gland. At the ovarian level, high-frequency LH pulses promote steroidogenesis and, in particular, the follicular phase rise in estradiol secretion from the developing preovulatory follicle.

The above basic hormonal relationships can now be integrated into a schema for estrous cycle generation (the major steps in the preovulatory sequence are illustrated in Fig. 3). During the midluteal phase of the cycle, the corpus luteum secretes copious quantities of progesterone, which applies a brake to the GnRH neurosecretory system. The resulting low frequency of GnRH and LH pulses (one pulse every 4 to 8 h) is not sufficient to initiate the preovulatory chain of events. Near the end of the luteal phase (~12 to 13 days after ovulation), progesterone secretion drops precipitously as the corpus luteum regresses (step 1 in Fig. 3). Within ~24 h after the onset of luteolysis, circulating progesterone reaches an undetectable level, liberating the GnRH neurosecretory system from frequency inhibition. Estradiol inhibits the amplitude of GnRH pulses but itself cannot reduce frequency. Thus, the brake on rhythmic GnRH secretion is removed; GnRH pulse frequency accelerates to one pulse every 30–60 min, and the pituitary responds with high-frequency LH pulses (step 2). This gonadotropic stimulus, in conjunction with the basal concentration of FSH, promotes maturation of the ovarian follicle and drives a sustained increase in estradiol synthesis (step 3). The circulating estradiol concentration thus rises steadily, eventually reaching a threshold for initiating two critically important events, estrous behavior and the preovulatory LH surge (step 4).

Two separate actions of estradiol are coordinated to induce the LH surge, one at the hypothalamus to

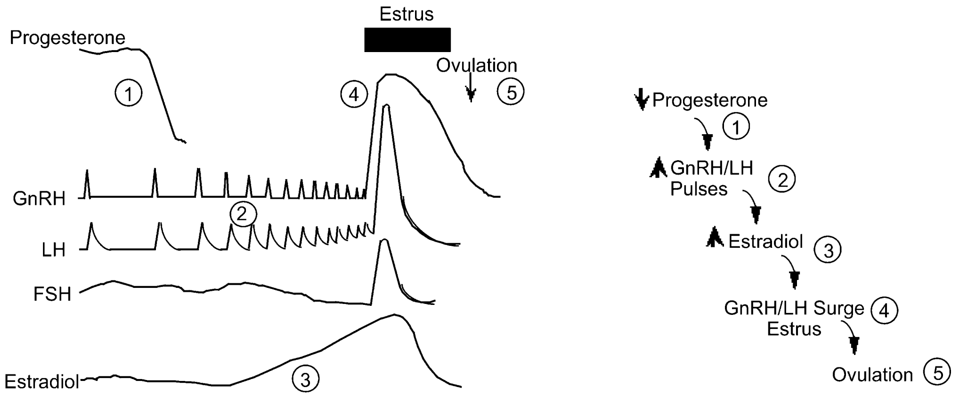


**FIGURE 3** Schematic representation of major steps in the preovulatory sequence of events in the ewe. The patterns of various reproductive hormones and the approximate times of estrous behavior and ovulation are shown on the left. The stepwise preovulatory sequence is shown at the right. See text for details. Reprinted from Battaglia *et al.* (2000), with permission.

induce a large and sustained (18–24 h) GnRH surge and the other at the pituitary to enhance responsiveness to GnRH. The LH surge, in turn, initiates a cascade of enzymatic and hemodynamic changes within the preovulatory follicle, resulting in digestion of the outer follicular wall and ovulation some 12 to 24 h later (step 5). Of interest, the GnRH surge also stimulates brain behavioral centers to increase the duration and intensity of sexual receptivity. This highly integrated coupling of ovarian, neuroendocrine, and behavioral events provides a means of coordinating the time of ovulation and insemination, thus maximizing chances for conception.

From the above perspective, the pulsatile rhythm of GnRH, and particularly its frequency, is critically important in generating the ovulatory cycle. Increased frequency initiates the preovulatory cascade. In situations where GnRH pulse frequency fails to increase, such as during pregnancy (when progesterone remains elevated) or during the nonbreeding season (see below), the estrous cycle ceases. Of key importance to cyclicity, estradiol itself does not inhibit frequency and this allows LH and estradiol to increase in parallel during the follicular phase. The end result is the GnRH and LH surges, estrous behavior, ovulation, and progression to the luteal phase of the cycle. With the luteal phase rise in progesterone secretion, GnRH pulse frequency is again inhibited until subsequent regression of the corpus luteum, initiating a new preovulatory sequence.

There is much interest in the mechanisms of the feedback actions of ovarian steroids on gonadotropin secretion. This is a point at which reproduction can be managed and fertility regulated, not only artificially but also naturally, as is the case with seasonal breeding. Within the present framework of interactions among endocrine rhythms, the following section addresses how steroid feedback responses, pulsatile GnRH secretion, and ovarian cyclicity are all influenced by a lower frequency rhythm that regulates seasonal reproduction.

## V. SEASONAL REPRODUCTION—EXPRESSION OF A CIRCANNUAL RHYTHM

### A. Description

Various strategies have evolved to enable seasonal reproduction. Among these are delayed fertilization, embryonic diapause, and an annually recurring decline and restoration of gonadal activity. Although vastly different mechanistically, these strategies all serve a common purpose, ensuring that young are born at the time optimal for their survival. In ewes, seasonal breeding is the consequence of an annual rhythm that causes periodic switching on and off of the estrous cycle. Typically, the breeding season begins in autumn (Fig. 4a). Unless pregnancy is established, estrous cycles persist until mid to late winter, at which time cyclicity ceases during a 6- to 7-month period of anestrus (the duration of anestrus and breeding season varies among different sheep breeds). Under natural conditions, pregnancy is usually established early in the breeding season. Given the 5-month gestation period, lambs are born in spring, which is early in the anestrous season, and thus the next opportunity for pregnancy must await the subsequent breeding season.

### B. Neuroendocrine Regulation

Given that the estrous cycle is a rhythm generated by a circular series of interrelated events, only one step in the sequence needs to be disrupted for the loop to be broken and cyclicity to cease. Extensive evidence indicates that the disrupted step is the development of sustained, high-frequency GnRH and LH pulses

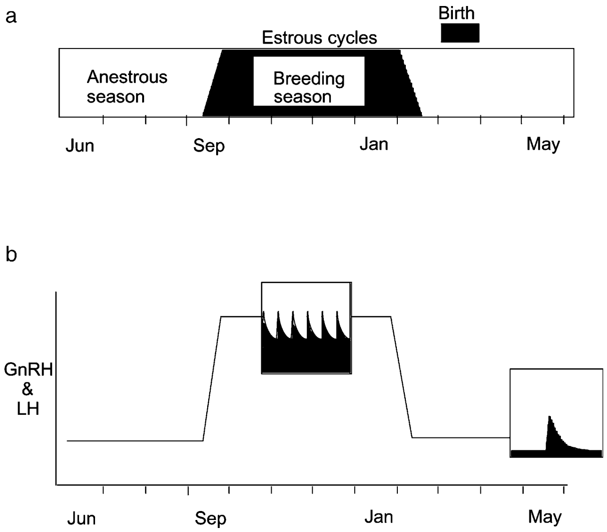


FIGURE 4 (a) Timing of breeding and anestrous seasons (expressed as occurrence of estrous cycles) and births in ewes in Northern Hemisphere temperature zone. (b) Schematic representation of an experiment demonstrating the seasonal change in responsiveness to the negative feedback action of estradiol in the ewe. Adapted from Legan *et al.* (1977). Changes in response to estradiol negative feedback as determined by LH and GnRH pulses in ovariectomized ewes treated with constant-release estradiol implants (ovx + E). See text for further details.

between luteolysis and the preovulatory LH surge (step 2 in the preovulatory sequence; see Fig. 3). Despite the absence of corpora lutea and elevated progesterone during the anestrous season, due to the absence of ovulation, GnRH pulses remain infrequent (only two to four pulses each day). Since highly frequent LH and thus GnRH pulses are essential for preovulatory follicular development and a sustained increase in estradiol secretion, there is no estradiol rise, LH surge, estrous behavior, or ovulation. Although some studies suggest that ewes also become less responsive to the behavioral and LH surge-inducing effects of estradiol, such changes are relatively minor and, in the case of the LH surge mechanism, there is also evidence that there is no seasonal change in response to estradiol. Rather, it is the pulsatile GnRH rhythm that ultimately dictates the seasonal reproductive state.

The lack of high-frequency GnRH pulses during anestrus is not due to a dysfunction of the GnRH system itself, because removal of negative feedback by ovariectomy causes GnRH pulses to accelerate to a high frequency. Rather, estradiol, which does not inhibit frequency during the breeding season, becomes highly effective at suppressing frequency during anestrus. This powerful inhibitory action of estradiol enables the follicle to restrict its own development during anestrus via a highly effective negative feedback loop involving the ovary and the hypothalamic GnRH pulse-generating mechanism. Because of this restricted follicular development and limited estradiol production, the preovulatory sequence is halted.

The profound seasonal change in responsiveness to estradiol negative feedback was disclosed in the 1970s by experiments in which ovariectomized ewes were treated with constant-release estradiol implants that clamped circulating estradiol chronically at an early follicular phase level (the experiment is summarized in Fig. 4b). Such ewes exhibit a high-amplitude annual cycle of circulating LH, with values being extremely low during the spring and summer and increasing some 50-fold during the autumn and winter. The seasonal rises and falls in LH, which coincide with the transitions between breeding and anestrous seasons in ovary-intact ewes, were subsequently found to result from a marked change in GnRH pulse frequency. During anestrus, estradiol powerfully inhibits GnRH pulse frequency, whereas, during the breeding season, it does not. In essence, the change in period of the pulsatile GnRH rhythm in the presence of estradiol constitutes the neuroendocrine basis for seasonal reproduction in the ewe.

## C. Neural Basis for Altered Estradiol Negative Feedback

The changes within the brain that lead to shifts in estradiol negative feedback effects are of great interest because they underlie a natural process of reversible fertility and infertility. Several potential mechanisms have been addressed, three of which are now considered briefly: (1) estradiol receptors, (2) neurotransmitter activity, and (3) neuroplasticity.

### 1. *Estradiol Receptors*

Since estradiol negative feedback requires interaction with target cells, a seasonal alteration in hypothalamic estradiol receptor is an attractive potential mechanism for the shift in feedback efficacy. This possibility, however, has been difficult to address experimentally because estradiol influences several hypothalamic functions, only one of which is inhibition of GnRH secretion, and it is not known which estrogen-sensitive hypothalamic area(s) and cell type(s) are relevant to suppression of GnRH pulse frequency. Complicating this matter further, estradiol feedback may be achieved indirectly via interneurons, rather than directly on GnRH neurons, and several different types of estradiol receptors exist (see section on pulsatile GnRH rhythm and Fig. 2d). Initial studies in this area did not reveal seasonal differences in estradiol binding or receptor levels in the hypothalamic tissue of ewes. More recent work, employing immunocytochemistry to localize estradiol receptor-α, did reveal a greater number of estradiol receptor-containing cells in the preoptic area of ewes during anestrus than during the breeding season. Although this difference is consistent with more potent negative feedback during anestrus, the neurotransmitter phenotype of the cells exhibiting increased estradiol receptor and their importance to inhibition of GnRH pulse frequency are not known.

### 2. *Neurotransmitters*

Seasonal alteration in neurotransmitter activity is of interest because estradiol can affect GnRH secretion indirectly via neurotransmitters linking estrogen-sensitive interneurons to GnRH cells. Among these, dopaminergic and serotonergic systems have received the greatest attention and, in particular, dopamine is implicated in the seasonal shift in estradiol negative feedback in the ewe. Multiple complementary approaches have revealed that a group of dopamine neurons having cell bodies located in the retrochiasmatic region of the hypothalamus (A15 dopaminergic cell group) are critically important to seasonality. These dopaminergic cells are preferentially activated by estradiol during anestrus and they are necessary for the inhibitory effect of estradiol on GnRH pulse frequency. The dopamine neurons themselves do not appear to contain the α form of the estradiol receptor, but they receive input from sites that do and they project to hypothalamic regions that contain GnRH neurons. Nevertheless, the specific cell types and pathways by which estradiol activates these dopamine neurons and whether or not they directly innervate GnRH neurons to regulate pulse frequency remain to be determined.

#### 3. *Neuroplasticity*

Investigation of seasonal morphological changes in the GnRH system was prompted by the demonstration that a marked seasonal plasticity occurs in brain regions that regulate other seasonal variations, most notably singing in birds. At the level of the GnRH system of mammals, structural rearrangements have been documented during the course of puberty, the estrous cycle, and reproductive aging. Recent observations in ewes have revealed a seasonal difference in synaptic input to GnRH neurons, the input being twofold greater during the breeding season than during anestrus. The seasonal difference in the innervation of GnRH neurons was identified in both ovary-intact ewes and ovariectomized ewes bearing constant-release estradiol implants. Thus, this finding reflects an intrinsic seasonal plasticity of the GnRH neurosecretory system rather than seasonal differences in ovarian hormone secretion. Complementing the change in synaptic input is the recent finding that a protein promoting neuroplasticity, the polysialylated form of neural cell adhesion molecule (PSA-NCAM), is closely associated anatomically with GnRH neurons of the ewe. This association is more prominent during the breeding season than during anestrus. Despite these interesting observations, the functional significance of the seasonal change in synaptic input to GnRH neurons, and of PSA-NCAM, to seasonal changes in pulsatile GnRH secretion is not known.

### D. Seasonal Reproductive Rhythm

Given the seasonal swings in estradiol feedback and the corresponding shifts in ovarian cyclicity, it is important to consider how endocrine rhythms participate in the generation and timing of seasonal cyclicity. Timing is considered in the depth in the next section. Suffice it to say here that a number of geophysical variables have the potential to influence the reproductive pattern of seasonal breeders. Among these, the annual change in day length is the most influential. Because photoperiodic changes are stable from year to year, they provide a highly reliable time cue for predicting future environmental conditions and thus for aligning the fertile period to the appropriate season. The seasonal breeding pattern of sheep, like that in other seasonal breeders, can readily be manipulated photoperiodically. For example, the pattern can be shifted so that the breeding season is 6 months out of phase or the period of the annual cycle can be accelerated so that two breeding seasons occur each year. At a neuroendocrine level, photoperiodic manipulations that influence the timing of the breeding season do so by altering the seasonal swings in response to the negative feedback action of estradiol on pulsatile GnRH secretion.

Importantly, endogenous rhythms provide the driving force for seasonal reproductive cycles in a number of long-lived seasonal breeders. In these species, repeated long-term cycles of reproduction and other biological processes (e.g., hibernation and molt) continue to be expressed when the influence of seasonal photoperiodic change is eliminated. For example, such cycles are evident during exposure to a constant photoperiod. In mammals, they also persist following blinding, pinealectomy, or denervation of the pineal gland, all of which functionally disconnect animals from their photoperiodic environment. The long-term cycles are referred to as circannual since their period approximates 1 year. Circannual cycles are considered to be driven by an endogenous rhythm because, in the absence of photoperiodic regulation, their period differs from 365 days and the various cycle stages (e.g., onset of the breeding season) drift out of phase with respect to time of the year (i.e., the rhythm is free-running).

The existence of a circannual reproductive rhythm is well documented in sheep. In ovariectomized ewes treated with constant-release estradiol implants, the rhythm is expressed as a free-running cycle in the serum LH concentration, which gradually shifts with respect to time of year. An example of a free-running, circannual LH cycle in a ewe maintained in a fixed photoperiod is shown in Fig. 5. As described above, the seasonal swings in LH in this animal model reflect changes in the frequency of the pulsatile GnRH rhythm.

The foregoing discussion indicates that seasonal shifts in the pulsatile GnRH rhythm of a circannual species such as sheep are not driven by seasonal changes in the environment. Rather, they are driven internally by a circannual rhythm. Photoperiod serves

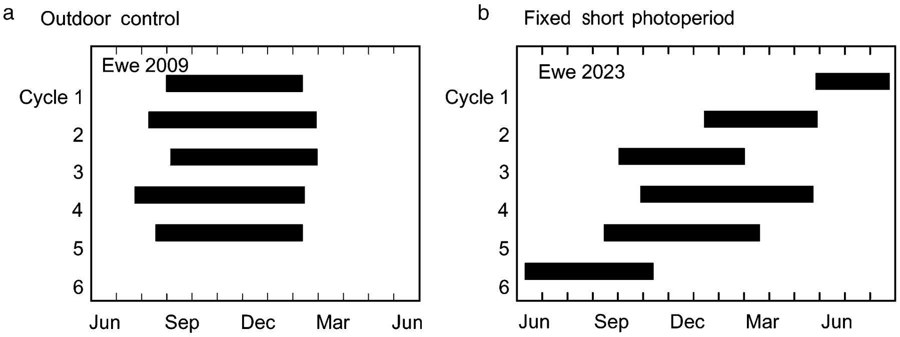


**FIGURE 5** Circannual cycle of reproductive neuroendocrine activity in ewes. (a) The yearly occurrence and timing of the seasonal LH cycle in an estradiol-treated ovariectomized ewe maintained outdoors in natural photoperiod for 5 years. High LH (solid bars) is indicative of the neuroendocrine breeding season (see Fig. 4b). (b) Results in a ewe maintained indoors in a fixed short photoperiod during the same time span. Note that the circannual cycle is entrained in the ewe maintained outdoors (no net change in timing of high-LH phase), whereas the cycle in the ewe kept in the fixed photoperiod is free-running. In the latter case, the average period of the circannual cycle is less than 365 days, so that the high-LH phase advances over successive cycles and six cycles are evident over the 5-year period compared to five in the outdoor control. Adapted from Karsch *et al.* (1989), with permission. See Fig. 4 for further representation of seasonal changes in GnRH and LH secretion in this animal model.

to set the period and phase of this rhythm (i.e., entrain the rhythm) so that reproductive activity occurs at the appropriate time. This entrainment process is achieved via interaction of the circannual mechanism with a rhythm that operates in yet another time domain, the circadian rhythm of melatonin secretion, as discussed in the following section.

## VI. CIRCANNUAL RHYTHM ENTRAINMENT—ROLE OF CIRCADIAN RHYTHMICITY

### A. Description

Circadian pacemakers generate 24 h rhythms and provide important timekeeping functions for all forms of life. Such rhythms drive a broad range of biochemical, behavioral, and physiological cycles, including secretory activity of endocrine glands, thereby enabling organisms to align daily activities to diurnal changes in their environment. Circadian rhythms also serve important timekeeping functions for rhythms that have a period longer than 24 h. For example, circadian rhythms determine when ovulation occurs in certain rodents, thus timing the ovarian cycle in these species. Circadian rhythms also participate in timing annual cycles; it is this influence that is crucial for entrainment of circannual rhythms.

### B. Role of Melatonin in Photoperiodic Time Measurement

The 24 h cycle of light and darkness entrains a circadian rhythm of melatonin secretion from the pineal gland, so that elevated secretion is confined to the hours of darkness and the duration of this elevation is directly proportional to length of the night. The importance of melatonin to photoperiodic regulation of seasonal processes was initially recognized by findings in the Syrian hamster. Pinealectomized hamsters, which lack the melatonin rhythm, fail to express short-day-induced testicular regression, but regression can be induced by replacement with melatonin. Subsequent work extended these findings to other seasonally breeding mammals and also demonstrated that melatonin is not simply inhibitory to reproduction. Rather, melatonin acts to mediate the effects of seasonal changes in photoperiod. Furthermore, the influence of melatonin extends beyond reproduction to a broad spectrum of seasonal processes. In essence, the circadian melatonin rhythm provides a hormonal code for photoperiod.

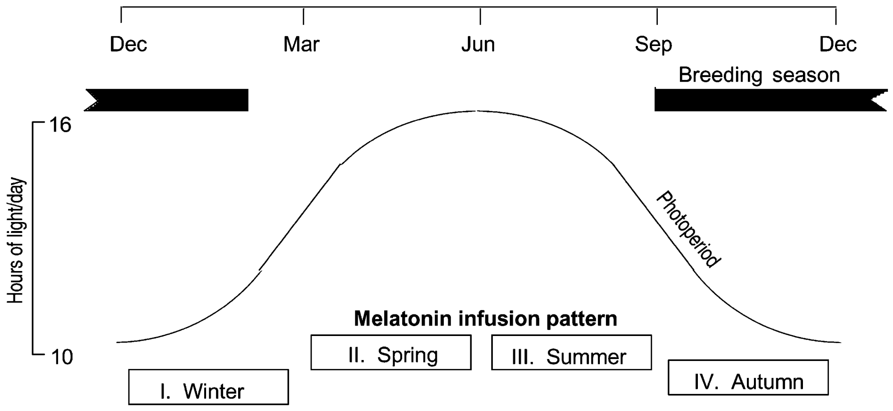
The mechanisms by which melatonin achieves its timekeeping function have been investigated from several perspectives including the entrainment of circannual rhythms. Before addressing this, it is pertinent to identify two characteristics of the circadian melatonin pattern that have been postulated to relay photoperiodic information. One is the phase relationship between nocturnal melatonin secretion and other circadian rhythms; this changes through the course of the year and thus could provide input for seasonal timing. The second is duration of the nocturnal increase in melatonin secretion, which varies seasonally in direct proportion to the hours of darkness and thus could provide a seasonal time cue.

Support for a role of both phase and duration has been obtained but in neither case has it been determined how these characteristics of the melatonin pattern are transcribed into altered seasonal functions.

## C. Melatonin Entrains the Circannual Reproductive Rhythm

The finding that pinealectomy causes circannual rhythms to free-run, in conjunction with observations that the circadian melatonin pattern provides a code for day length, has prompted investigation into the role of melatonin in circannual rhythm entrainment. This line of research has been facilitated by the development of experimental paradigms in which circadian delivery of melatonin, by timed infusion, can mimic neuroendocrine effects of photoperiod in animals rendered photoperiodically unresponsive by pinealectomy. Importantly, this approach has revealed that nocturnal melatonin infusions of differing durations, which match the duration of endogenous melatonin under specific photoperiods, can restore responses characteristic of those specific photoperiods.

Using this experimental paradigm, the involvement of the circadian rhythm of melatonin secretion in entraining the circannual rhythm of reproductive neuroendocrine activity has been investigated in pinealectomized ewes. Initial work demonstrated that the circadian delivery of melatonin can entrain the circannual reproductive rhythm, regulating both period and phase with respect to the seasons. In addition, exposure to melatonin for as little as 2 months each year was found to be fully sufficient for entrainment. Next, by gradually changing the duration of the nocturnal melatonin infusions to mimic the gradually changing melatonin patterns secreted during different seasons, it was determined that a seasonal specificity exists with respect to the melatonin patterns that entrain the rhythm. Patterns mimicking those secreted naturally during summer are most effective, whereas those secreted during the winter are ineffective (see Fig. 6 for a more complete synopsis of those findings). Finally, the winter melatonin pattern, which does not entrain the rhythm when delivered



**Influence of melatonin infusion on circannual rhythm**

| | |
|---|---|
| I. Winter pattern: | Not entrained |
| II. Spring pattern: | Partly entrained (rhythm phase advanced) |
| III. Summer pattern: | Entrained |
| IV. Autumn pattern: | Partly entrained (rhythm phase delayed) |

**FIGURE 6** Summary of a study to determine the influence of four different melatonin infusion patterns, which mimicked endogenous circadian melatonin patterns during the four seasons, on entraining the circannual rhythm of reproductive neuroendocrine activity in pinealectomized ewes. Separate groups of sheep each received one of four season-specific melatonin infusion patterns [(I) winter; (II) spring; (III) summer; (IV) autumn] for 3 months during the corresponding season each year. For example, the spring melatonin pattern (II) consisted of a gradually decreasing duration of nightly melatonin infusion between the spring equinox and the summer solstice, mimicking the endogenous melatonin secretory pattern at that time of the year. Following the 3-month infusions, no melatonin was given for the remaining 9 months of the year. This yearly treatment cycle was repeated over a 3-year period. The efficacy of each treatment in entraining the circannual rhythm of reproductive neuroendocrine activity is indicated at the bottom. The black bars near the top indicate timing of the natural breeding season and the curved line depicts the photoperiod. Adapted from Woodfill *et al.* (1994).

in winter, is also ineffective when infused in summer, whereas the summer melatonin pattern is effective when infused during other rhythm stages. This supports the concept that the specific characteristics of the circadian pattern of melatonin secretion appear to be key to circannual rhythm entrainment.

The capability of melatonin to entrain the circannual rhythm of reproductive neuroendocrine activity in a season-specific fashion only begins to scratch the surface of the entrainment process. Clearly, it is important to know how and where circannual rhythms are generated and how melatonin interacts with the circannual mechanism. Recent work provides evidence that the melatonin relevant to seasonal GnRH regulation in sheep is secreted into the third cerebral ventricle and that it interacts with receptors located in the premamillary region of the hypothalamus. These findings provide an important clue as to where to look for mechanisms that generate and entrain the circannual reproductive rhythm of this species. It is also important to note that, beyond reproduction, melatonin may act elsewhere to regulate seasonal cycles of other neuroendocrine activities. For example, melatonin appears to mediate photoperiodic regulation of prolactin secretion in sheep by acting on the pars tuberalis of the pituitary gland.

## VII. SUMMARY AND FINAL CONSIDERATIONS

This article has examined how different types of endocrine rhythms are integrated to regulate a critically important biological process—reproduction. At the outset, a conceptual framework was presented: the seasonal expression of ovulatory cycles reflects a finely tuned interplay among endocrine rhythms that operate on different time domains (see Fig. 1). This concept was exemplified in this article by considering evidence that a circadian rhythm (melatonin secretion) entrains a circannual rhythm (neuroendocrine feedback responsiveness) and this dictates the frequency of a circhoral rhythm (GnRH pulses) that, in turn, drives a biweekly rhythm of ovarian activity (estrous cycle). Collectively, this integrated system of rhythms dictates if and when ovulatory cycles occur.

In closing, it is important to point out that the schema developed here is almost certainly overly simplistic. Other interactions among these endocrine rhythms are likely to contribute to the coordinated activity of the system. Some of these are illustrated by the dashed lines in Fig. 1. For example, estradiol can cause phase shifts of circannual cycles, as revealed by studies in ground squirrels. In this manner, the seasonal changes in ovarian activity themselves may influence the expression of the underlying circannual rhythm. Furthermore, under conditions in which circannual rhythms free-run, the expression of circadian rhythms has been found to change on a circannual basis. One such change of particular interest here is the duration of the nocturnal secretion of melatonin in sheep. Thus, the hormonal message that entrains the circannual reproductive rhythm itself appears to be reciprocally regulated by the circannual mechanism. Such findings document the enormous complexity of the interplay of rhythmic processes and they serve to emphasize that much still remains to be learned about the generation, regulation, and integration of endocrine rhythms.

## Glossary

**circadian rhythm** Endogenous oscillation having a period of ~24 h.

**circannual rhythm** Endogenous oscillation having a period of ~365 days.

**circhoral rhythm** Endogenous oscillation having a period of ~1 h.

**endocrine rhythm** An oscillation of hormone secretion that is generated endogenously.

**entrainment** Regulation of rhythm period and phase by time cues in the external environment.

**free-run** Persistence of a rhythm in the absence of entrainment.

**period** Interval between repeated occurrences of a rhythm.

**phase** Characteristic of a rhythm at any given point in its progression, for example, onset of the breeding season in the case of the seasonal reproductive rhythm.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Gonadotropin-Releasing Hormone (GnRH) • Gonadotropin-Releasing Hormone Neuron • Growth Hormone (GH) • Neuropeptides and Control of Anterior Pituitary • Ovulation • Prolactin • Stress and Reproduction**

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# Endometrial Remodeling

Lois A. Salamonsen and Rebecca L. Jones
*Prince Henry's Institute of Medical Research, Australia*

I. INTRODUCTION
II. MORPHOLOGICAL CHANGES ACROSS THE MENSTRUAL CYCLE
III. CYCLE-RELATED CHANGES IN THE ENDOMETRIAL EXTRACELLULAR MATRIX
IV. ROLE OF STEROID HORMONES IN MEDIATING ENDOMETRIAL REMODELING
V. STEROID-REGULATED PARACRINE FACTORS IN HUMAN ENDOMETRIUM
VI. PARACRINE REGULATION OF ENDOMETRIAL REMODELING EVENTS
VII. SUMMARY

**The human endometrium undergoes remarkable remodeling across the menstrual cycle, with phases of regeneration and proliferation, differentiation, and breakdown and shedding. It is one of the few adult nonpathological tissues that actively remodels and thus produces a wide range of growth factors, cytokines, and enzymes that are generally associated with embryonic development or tumorigenesis. The remodeling is driven overall by the maternal ovarian hormones estrogen and progesterone, but their actions are mediated by a myriad of local regulators acting in concert. The changes across the menstrual cycle and their regulation by steroid hormones will be the focus of this article.**

## I. INTRODUCTION

The primary function of the endometrium is to provide an environment that is permissive to attachment of a conceptus but that also regulates subsequent trophoblast invasion, enabling optimal development of an intimate contact between maternal and fetal blood supplies. As it is important that embryos implant only when they are at the correct stage of development, the endometrium is nonreceptive to embryo implantation for the majority of the menstrual cycle. Implantation occurs only when there is perfect synchrony between embryonic and endometrial development, during the brief window of endometrial receptivity. The mechanisms of implantation differ widely between different mammalian species: the extent of endometrial preparation or remodeling required also varies. Human implantation involves a very high degree of trophoblast invasion into the maternal blood vessels and myometrium, and thus, the human endometrium undergoes more extensive preparative remodeling and differentiation than that of any other species, to tightly regulate placentation. Many of these changes are irreversible, and thus there is a requirement for shedding of the endometrium during menstruation, if pregnancy is not achieved.

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## II. MORPHOLOGICAL CHANGES ACROSS THE MENSTRUAL CYCLE

The human endometrium comprises two histologically and functionally distinct layers, the basalis, which is adjacent to the myometrium, and the functionalis, which extends from the basalis to the uterine lumen. During each menstrual cycle, the basalis is retained, whereas the functionalis undergoes cyclical remodeling. The endometrium is composed of epithelial, stromal, vascular, and lymphomyeloid cells, surrounded by a complex extracellular matrix (ECM). The normal menstrual cycle can vary from 21 to 35 days, with menstruation lasting for 3–7 days. In the normalized cycle of 28 days, day 1 is defined as the first day of menstrual bleeding. The major phases are the menstrual phase, the proliferative (follicular) phase, during which tissue regrowth occurs, and the secretory (luteal) phase when differentiative changes in preparation for implantation predominate (Fig. 1). Normal ovulatory menstrual cycles are associated with consistent morphological changes. Clearly defined morphological parameters have enabled the classification of the histological changes in the proliferative and secretory phases, to provide a method for accurately dating the endometrial biopsy.

### A. Proliferative Phase (Normalized Cycle: Days 5–13)

The proliferative phase is of variable length and is overall regulated by estrogen originating from the developing ovarian follicle. Estrogen levels rise as the phase progresses, to reach a peak just prior to ovulation. Reepithelialization of the denuded endometrial surface is initiated during the menstrual phase in parallel with tissue breakdown in adjacent areas. This resembles tissue repair in the fetus, which, unlike that in the adult, is devoid of scarring. The damaged endometrial vessels are also repaired within 5 days of menstrual onset. Once the luminal epithelium is restored, there is a rapid proliferation of all resident cell types—stromal cells, glandular epithelium, and blood vessels—and new ECM is laid down by synthesis from these cells. Stromal edema is apparent in the midproliferative phase. The overall result is thickening of the endometrial layer.

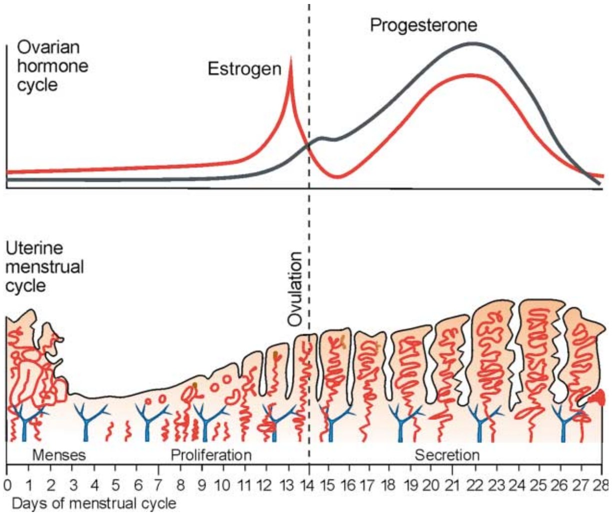

**FIGURE 1** Ovarian hormone fluctuations and endometrial remodeling across a standardized 28-day menstrual cycle.

### B. Secretory Phase (Normalized Cycle: Days 14–28)

Following ovulation (at approximately day 14 of an idealized 28-day cycle), the endometrium undergoes functional differentiation events under the control of progesterone released from the corpus luteum, to provide a suitable environment for embryo implantation. The sequential changes in the appearance of the endometrium during the secretory phase enable accurate assessment of the timing after the luteinizing hormone (LH) surge, and subdivision into early, mid, and late secretory phases. The early secretory phase is characterized by the development of subnuclear vacuoles in epithelial cells, resulting in an altered intracellular location of the nuclei during the early proliferative phase. Over the early to midsecretory phases, epithelial glands become increasingly tortuous and have increased secretory activity. During the midsecretory phase, there is prominent stromal edema, accompanying the development and coiling of the specialized spiral arterioles. After day 22, the stromal cells begin a differentiation process known as decidualization, which is a prerequisite for successful implantation and placentation. This involves widespread morphological changes and tissue remodeling, as fibroblast-type cells become enlarged and rounded and deposit a pericellular matrix. The resultant decidual cells have a markedly different phenotype than the fibroblasts from which they are derived, producing a wide array of growth factors and cytokines that act both to promote and to regulate trophoblast invasion during embryo implantation. Elevated numbers of leukocytes (particularly macrophages and the specialized uterine natural killer cells) are present in the endometrial stroma as decidualization commences (Table 1). If pregnancy occurs, decidualization progresses

**TABLE 1** **Relative Distributions of Specific Inflammatory Cells in the Functional Endometrium at Three Stages of the Normal Menstrual Cycle**

| Cell type | Proliferative phase days 10–12 | Secretory phase days 22–23 | Menses days 26–28 |
|---|---|---|---|
| Macrophages | + | ++ | +++ |
| Eosinophils | − | − | ++ |
| Neutrophils | − | − | +++ |
| Mast cells | ++ | ++ | ++ |
| T lymphocytes | + | + | + |
| B lymphocytes | −/+ | −/+ | + |
| Uterine NK cells | − | +/++ | +++ |

*Note*. −, +, ++, and +++ represent values of 0, 1–2, 3–5, and 6–15% of total endometrial cells, respectively.

throughout the endometrium, providing the maternal component of the placenta.

## C. Menstrual Phase (Normalized Cycle: Days 1–4)

In the absence of a pregnancy, falling progesterone levels in the late secretory phase trigger endometrial regression. Menstruation involves the partial breakdown and loss of the functionalis layer of the endometrium that occurs in association with uterine bleeding. Interestingly, other organs such as the breast, vagina, and oviduct contain the same complement of steroid receptors and undergo considerable tissue remodeling, but none break down, suggesting that it is the unique characteristics of decidualized stroma, spiral arterioles, and leukocyte infiltrate that define the endometrium as a tissue targeted for destruction.

Tissue destruction at menstruation is very focal and reepithelialization occurs at some foci coincident with tissue degradation while bleeding is still in progress at other sites, indicating the importance of local molecular triggers. There is a further influx of leukocytes, primarily polymorphonuclear leukocytes and macrophages, in the premenstrual phase and these are postulated to play a major role in the local initiation of tissue breakdown (Table 1). Even before menstruation, widespread degeneration is seen in the basal lamina supporting the decidualized cells and the endothelium of blood vessels. This is most likely a result of extensive degradation of the ECM; small lesions are apparent by scanning electron microscopy in the luminal epithelium on day 28 of the cycle. The classic concept of menstruation originated from studies in the rhesus monkey in the 1940s and proposed that it is an event resulting from vasoconstriction/reperfusion. It is now, however, widely accepted that menstruation is a result of both vascular and inflammatory events driven by local regulatory factors and that the tissue destruction is caused by the action of matrix-degrading enzymes.

## III. CYCLE-RELATED CHANGES IN THE ENDOMETRIAL EXTRACELLULAR MATRIX

The endometrial interstitial ECM is primarily a product of stromal fibroblasts and comprises a variety of collagens, glycosaminoglycans, and proteoglycans. As in other tissues, the basal lamina is associated with epithelial and vascular structures. The ECM components demonstrate spatial variation within the endometrium and also undergo specific cyclical changes during the menstrual cycle; however, the regulatory mechanisms involved are unclear (Table 2). As the proliferative phase progresses, an initial fine network of reticulum fibers in the functionalis progressively becomes denser and thicker. This network comprises collagens type I, III, V, and VI, along with vitronectin, which stains most densely adjacent to the glandular basal lamina. A peak of hyaluronan (whose extreme hydrophilic nature contributes to the hydration of tissue) is seen in the midproliferative phase, coincident with stromal edema. Also during the proliferative phase, components of the basal lamina (collagen type IV, laminin, nidogen) increase with restoration of the epithelium and development of the glands. During the midsecretory phase, a number of important alterations occur in the ECM of the functionalis, at the time when implantation would occur and immediately prior to decidualization. The stroma becomes more edematous in concert with a second peak of hyaluronan, and the fibrillar components become more loosely arranged. Collagen V epitopes become unmasked, and a system of matrix channels is seen between decidual cells. There is also a progressive loss of collagen type VI (an interfibrillar cross-linker). The decidual cells lay down components of basal lamina that surround these cells,

TABLE 2 **Components of Endometrial Extracellular Matrix**

| | | Variation during menstrual cycle | | |
|---|---|---|---|---|
| **Matrix component** | | **Proliferative** | **Mid secretory** | **Late secretory** |
| **Interstitial components** | | | | |
| Fibronectin | | ++ | +++ | ++ |
| Vitronectin | | + | − | − |
| Collagen type I | | +++ | ++ | ++ |
| Collagen type III | | +++ | ++ | ++ |
| Collagen type V | | +++ | + | + |
| Collagen type VI | | +++ | ++ to +/− | − |
| Tenascin | | +++ | + Perivascular only | + Perivascular only |
| Chondroitin and dermatan sulfate proteoglycans | | | Increase in secretory phase? | Increase in secretory phase? |
| Keratan sulfate | | | Increase in secretory phase? | Increase in secretory phase? |
| Hyaluronic acid | | Midproliferative peak | ++ | +/− |
| **Basal lamina components** | Site | | | |
| Laminin | | | | |
| | G/V | +++ | +++ | +++ |
| | S | − | − to −/+ | − to + |
| Collagen type IV | | | | |
| | G/V | +++ | +++ | +++ |
| | S | − | − | − to + |
| Heparan sulfate proteoglycan | | | | |
| | G/V | +++ | +++ | +++ |
| | S | − | − | − to + |
| Entacin (nidogen) | | | | |
| | G/V | +++ | +++ | +++ |
| | S | − | − | − to + |

*Note.* G, glandular basal lamina; V, blood vessel walls; S, pericellular matrix of stromal cells; −, not detected; −/+, barely detectable; + to +++, increasing levels.

reflecting the change from a mesenchymal type fibroblast to a more epithelial-like phenotype. Endometrial regression prior to menstruation correlates with a loss of hyaluronan and water of hydration. Extensive destruction of the both fibrillar and basement membrane ECM is observed immediately prior to and during menstruation.

## IV. ROLE OF STEROID HORMONES IN MEDIATING ENDOMETRIAL REMODELING

All of the remodeling events throughout the menstrual cycle are governed by estrogen and progesterone, with estrogen stimulating the regrowth and proliferation after menses and progesterone driving differentiation in preparation for implantation. Steroid hormone action is dependent on the presence of specific ligand-activated nuclear receptors in target tissues.

### A. Estrogen Receptors

Two receptors for estrogen have been described, ER-α and ER-β. There is a high degree of sequence homology between the two and both stimulate the transcription of ER target genes. The most significant disparity between the two receptors lies in their tissue distribution. In the human endometrium, both ER-α and ER-β are expressed. ER-α is found in both the epithelial glands and the stroma of the functionalis layer, is maximal during the late proliferative phase, and declines during the secretory phase (Table 3). The epithelium also expresses ER-β with a decline during the secretory phase. ER-β is not expressed in the stroma. In the vascular endothelium, only ER-β is present, whereas both subtypes are found in the perivascular cells surrounding endometrial blood vessels. The functional importance of ER-α for human fertility has been highlighted by studies using mice in which both ER-α and ER-β were separately disrupted (ERKO and BERKO, respectively). The loss of ER-α expression in the uteri of ERKO mice is associated with the loss of estrogen responsiveness, whereas mice lacking ER-β exhibit a relatively normal uterine phenotype and are fertile.

**TABLE 3 Steroid Hormone Receptors in Endometrial Glands and Stroma during the Human Menstrual Cycle**

| | Menstrual | | Proliferative | | Secretory | |
|---|---|---|---|---|---|---|
| Receptor | Epi | Str | Epi | Str | Epi | Str |
| ER-α | +/− | +/− | ++ | + | +/− | +/− |
| ER-β | +/− | +/− | + | +/− | +/− | +/− |
| PRA | − | + | +++ | +++ | − | + |
| PRB | + | − | +++ | + | + | − |
| AR | − | + | − | ++ | − | + |

*Note*. Epi, epithelial glands; Str, stromal cells; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor.

## B. Progesterone Receptors

The human progesterone receptor (PR) is also expressed as two isoforms, PRA and PRB. They are products of a single gene, translated from individual mRNA species under the control of different promoters, and they differ only in that the smaller isoform PRA lacks 164 amino acids from the N-terminus. On the basis of *in vitro* studies, it appears that the two proteins are functionally different. PRB is the more transcriptionally active isoform, whereas the truncated PRA can act as a dominant repressor of PRB activation of progestin-sensitive genes. Thus, it is likely that the relative levels of PRA and PRB within target cells determine the nature and magnitude of the response to progesterone. In the normal endometrium during the menstrual cycle, PRA and PRB are coexpressed in target cells. PR expression is stimulated by estrogen in the proliferative phase, but there is selective down-regulation by progesterone itself in the secretory phase. Before ovulation, PRA and PRB are expressed at equivalent levels in glandular epithelium. PRB, but not PRA, persists in the glands during the midsecretory phase, suggesting that PRB is of utmost importance in glandular secretion at this time (Table 3). In the stroma, the predominance of the PRA isoform throughout the cycle implicates this isoform in events such as stromal mitosis, decidualization, and regulation of ECM composition. PRs are also expressed on endometrial endothelial cells but are absent from leukocytes in the endometrium. Mice with a null mutation of PR display uterine hyperplasia and inflammation.

## C. Androgen Receptors

Androgen receptors (ARs) are also expressed by the human endometrium. Two forms of AR exist, ARA and ARB. ARs predominate in stroma during the proliferative phase; little expression is seen in the epithelium (Table 3). Anti-progestins such as mifepristone (RU486) induce ARs in the glands and enhance their levels in the stroma. Importantly, many progestins used in contraceptive preparations, particularly levonorgestrel, exhibit potent androgenic actions, and thus understanding the effects of androgen action on the endometrium is of clinical importance.

# V. STEROID-REGULATED PARACRINE FACTORS IN HUMAN ENDOMETRIUM

Estrogen and progesterone drive endometrial remodeling through the downstream regulation of a wide range of cytokines, growth factors, and enzymes, which act locally to mediate cellular events. The local regulation of proliferation, differentiation, and breakdown is evident in the focal nature of these changes. Many cytokines and growth factors that act as paracrine and autocrine regulators are produced within the endometrium by one or more cellular components and display cyclical variation consistent with their actions on the endometrium.

## A. Cytokines and Growth Factors

A range of cytokines and growth factors, whose expression is generally limited to pathological conditions in the adult, are produced by the endometrium and are essential for the complex cellular interactions throughout the menstrual cycle. These include proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and IL-6; leukocyte-attracting chemokines, such as IL-8, monocyte chemoattractant protein-1 (MCP-1), and eotaxin; immunomodulatory cytokines, such as IL-4, IL-10, IL-11, leukemia inhibitory factor (LIF), colony-stimulating factor 1 (CSF-1), and granulocyte/macrophage colony-stimulating factor (GM-CSF); and growth factors, such as epidermal growth factor, insulin-like growth factors (IGFs), transforming growth factor-βs (TGF-βs), and activins.

Cytokines are produced by epithelial cells, vascular endothelium, and decidualized stroma, in addition to infiltrating leukocytes, with cyclical variation consistent with regulation by steroid hormones and the paracrine environment (Fig. 2). A number of cytokines have been shown to regulate endometrial function *in vivo* or *in vitro*. Among the known functions of these regulators are modulation of proliferation, induction of other cytokines/growth factors and adhesion molecules, stimulation of extracellular matrix molecule synthesis, alteration of protease/protease inhibitor synthesis, activation

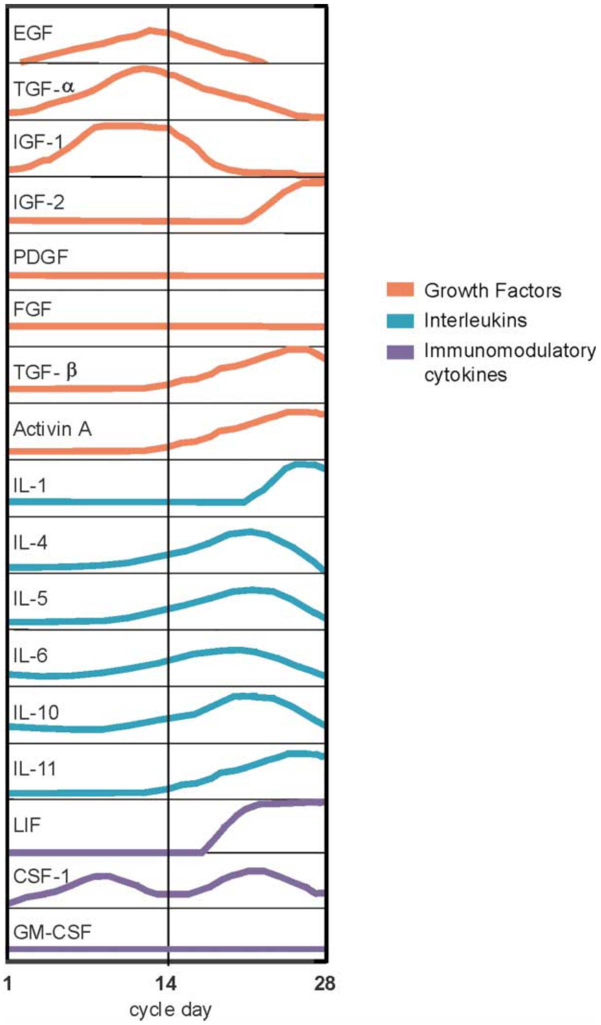

**FIGURE 2** Relative amounts of cytokine and growth factor mRNA and protein levels in human endometrium during the menstrual cycle. Levels illustrated are relative to levels during the early proliferative phase. Adapted from Giudice, L. C. (1995). *Trends Endocrinol.* **6**(2), 60–69, with permission from Elsevier Science.

of lymphomyeloid cells, and regulation of differentiation.

## B. Angiogenic and Vasoactive Factors

Numerous angiogenic promoters and their receptors, along with inhibitors of angiogenesis, have been identified in human endometrium, some with cyclical variation (Table 4). Furthermore, molecules containing angiogenesis inhibitors that are released by proteases acting at cryptic sites are also abundant in the endometrium; these include plasminogen (the source of angiostatin) and collagen IV (the source of canstatin).

Vasoactive agents of importance in the endometrium include prostaglandins (PGs), endothelin (ET), and nitric oxide (NO). PGs are produced within the endometrium by both epithelial cells and decidualized stroma and are present in the menstrual fluid at high concentrations. Both PG synthesis (via cyclooxygenases 1 and 2) and metabolism (by PG dehydrogenases) are influenced by estrogen and progesterone. The major cellular source of ET, a potent vasoconstrictor, is the epithelium, although endothelium and decidualized stroma are additional sites of production. ET mRNA expression varies across the menstrual cycle, reaching maximal levels in the premenstrual phase. NO relaxes myometrial and vascular smooth muscle and thus is a strong candidate for mediating various steroid hormone and cytokine effects in the endometrium and decidua in the processes of developing receptivity, implantation, and menstruation. Both inducible NO synthase and endothelial NO synthase are present in endometrium, in a variety of cell types including inflammatory cells.

## C. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are the enzymes that degrade components of both interstitial and basement membrane ECM. Their synthesis is negligible in normal connective tissue. MMP activity is tightly regulated; most MMPs are secreted as latent zymogens that require activation extracellularly. Their local activity can be inhibited by tissue inhibitors of MMPs (TIMPs) by the formation of 1:1 complexes. Growth factors, cytokines, steroid hormones (including progesterone and androgens), and other regulatory molecules differentially regulate the genes for MMPs. A number of MMPs have been identified within the endometrium. Their pattern of expression during the normal menstrual cycle is consistent with local actions in remodeling processes. In particular, a dramatic increase in their expression and activation premenstrually, at foci within the functionalis, supports a pivotal and critical role for these degradative enzymes in the process of normal menstruation.

# VI. PARACRINE REGULATION OF ENDOMETRIAL REMODELING EVENTS

## A. Proliferation, Angiogenesis, and Apoptosis

Significant epithelial cell mitotic activity and DNA synthesis very early in the cycle indicate

TABLE 4 Angiogenic Promoters/Receptors and Inhibitors Identified in Human Endometrium

| | Cellular location | | | |
|---|---|---|---|---|
| Factor | Stroma | Glands | Blood vessels | Phase of maximal expression |
| **Angiogenesis promoters** | | | | |
| VEGF | + | ++ | | Secretory |
| VEGF R1 | + | + | +++ | Secretory |
| VEGF R2 | + | | +++ | Proliferative |
| FGF-1 | + | +++ | | No variation |
| FGF-2 | + | +++ | | No variation |
| FGF-R1 | | +++ | | No variation |
| | + | | | Proliferative |
| PD-ECGF | + | | | Proliferative |
| | | ++ | | Secretory/menstrual |
| **Angiogenesis inhibitors** | | | | |
| TSP-1 | NA | NA | NA | Secretory |
| METH-1 | + | − | | NA |
| Soluble VEGF R1 | NA | NA | NA | Late secretory |

the regeneration of epithelium from remaining glands and persisting areas of surface epithelium rather than from stem cells during the repair phase (days 3–4). Mitotic activity is a key feature of all endometrial components during the proliferative phase but declines during the secretory phase, persisting at low levels in surface epithelia and predecidual cells only. IGFs are likely candidates for conveying estrogen action throughout the endometrium, with the balance of ligand (IGFs) and binding proteins shifting from the proliferative to the secretory phase, corresponding to the stimulation and inhibition of estrogen-driven proliferation. Other growth factors, including EGF and TGF-β are prevalent in proliferating endometrium. Following ovulation, progesterone plays a key role in regulating endometrial proliferation by suppressing estrogen-driven mitosis in both glands and stroma. Specific mechanisms proposed include down-regulation of estrogen receptors, induction of the enzyme catalyzing conversion of estradiol to the less active estrone, and the decrease of estrogen-induced specific gene expression.

Angiogenesis, including proliferation of endothelial cells, occurs in three episodes during the menstrual cycle, representing initial repair during the early proliferative phase, estrogen-driven angiogenesis during the midproliferative phase, and progesterone-driven growth of the coiled arterioles during the secretory phase. Dynamic expression of epithelial and endothelial vascular endothelial growth factor and its receptors, as well as other angiogenic agents (Table 4), throughout the cycle indicates that complex and distinct mechanisms are involved in the regeneration of blood vessels and the remodeling of spiral arterioles.

Coincident with cellular proliferation is the controlled apoptosis of individual epithelial cells, presumably those unable to pass successfully through the cell cycle due to DNA damage. Apoptotic incidence increases later in the cycle, during the receptive phase (day 19–20) and in normal late secretory and menstrual endometrium. Members of the Bcl family protect against apoptosis and Bax protein increases the apoptotic susceptibility of cells. Bcl-2 expression is prominent during the proliferative phase but decreases toward the secretory phase and is absent or negligible in the midsecretory phase. Bax expression also decreases after the proliferative phase, though it remains detectable throughout the entire secretory phase, indicating that the decreased Bcl-2:Bax ratio determines the susceptibility to apoptosis. A number of other apoptotic-regulatory factors such as the Fas/Fas ligand system are also expressed in the endometrium but their contribution to the apoptosis trigger remains to be determined. Given that a large proportion of the cells shed at menstruation are viable (cells from menstrual fluid can be cultured and endometriosis is postulated to arise from retrograde menstruation), the contribution of apoptosis to this process must be limited.

## B. Decidualization

Decidualization is a major remodeling event and is critical for the modulation of trophoblast invasion and optimal placentation. Progesterone initiates the decidual reaction in estrogen-primed stromal cells, and products of epithelial cells, leukocytes, and the decidualized stromal cells themselves have contributory actions in mediating the differentiation and remodeling processes. Major differences exist in the initiation and progression of decidualization between humans and most other mammalian species, meaning that studies on human endometrium are critical. Some key factors involved in mediating stromal decidualization have been identified in mouse knockout models, in which the endometrium fails to decidualize and thus implantation is blocked. These include IL-11, prolactin, and prostaglandins. These factors have subsequently been shown to be also important for the decidual transformation in human endometrium. *In vitro* models of decidualization have been widely utilized to delineate the roles of paracrine agents in the regulation of decidualization in the human uterus. These generally involve stimulating decidual transformation of isolated endometrial cells with combined estrogen and progesterone or with cAMP. Thus, corticotrophin-releasing hormone, activin A, IL-11 and prostaglandin $E_2$ have been shown to promote decidualization, whereas IL-1, TNFα, and macrophage CSF play inhibitory roles. MMPs (particularly the gelatinases MMP-2 and MMP-9) and TIMPs play roles in the tissue remodeling associated with decidualization and help restrict cytotrophoblast invasion during implantation. Recent microarray analysis identified a range of genes regulated during *in vitro* decidualization of human stromal cells: these included cytokines, growth factors, nuclear transcription factors, and mediators of the cAMP signal transduction pathway. Further study of these factors will contribute to a greater understanding of the mechanisms involved in decidualization in the human uterus.

## C. Menstruation

Menstruation is triggered in a nonconception cycle by the withdrawal of progesterone from the decidualizing endometrium, resulting in inflammation, tissue destruction, and shedding. Steroid-regulated chemokines, including IL-8, MCP-1, MCP-2, RANTES, and eotaxin, may be responsible for the chemoattraction and activation of neutrophils, macrophages, and eosinophils into the endometrium. Activated leukocytes distributed throughout the stroma are a source of pro-inflammatory cytokines, PGs, and MMPs, which together have the potential to instigate cascades of inflammation and breakdown at localized foci. PGs have integral roles during menstruation, as inflammatory and vasoactive mediators augmenting leukocyte infiltration. Administration of intrauterine $PGF_{2\alpha}$ causes menses and increases uterine contractility as it is overall a vasoconstrictor. By contrast, $PGI_2$ causes vasodilatation. COX-2, the inducible cyclooxygenase isoform, is up-regulated with progesterone withdrawal, coincident with falling expression of the progesterone-dependent metabolizing enzyme PGDH, resulting in elevated concentrations of bioactive PGs in the endometrium and menstrual fluid. ET-1, a potent vasoconstrictor, is made and released by human endometrium and can act on both epithelial cells and endothelial cells. Its production increases around the time of menstruation, suggesting a paracrine role in endometrial bleeding and/or repair.

MMPs have the capacity to digest all components of the endometrial ECM, and there are dramatic increases in MMP mRNA and protein levels between cycle days 26 and 4. MMP-1, -2, -3, and -9 in particular have been shown to be increased in association with endometrial degradation while MMP-7 appears more likely to have a role in endometrial repair. Active forms of the enzymes have been demonstrated in perimenstrual and menstrual endometrium. TIMPs are also present in the endometrium but smaller cyclical changes are observed, suggesting that the major regulation of MMP action is at the levels of MMP expression and activation. Although progesterone is a negative transcriptional regulator of most MMPs, the very focal nature of their expression suggests local regulation. Leukocytes scattered throughout the stroma produce MMPs and other enzymes, such as mast cell tryptase and neutrophil elastase, which can activate certain MMPs and thus contribute to the cascade of matrix-degrading activity at menstruation.

## VII. SUMMARY

Estrogen and progesterone overall regulate endometrial remodeling across the menstrual cycle, although many of their actions are indirect and are mediated by an array of paracrine agents produced locally by the various cellular components of the endometrium. Cytokines and growth factors form complex networks that facilitate communication between epithelial and stromal cells, leading to the regulation of decidualization and endometrial receptivity for embryo implantation. Interactions between leukocytes, endothelial components, and stromal components are necessary for the initiation of menstruation. Identification of the molecules involved in mediating these processes in the human endometrium is essential for the future development of novel contraceptive agents for women, for detecting endometrial causes of infertility, and for the treatment of infertility.

## Glossary

**chemokine** A chemotactic cytokine that directs the migration of leukocytes.

**decidualization** The differentiation of stromal fibroblasts into decidual cells.

**extracellular matrix** The complex, collagenous matrices to which cells are attached within a tissue; it includes both interstitial matrix and basal lamina.

**menstruation** The process whereby the functional layer of the endometrium is shed, accompanied by bleeding at the end of each nonpregnant cycle.

**steroid hormone** One of a group of steroids that function as hormones, includes estrogens, progestogens, and androgens.

## See Also the Following Articles

**Androgen Receptor Structure and Function • CC, C, and CX$_3$C Chemokines • CXC Chemokines • Decidualization • Endometriosis • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Extracellular Matrix and Follicle Development • Implantation • Ovulation • Progesterone Action in the Female Reproductive Track**

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# Endometriosis

KHALED ZEITOUN* AND SERDAR E. BULUN†

**Columbia University • †University of Illinois*

I. EPIDEMIOLOGY
II. ETIOLOGY
III. ESTROGEN BIOSYNTHESIS AND METABOLISM IN ENDOMETRIOSIS
IV. ESTROGEN AND PROGESTERONE RECEPTORS
V. CLASSIFICATION
VI. DIAGNOSTIC TESTS
VII. SYMPTOMS
VIII. CLINICAL EXAMINATION
IX. TREATMENT

**Endometriosis is a heterogeneous and complex disorder that is characterized by the presence of endometrial glands and stroma outside of the endometrial cavity. Adenomyosis arising as the result of infiltration or invagination of the myometrium by endometrial glands is**

involved in mediating these processes in the human endometrium is essential for the future development of novel contraceptive agents for women, for detecting endometrial causes of infertility, and for the treatment of infertility.

## Glossary

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**decidualization** The differentiation of stromal fibroblasts into decidual cells.

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**steroid hormone** One of a group of steroids that function as hormones, includes estrogens, progestogens, and androgens.

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**Androgen Receptor Structure and Function • CC, C, and $CX_3C$ Chemokines • CXC Chemokines • Decidualization • Endometriosis • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Extracellular Matrix and Follicle Development • Implantation • Ovulation • Progesterone Action in the Female Reproductive Track**

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# Endometriosis

Khaled Zeitoun* and Serdar E. Bulun†

*Columbia University • †University of Illinois

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**Endometriosis is a heterogeneous and complex disorder that is characterized by the presence of endometrial glands and stroma outside of the endometrial cavity. Adenomyosis arising as the result of infiltration or invagination of the myometrium by endometrial glands is**

**considered a different entity from endometriosis. Both conditions affect women throughout the world, resulting in infertility, pelvic pain, and decreased quality of life.**

## I. EPIDEMIOLOGY

The exact incidence or prevalence of endometriosis is unknown. It is estimated that 2–10% of women of reproductive age are afflicted with this disease. Diagnosis has improved because of widespread use of laparoscopy and also with increased awareness of the disease. At laparoscopy for chronic pelvic pain and infertility, endometriosis is detected in 25 and 33% of patients, respectively.

Endometriosis is typically diagnosed at the age of approximately 30 to 35 years. This disorder is believed to begin after puberty with establishment of menses and to resolve with menopause. However, it is not uncommon for patients to develop endometriosis during the two extremes of the female reproductive life. Approximately 10% of cases are diagnosed in teenagers and up to 5% in postmenopausal women. Significant endometriosis diagnosed at the time of puberty or shortly afterward is primarily associated with obstructive Müllerian anomalies. However, there are also reports of severe cases in adolescents with normal pelvic anatomy.

The natural history of endometriosis is subject to speculation and debate. Establishment of endometriotic lesions and their clinical courses are often not well defined secondary to the multiple factors affecting the initiation and progression of the disease. Nonetheless, endometriosis remains one of the most common gynecologic disorders resulting in hospitalization and is a leading cause of pelvic pain and infertility.

## II. ETIOLOGY

Multiple theories have been developed in an attempt to explain the etiology of endometriosis. Unfortunately, no single theory is sufficient for the explanation of highly variant pathologic and clinical presentations of endometriosis. Endometriosis may actually represent a spectrum of abnormalities where the exact line between normal and disease states is not completely defined. One of the most widely accepted theories explaining the development of endometriosis is the implantation of viable endometrium at ectopic sites. Sampson hypothesized that during menstruation, retrograde bleeding deposits endometrial fragments into the peritoneal cavity via the oviducts. In animal models, menstrual efflux that was diverted into the peritoneal cavity developed into endometriotic lesions. Furthermore, endometrial cells present in human menstrual efflux are viable and can be grown in tissue cultures as well as in immunologically compromised animals. The predominance of endometriotic lesions in the dependent parts of the pelvis and the common association of endometriosis with obstructive Müllerian anomalies lend clinical support to Sampson's theory. However, retrograde menstruation alone does not explain why only 5–10% of women develop pelvic endometriosis when menstrual reflux is believed to be a universal phenomenon.

Other forms of implantation are also possible. Iatrogenic implantation of endometrium in abdominal and episiotomy scars at the time of delivery resulting in endometriosis has been reported. Also, lymphatic or hematogenous dissemination of endometrium explains some cases of endometriotic implants found in lymph nodes or in distant sites such as the lungs or extremities.

Coelomic metaplasia of the peritoneal lining and growth of lesions from embryonic rests within the pelvis and peritoneal cavity may also explain the development of endometriosis when endometrial implantation is not possible. Endometrioid tumors of the ovary suggest the capacity for coelomic epithelium, lining the ovary and the peritoneal cavity, to transform into endometrial tissue. However, an initiating factor such as exposure to estrogen is needed.

The presence of immunologic abnormalities in patients with endometriosis has been suggested by studies demonstrating alterations in cell-mediated immunity and by demonstration of antibodies against the endometrium. The absence of associated immune defects in cases with endometriosis, the complexity of the immune system, and the inconclusive nature of these studies cast doubts on theories implicating the immune system. However, a subtle immunological defect in the clearance of endometrial fragments at the time of retrograde menstruation might contribute to the initiation of the disease process.

A genetic basis for endometriosis is suggested by the increased incidence in women with a family history of this disease. In these individuals, endometriosis develops earlier in life and is found to be more severe. The inheritance pattern is probably polygenic or multifactorial.

After demonstration of an increased incidence of severe endometriosis in a colony of rhesus monkeys chronically exposed to the environmental toxin dioxin by Rier *et al.* in 1993, there was enthusiasm in the scientific community for clarifying the association between environmental toxins and endometriosis. This was enhanced by the observation that elevated dioxin levels have been reported in a significantly higher number of patients with endometriosis compared to healthy controls in a population of women with infertility. These and other studies suggested an association between dioxin exposure and development of endometriosis in women.

Since a single theory is not sufficient to explain all pathophysiologic aspects of endometriosis, it is now proposed that a multitude of factors might be involved in the etiology of this enigmatic and complex disorder. Retrograde menstruation transports viable endometrium into the peritoneal cavity but may also act as an initiating factor for metaplasia of the coelomic epithelium into endometriotic lesions. At the same time, defective clearance of shed endometrial fragments due to a local or general immunologic defect may further contribute to the development of endometriosis. An individual's genetic predisposition or environmental toxins may impede clearance of the endometrial fragments from the peritoneal cavity or enhance implantation and then growth of lesions. These environmental factors may also enhance metaplasia of the coelomic endothelium into endometrial-like tissue. In all circumstances, estrogen is an important factor for the progression and persistence of the disease. With further investigation, more information about the pathophysiologic origin of endometriosis may become available in the future.

## III. ESTROGEN BIOSYNTHESIS AND METABOLISM IN ENDOMETRIOSIS

Several molecular aberrations were found in endometriotic lesions (in contrast to eutopic endometrium) that favor increased local concentrations of estradiol. These aberrations are important in view of the fact that endometriosis is an estrogen-dependent disease.

The conversion of androstenedione and testosterone to estrone and estradiol is catalyzed by aromatase P450, which is expressed in a number of human tissues and cells such as ovarian granulosa cells, placental syncytiotrophoblasts, adipose and skin fibroblasts, and the brain. In a woman of reproductive age, the ovary is the most important site of estrogen biosynthesis. On the other hand, estrogen formation can also take place in extraglandular tissues such as adipose and skin. The latter source of estrogen is important when ovarian estrogen formation is decreased in the postmenopausal period and also when endometriosis is treated by agents that inhibit ovarian estrogen production. Recently, it was also demonstrated that endometriotic stromal cells aberrantly express aromatase, which converts $C_{19}$ steroids to estrogens within the lesions (Fig. 1). Aromatase activity in these cells is regulated via cyclic AMP and vigorously stimulated by prostaglandin E2 (PGE2). Estrogen stimulates cyclooxygenase-2, giving rise to increased PGE2 formation. Thus, this positive feedback loop serves to produce increasing quantities of estradiol and PGE2 in endometriosis. Furthermore, the lack of aromatase expression in eutopic endometrium seems to be maintained by binding of an inhibitory transcription factor, COUP-TF (chick ovalbumin upstream promoter-transcription factor), to the aromatase promoter. In endometriosis, however, an aberrantly expressed factor, SF-1, displaces COUP-TF to bind to this same promoter and activates aromatase expression and thus local estrogen biosynthesis (Fig. 2).

In addition, endometriotic glandular cells are deficient in 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2, which inactivates estradiol by converting it to estrone in the eutopic endometrium in response to progesterone. A deficiency of this enzyme in endometriosis impairs the inactivation of estradiol

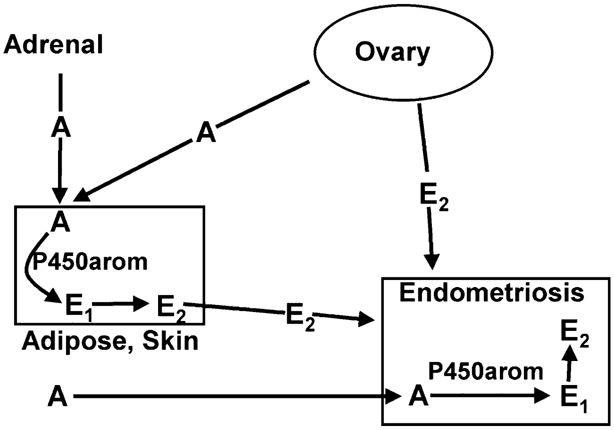

FIGURE 1 Origin of estradiol in endometriosis: Androstenedione (A) of adrenal and ovarian origin is the principal precursor of estrogen in the ovary, adipose, skin, and also endometriotic lesions. Estradiol ($E_2$) from the ovary and peripheral tissues reaches the endometriotic lesions via the bloodstream. Aromatase (P450arom) in the stromal cell also catalyzes the conversion of androstenedione to estrone ($E_1$), which is further reduced to $E_2$ by 17β-HSD type 1 in the endometriotic tissue. Both circulating estradiol and locally produced estradiol will lead to maintenance and growth of the lesions.

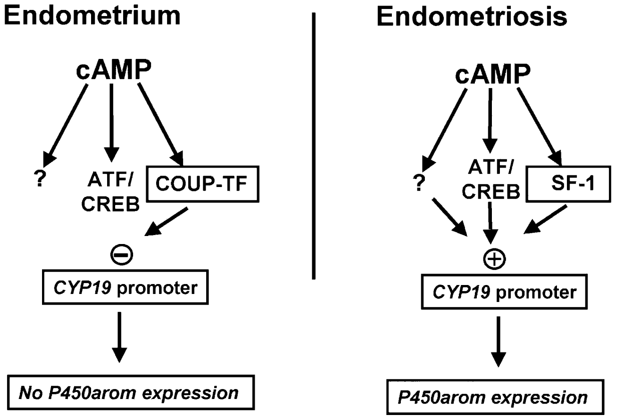

FIGURE 2 Proposed mechanism of regulation of aromatase (P450arom) expression by SF-1 and COUP-TF in eutopic endometrium and endometriosis: COUP-TF binds to a specific regulatory DNA sequence upstream of the aromatase promoter II in eutopic endometrial stromal cells in the absence of any competition by SF-1, since SF-1 expression is not detected in the majority of endometrial samples. Thus, COUP-TF exerts its inhibitory effect on the complex of general transcription factors, which binds to the TATA box. In the endometriotic stromal cell, however, SF-1 is present and binds to the identical DNA sequence with a higher affinity than that of COUP-TF. Upon replacing COUP-TF, SF-1 synergizes with other transcription factors such as a member of the activating transcription factor/cAMP-responsive element-binding protein (ATF/CREB) family of transcription factors and others to initiate the transcription of the aromatase gene in response to cAMP.

and may be a consequence of progesterone insensitivity. Aforementioned molecular aberrations that increase local estradiol concentrations may be important in the etiology of endometriosis and also offer an explanation for the high failure rates obtained with current treatment modalities. These molecules may be targeted to develop novel therapeutic strategies.

## IV. ESTROGEN AND PROGESTERONE RECEPTORS

In order to better understand the behavior of endometriosis, many studies have focused on comparing and contrasting eutopic versus ectopic endometrial tissues in terms of their estrogen receptor (ER) and progesterone receptor (PR) profile. To date, the presence of estrogen and progesterone receptors in the glandular epithelium and the stroma of ectopic endometriotic tissue has been confirmed by multiple studies. However, investigations on the biologic behavior of these receptors have not yielded consistent results.

When compared to the eutopic endometrium, it is still unclear whether ectopic endometrium has higher, similar, or lower ER/PR contents. Earlier studies have mostly found levels of ERs in endometriotic tissues that are lower than or similar to those in the endometrium. More recently, Jones and colleagues found that ectopic ER content is actually higher than that in the eutopic endometrial tissue. Although the scientific methodology of this study is purportedly more sensitive, the investigators also sampled ectopic endometrial tissue mostly from ovaries rather than from pelvic implants as was done in the previous studies. Thus, this variation may account for the observed difference in ER content.

The presence of cyclic variation in ER/PR contents in ectopic endometrial tissue that is normally exhibited in eutopic tissue has also been studied extensively. Although some authors described no predictable cyclic menstrual pattern in the ectopic ER, others have found that ERs in endometriotic implants have a predictable decline during the secretory phase of the menstrual cycle. Similarly, ectopic PRs have been found to either decline during the late secretory phase in some studies or remain elevated throughout the secretory phase in other studies.

Although these conflicting results can perhaps be partially attributed to the variations in laboratory methods, i.e., tissue preservation or immunohistochemical staining methods, true biologic differences among endometriotic implants have been documented. Taking simultaneous biopsies from the endometrium and multiple endometriotic implants from the same patient, Howell found marked heterogeneity in the expression of estrogen and progesterone receptors in endometriosis. These differences could be demonstrated when comparing endometriotic lesions with the corresponding endometrium and also between samples of endometriosis collected from different sites within the same patient. Hence, it is possible that microenvironmental factors related to the site, depth, and degree of fibrosis of each individual lesion could determine the amount of steroid hormone stimulation reaching the lesion. This could account for the observed differences in ER/PR profiles between endometriosis and the endometrium. More importantly, it may be responsible for the differences among endometriotic lesions from different sites within the same patient. Therefore, the ER/PR profile of a single endometriosis sample from a patient cannot be assumed to represent the status of all endometriosis lesions within the patient. In a recent study, it was also shown that the main progesterone receptor in extraovarian endometriotic lesions is progesterone receptor A, whereas progesterone receptor B (PRB) is the receptor predominantly expressed in normal endometrium. A deficiency of PRB in endometriosis might cause an impaired response to progesterone and hence progesterone resistance. Clinically, this heterogeneity in ER/PR content may account for the disparate response of endometriosis to medical treatment among different patients.

## V. CLASSIFICATION

Several classification systems have been suggested over the years to stage endometriosis according to the severity of the disease. The goal was to divide the disease into stages that correlated with pregnancy rate or response to treatment. This goal was, however, never completely realized. The staging systems were complex and not widely used, and studies did not show a good correlation between pregnancy rates and stage of the disease.

The American Fertility Society (AFS) original and revised classifications were an effort to predict the probability of pregnancy following treatment of endometriosis, according to disease severity. The revised AFS classification introduced in 1985 divided endometriosis into four stages (minimal, mild, moderate, and severe). In this classification, higher scores were given to deep endometriosis, dense adhesions, and obliteration of the cul-de-sac. Numerous studies could not demonstrate a difference in pregnancy rates after treatment according to stage.

A new attempt at scoring the disease was made with the revised American Society for Reproductive Medicine (ASRM) classification of endometriosis in 1996. The new classification encouraged the recording of lesion color and morphology. Like previous AFS classifications, it contained a standardized form to record findings. Again, data did not support any improvement in prediction of pregnancy after treatment in the ASRM classification.

Despite the subjective nature of laparoscopic evaluation and the limitations of the ASRM system, it is hoped that accumulation of data from the current scoring systems and accurate morphological description may permit future development of a classification system that would be more helpful in predicting pregnancy. Future classifications might include serum or tissue markers for endometriosis in addition to anatomical description of the lesions as recent data indicate the presence of multiple molecular aberrations in endometriosis, some of which might affect fertility.

## VI. DIAGNOSTIC TESTS

The importance of an accurate noninvasive test for a common disorder like endometriosis cannot be overemphasized. Unfortunately available nonsurgical tests are still too insensitive or nonspecific.

### A. CA-125 Measurement

CA-125 is a cell surface antigen and a useful marker in monitoring patients with epithelial ovarian cancer. This antigen is found in coelomic epithelial-derived tissues and thus is also expressed by the endometrium. Elevated CA-125 serum levels were demonstrated in patients with endometriosis especially in advanced stages of the disease. The use of this assay as a screening test for diagnosis was, however, disappointing because of low sensitivity (between 20 and 80%). Its use in monitoring treatment and recurrences is also limited. CA-125 elevations can also occur due to other concurrent conditions such as pregnancy, menstruation, uterine leiomyomata, and pelvic inflammatory disease. In conclusion, there is no accurate serum marker for diagnosing endometriosis at present. However, several molecular aberrations in this abnormal tissue are currently being studied. The characterization of a marker that could predict the presence of the disease and be used to monitor therapy will be invaluable.

### B. Pelvic Imaging

Ultrasound is the preferred imaging method for endometriomas. It is best performed using the vaginal probe because of better resolution. Ultrasound, however, is not very useful in the detection of endometriotic implants and cannot differentiate these from other masses. In contrast, endometriomas are diagnosed with excellent sensitivity and specificity. The use of Doppler flow studies to differentiate endometriosis from other ovarian masses, especially malignant tumors, has also been described. A specific ultrasound description is reported with pericystic vessels, scanty blood supply, high resistance index, and an avascular cavity. The role of Doppler studies is, however, not well defined.

The use of magnetic resonance imaging (MRI) has been used for diagnosis of endometriosis. MRI might be more useful in the detection of endometriotic implants than ultrasound. However, the sensitivity and specificity for detection of endometriomas are not better. At present, the use of MRI for diagnosis of endometriosis has a very limited role. Other imaging studies including computerized tomography (CT) scans, barium enemas, and chest X rays may be needed in complicated and advanced cases involving extra-abdominal sites or bowel. These studies, however, are nonspecific, and endometriotic lesions cannot be differentiated from other disease states with certainty.

### C. Laparoscopy

Despite attempts to use imaging studies to diagnose endometriosis, surgical diagnosis remains the gold standard. A definite diagnosis of endometriosis will usually need pelvic and abdominal exploration. Laparoscopy has assisted in the pathologic diagnosis of endometriosis while decreasing patient morbidity and has replaced laparotomy in all but very rare cases. Tissue biopsy is recommended to obtain histologic confirmation of endometriosis; however, in experienced hands, the presence of typical lesions is usually sufficient for diagnosis. The recent appreciation and description of different lesion types are probably among the causes for the increase in frequency of diagnosis. Atypical lesions such as clear papules might be difficult to detect, and a thorough examination by a surgeon familiar with the disease is ideal. Also, techniques such as infusion of crystalloids into the cul-de-sac or painting of peritoneal surfaces with blood-tinged peritoneal fluid can be used to enhance the detection of these subtle lesions.

Deep infiltrating lesions are sometimes difficult to visualize because they are extraperitoneal. However, they should be suspected and looked for. Careful examination under anesthesia and palpation with a probe might aid in detecting these active lesions. Endometriomas are usually easily detected by laparoscopy, but sometimes aspiration of a suspicious cyst is helpful in confirming the diagnosis before excision. Furthermore, inspection of the bowel is mandatory because a significant number of cases contain bowel lesions.

## VII. SYMPTOMS

Endometriosis is a leading cause of pelvic pain and is strongly associated with infertility. However, endometriosis has also been implicated in a variety of other disorders including spontaneous abortions and abnormal uterine bleeding. In many instances, these associations could not be confirmed by more recent studies.

### A. Pain

Pain is a common symptom attributed to endometriosis. Actually, more than half of the patients presenting with pelvic pain and dysmenorrhea are diagnosed with endometriosis. The typical endometriosis-associated pain starts 1 to 2 days before onset of bleeding and lasts throughout menstruation, but it should be suspected in secondary or worsening primary dysmenorrhea. Dyspareunia is also a symptom of endometriosis and is usually associated with endometriosis of the uterosacral ligaments, deep pelvic implants, lesions of the rectovaginal septum, or a fixed retroverted uterus. The presence of noncyclic pelvic pain, backache, and rectal pressure is also commonly described. Endometriosis might present by acute abdominal pain in 3% of cases and one of the reasons is a leaking or ruptured endometrioma. Pain related to specific organ involvement such as bowel (e.g., dyschezia), lung (chest pain), or nerves (sciatica) is also described. Often endometriosis-related pain does not correlate with the severity of the lesion and the actual pathophysiology of the pain in milder stages is not well understood but may be related to prostaglandin production in the lesions.

### B. Infertility

Infertility is another symptom commonly attributed to endometriosis. Up to 30% of cases of infertility are associated with laparoscopically proven endometriosis but the disease has been detected in only a small percentage of fertile patients undergoing tubal ligation. The exact incidence of endometriosis is, however, unknown and so the percentage of cases associated with infertility is difficult to assess. Furthermore, no correlation has been demonstrated between the different stages of endometriosis and pregnancy rates or response to therapy in infertile patients. Another factor complicating the picture is that the exact mechanism causing infertility in milder forms of endometriosis is unknown; thus, doubts still exist as to whether endometriosis is a direct cause of infertility.

Factors implicated in causing endometriosis-associated infertility include anatomic distortion and fibrosis of the fallopian tubes in severe cases, anovulation, luteal-phase defects, luteinized unruptured follicle syndrome, autoimmune factors, spontaneous early abortions, altered peritoneal fluid environment by prostaglandins or inflammatory cells, and hyperprolactinemia.

## C. Spontaneous Abortion

The older studies indicating an increased rate of spontaneous abortion in patients with endometriosis could not be validated by more recent, controlled studies.

## D. Abnormal Uterine Bleeding

Many different patterns of abnormal bleeding in endometriosis have been described, including premenstrual spotting, hypermenorrhea, and oligomenorrhea. The incidence of anovulation and luteal-phase defects is, however, similar in endometriosis patients compared to women without the disease. Available data do not support an association between endometriosis and abnormal uterine bleeding.

## E. Other Symptoms

Other uncommon symptoms related to specific organ involvement are sometimes seen, such as recurrent pneumothorax or hemoptysis in lung involvement, rectal bleeding or hematuria in rectal or bladder involvement, ureteric obstruction, and skin, perineal, or vaginal masses.

# VIII. CLINICAL EXAMINATION

Physical findings in endometriosis are not specific and in many instances do not correlate with the disease severity confirmed at laparoscopy. In many cases, minimal endometriosis lesions are found despite significant pelvic pathology and severe symptoms.

Cul-de-sac tenderness is one of the most common findings. Nodularity along the uterosacral ligaments and in the cul-de-sac is also very suggestive. The lesions will be more prominent at the time of menstruation and tenderness will usually increase. Significant endometriomas will be appreciated as adnexal masses, but other lesions such as ovarian tumors may give a similar picture.

Severe adhesions obliterating the cul-de-sac can cause the uterus to remain in a retroverted position, and movement of the uterus during examination will elicit tenderness. Deep lesions in the rectovaginal septum are better appreciated by a combined rectovaginal examination. This is important because they might be missed during laparoscopy.

# IX. TREATMENT

The inadequate number of prospective, randomized, controlled studies together with our ignorance about the natural course of endometriosis makes assessment of treatment modalities a difficult task. Moreover, treatment often depends on the presentation of the disease in individual patients, their ages, and their reproductive desires. Unfortunately, no ideal long-term treatment is available at present and many of the current options are temporarily curative or associated with significant morbidity.

## A. Expectant Management/Prophylactic Treatment

Expectant management has the advantage of being cheaper and avoids the side effects of therapy. On the other hand, surgical ablation of the lesions might prevent or slow the progression of the disease in at least some cases. Until recently, there was no evidence that medical or surgical therapy is superior to expectant management in infertile patients with milder forms of endometriosis. In 1998, a Canadian prospective randomized study demonstrated that surgical ablation of lesions in minimal and mild cases of endometriosis enhanced fertility in this group of patients. Although this is only one study, it nonetheless points in favor of ablation of even milder lesions discovered during laparoscopy. In younger patients diagnosed with endometriosis and not yet seeking pregnancy, the long-term use of oral contraceptives in an attempt to suppress the disease or slow progression might be a reasonable option. Data demonstrating the long-term efficacy of such an approach, however, are not available.

## B. Medical Suppression

The medical options available for treatment of endometriosis include danazol, progestins, gonadotropin-releasing hormone (GnRH) agonists, and experimental modalities such as mifepristone and aromatase inhibitors. All medical modalities are of proven effectiveness in managing pelvic pain and in decreasing the size of the endometriotic lesions. For treating infertility, these medical therapies have no proven beneficial effect. In fact, they may be a cause of delay in pregnancy due to their suppressive effect on ovulation. The effect of medical suppression is not permanent and recurrence rates after discontinuation of therapy are high. The different medications used are not expected to have any effect on adhesions or larger endometriomas.

## C. Danazol

Danazol is an isoxazol derivative of 17$\alpha$-ethynyltestosterone and is well absorbed and active when given orally. It has a central effect by which it attenuates the midcycle gonadotropin surge and this is associated with anovulation and a reduction of estradiol levels. The drug also inhibits steroidogenic enzymes within the ovary. Danazol's binding to the androgen receptor may have a local suppressive effect on endometriosis lesions but is also the cause of some of its most disturbing side effects.

Danazol is usually used in divided doses ranging from 400 to 800 mg per day for 6 to 9 months. The drug is very effective, relieving pelvic pain associated with endometriosis in approximately 90% of cases. Reports have also indicated its success in reducing the size of endometriotic lesions. The effectiveness of therapy is directly related to induction of amenorrhea. Side effects of danazol are related to both its androgenic effect and the resulting hypoestrogenic environment.

Danazol's side effects include weight gain, mood changes, depression, oily skin, muscle cramps, headaches, edema, altered appetite, acne, hirsutism, fatigue, decreased breast size, hot flashes, voice changes, and irregular bleeding. The drug has an adverse effect on the lipoprotein profile, resulting in a decrease of high-density lipoproteins (HDLs) and an increase of low-density lipoproteins, and may be associated with increased liver enzymes. Pregnancy must be excluded because of the potential for virilization of a female fetus. The majority of patients taking the medication will experience side effects and at least 20% will discontinue the drug because of them. Recurrence rates are as high as 30% in 2 years after cessation of therapy. Danazol was once a very popular drug for the treatment of endometriosis but its significant side effects limit its current use in the advent of GnRH agonists.

### D. Gestrinone

Gestrinone is a 19-nortestosterone derivative with progesterone agonist and antagonist effects. Its use results in amenorrhea and endometrial atrophy. It has been studied extensively in Europe for the treatment of endometriosis. It has a long half-life and is given orally 1 to 3 times a week (2.5 to 7.5 mg). The potential side effects are similar to those of danazol but are much milder and better tolerated. There is no adverse effect on lipoproteins or liver function tests.

### E. Progestins

Progestins are believed to act by inducing decidualization and atrophy of the endometrial tissue within endometriotic implants. Progestins also interfere with follicular growth probably via a central mechanism on the pituitary–hypothalamic unit, but a local ovarian effect is also possible. This will result in decreased estrogen levels.

Progestins can be given orally, e.g., medroxyprogesterone acetate 20–30 mg orally per day or via the intramuscular (im) route as in case of Depo-medroxyprogesterone acetate at a dose of 150 mg im every 1 to 3 months. Norethindrone acetate, an orally active 19-nortestosterone progestin, was also successful in controlling painful endometriosis in small studies. The side effects of progestins include abnormal bleeding, mood changes, depression, headaches, weight gain, bloating, nausea, and a decrease in HDLs. Overall, progestins are better tolerated than danazol, and few patients discontinue their medications due to their side effects. Progestins do have a beneficial effect on pain and lead to regression of lesions. However, the effect on pregnancy rate is no different than expectant management. Progestins are an effective and cheap alternative for patients who do not tolerate danazol, GnRH agonists, or oral contraceptives.

### F. Oral Contraceptives

Oral contraceptive-induced amenorrhea has been used to treat endometriosis, and this regimen was termed "pseudo-pregnancy." Initially, high-dose oral contraceptive pills were preferred, but the more recent low-dose estrogen pills seem to have similar effects on endometriosis. Oral contraceptives can be used in a continuous fashion, resulting in amenorrhea, or in a cyclic fashion. The therapeutic effect is probably due to the progestin in the pill causing decidualization and atrophy of the endometriotic lesions. The common side effects of oral contraceptives may be more severe in larger doses or in continuous regimens.

Oral contraceptives are a low-cost option for suppressive therapy and seem to be effective in alleviating pelvic pain. The role of oral contraceptive therapy for decreasing recurrence is less clear, but it seems to be a reasonable option in cases where pregnancy is not desired at the time of therapy.

### G. Gonadotropin-Releasing Hormone Agonists

Continuous administration of GnRH agonists downregulates the pituitary–hypothalamic unit, leading to decreased gonadotropin levels. The resultant hypoestrogenic environment due to ovarian inactivity seems to cause regression of endometriotic implants. GnRH agonists are modified GnRH peptides with a longer half-life and greater potency than the naturally occurring molecule. GnRH agonists can be used as a nasal spray (Nafarelin at a dose of 200 to 400 mg twice daily) or as an injection (the depot form of Leuprolide acetate at a dose of 3.75 mg every 4 weeks). Also available are a long-acting injectable form of Leuprolide administered every 3 months and a subcutaneous implant (goserelin acetate).

GnRH agonists are used widely and have replaced danazol for the treatment of endometriosis. GnRH agonists do not have androgenic or progestogenic side effects and have no reported adverse effect on the lipid profile. The hypoestrogenic effect of GnRH agonists is, however, more profound and is the primary cause of the side effects from this agent. These include vaginal dryness, hot flashes, abnormal vaginal bleeding, insomnia, depression, libido changes, headache, fatigue, and skin changes. The side effect of greatest concern is the decrease of bone density with GnRH agonists. A decrease in trabecular bone density of up to 6% in 6 months has been reported. Although this effect is considered reversible after discontinuation of therapy, it limits the long-term use of this agent. Recent studies have also indicated the relative safety of a second 6-month course of GnRH agonist in cases with recurrence of symptoms. The long-term effect of multiple courses in these young women is, however, not known, and there is a potential for delayed adverse effects on bone integrity.

Add-back therapy combines GnRH agonists with other agents such as estrogen and progestins or anti-resorptive agents such as biphosphonates. The goal is to decrease vasomotor symptoms and detrimental effects on bone density in order to use GnRH agonists for prolonged periods of time. Medroxyprogesterone was not effective when used as an add-back to GnRH agonists. Although norethindrone decreased side effects without changing the therapeutic efficacy, it had an adverse effect on lipoproteins. Although the use of an estrogen/progestin combination add-back therapy would be excellent for vasomotor symptoms and can help preserve bone density, it may also decrease the efficiency of GnRH agonists in controlling endometriosis. Further investigations are needed to study the impact of this line of therapy. In addition, preliminary studies using biphosphonates and norethindrone showed a beneficial effect in preserving bone mass.

GnRH agonist treatment results in amelioration of pain in up to 90% of patients during therapy, comparable to the efficacy of danazol. Reduction of lesion size is also reported. Side effects are more tolerable to patients than those of danazol. Similar to danazol, there was no beneficial effect on pregnancy rates following discontinuation of GnRH agonist therapy. Also, significant recurrence rates were reported, as with other medical lines of management.

## H. Other Modalities

The anti-progesterone mefiprostone (RU486) was reported to have a beneficial effect on endometriosis in small studies. Because RU486 disrupts the endometrium along with the decidua in early pregnancy, it was hypothesized that RU486 might arrest or cause regression of endometriosis. In addition, there is preliminary evidence suggesting that RU486's antioxidant effect is also responsible for the suppression of endometriosis. Clinically, RU486 has provided symptomatic relief to patients with symptomatic endometriosis based on several small studies. Although this group of agents might prove to be of benefit, a major concern is the consequence of a long-term anti-progesterone effect. Although no cases of hyperplasia or atypia have been found in the clinical trials, all patients who received long-term treatment with RU486 exhibited abnormal endometrial pathology consistent with chronically unopposed estrogen. Thus, the long-term use of these agents will require careful follow-up.

Recently, the aromatase inhibitor amastrozole was used in combination with alendronate and calcium to successfully treat a patient with severe recurrent endometriosis after total hysterectomy and salpingo-oophorectomy resistant to large doses of progestins. Amastrozole caused a profound decrease of estrogen levels and consequently resulted in the disappearance of a recurrent fungating endometriotic lesion in the vaginal vault. The successful alleviation of severe chronic pain in this patient was impressive.

Bone loss, however, was significant (from 2.5 to 6.5% in 9 months) despite addition of a bisphosphonate. The effect of aromatase enzyme inhibitors merits further study in a larger number of patients. It might have a role in the treatment of resistant cases of endometriosis unresponsive to other medical modalities. It might also increase the pain-free interval after discontinuation of therapy.

## I. Surgical Treatment

Surgical therapy is the most common treatment modality used in endometriosis. Most cases affected with endometriosis are subjected to diagnostic laparoscopy, as this is still the only means of obtaining a definite diagnosis. Since it is now possible to accomplish surgical resection and/or ablation in nearly all cases via laparoscopy, this route is preferred to laparotomy except in very special circumstances. Surgical therapy for endometriosis is effective in relieving symptoms of pain in debilitating lesions. Actually, large lesions such as endometriomas and adhesions can be treated only surgically, as medical therapy is not effective.

In contrast to medical treatment, surgical treatment of endometriosis did prove of value in improving pregnancy rates. Surgical excision of endometriosis was considered beneficial in more severe cases of endometriosis, but the effect of surgery on milder stages of the disease was controversial. Recently, a prospective randomized study comparing surgical ablation of minimal and mild endometriosis to expectant management demonstrated a higher pregnancy rate in the surgically treated group. This study provides preliminary evidence that even milder lesions should probably be ablated when discovered at diagnostic laparoscopy.

Recurrence after surgery will depend on the severity of the disease and the completeness of the procedure. Recurrence rates are approximately 20% over 5 years following complete surgical excision or ablation.

The role of medical treatment modalities used with surgery is controversial. Preoperative medical suppression may be used in an attempt to decrease lesion size and vascularity before surgery. The disadvantage of this approach is potential changes in the appearance of lesions, making diagnosis and treatment more difficult as a result. A delay in diagnosis will consequently also delay pregnancy.

Postoperative medical therapy might be used for patients with pain after incomplete excision especially in severe disease. Postoperative medical therapy should never be used in patients seeking pregnancy as this will delay pregnancy throughout the duration of therapy. In conclusion, a beneficial effect of perioperative medical therapy is not supported by available data and thus its use should be restricted to special circumstances. Cases with intractable pain awaiting surgery might benefit from such an approach.

## J. Principles of Surgery in Endometriosis

Surgery for endometriosis might range from a simple ablation of small superficial lesions to complicated cases with massive adhesions involving nearby organs such as the bowel and the urinary tract.

- The goal during surgery is complete extrication of the disease whenever possible. Adequate definition of the extent of the lesions is important.
- Visualization should be adequate and careful exploration of the pelvis is a must.
- In many cases, anatomy is distorted and identification of vital organs such as rectum, ureters, and bladder is mandatory to prevent injury.
- Endometriotic lesions are in many instances deeper than they appear, and this is important for complete excision or ablation of the lesions.
- The surgeon must be familiar with the use of electrocautery and laser as well as sharp dissection using laparoscopic scissors.
- It is sometimes important to recognize the need for help from a gastrointestinal surgeon or urologist in complicated cases.
- Adequate preoperative bowel preparation might be necessary especially in more advanced endometriosis.

In patients not desiring future fertility, removal of the uterus and the ovaries together with excision of the lesions might be the reasonable and definitive treatment. Recurrence rates after hysterectomy with or without salpingo-oophorectomy are low and range from 2 to 6%.

## K. The Role of Assisted Reproduction

Controlled ovarian hyperstimulation (COH) with gonadotropins or clomiphene citrate followed by intrauterine insemination (IUI) enhances monthly fecundity rates in endometriosis patients with patent fallopian tubes. The effect on long-term pregnancy rates is, however, controversial, with no demonstrated improvement over expectant management in most studies. Ovarian stimulation with intrauterine insemination seems to have better results than stimulation with timed intercourse. From the data available, the routine use of COH/IUI is probably not recommended but may be of value in older couples or if pregnancy is delayed after surgical excision.

Most patients with endometriosis have a least one patent fallopian tube. In these patients, gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer, or tubal embryo transfer are possible. Several groups reported excellent success rates with GIFT in patients with endometriosis seeking pregnancy. Nowadays, with the decreasing frequency of GIFT and increased information gained by *in vitro* fertilization (IVF), GIFT might have a role in only a few centers experienced with the procedure.

*In vitro* fertilization and embryo transfer (IVF/ET) is probably the preferred alternative when advanced assisted reproductive technology is needed. IVF/ET should be considered after 1 year of follow-up or after failure of conventional therapy. The procedure should be used earlier in cases with extensive tubal damage, in older patients, and in cases with associated factors leading to infertility.

The effect of endometriosis on pregnancy rates with IVF/ET is another debated issue, especially in more severe forms of the disease. Endometriosis was implicated in decreased ovarian response, interference with monitoring, decreased fertilization/cleavage rates, decreased pregnancy rates, and increased early abortions. Recent studies, however, suggest no adverse effect of endometriosis on pregnancy rates with IVF/ET. Down-regulation of the ovary with a GnRH agonist for 6 months before IVF/ET has also been suggested by some to improve outcome, but currently there are no conclusive data to recommend this.

In conclusion, COH/IUI seems to accelerate the occurrence of pregnancy but does not change overall fertility. With improved techniques in IVF/ET, the pregnancy rates in endometriosis, even in severe cases, seem to be similar to those for tubal factor infertility. IVF/ET should be utilized after an adequate trial of less invasive procedures.

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## Glossary

**adenomyosis** The infiltration or invagination of the myometrium with endometrial glands.

**endometriosis** The presence of endometrial glands and stroma outside their normal location within the endometrial cavity.

**endometrium** The inner lining of the uterine cavity.

**gonadotropin- releasing hormone agonists** Synthetic hormones that suppress the pituitary gonadotropin secretion leading to secondary ovarian suppression.

**infertility** The failure to achieve pregnancy within a year of unprotected intercourse.

**menstruation** The monthly shedding of the nonpregnant endometrium.

**retrograde menstruation** The flow of menstrual effluent via the fallopian tubes into the peritoneal cavity.

## See Also the Following Articles

**Decidualization • Endometrial Remodeling • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Gonadotropin-Releasing Hormone Pharmacology: Agonists and Antagonists • Placental Immunology • Progesterone Action in the Female Reproductive Tract**

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**selective estrogen receptor modulators** Natural or synthetic ligands that exhibit either estrogen agonist or antagonist activity among different cells and tissues. Most likely, this is due to the differential expression of cell- and tissue-specific factors, such as co-activator and co-repressor proteins.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Estrogen and Progesterone Receptors in Breast Cancer • Phytoestrogens • SERMs (Selective Estrogen Receptor Modulators) • Sexual Differentiation, Molecular and Hormone Dependent Events in • Sexual Differentiation of the Brain**

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# Environmental Disrupters of Thyroid Hormone Action

Françoise Brucker-Davis
*Hôpital l'Archet 1, Nice, France*

I. INTRODUCTION
II. NATURAL ENVIRONMENTAL THYROID DISRUPTERS
III. SYNTHETIC ENVIRONMENTAL THYROID DISRUPTERS
IV. MECHANISMS OF THYROID DISRUPTION
V. FACTORS PREDISPOSING TO THYROID DISRUPTION
VI. CLINICAL IMPACT OF THYROID DISRUPTERS

**The thyroid gland is easily disturbed by numerous external factors, both natural and synthetic. Endemic environmental goitrogenesis, the enlargement of the thyroid gland, has long been associated with diets deficient in iodine or with diets rich in goitrogenic substances (phytogoitrogens). In addition to goiter development, thyroid hormone imbalance and/or exposure to thyroid-toxic chemicals during pregnancy can have devastating effects on fetal brain development, as illustrated by the endemic cretinism observed in areas where native diets are iodine deficient.**

## I. INTRODUCTION

The goiter, or enlarged thyroid gland, has long been recognized to be the result of a dietary deficiency of iodine-containing foods. The question of environmental thyroid disruption, however, has been recently assessed in the more global context of the impact of environmental synthetic chemicals on endocrine system function. Many synthetic chemicals have been found to have deleterious effects on the thyroid, both *in vitro* and *in vivo* in fauna, laboratory animals, and humans. The clinical significance of exposure to environmental chemicals and contaminants with respect to normal thyroid development and function is currently under investigation. It is important to determine whether, beyond their goitrogenic effects, these chemicals may also have an effect on thyroid tumorigenesis and cognitive functions.

## II. NATURAL ENVIRONMENTAL THYROID DISRUPTERS

The primary natural thyroid disrupters, excluding dietary iodine deficiency, are goitrogens found in food or water supplies as a result of bacterial contamination or mineral compound decomposition (Table 1). Goitrogenic substances are also found in many vegetables: thiocyanates and isothiocyanates in Cruciferae; goitrin in turnips; cyanogenic glucosides in cassava or sweet potatoes; disulfides in onion and garlic; and flavonoids in millet, sorghum, and beans. In addition, degradation of humic substances, i.e., soil decomposition of plant and animal tissues, leads to the production of resorcinol, a phenol derivative with potent antithyroid effects. Distinguishing between natural mineral compounds (such as coals or shales) and synthetic contamination is sometimes fuzzy, because mineral compound decomposition and industrial manufacturing processes may produce similar chemical products.

## III. SYNTHETIC ENVIRONMENTAL THYROID DISRUPTERS

The twentieth century witnessed vast developments in the chemical industry, particularly following the end of the Second World War. Thousands of new compounds are now produced annually for both agricultural and industrial purposes. Few if any of the routinely manufactured chemicals have been tested for their endocrine effects, and in cases in which tests have been made, the test doses are not relevant to endocrine systems or to levels of probable environmental exposure.

Synthetic chemicals are used to make every imaginable type of product: computers, automobiles, toys, clothing, food containers, cosmetics, and perfumes. The chemicals, as the manufactured products are discarded and break down, are eventually released into the environment and are found in water, soils, and foodstuffs, including vegetables, fruits, diary products, meats, and fish. Evidence of the ubiquity of contaminating chemicals is supported by their presence in urine, adipose tissue, and even amniotic fluid. They are found in most cord blood samples, proof of human *in utero* transplacental contamination. Additionally, early postnatal exposure can occur through maternal breast milk, potentially impeding the well-known benefits of breast-feeding.

Some compounds, such as pesticides, are released intentionally into the environment and are designed to be toxic. Others, such as plastics and industrial compounds, until recently were considered benign. They are released unintentionally as a result of pollution. A number of synthetic chemicals are persistent, in some cases by design, and are able to travel long distances under the influence of winds and water currents. They also bioconcentrate and biomagnify in live organisms as they move up the food chain.

**TABLE 1 Chemicals with Thyroid-Disrupting Properties**

| Family | Name[a] | Natural or synthetic[b] |
|---|---|---|
| Sulfurated organics | Thiocyanate, isothiocyanate, goitrin, disulfides | N |
| Flavonoids | Glycosides, aglycones | N |
| Phenol derivatives | Resorcinol, DNP, pyrogallol | N, S |
| Pyridines and hydroxypyridines | — | N, S |
| Phthalate esters and metabolites | — | N, S |
| Polyhalogenated hydrocarbons | PCB, PBB, dioxin | S |
| Polycyclic aromatic hydrocarbons | Benzopyrene, methylcolanthrene, dimethylbenzanthracene | N, S |
| Pesticides | | |
| Chlorinated | DDT and others | S |
| Others | Amides, benzonitriles, carbamates, organophosphates, pyrethroid, pyridinoxy, thiocarbamates, thiourea, triazine, triazole | S |
| Heavy metals, inorganics | Hg, Pb, I, Cd, perchlorate | N |

[a] Abbreviations: DNP, dinitrophenol; DDT, dichlorodiphenyldichloroethane; PCB, polychlorinated biphenyl; PBB, polybrominated biphenyl; Hg, mercury; Pb, lead; I, iodine; Li, lithium; Cd, cadmium.

[b] N, Naturally occurring even though it may be used for industrial or other anthropological purpose; S, synthetic.

Importantly, animal and human species have been exposed to these chemicals for less than a century. This extremely short period of time with respect to the evolution of species has not allowed for adaptation. Indeed, differences in how humans and animals handle man-made compounds and natural compounds involve detoxification and metabolism pathways and binding affinities for protein carriers.

Many synthetic chemicals have thyroid system effects (see Table 1). Interestingly, some chemicals, e.g., dichlorodiphenyldichloroethane (DDT) and polychlorinated biphenyls (PCBs) or their metabolites, also have estrogenic and antiandrogenic activities. Field studies in fauna have supported evidence of environmental thyroid disruption in salmon and birds from the Great Lakes and in the Florida panther. However, no specific correlation has been established between these observations and a given chemical, because environmental exposure has been to a mixture of chemicals. On the other hand, experimental studies in laboratory rodents and birds, for example, have shown specific thyroid system effects of many compounds, either alone or in mixtures.

## IV. MECHANISMS OF THYROID DISRUPTION

Chemicals may disrupt the thyroid economy at virtually all stages, as shown in Table 2. This complicates the screening of chemicals for their impact on the thyroid gland. Disruption is primarily at the level of thyroid gland morphology and thyroid hormone metabolism. An effect at one stage of thyroid function or development also induces compensatory mechanisms at other stages. For example, increased glucuronidation of thyroxine (T4) by PCBs results in increased thyroid gland production of thyroid hormones to keep up with the elimination. The effects of some chemicals may be profound: the antithyroperoxidase (antiTPO) activity of resorcinol is 26 times the activity of propylthiouracil, one of the main medications for treating thyroid overactivity. Many halogenated compounds compete with natural hormones for binding to protein carriers (transthyretin, and to a lesser degree thyroxine-binding globulin), although the clinical consequences are unclear. Preliminary data suggest a possible effect of PCBs on thyroid hormone regulated genes.

## V. FACTORS PREDISPOSING TO THYROID DISRUPTION

The nature of thyroid system disruption by environmental chemicals depends on the chemical and on the exposed individual. Among the factors linked to the chemical are its persistence and environmental concentrations, as well as its ability to cross the placenta and the blood–brain barrier and whether it is eliminated in breast milk. Chemical structure occasionally indicates a possible thyroid effect. For example, DDT, PCBs, and thyroid hormones have similar structures; compounds with structures unrelated to thyroid hormone structure are less predictable (Fig. 1). In addition, the compounding effects of iodine deficiency and/or the presence of co-contaminants are important.

Inherent differences in individuals and species exposed to chemicals dictate thyroid effects. For example, compared to humans, rats are more susceptible to goiter; there are also differences based

TABLE 2 Mechanisms of Thyroid Disruption

| Level of disruption | Examples of chemicals | Frequency |
|---|---|---|
| Thyroid hormone synthesis | | |
| Iodine uptake | Iodine, thiocyanate, sulfurated agents, aldrin, perchlorate | +++ |
| AntiTPO[a] action | Thiourea, triazole, phenols, phtalate | ++ |
| Inhibition of thyroid hormone secretion | Iodine, PCBs? | + |
| Transport of thyroid hormone | Polyhalogenated, chlorinated pesticides; phenol, phthalate | +++ |
| Metabolism | | |
| Deiodinase, glucuronyltransferase, sulfatase | Pesticides, heavy metals, polyhalogenated pesticides, polycyclic aromatics, polyhalogenated PCBs, pentachlorophenols | +++ |
| Central effect | Lead, dinitrophenol, PCBs?, pentachlorophenol? | +/− |
| Autoimmunity | PBBs?, methylcholanthrene, furan? | +? |
| Tumorigenesis[b] | Iodine isotopes, acetochlor | +/− |
| Genomic effect | PCBs? | +/− ? |

[a] AntiTPO, Antithyroperoxidase.
[b] Independently of effect through hormonal effects.

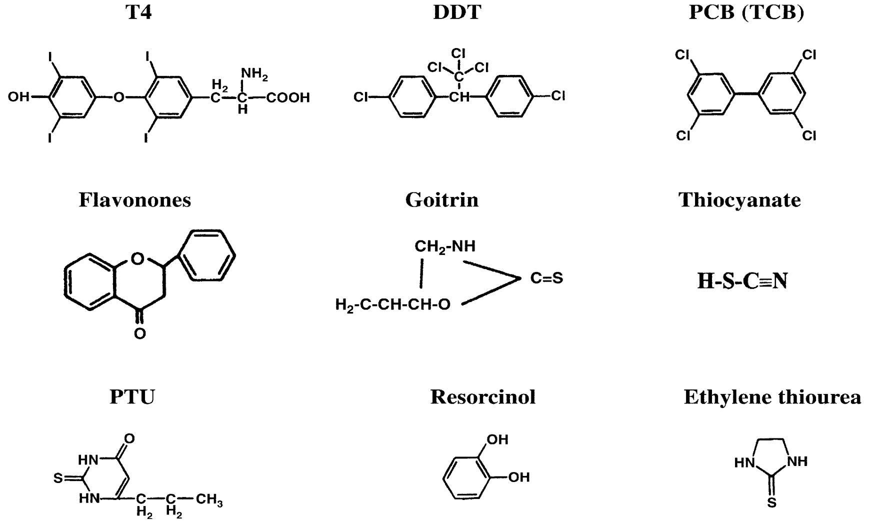

FIGURE 1 Comparison of the chemical structure of thyroxine (T4) with the structures of selected pesticides and industrial and natural chemicals with documented thyroid activity. DDT, Dichlorodiphenyldichloroethane; TCB, tetrachlorobiphenyl; PTU, propylthiouracil.

on sex (women are more susceptible than men) genetic susceptibility and endogenous thyroid status (borderline state of dysthyroidism). Diets rich in goitrogenic substances play an obvious role. Most importantly, the effect on fetal thyroid economy will depend on the timing of exposure and on maternal thyroid status.

## VI. CLINICAL IMPACT OF THYROID DISRUPTERS

Although the deleterious effects of natural thyroid disrupters are well documented, much less is known regarding the effects of synthetic chemicals. The potential effects are different in individual adults ("activational phenotype") and fetuses ("developmental phenotype"). However, it is fair to say that there is currently no consensus on the real impact of synthetic thyroid disrupters in humans.

In adults, the potential risks of chemical exposure are goiter, thyroid cancer, and autoimmune thyroid disease. There are some scanty reports of thyroid abnormalities observed in the context of occupational exposure to pesticides or industrial chemicals. Those suggest at best a minor effect on genetically predisposed individuals, for both the occurrence of goiter and/or autoimmune thyroid disease. The main interest of these studies is that they involve mainly men, who are supposedly less likely than women to develop thyroid diseases.

In fetuses, the potential phenotype involves cognitive functions and behavior. Several studies have found neurological impairments linked to *in utero* exposure to synthetic chemicals such as PCBs or dioxins. However, even though those compounds have well-documented experimental thyroid effects, it is not clear whether the observed neurocognitive effects in the children are linked to those thyroid effects or to more direct neurotoxic effects.

Thus, the impact of chemical thyroid disruption following environmental exposure is largely unknown in human adults and fetuses, mainly because of methodological difficulties. There are no comprehensive data on human exposure and the number of potential chemical culprits is large. Consequently, analysis is limited to the resultant effects of a mixture of chemicals. In addition, thyroid disruption may be only one of the toxicity pathways of chemicals, because many compounds express different properties (direct neurotoxicity, estrogenicity, etc.).

## Glossary

**endemic cretinism** Mental retardation, associated with various neurological phenotypes, due to the deleterious effect of iodine deficiency on fetal brain development. This pathology occurs in areas where normal diets are iodine deficient, in the absence of an efficient program of iodination.

**endocrine disrupter** Exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones that are responsible for maintenance of homeostasis and regulation of developmental processes. Disrupters can directly affect an exposed individual and can affect fetuses through *in utero* exposure.

**goiter** Thyroid gland enlargement, the most common thyroid abnormality, is present in about 200 million people worldwide.

**polyhalogenated hydrocarbons** Synthetic chemicals, chlorinated or brominated, such as polychlorinated biphenyls, dioxin, or polybrominated biphenyls.

## See Also the Following Articles

**Environmental Disruptors of Sex Hormone Action • Iodine • Thyroid and Reproduction • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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# Ephrins and Their Receptors

MARTIN LACKMANN* AND ANDREW W. BOYD†

*Ludwig Institute for Cancer Research, Australia •
†Queensland Institute for Medical Research, Australia*

I. INTRODUCTION
II. MOLECULAR PROPERTIES OF EPHRINS AND Eph RECEPTORS
III. SIGNALING FUNCTIONS OF EPHRINS AND Eph RECEPTORS
IV. BIOLOGICAL ACTIVITIES OF EPHRINS AND Eph RECEPTORS
V. SUMMARY

**Ephrins are cell-surface-associated ligands for a family of ephrin receptors (Eph). Ephrins and Ephs act as guidance cues to facilitate and direct the movement of cells and cell layers during various developmental processes.**

## I. INTRODUCTION

Ephrins are cell-surface-associated ligands for ephrin receptor (Eph) tyrosine kinases (RTKs), the largest subgroup of the RTK superfamily. In contrast to other cytokine receptors, Eph RTKs (Ephs) have been discovered in the past decade as orphan receptors mostly by homology (complementary DNA) cloning approaches. The founding family member, EphA1, was isolated in a low-stringency screen with a v-*fps* oncogene cDNA probe from an erythropoietin-producing hepatocellular (EPH) carcinoma cell line; EPH receptor-interacting proteins (i.e., ephrins) were isolated in a simultaneous effort by several laboratories to unravel Eph RTK function. Although several

# Environmental Disruptors of Sex Hormone Action

Caroline M. Markey, Ana M. Soto, and Carlos Sonnenschein
*Tufts University School of Medicine*

I. INTRODUCTION
II. HISTORICAL PERSPECTIVE
III. ENDOCRINE DISRUPTING CHEMICALS
IV. MODE OF ACTION
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**The field of "environmental endocrine disruption" has emerged from repeated observations, made by biologists and physicians, that a link exists between the exposure of wildlife and humans to synthetic chemicals and the development of reproductive, neurological, and endocrine abnormalities.**

## I. INTRODUCTION

Since the beginning of World War II to the present date, industrialized nations have been actively engaged in the development and manufacture of a plethora of synthetic chemicals, a subset of which are hormonally active. These chemicals have been introduced into the environment deliberately for use in agriculture and medicine, inadvertently as by-products of industrial use, or irresponsibly as waste released into rivers, lakes, and the atmosphere. It is noteworthy that, within this period, there has been a rising incidence of hormone-related conditions such as early onset of puberty and diseases such as breast cancer, ovarian tumors, cervico-vaginal adenocarcinoma, endometriosis and uterine leiomyomas in women, and decreased sperm production, cryptorchidism, hypospadias, and increased testicular cancer in men. This article aims to provide a historical account of the relatively new field of "environmental endocrine disruptors" and its impact on wildlife and humans at different levels of biological complexity.

## II. HISTORICAL PERSPECTIVE

The Second World War stimulated the advent of technological breakthroughs in the nuclear, chemical and agricultural industries, the magnitude of which precipitated the introduction of the terms the "Second Industrial Revolution" and the "Green Revolution" into our vernacular. The excitement of man's ability to manipulate nature to his perceived gain would not be shadowed by the specter of negative consequences until many years later.

The first indication that some of these new chemicals demonstrated biological activity other than that of their designed intent was observed with the pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT), the results of which were published in 1950. However, it was not until the repeated observations of acute death, spasms, altered behavior, eggshell thinning, impeded hatching, and a dramatic decline in the population of several bird species, most notably the American bald eagle and peregrine falcon, that the harmful effects of DDT were acknowledged in the United States of America. Rachel Carson, through her 1962 book *Silent Spring*, warned that the adverse effects observed in wildlife were indicators of deleterious health effects in humans. Motivated by fears that some of these chemicals may induce cancer in humans, the U.S. government banned the agricultural use of DDT in 1973 and the industrial use of polychlorinated biphenyls (PCBs) in 1977. DDT is still being used in some Latin American countries, Asia, and Africa under the pretext that it is the most effective and inexpensive way to run sanitation campaigns against malaria. Although the World Health Organization recommends the use of DDT for malarial outbreaks only, public health experts do not uniformly endorse its use since it is acknowledged that this chemical can target only adult insects, not larvae, and its repeated use has resulted in resistance.

The policy of banning DDT had the effect of reestablishing the bald eagle population, and it allowed the more subtle effects of DDT exposure to be revealed. The metabolites of this chemical still remained in the environment due to its half-life of 58 years, and its ability to induce a diversity of developmental malformations in hatchling birds became apparent particularly in the Great Lakes region of the United States. The growing awareness that lower levels of DDT, and other agricultural and industrial chemicals of a hormonally active nature, could have profound effects on the development and reproduction of wildlife species and humans stimulated Dr. Theo Colborn, currently at the World Wildlife Fund, to convene the 1991 Wingspread Conference in Wisconsin. Its participants concluded that "a large number of manmade chemicals that have been released into the environment…have the potential to disrupt the endocrine system of animals, including humans." The participants also observed that some of the developmental defects documented in the genital tract of wildlife species had already been seen in the daughters and sons of women who had been exposed to the synthetic estrogen diethylstilbestrol (DES) during pregnancy. This chemical, which caused functional and anatomical anomalies of the genital tract and induced clear cell adenocarcinoma of the vagina in exposed daughters, had been administered therapeutically to prevent spontaneous abortion between the years 1948 and 1971 in the United States, Europe, and Australia. The DES phenomenon provided a template for the potential effects that other hormonally active agents could have on human health.

On the basis of these and additional findings, amendments to the 1996 Safe Drinking Water Act and the 1996 Food Quality Protection Act required the U.S. Environmental Protection Agency (EPA) to develop a program aimed at screening and testing chemicals used in large volumes that were suspected of contaminating water and food in order to assess their potential activity as endocrine disruptors. At that time, the EPA defined an endocrine disruptor as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior."

## III. ENDOCRINE DISRUPTING CHEMICALS

### A. Synthetic and Natural Chemicals

Estimates suggest that approximately 75,000 synthetic chemicals are currently manufactured in the United States. The first of the synthetic chemicals that alerted biologists to their hormonal effects in wildlife were the organochlorines DDT, PCBs, and kepone, all of which show estrogenic activity. This list of estrogenic chemicals has grown to include the contraceptive pill ethinylestradiol, the pesticides endosulfan, dieldrin, toxaphene, and lindane, antioxidants such as *p*-hydroxyanisol and various alkylphenols, disinfectants such as *o*-phenyl-phenol, the ultraviolet screening agent 3-(4-methylbenzylidene) camphor, and some of the various PCB congeners. Similarly, the alkylphenols, including nonylphenol and octylphenol, which are used in industrial processes, industrial detergents, and personal hygiene products, have been added to this list, as have bisphenol A (BPA), which is used in epoxy resins (dental materials) and polycarbonate plastics (food and beverage containers), and phthalates, which are used as plasticizers. Natural plant compounds, such as the phytoestrogens coumestrol, genistein, and zearalenone, also show significant estrogenic activity. These products are ingested commonly in soy-based infant formula and other food products, encouraging some researchers to voice concern about the indiscriminate consumption of these estrogens during early childhood.

In addition to these xenoestrogens, it has become apparent that certain compounds released into the environment induce effects that can be characterized as anti-estrogenic or anti-androgenic. Procymidone and metabolites of the fungicide vinclozolin act as androgen antagonists by competitively binding mammalian androgen receptor (AR), which inhibits AR binding to DNA and thus androgen-dependent gene expression. In some instances, compounds such as the DDT metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) and methoxychlor metabolites can induce a combination of hormonal effects that are both estrogenic and anti-androgenic. Although classically considered estrogenic, DDT, BPA, and butyl benzyl phthalate have been shown to possess some anti-androgenic qualities, based on the findings that they induce changes in reproductive endpoints that are believed to be androgen-dependent. For example, *in utero* exposure of mice to low doses of BPA decreases anogenital distance in males.

Some endocrine disrupting chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated aromatic hydrocarbon that binds the aryl hydrocarbon receptor (AhR), affect androgen, estrogen, thyroid, and glucocorticoid functions. A known human carcinogen, TCDD is a trace contaminant of industrial processes and is generated during the production of chlorinated products such as herbicides and wood preservatives, the incineration of trash containing papers and plastics, and the burning of fossil fuels. Polycyclic aromatic hydrocarbons, which are found in diesel exhaust, cigarette smoke, and charbroiled foods, also bind the AhR. In addition to the estrogenic PCB congeners mentioned above, PCB congeners with zero or one ortho chlorine, two para chlorines, and at least two meta chlorines adopt a planar conformation similar to that of TCDD and bind the AhR. This complexity of effects highlights the growing belief that many of these endocrine disrupting chemicals alter hormone action through multiple mechanisms. A further confounding factor in this categorization of chemicals is that some can have a different effect depending upon the tissue in which exposure occurs. For example, tamoxifen, which shows anti-estrogenic behavior in breast tissue, and as such is used for breast cancer therapy, has estrogenic behavior in the uterus, which can cause the growth of endometrial lesions in these patients.

Hormonally active agents such as estrogens (ethynylestradiol and Zeranol), progestagens (medroxyprogesterone acetate and pregnenedione), and androgen agonists (Trenbolone and methyltestosterone) are used in food-producing animals, such as beef cattle, within the United States for improved feed efficiency and weight gain. In contrast, the European Union has banned the importation of hormone-treated beef into its territory based on the tenets of the precautionary principle.

### B. Metabolism, Pharmacokinetics, and Pharmacodynamics

The categorization of the hormonal activity of chemicals has been based largely upon a battery of *in vitro* and *in vivo* assays. Regarding estrogenic activity, the most notable of the bioassays are the (1) estrogen receptor (ER) competitive binding assay, (2) yeast-based reporter gene assay, (3) E-SCREEN assay, and (4) mouse uterotropic assay. These assays measure the ability of a suspected hormonally active chemical to (1) competitively bind the mouse uterine ER, (2) transcriptionally and translationally activate a reporter gene construct via an estrogen-response element, (3) induce the proliferation of estrogen-responsive human MCF-7 cells, and (4) induce increased uterine wet weight in the prepubertal or adult ovariectomized mouse, respectively. Although these methods have certainly provided insight into the hormonal nature of both synthetic and natural chemicals, they are not equivalent. The ER binding, reporter gene, and E-SCREEN assays are consistent in detecting estrogenic activity, albeit with different sensitivities; the uterotropic assay, although quite insensitive, is able to detect pro-estrogens, that is, chemicals that do not bind to the ER until they undergo metabolism. For example, methoxychlor is able to induce a positive uterotropic effect (increased uterine weight) but shows neither positive competitive binding of ER nor transcriptional activation of the ERE-reporter gene construct. This is due to the fact that "pro-estrogens" require metabolic activation in the liver, inducible only in an *in vivo* model, to attain their hormonal character. Thus, *in vitro* assays, though valuable in the characterization of receptor-binding chemicals, do not take into account the metabolism, pharmacokinetics, and pharmacodynamics of compounds originally suspected of being estrogenic as do *in vivo* models. These latter assays provide the most pertinent indicators of possible endocrine effects in wildlife species and humans. Of equal importance is the binding affinity of hormonally active chemicals to various serum proteins, including albumin, sex hormone-binding globulin, corticosteroid-binding globulin, and α-fetoprotein, in terms of assessing the chemicals' potency. For example, although BPA is considered a weak estrogen, it binds α-fetoprotein with negligible affinity relative to estradiol, thereby making it more available systemically to bind ER. This protein acts as an estrogen sink in the rodent fetus and neonate, protecting them against high levels of maternal estrogens, which unbound have a detrimental impact on development.

Yet, even *in vivo* models demonstrate that there are species- and strain-specific differences in terms of the effects that endocrine disrupting chemicals have on tissues and organs. For example, administration of approximately 0.3 mg/kg BPA per day to Fisher 344 rats for 3 days will induce a 2.5-fold increase in the height of uterine epithelial cells, an increase in uterine wet weight, and proliferation and cornification of the vaginal epithelium. The exact same treatment has no effect on these parameters in Sprague–Dawley rats.

## IV. MODE OF ACTION

Endocrine disrupting chemicals are believed to exert their effect by (1) mimicking normal, endogenous hormones such as estrogens and androgens, (2) antagonizing endogenous hormones, (3) altering the pattern of synthesis and metabolism of endogenous hormones, and (4) modifying hormone receptor levels. Natural, endogenous estrogens are involved in the development and maintenance of the female reproductive tract and secondary sexual characteristics and regulation of the menstrual cycle, pregnancy, and lactation. At the cellular level, these endogenous hormones promote cell proliferation and the synthesis and secretion of cell-specific proteins in reproductive tissues such as the ovary, oviduct, uterus, vagina, hypothalamus, pituitary, and mammary gland. Similarly, androgens are involved in the development and maintenance of the male reproductive tract and secondary sexual characteristics through comparable cellular mechanisms, which function in the testis, epididymis, vas deferens, prostate, seminal vesicle, hypothalamus, and pituitary. These effects are mediated, for the most part, by ER-α and ER-β in the female, and AR in the male. However, various tissues of the male reproductive tract also express ER just as various tissues of the female reproductive tract express AR. In both sexes, nonreproductive organs, including the thyroid, cardiovascular system, and bone, also express ER.

On the basis of even this simplistic description of the role of estrogens and androgens in development and reproduction, it can be appreciated that exposure to chemicals that possess the ability to mimic, antagonize, or modulate endogenous hormones has a potentially striking impact. Although the environmental chemicals are usually less potent than endogenous hormones, such as estradiol, it is now clear that they act additively with them. This explains how low, seemingly insignificant levels of xenoestrogens, such as the "weak" BPA, may have an impact when added to the already significant levels of endogenous steroidal hormones. Further, several endocrine disruptors have unequivocally been shown to induce nonmonotonic dose-response curves of biological effects; that is, lower doses induce a more profound effect than higher doses ("low-dose effect"). This phenomenon, which has been known for a long time regarding sex steroid action, was first described for endocrine disrupting agents in the mouse prostate, using organ weight as the endpoint, in response to fetal exposure to increasing doses of DES, and later by the "weak" xenoestrogen BPA. Endogenous hormones act on hormone-sensitive endpoints in this manner, as evidenced by the observation that prostate cells undergo increased cell proliferation in response to low doses of androgen but decreased cell proliferation in response to high doses of androgens, a phenomenon that is due to the operation of different and discrete pathways at low and high doses. In other instances, low-dose effects have been observed to occur due to down-regulation of the receptor by the ligand.

The deleterious effects of endocrine disrupting chemicals also depend on the period during which an organism is exposed to the chemicals. The developing organism is critically sensitive to both endogenous and exogenous hormones. The "critical window" of exposure differs depending upon the time at which specific developmental events occur in particular tissues or organs. For example, clear cell adenocarcinoma of the vagina was observed in daughters of pregnant women exposed *in utero* to DES, but only if exposure occurred before the 13th week of gestation. It is clear that the developing fetus and neonate are profoundly sensitive to hormonally active chemicals, as demonstrated also by the observation that mammary gland dysgenesis occurs in the adult following fetal exposure to concentrations of BPA that are 4000-fold lower than those required to induce a uterotropic effect in the prepubertal mouse. The finding in 1998 that the DDT metabolite DDE and other chemicals such as PCBs and phytoestrogens are the most frequently recovered contaminant in amniotic fluid of women ages 35 years and over in the United States attests to the need for concern. This occurred despite a restriction in the use of the parental chemical DDT in 1973.

## A. Receptor Mediation

The ER knockout (ERKO) and AR knockout mouse models have confirmed the mediating role played by estrogen and androgen receptors on the effects of sex hormones. By analogy, the sex steroid receptors were also thought to be the mediators of effects of endocrine disrupting chemicals on sex hormone-sensitive tissues. Many *in vitro* studies revealed that suspected xenoestrogens bind to ER-α, ER-β, and AR. It was then concluded that certain endocrine disrupting chemicals, such as DES and ethinyl estradiol, which showed high binding affinity for ERs, were potent estrogens, whereas BPA and alkylphenols, which showed low binding affinity, were called "weak" estrogens. The latter chemicals were, however, full agonists, able to elicit a response of magnitude similar to that elicited by the former chemicals. Further, these studies revealed that some endocrine disruptors, such as genistein, had an approximately 50-fold higher binding affinity for ER-β than for ER-α; this provided insight into how specific organs may be affected by various chemicals based on their distribution patterns of sex steroid hormone receptors. Although this information is factual, it overemphasized the importance of models in which xenoestrogen activity is measured in the absence of endogenous estrogens. For example, BPA was considered to be a weak estrogen on the basis of the receptor-binding assay, yet subsequent studies have revealed that fetal exposure to low doses of this chemical had significant effects on the development of the male and female reproductive tracts and mammary glands.

Certain endocrine disrupting chemicals act as selective ER modulators (SERMs); that is, their agonist and/or antagonist activity may differ among cells and tissues. As described earlier in this article, tamoxifen has this capacity, and other chemicals are now being added to the list of SERMs. This is prompting a reevaluation of the pharmacological classification of ER ligands. Several studies including transgenic mouse models, in which a reporter of ER-α and ER-β activity is expressed, have confirmed that ER activity is found not only in the brain and the reproductive system but also in the liver, the kidney, and the thyroid and adrenal glands. These findings suggest that these organs are additional potential sites for endocrine disruptor activity. Further, the study revealed that the pituitary gland is 25-fold more sensitive to DES than the uterus and that BPA is a potent agonist in stimulating ER transcriptional activity in the pituitary but induces a limited uterotropic effect. These data indicate that considerable differences in the efficacy and potency of different ER ligands, such as the endocrine disrupting chemicals, may be influenced by the differential expression of cell- and tissue-specific factors, such as co-activator and co-repressor proteins.

### B. Nonreceptor Mediation

Transgenic ERKO models have revealed that certain estrogenic chemicals may exert their effect through alternative pathways that do not involve ER-α or ER-β. This was shown to occur with the catecholestrogen 4-hydroxyestradiol-17β (10 μg/kg) and the environmental estrogens kepone (15 mg/kg) and methoxychlor (1.8–60 mg/kg), which in ERKO mice are able to up-regulate uterine expression of the estrogen-responsive lactoferrin and glucose-6-phosphate dehydrogenase (G6PD) genes. In both studies, treatment of the ERKO mice with estradiol had little or no effect. It is possible that the observed effects were mediated through other nuclear receptors, such as ER-γ, or may have been due to the effect of alternatively spliced forms of ER that contain the ligand-binding domain. Nonetheless, these data raise the possibility that endocrine disruptors may exert their effects on hormone-sensitive tissues by additional independent signaling pathways that exclude the classical sex steroid hormone receptors. Some phthalates, such as dibutyl phthalate, and the herbicide atrazine show non-receptor-mediated effects on the androgen system. Atrazine, the most commonly used herbicide in the United States and possibly the world, acts by increasing aromatase activity, resulting in the promoted conversion of testosterone to estrogens, as shown in the alligator. This non-receptor-mediated mechanism has been postulated to induce demasculinization of laryngeal size and hermaphroditism in African clawed frogs (*Xenopus laevis*) following larval exposure to environmentally relevant levels of atrazine.

## V. EFFECTS AT THE MOLECULAR, CELLULAR, AND TISSUE LEVELS

### A. Developmental Genes

Although significant progress has been made in establishing that environmental chemicals induce developmental and reproductive anomalies through either receptor-mediated or non-receptor-mediated pathways, limited information is available describing the signals that link these two events. Members of the *homeobox* (Hox) and *wingless* (Wnt) family of genes have recently been identified as hormone-sensitive candidates that relay information on tissue patterning within the developing uterus, vagina, and mammary gland, particularly as regards the relationship between the epithelial and the stromal compartments, which is critical to normal development. Hoxa-9, hoxa-10, hoxa-11, and hoxa-13 are all expressed along the paramesonephric duct in the embryonic mouse, and by birth and into adulthood, these genes establish a spatial co-linearity such that they are expressed in the fallopian tubes, uterus, uterus and uterine cervix, and upper vagina, respectively. In late gestation, hoxd-10 and hoxd-11, hoxd-12, and hoxd-13 are also expressed in the oviduct, uterus, and posterior uterus/vagina, respectively. Wnt 4, Wnt 5a, and Wnt 7a are expressed in specific mesenchymo-epithelial patterns during perinatal development of the uterus, and Msx 1, Msx 2, and Wnt 10b are expressed during prenatal mammary gland morphogenesis.

Recently, the expression of some of these developmental genes was shown to oscillate in response to changes in circulating levels of steroid hormones during the murine estrous cycle or down-regulate following ovariectomy. Therefore, the subsequent findings that prenatal exposure to the potent estrogen DES altered the expression of Wnt 7a and Hoxa-10 during uterine morphogenesis in the mouse established an important correlation between chemical exposure and the ensuing developmental abnormalities associated with endocrine disruption. This link was made even more definitive when the phenotype of mice carrying specific Hox and Wnt null mutations was observed to be strikingly similar to that of human and mouse “DES daughters.” The Wnt genes are associated with cellular responses such as cell proliferation, apoptosis, and cell–cell communication (through the β-catenin/E-cadherin complex), and as such, changing patterns of expression due to environmental chemical exposure can provide insight into how these chemicals influence cell fate determination and tissue morphogenesis in hormone-sensitive organs.

### B. Cell Cycle Dynamics and Sex Steroid Receptor Expression

Induction of cell proliferation is considered the hallmark of estrogen action in the female genital tract. Thus, it has been adopted as a critical determinant of whether an environmental chemical is an estrogen. The mechanisms underlying this effect have not been completely elucidated; however, data gathered using diverse models such as established cell lines, primary cultures, and organ cultures indicate that the proliferative effect of estrogens is not directly stimulatory. Nonetheless, estradiol and DES have triggered opposite effects on cell number (and presumably cell proliferation) depending upon the time of exposure, the dose used, and the cell or tissue in which this parameter is measured. Similarly, apoptosis and the expression of sex steroid receptors within the female reproductive tract are modulated differentially depending upon the circumstances of exposure. For example, neonatal exposure of rodents to high doses of estradiol results in a severely hypoplastic mammary gland in adulthood characterized by reduced ductal-alveolar development, but the same exposure regimen with low doses of this chemical results in a hyperplastic mammary gland associated with increased lactational activity. Furthermore, prenatal exposure to estradiol or DES alters the timing of developmental events in the mouse uterus and vagina, specifically the precocious development of glands, which in adulthood become the very site at which adenocarcinoma develops. Prenatal exposure to TCDD results in the persistence of terminal end buds in the mammary glands, the same structural site in which a dramatic reduction of apoptosis is observed following prenatal exposure to BPA; these structures are the sites at which carcinogenesis develops in rodents and humans. All of these hormone-induced modulations of proliferation and apoptosis suggest that environmental chemicals can induce changes in the timing of developmental events, such that tissue organization is permanently altered; in turn, this may predispose these organs to disease or carcinogenesis.

Hormone-induced changes in the expression of sex steroid receptors have a profound impact on disease and normal functioning of tissues. Approximately 70% of human breast tumors exhibit an overexpression of ER. Transgenic mouse models in which ER expression is up-regulated by 25% have been shown to develop uterine adenocarcinoma at a far earlier stage in adult life relative to the wild type following neonatal exposure to DES. Subsequently, the finding that prenatal exposure to low doses of BPA induces a significant increase in the expression of both ER and PR within the lumenal epithelium of the adult mouse uterus is of concern, as this may predispose the organ to disease. The down-regulation of sex steroid receptors within reproductive organs can elicit an impact of equal importance since such a phenomenon can compromise fertility. For example, neonatal exposure of mice to DES affects the ability of the uterus to respond to estradiol; lower DES doses induce an enhanced response and higher doses induce a dampened response (uterotropic assay). In summary, these findings highlight the potential for environmental chemicals to induce alterations in normal relationships between proliferation, apoptosis, and expression of sex steroid receptors and reveal how such events may predispose organisms to infertility, disease, and/or carcinogenesis.

## VI. ENVIRONMENTAL EXPOSURE OF WILDLIFE

Lakes and rivers are one of the primary sites of chemical contamination for many wildlife species. This can occur either directly through skin absorption and respiration within chemical-laden waters, as is the case with invertebrates, fishes, and amphibians, or indirectly through preying upon these aquatic species. One of the earliest observations of chemical toxicity of aquatic species was made in 1964, when the high incidence of tumors in fish within the Los Angeles harbor, Southern Californian waters, and a Maryland lake was believed to be associated with exposure to sewage outlets. Other reports in sites throughout the United States unveiled a link between neoplasms in fish and high levels of PCBs from heavy manufacturing industries, chemical plants, wood product plants, and large shipping industries. These chemicals also induced developmental and reproductive abnormalities in fish by their ability to disrupt the endocrine system. This was first noted in the Great Lakes region that lies between Canada and the United States and also in rivers in the United Kingdom. In this latter case, the incidence of hermaphroditism and feminization observed in male fish residing in waters downstream of industrial effluent outlets from wool mills was reversed when the offending mills switched voluntarily from using estrogenic alkylphenol polyethoxylates to nonestrogenic detergents (alkyl ethoxylates).

Aquatic species act as "sentinels" of endocrine disrupting activity since changes in their development, reproduction, and ultimately population size provide the first indication of damage to an ecosystem. This was realized too late for the human populations residing in the Great Lakes region who relied upon local fish as a food source; their infants subsequently showed increasing evidence of motor and cognitive skill dysfunction. Aquatic species are the first organisms to suffer the effects of pesticide- and herbicide-laden water run-off from nearby agricultural areas and the direct dumping of industrial waste products, such as PCBs, into local waterways. Similarly, the use of biocides, such as tributyltin, in marine paints and wood preservatives increases the exposure of aquatic species to endocrine disrupting chemicals. Organisms that rely on algae in lakes and ponds as a food source, such as the crustacean *Daphnia magna*, show a reduced diversity in populations when exposed to hormonal chemicals, in part due to the effects of these chemicals on sex ratios. Laboratory experiments have shown that exposure of these invertebrates to fungicides, detergents, and agricultural effluents induces metabolic androgenization by significantly inhibiting the metabolic clearance of testosterone while enhancing the conversion of testosterone to other androgenic steroid hormones. Similarly, snails respond to tributyltin exposure by developing a hermaphroditic phenotype. The worldwide decline in frog populations has been attributed to chemical exposure, in large part from the herbicide atrazine to which amphibians are critically sensitive. Exposure of male frogs to this chemical causes the development of ovaries and reduces testosterone levels by increasing the production of aromatase, which catalyzes the conversion of testosterone to estrogen. Atrazine was one of numerous chemicals that were found to contaminate certain lakes in Florida and were directly associated with developmental abnormalities in alligators, such as micro-penis in the males. Chemically induced effects in lower invertebrates and vertebrates have repercussions in species that are higher on the food chain, such as birds, mammals, and ultimately humans, by the phenomenon known as biomagnification. An initial concentration of the persistent organochlorine DDT, which bioaccumulates within adipose tissue and organs, magnifies 10-fold each time it moves up the food chain. However, certain chemicals make it into the food chain by different means. PCBs and alkyl phenols from industrial effluent and pesticides and herbicides from agricultural water run-off often make it into groundwater via percolation through soil and into the air due to their volatility. Similarly, pesticides are sprayed directly onto fruit and vegetables, and plants have also been shown to take up endocrine disrupting chemicals from water.

BPA is released into the environment by a variety of means, including air, water, and soil. Humans are exposed directly to this estrogenic chemical through leaching from dental materials, food and beverage containers, and babies' formula bottles. It is also used in the manufacture of a multitude of products including optical lenses, adhesives, powder paints, building materials, compact disks, and for the encapsulation of electrical and electronic parts. Although humans may be the primary targets of BPA exposure, recent evidence has revealed that it is also present in lakes and rivers in concentrations that have experimentally generated "superfemale" phenotypes in prosobranch mollusk species; these exhibit additional reproductive organs such as two vaginas.

## VII. IMPLICATIONS FOR HUMAN DEVELOPMENT AND CARCINOGENESIS

Descriptions of the effects of fetal exposure to endocrine disruptors on the female and male reproductive system have predominated in the literature; however, two hormone-dependent systems that are beginning to receive attention are the mammary glands and the thyroid gland. These will be focused on next due to their importance in potentially linking exposure to endocrine disruptors with carcinogenesis and neurological/motor dysfunction.

### A. Mammary Glands

Estrogen exposure throughout a woman's life is a major risk factor for the development of breast cancer. Early age of menarche, late age of menopause, and obesity in the postmenopausal period (and thus increased aromatase activity and estrogen production) all correlate positively with breast cancer incidence. Also, the intrauterine milieu has an influential role since differences in intrauterine levels of estrogens correlate positively (twin births) and negatively (preeclampsia) with breast cancer incidence in the daughters from these pregnancies. Hence, fetal exposure to hormonally active chemicals may predispose humans to breast cancer. Various epidemiological case-control and cohort North American and European studies have revealed a positive correlation between blood serum levels of dieldrin, DDT, and PCBs in women and breast cancer incidence. Controversy abounds on this interpretation of the data mainly because none of these chemicals can be construed to be a marker of a total xenoestrogen exposure. It is worth noting that women who were exposed therapeutically to DES while pregnant between the years 1948 and 1971 now show a higher incidence of breast cancer.

Studies on rodents have shed light on fetal and neonatal exposure to estrogens during development and carcinogenesis of the mammary gland. However, it should be highlighted that tumors would not generally develop in the absence of either the mouse mammary tumor virus in mice or a 9,10-Dimethyl-1, 2-benzanthracene/N-methyl-N-Nitrosourea (DMBA/NMU) insult in both mice and rats. Using these models, several protocols of perinatal exposure to DES have resulted in an increased incidence of mammary tumors. In addition, fetal exposure to certain hormonally active chemicals has been shown to result in changes in the timing of developmental events and ultimately tissue architecture within the mammary gland, alterations that may predispose it to neoplastic transformation. *In utero* exposure of mice to 25 and 250 μg/kg BPA induces alterations in DNA synthesis within the epithelium and stroma, a striking reduction in apoptosis within the terminal end buds, and increased expression of progesterone receptors at puberty (postnatal day 30). These developmental changes preceded permanent changes that were characterized by a striking proliferation of all epithelial structures (ducts, terminal ducts, terminal ends, and alveolar buds) and increased expression of ER at 6 months of age. This disruption of the relationship between the epithelial and stromal tissue compartments and the relationship between DNA synthesis and apoptosis has been shown to predispose the mammary gland to carcinogenesis.

Other endocrine disruptors have been shown to alter mammary gland development and favor carcinogenesis. Perinatal exposure of rats to TCDD has been shown to induce the impairment of ductal growth, side branching, and alveolar development, accompanied by a persistence of terminal end buds and increased expression of ER. These animals also developed a higher incidence of chemically induced mammary adenocarcinomas. Typically present only at puberty, terminal end buds are very active sites of DNA synthesis and apoptosis, and thus their persistence into adulthood appears to provide a structural template for the development of carcinogenesis, which has been shown to arise specifically from these sites in both rodents and humans. Furthermore, neonatal exposure of rats to a combination of the organochlorines DDT, DDE, and 19 PCBs favored the development of chemically induced mammary tumors. This occurred following exposure to 100 × the concentration of endocrine disrupting chemicals that are found in the breast milk of Canadian women.

Mice are not susceptible to the spontaneous development of mammary tumors the way humans are, and in addition, humans are exposed to more than one environmental chemical throughout their lives. Thus, if a morphological template for the development of mammary gland carcinogenesis is established through the influence of endocrine disrupting chemicals, additional hormonal insults may initiate neoplastic transformation. The multitude of environmental chemicals to which humans are all exposed involuntarily, in addition to medically used hormones (hormonal contraceptives or hormone replacement therapy), may be a cumulative cause in the increase of breast cancer incidence that has been observed during the past 50 years.

## B. Thyroid Gland

The critical role of thyroid hormone in normal brain development has been revealed by the intellectual deficits that characterize congenital hypothyroidism, most notably mental retardation, deaf-mutism, and motor dysfunction. Thus, reports that a large number of endocrine disrupting chemicals can alter thyroid function are of grave concern. Epidemiological studies of pregnant women and newborns exposed to PCB-contaminated fish from the Great Lakes have demonstrated an association between PCB exposure and the development of cognitive and behavioral changes in children. Other studies have shown an inverse relationship between serum levels of PCBs in infants, children, and adults and levels of triiodothyronine and thyroxine (T4) and a positive correlation between serum PCBs and thyrotropin (TSH); some conflicting data have also been reported.

The mechanisms by which PCBs impact thyroid function are becoming apparent by *in vitro* and *in vivo* studies. Exposure to PCB congeners has been shown to (1) competitively bind thyroid hormone-binding proteins, thus potentially reducing the carrying capacity of the blood for T4, (2) reduce the serum half-life of T4 by increasing the rate of biliary excretion of T4 and facilitating clearance through liver metabolism, and (3) reduce the ability of the thyroid gland to respond to TSH. Furthermore, PCB congeners may affect brain development by directly interacting with the thyroid hormone receptors α and β; these mediate the majority of the biological actions of thyroid hormone. In rats, a number of thyroid hormone-responsive endpoints, such as the expression of RC3/neurogranin mRNA in the forebrain and myelin basic protein mRNA in the cerebellum, are up-regulated in a dose-dependent manner by postnatal exposure to Arochlor 1254. Similarly, fetal exposure to the same chemical has been shown to increase expression of RC3/neurogranin and Oct-1 mRNA in the embryonic day 16 fetal cortex and also the *notch* signaling gene HES-1; this increases gliogenesis at the expense of neurogenesis. If PCB congeners do indeed alter the balance of neuron to glia production in the brain, this finding may contribute to the understanding of how environmental chemicals can alter brain development and subsequently cognitive and motor function in humans.

## VIII. HUMAN DISEASE AND FERTILITY

The observation that the average sperm count has significantly declined in men from various countries in Europe and in the United States, though still controversial, led to the hypothesis that exposure to environmental chemicals may be associated with an increase in the incidence of male genital tract abnormalities and infertility. This hypothesis is supported by trends in these endpoints in several European countries as well as the United States. Studies on cohorts exposed through industrial accidents (Seveso, Italy; Yu-Cheng, Taiwan; Yucho, Japan) and in occupational settings to high levels of hormonally active chemicals, as well as rodent models, lend further support to this hypothesis, as do the limited data on men exposed *in utero* to DES ("DES sons"). The impact of exposure to estrogens on female reproductive health was made abundantly clear by the case of the DES daughters, as mentioned earlier in this article. The increased incidence of endocrine-related diseases, such as uterine cervix adenocarcinoma, specific ovarian tumors, and uterine leiomyomas, that have been observed in human populations over the past few decades also suggest a link with chemical exposure. As with males, numerous animal studies have demonstrated that *in utero* exposure to a variety of hormonally active chemicals induces developmental and reproductive abnormalities, which range from precocious puberty to the onset of endometriosis.

Recent work has demonstrated that perinatal exposure of mice to the phytoestrogen genistein, at doses comparable to those used in soy-based infant formula, induces an increased incidence of uterine adenocarcinoma in adults. Therefore, the increasingly popular consumption of this natural, unregulated estrogen by women and infants is becoming a cause for alarm. Western populations enthusiastically adopted the use of soy products in their diet, based upon the fact that Asians, who traditionally eat a high-soy-content diet, have a significantly lower incidence of both breast and prostate cancer. However, the Asian and Western diets differ in many other aspects, which preclude for the moment making a correlation between cancer incidence rates and one specific dietary component. Yet, average measurements of the levels of genistein (one soy component) revealed that Western diets contain 5 parts per million (ppm), Asian diets contain 100 ppm, and Western infant soy-based formula contains 500 ppm. This discrepancy highlights the need to practice caution when it comes to extrapolations of anecdotal evidence to human health policy.

Endocrine disruption seems to have consequences that are transmitted transgenerationally. For example, daughters of rats that were exposed to DES during perinatal development have a significantly greater incidence of malignant reproductive tract tumors, including uterine adenocarcinoma, compared to daughters of vehicle-exposed controls. The mechanisms underlying these effects are unknown, although it has been suggested that they may be mediated by epigenetic phenomena such as DNA methylation. As with all the anomalies described above, the evolutionary consequences of such developmental and reproductive alterations in human populations are unknown at this stage, but there is adequate evidence, based on wildlife indices, to suggest that population size and diversity would be impacted significantly.

### A. Susceptible Populations or Individuals

Evidence that individuals might show a different response to the same chemical exposure was first established in studies that showed a differential response in the prostate weight of mice following prenatal estradiol exposure; the difference depended upon the intrauterine position of the developing mouse.

Specifically, males situated *in utero* between two females showed a significantly greater increase in their adult prostate weight than those males situated between two males when exposed prenatally to estrogens. Minor differences in the local concentrations of intrauterine estradiol, which appeared to be gender-specific, were determined as the basis for different adult outcomes. This phenomenon should be taken into consideration when dealing with xenoestrogen exposure. For example, the positional effect has been shown to act additively with fetal BPA exposure in determining the age at which puberty initiates in female offspring. In this regard, one major issue that has arisen in the field of environmental endocrine disruption is that wildlife populations and outbred rodent strains are composed of nonidentical individuals and thus vary in their susceptibility to any particular noxious agent. This was made clear by a study showing that different strains of mice (inbred and outbred) responded very differently (in terms of testicular parameters) to estradiol exposure.

A new area of molecular investigation has suggested that the phenomenon of single-nucleotide polymorphisms (SNPs) may be implicated in the differential response of individual animals and humans to toxicological insult. SNPs are common DNA sequence variations that exist between genders, races, ethnicities, and individuals. Although most of these sequence variations do not affect protein function directly, they may have an indirect influence by altering the function of regulatory sequences that control gene expression or the stability and processing of the mRNA transcript. These subtle differences may contribute to the future understanding of how exposure to endocrine disrupting chemicals impacts wildlife and human health.

## IX. CONCLUDING REMARKS

The Second Industrial Revolution and the Green Revolution, which heralded the massive synthesis and release of chemicals into our environment, are having a profound and continued effect on the development, reproductive health, and disease status of wildlife and humans. As a result of the hormonal activity of many chemicals, phenomena such as changes in the sex ratio, gender status, and genetic diversity of aquatic species, birds, and mammals have emerged. The implications for human health are serious and well founded, since there is increasing evidence of the presence of pesticides, herbicides, plasticizers, and other industrial chemicals in human breast milk, amniotic fluid, blood serum, and fat samples. Some of these compounds, such as the chlorinated compounds, are persistent and therefore bioaccumulate. A plethora of experimental data collected in rodents reveal that fetal exposure to similar doses of these chemicals can induce imbalances in gene expression, cell proliferation and apoptosis, communication between tissue compartments, and changes in sex steroid receptor expression in many hormone-sensitive organs. These changes often translate into morphological and functional alterations. The resultant susceptibility of these experimental species to developmental and behavioral anomalies, diseases of the reproductive tract and other organs, compromised fertility, immune deficiencies, and in some instances, carcinogenesis portends comparable vulnerability in humans. If one agrees with the oft-quoted maxim by Theodosius Dobzhanski that “nothing makes sense in Biology if not in the context of evolution,” it becomes inescapable to conclude that all living things are participants of a man-made evolutionary experiment. Scientists have presented sufficient evidence that the outcome of this experiment may not be to the benefit of humans and the ecosystem on which all humans depend. Thoughtful, conscientious citizens and responsible politicians must now bear the responsibility of offering less risky alternatives to provide a balanced accommodation between a reasonable standard of living for humans and the perils that endocrine disruptors pose to all.

## Glossary

**aryl hydrocarbon receptor** An intracellular protein expressed in several tissues of most mammals, including humans, whose three-dimensional structure and endogenous ligand are unknown. It is assumed that this receptor mediates the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and structurally related halogenated aromatic hydrocarbons, including polychlorinated biphenyls.

**biomagnification** The phenomenon in which the tissue concentrations of some pollutants are increased as they transfer from one organism to another through the food chain due to their lipophilic nature.

**endocrine disruptor** An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body. These hormones are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.

**hermaphroditism** Derived from the Greek god and goddess Hermes and Aphrodite, this phenomenon describes the developmental condition in which an individual displays both male and female sexual phenotypes.

**selective estrogen receptor modulators** Natural or synthetic ligands that exhibit either estrogen agonist or antagonist activity among different cells and tissues. Most likely, this is due to the differential expression of cell- and tissue-specific factors, such as co-activator and co-repressor proteins.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Estrogen and Progesterone Receptors in Breast Cancer • Phytoestrogens • SERMs (Selective Estrogen Receptor Modulators) • Sexual Differentiation, Molecular and Hormone Dependent Events in • Sexual Differentiation of the Brain**

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# Environmental Disrupters of Thyroid Hormone Action

FRANÇOISE BRUCKER-DAVIS
*Hôpital l'Archet 1, Nice, France*

I. INTRODUCTION
II. NATURAL ENVIRONMENTAL THYROID DISRUPTERS
III. SYNTHETIC ENVIRONMENTAL THYROID DISRUPTERS
IV. MECHANISMS OF THYROID DISRUPTION
V. FACTORS PREDISPOSING TO THYROID DISRUPTION
VI. CLINICAL IMPACT OF THYROID DISRUPTERS

**The thyroid gland is easily disturbed by numerous external factors, both natural and synthetic. Endemic environmental goitrogenesis, the enlargement of the thyroid gland, has long been associated with diets deficient in iodine or with diets rich in goitrogenic substances (phytogoitrogens). In addition to goiter development, thyroid hormone imbalance and/or exposure to thyroid-toxic chemicals during pregnancy can have devastating effects on fetal brain development, as illustrated by the endemic cretinism observed in areas where native diets are iodine deficient.**

## I. INTRODUCTION

The goiter, or enlarged thyroid gland, has long been recognized to be the result of a dietary deficiency of iodine-containing foods. The question of environmental thyroid disruption, however, has been recently assessed in the more global context of the impact of environmental synthetic chemicals on endocrine system function. Many synthetic chemicals have been found to have deleterious effects on the thyroid, both *in vitro* and *in vivo* in fauna, laboratory animals, action, or elimination of the natural hormones that are responsible for maintenance of homeostasis and regulation of developmental processes. Disrupters can directly affect an exposed individual and can affect fetuses through *in utero* exposure.

**goiter** Thyroid gland enlargement, the most common thyroid abnormality, is present in about 200 million people worldwide.

**polyhalogenated hydrocarbons** Synthetic chemicals, chlorinated or brominated, such as polychlorinated biphenyls, dioxin, or polybrominated biphenyls.

## See Also the Following Articles

**Environmental Disruptors of Sex Hormone Action • Iodine • Thyroid and Reproduction • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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# Ephrins and Their Receptors

Martin Lackmann* and Andrew W. Boyd†

**Ludwig Institute for Cancer Research, Australia* • *†Queensland Institute for Medical Research, Australia*

I. INTRODUCTION
II. MOLECULAR PROPERTIES OF EPHRINS AND Eph RECEPTORS
III. SIGNALING FUNCTIONS OF EPHRINS AND Eph RECEPTORS
IV. BIOLOGICAL ACTIVITIES OF EPHRINS AND Eph RECEPTORS
V. SUMMARY

**Ephrins are cell-surface-associated ligands for a family of ephrin receptors (Eph). Ephrins and Ephs act as guidance cues to facilitate and direct the movement of cells and cell layers during various developmental processes.**

## I. INTRODUCTION

Ephrins are cell-surface-associated ligands for ephrin receptor (Eph) tyrosine kinases (RTKs), the largest subgroup of the RTK superfamily. In contrast to other cytokine receptors, Eph RTKs (Ephs) have been discovered in the past decade as orphan receptors mostly by homology (complementary DNA) cloning approaches. The founding family member, EphA1, was isolated in a low-stringency screen with a v-*fps* oncogene cDNA probe from an erythropoietin-producing hepatocellular (EPH) carcinoma cell line; EPH receptor-interacting proteins (i.e., ephrins) were isolated in a simultaneous effort by several laboratories to unravel Eph RTK function. Although several ephrins, including the first identified EphA2 ligand, now known as type A1 ephrin, were isolated as soluble proteins, it rapidly became apparent that ephrins are produced as cell-associated proteins. Structures and functions of the 14 transmembrane Ephs and the 9 cell-membrane-associated ephrins (Fig. 1) are highly conserved in metazoans, ranging from *Caenorhabditis elegans* to humans. Ephs and ephrins are dynamically expressed in spatially restricted patterns at discrete phases during vertebrate embryogenesis; they guide the migration and positioning of cells and cell layers during gastrulation and during the development of nervous system and vasculature. In contrast to other RTK systems, Eph signaling does not promote proliferation and differentiation; rather, engagement of ephrins with corresponding Ephs on opposing cells triggers cell morphological changes that underlie cell adhesion and cell repulsion. There is little indication for a role of Ephs and ephrins in normal adult tissue, although their unscheduled overexpression in many human cancers emphasizes emerging functions in tumor progression and metastasis.

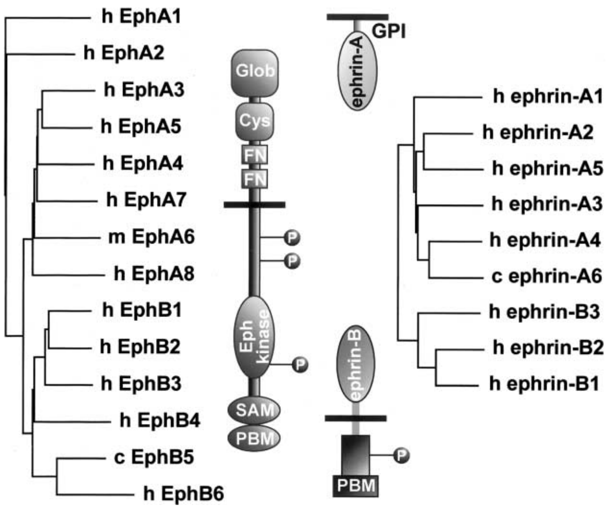

**FIGURE 1** Protein domains and sequence homology of ephrin receptors (Eph) and ephrin families. The sequence homology trees were derived from alignment of the human (h) sequences except for (m, mouse) EphA6 and (c, chicken) EphB5 and type A6 ephrin (ephrin-A6), for which no human sequences were available. Glob, Globular ligand-binding domain; Cys, cysteine-rich epidermal growth factor-like domain; FN, fibronectin type III motif; ▬, cell membrane; SAM, sterile α-motif domain; PBM, PDZ-binding domain; GPI, glycosyl phosphatidylinositol membrane anchor; P, phosphotyrosine. We are grateful for Dr. Nathan Hall, Ludwig Institute of Cancer Research (Melbourne), for providing the Eph and ephrin alignments.

## II. MOLECULAR PROPERTIES OF EPHRINS AND Eph RECEPTORS

### A. Organization of Subfamilies

Ephrins are synthesized as cell-surface-bound proteins, with their mode of membrane attachment providing the criterion for classification into two subfamilies, type A and type B (Fig. 1). Type A ephrins (ephrins A1–A6) are anchored to the outer leaflet of the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety, whereas type B ephrins (ephrins B1–B3) contain a membrane-spanning domain and a short cytoplasmic tail. Likewise, Eph RTKs are subdivided, on the basis of distinct structural differences in their ligand-binding domain (see Section II.C), into eight type A receptors and six type B receptors. This structural subdivision of Ephs and ephrins correlates with binding specificities of ephrins to Eph receptors. With the exception of EphA4, which binds certain type A and type B ephrins with comparable affinities, type A ephrins bind preferentially to EphA receptors and transmembrane, type B ephrins bind EphB receptors. Accordingly, type A or type B receptors and ligands are named in chronological order of their discovery and depending on their preference for either GPI-linked (A) or transmembrane (B) ephrins, respectively. Within the two subclasses, individual ephrins bind multiple Eph RTKs and vice versa, whereby an ordering of binding affinities indicates preferred interactions between certain Eph RTKs and ephrins. *In vivo*, interactions between cell-surface-tethered Eph RTKs and ephrins are necessarily multivalent and of high avidity, and even weak interactions between the individual proteins can be physiologically relevant. However, different Eph/ephrin signaling complexes are not functionally interchangeable. As a consequence, the formation of biologically relevant Eph/ephrin complexes and the "net biological response" are determined by both the expression profiles and the affinity of competing receptor/ligand interactions.

### B. Ephrins: Protein Origin and Structure

Nine closely related ephrins, with sequence similarities of 30–70% in their core sequences, have been identified in vertebrates. Orthologues have been unambiguously defined in mammals and birds and, in addition to a high sequence identity (~80–95%), are functionally interchangeable. Ephrins have also been identified and characterized functionally in the frog (*Xenopus laevis*), the zebrafish (*Brachydanio rerio*), nematodes (*Caenorhabditis elegans*), and flies

(*Drosophila melanogaster*), suggesting that ephrins have developed from an ancestral ephrin protein dating back to the origin of metazoans. At least some of these proteins are likely orthologues of particular mammalian molecules, and human or mouse ephrins exert physiological functions in zebrafish animal studies. Analysis of the full genomes reveals four GPI-linked ephrins in *C. elegans*, but only one ephrin in *Drosophila*. Thus, despite development of a more complex body plan, the fly requires fewer Eph and ephrin genes than does the worm. Both type A and type B ephrins share a highly conserved, N-glycosylated, receptor-binding core domain of approximately 125 amino acids that has no sequence similarities to other proteins. The protein structure of their 28- to 30-kDa extracellular domain resembles that of copper-binding plant cupredoxins. However, ephrins do not bind metal ions and differ structurally by two distinct loops that are involved in the formation of receptor/ligand dimers and higher order oligomers (see Section II.D). The ephrin core domain folds into a β-barrel of eight parallel and antiparallel β-strands, arranged in a Greek key topology and stabilized by two intermolecular disulfide bonds. This protein fold is interspersed by two short α-helices and a single-turn $3_{10}$-helix, which is missing in type A ephrins.

The receptor-binding domain is followed by a less conserved spacer region that in type A ephrins contains a disintegrin and a metalloprotease (ADAM) cleavage site essential for Eph/ephrin-mediated cell repulsion (see Section III.A) and the GPI cleavage/transfer consensus sequence. In type B ephrins, a single transmembrane-spanning segment connects to a short, essentially invariant, cytoplasmic domain containing several tyrosine phosphorylation and one PDZ domain binding motifs, which serve as docking sites for cytoplasmic ligands during "reverse signaling" by type B ephrins (see Section III.B).

## C. Ephs: Protein Origin and Structure

Ephs have diversified in vertebrates into a closely related family of 14 receptors, related by highly homologous core regions in their extracellular and cytoplasmic domains (Fig. 1). An Eph RTK has been cloned from the freshwater sponge *Ephydatia fluviatilis*, indicating the origin of this protein family before the parazoan-eumetazoan evolutionary split. A characteristic arrangement of protein domains (Fig. 1) is conserved from the single Eph receptor found in *Drosophila* and *C. elegans* to all the classes found in vertebrates. A failure to find homologues in some vertebrate species suggests that not all 14 Ephs occur in all vertebrates. Ephs consist of modular extracellular and intracellular subdomains common to many other receptor tyrosine kinase subfamilies, with the exemption of the N-terminal, ligand-binding domain, which is unique to the Eph family (Fig. 1). The genomic organization of several vertebrate Ephs indicates composition of at least 16 exons and highly conserved exon/intron boundaries that correlate with the protein subdomain structure.

The EphB2 ligand-binding domain, solved by X-ray crystallography, contains two antiparallel β-sheets of six concave and five convex β-strands that form a compact β-sandwich. Its topology has remarkable structural similarity to the carbohydrate-binding domain of lectins, in particular influenza virus hemagglutinin. However, the two families are not related by sequence and Eph/ephrin interactions are independent of carbohydrate moieties. Type A Ephs differ structurally from type B Ephs by a four-residue truncation within one of the loops connecting the β-strands of the core structure. This "specificity loop" is part of the low-affinity ligand-binding interface, which facilitates oligomerization of Eph/ephrin complexes (see Section II.D). The Eph ligand-binding domain is followed by a cysteine-rich epidermal growth factor (EGF)-like motif that is involved in ligand-independent receptor dimerization and two typical fibronectin-type III repeats preceding its single membrane-spanning segment.

The cytoplasmic domain is composed of a short 50- to 65-residue juxtamembrane region, which regulates kinase activity and provides biologically important docking sites for SH2 domain-containing cytoplasmic ligands (Fig. 2). It is followed by an uninterrupted kinase domain, an α-helical sterile α-motif (SAM), and a C-terminal PDZ domain binding motif. The EphB2 kinase domain conforms to a prototypic protein kinase structure, consisting of a small N-terminal lobe that, in the catalytically active conformation, clamps onto the bigger C-terminal lobe to form the interfacial nucleotide-binding site and catalytic cleft. Importantly, and characteristic for the regulation of Eph RTK activity (see Section III.A), the C-terminal half of the nonphosphorylated juxtamembrane region in the latent Eph kinase interacts tightly with the N-terminal lobe of the kinase domain, thereby stabilizing an inactive conformation of the catalytic cleft.

The Eph SAM domain is a protein/protein interaction motif common to various cytosolic signaling proteins. Although a homodimeric configuration of the EphA4 SAM domain in its crystal

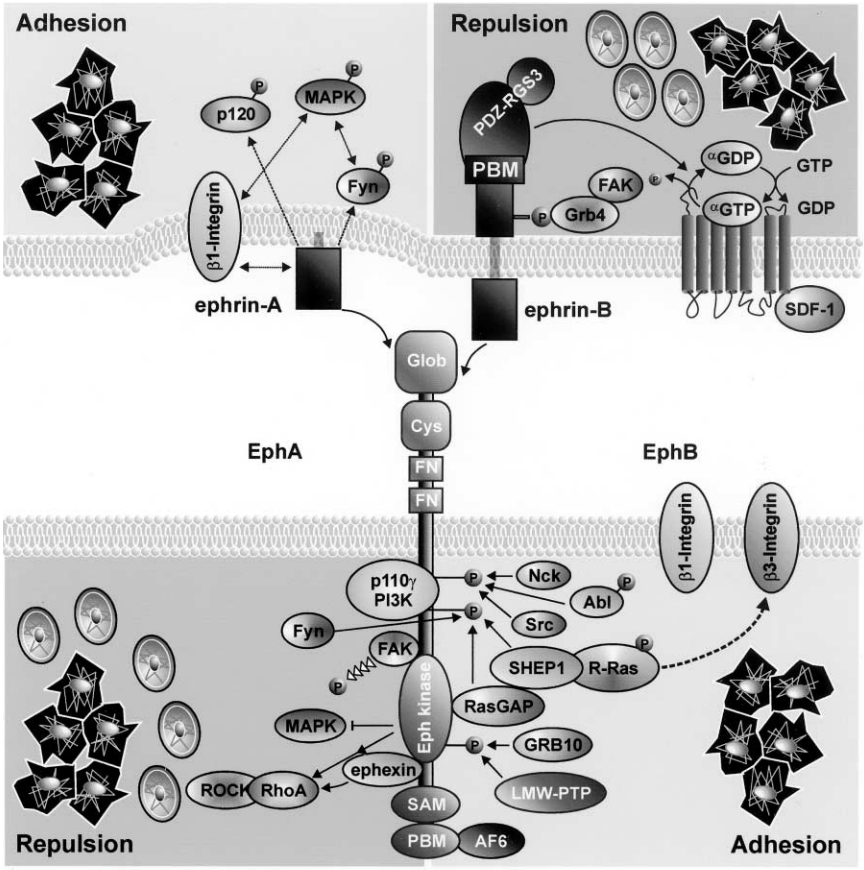
Adhesion
P
p120
P
MAPK
P
Fyn
β1-Integrin
ephrin-A
Repulsion
PDZ-RGS3
PBM
FAK
P
P
Grb4
αGDP
GTP
αGTP
GDP
SDF-1
ephrin-B
Glob
Cys
EphA
EphB
FN
FN
β1-Integrin
β3-Integrin
p110γ
PI3K
P
Nck
Abl
P
P
Src
Fyn
FAK
P
SHEP1
R-Ras
P
RasGAP
Eph kinase
MAPK
P
GRB10
ephexin
LMW-PTP
ROCK
RhoA
SAM
PBM
AF6
Repulsion
Adhesion

**FIGURE 2** Signaling components of ephrins and Ephs that modulate cell shape, adhesion, and motility. Motifs of Eph and ephrin proteins are labeled as in Fig. 1, showing the signaling proteins mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), and Abl, and the Src family kinases, Src and Fyn. Heterotrimeric GTP-binding protein (G-protein) signaling components include the regulator of G-protein signaling (RGS3) and the chemokine receptor and its ligand SDF-1. Small guanosine triphosphatase pathway components include RhoA, R-Ras, Ras GTPase-activating protein (RasGAP), Rho-associated kinase (ROCK), and Eph receptor-interacting nucleotide exchange factor (ephexin). Other Eph/ephrin signaling proteins include low-molecular-weight protein tyrosine phosphatase (LMW-PTP), the SH2 and SH3 adapter proteins Nck and Grb4, SH2 domain-containing Eph receptor-binding protein 1 (SHEP1), phosphatidylinositol 3′-kinase (the p110γ isoform of phosphatidylinositol 3-kinase), the acute myeloid leukemia-1/chromosome 6 fusion protein (AF6), and p120, a 120-kDa tyrosine phosphorylated raft protein. Reprinted from Boyd and Lackmann (2001), with permission.

structure is thought to suggest involvement in Eph receptor dimerization, its function in Eph signaling is currently unknown.

## D. Receptor–Ligand Interactions and Binding Affinities

Ephrins bind Eph receptors in a strict one-to-one stoichiometry and require membrane attachment and/or clustering to trigger biological responses. Within each subclass, receptors and ligands interact with a distinct ordering of affinities, with dissociation constants ranging from 5 to 500 n*M*. However, *in vivo* the interactions between membrane-bound ephrins and transmembrane Ephs are multivalent and of high avidity. In many cases, corresponding Eph receptor and ephrin classes are expressed in reciprocally graded and apparently mutually exclusive distributions in adjacent cell populations. Thus, receptor-bearing and ligand-presenting cells encounter each other only at the border of compartments, whereby Eph/ephrin interactions trigger responses, often cell repulsion, in both types of cells. Simultaneous expression of different type A or type B receptors and corresponding ligands occurs frequently during vertebrate development, leading to competing interactions between various receptor/ligand combinations. This interaction scheme serves to relay positional information: the location of an Eph-expressing cell (or axon) in a given microenvironment of ephrin expression is determined, through repulsive and/or adhesive responses, by affinity and abundance of its Eph receptor population in relation to that of neighboring cells (Fig. 3).

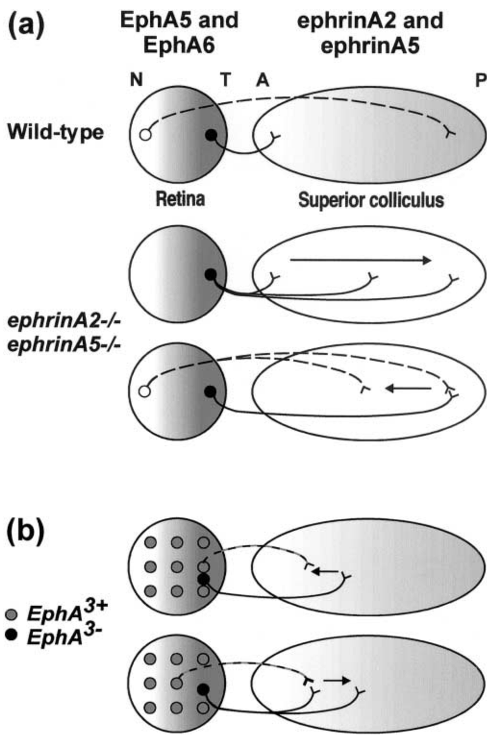

**FIGURE 3** Eph-mediated topographic mapping in the vertebrate visual system. Shading indicates graded expression of EphA5 and EphA6 in the retina and of types A2 and A5 ephrin in the superior colliculus. (a) EphA5 and EphA6 are expressed in the mouse retina (EphA3 in the chicken) in a high temporal (T, solid line), low nasal (N, dashed line) gradient and are positioned by a corresponding high posterior (P) to low anterior (A) gradient of types A5 and A2 ephrin in the superior colliculus. Temporal axons (high EphA5/EphA6), repelled by low-level expression of types A2 and A5 ephrin, position to anterior positions, whereas nasal axons (low EphA5/EphA6) require high ephrin concentrations for repulsion and project to posterior positions. Lack of type A2 ephrin and type A5 ephrin allows axons to move to more posterior positions (→). Competition forces nasal axons to project more anteriorly (←). (b) Forced, uniform overexpression of EphA3 (which interacts with both ephrins) in 50% of retinal ganglion cells not only stops those axons earlier (more anterior position, ←), but, due to competition for ephrin-expressing targets, pushes neighboring EphA3$^{-}$ retinal ganglion cell axons to more posterior positions (→). Reprinted from Klein (2001), with permission from Elsevier Science.

Combined findings from kinetic and *in vivo* experiments suggest a stepwise mechanism of Eph receptor activation: initial high-affinity interactions between membrane-associated ephrin clusters and Eph receptors facilitate accumulation of receptors to a critical concentration, which triggers oligomerization through the cysteine-rich domain. Receptor transphosphorylation occurs also at high local receptor concentration in the absence of ligand, whereby ligand-independent receptor dimerization is mediated predominantly by the cysteine-rich Eph domain. These findings are in agreement with structural features of the complex between the EphB2 ligand-binding domain and ephrin-B2, indicating two distinct interfaces that furnish the receptor/ligand interaction. An extensive high-affinity Eph/ephrin binding interface occupies 13 and 16% of the receptor and ligand surface areas, respectively. It includes an extended, hydrophobic loop of the ligand, which inserts into a deep channel defined by receptor β-strands. A smaller, low-affinity oligomerization interface occupies only 5–6% of mostly polar receptor and ligand surface areas, and includes interactions between a ligand β-strand and the Eph receptor specificity loop. Molecular details of the involvement of the cysteine-rich dimerization domain with higher order oligomers await elucidation of its structure.

## III. SIGNALING FUNCTIONS OF EPHRINS AND Eph RECEPTORS

### A. Receptor (Forward) Signaling

Receptor dimerization or oligomerization (clustering) leads to a release of the conformational constraint imposed by the juxtamembrane domain and to transphosphorylation of multiple receptor tyrosine residues. In particular, the phosphorylated juxtamembrane tyrosines no longer associate with the N-terminal kinase lobe and are docking sites for downstream SH2 domain signaling molecules. Eph signals modify the cytoskeletal cell architecture through recruitment and activation of proteins directly involved in regulating cytoskeletal organization, including Fyn, Src, Abl, Ras GTPase-activating protein (RasGAP), Rho, Rac, SHEP1, SHP2, Cbl, Crk, and Nck (Fig. 2). Their involvement in signaling pathways of downstream-activated integrin receptors supports evidence from functional experiments for a direct communication between activated Eph receptors and mechanisms that regulate cell adhesion and cytoskeletal plasticity. Eph/ephrin signals also directly modulate integrin function and are regulated by cadherins. Eph RTK signaling commonly results in loss of actin stress fibers, focal adhesions, cell processes, and cell attachment, and culminates in contact repulsion. However, initially, high-avidity interactions between Ephs and ephrins tether the reacting cells, implying a mechanism that disrupts this bond before opposing cells can disengage. Indeed, clustered Ephs activate an ephrin-associated ADAM protease that specifically releases the ephrin exodomain from the cell membrane to allow repulsion to proceed.

Not all biological responses of ephrin-tethered Ephs are mediated by tyrosine kinase signaling and trigger cell repulsion. Kinase-deficient Eph splice variants, which are expressed during mouse development, and kinase-independent signaling functions of cytoplasmic domains of certain Ephs switch the cell response from repulsion to cell adhesion.

### B. Ligand (Reverse) Signaling

Evidence for essential signaling functions of ephrins stems primarily from *in vivo* studies of animals with mutations/deletions in Eph/ephrin components. The almost invariant 33-amino-acid C-terminal tail of type B ephrins contains five tyrosines that become phosphorylated on receptor binding and is followed by an invariant PDZ-binding motif. Several noncatalytic adapter proteins containing multiple PDZ domains have been identified as cytoplasmic ligands for the conserved ephrin B PDZ-binding motif. Importantly, type B ephrin signaling regulates cerebellar granular cell migration by affecting the chemoattraction of the cells to SDF-1: via its PDZ motif EphB-clustered ephrin type B recruits the GTPase-activating protein PDZ-RGS3. The GAP activity of its RGS domain inhibits heterotrimeric G-protein-mediated signaling of the SDF-1 chemoattractant receptor, CXCR4, thereby abrogating its chemotactic response to SDF-1. GRAB4 is the first known SH2 domain signaling molecule known to bind to clustered, phosphorylated type B1 ephrin. GRAB4 binding results in the recruitment of a signaling complex containing Cbl-associated protein (CAP), Abl-interacting protein-1, dynamin, PAK1, and axin; activation of FAK; and subsequent loss of focal adhesions and cell rounding.

Although it is clear that Eph binding to GPI-anchored ephrins triggers cellular responses, the involved mechanisms remain unexplored. Increased adhesion of type A8 ephrin-expressing cells to fibronectin due to up-regulation of β1-integrin involves recruitment and activation of Src and mitogen-activated protein (MAP) kinase pathways to ephrin clusters localized within lipid raft membrane microdomains.

### C. Bidirectional Signaling

Once initiated, Eph and ephrin signal transduction mechanisms converge on the regulation of processes involved in cell shape, adhesion, and movement. Perhaps the best documented example for bidirectional signaling by Eph receptors and type B ephrin ligands is the formation of hindbrain segments and somites during zebrafish development. Studies with truncated, dominant-negative ephrins or Ephs have demonstrated that repulsive and deadhesive responses from differentially activated Ephs and ephrins segregate mixed cell populations into ligand-and receptor-expressing domains. Importantly, only bidirectional activation of both Eph- and type B ephrin-expressing cells can prevent cell intermingling, which still occurs if signaling by one of the components is defective. Studies in mice with targeted deletion of type B Eph receptors and ephrins indicate that their bidirectional signals are also essential for the development of the vascular system. Although the involved molecular mechanisms require further investigation, effective partition of cells fated to form arteries from cells fated to form veins requires signals from EphB receptors and "reverse signals" from type B ephrin ligands.

## IV. BIOLOGICAL ACTIVITIES OF EPHRINS AND Eph RECEPTORS

### A. Functions During Embryogenesis

Signaling by Ephs and ephrins is important in the positioning of cells and cell populations during many developmental processes, including gastrulation, patterning of the neural tube and paraxial mesoderm, and development of the neural and vascular network. Eph signaling during vasculogenesis is essential, and disruption of mouse type B2 ephrin (EphB4 or EphB2/EphB3; double knockout) genes results in early (day 10) embryonal death due to arrest of vasculogenesis during development of the primitive vascular plexus and lack of angiogenic remodeling.

Analysis of *C. elegans* Eph and ephrin mutants indicates importance of kinase-dependent and kinase-independent Eph signaling for neuronal cells during gastrulation cleft closure, and for epidermal cells to complete the ventral closure of the epidermis. Likewise, navigation of cells during zebrafish and

*Xenopus* gastrulation and boundary formation during zebrafish segmentation is dependent on Eph/ephrin signals, and in the mouse fusion of the neural folds at the dorsal midline requires adhesion between cells expressing type A5 ephrin and a kinase-deficient splice variant of EphA7. Possibly the most prominent role of Eph/ephrin signaling in vertebrates is found during development of the nervous systems, and most of the receptors and ligands are expressed here, in strictly spatially and temporally defined patterns. The migration of neural crest cells and their targeting to the correct destination are governed by Eph/ephrin activities. Targeted disruptions in mouse Eph and ephrin genes demonstrate essential guidance functions for limb motor axons (EphA4), corticospinal neurons (EphA4, type B3 ephrin), superior colliculus axons (EphA8), anterior commissure and corpus callosum axons (EphB2, EphB3), and retinal ganglion cell (RGC) axons (EphA3, EphA5, EphA6, EphB2). The current model of Eph/ephrin signaling in directing pathfinding suggests that the migration of an Eph receptor-bearing cell or growth cone into a gradient of ephrin expression leads to cell-contact-dependent Eph receptor activation. The resulting signals instruct the cell or growth cone to change direction and avoid the ligand-rich area. The molecular mechanism underlying the formation of topographic maps in the visual system demonstrates this principle (Fig. 3). Interestingly, the single *Drosophila* Eph receptor functions in a similar manner during development of a topographic map in the developing fly visual system.

## B. Functions in Pathology

Generally, expression of Eph RTKs in adult tissues is low or undetectable and, apart from postulated functions in synaptic plasticity, neuroblast migration, and adult angiogenesis, there is no evidence to suggest roles in adult tissues. However, high expression levels of several Eph RTKs and ephrins, including EphA1, EphA2, EphA3, EphB2, EphB4, type A1 ephrin, type A5 ephrin, and type B2 ephrin, are found in various tumors and tumor cell lines and in many cases correlate with invasive potential and metastasis. EphA1, EphA3, and EphB4 have been isolated from tumor cell lines, but their elevated expression is clearly not due to gene amplification or rearrangement. Although most studies to date provide circumstantial evidence for Eph functions in oncogenesis, several findings suggest that Eph/ephrin overexpression in tumors is prometastatic due to mechanisms that are normally executed during embryogenesis. EphA2/type A1 ephrin and type B2 ephrin signaling is involved in tumor neovascularization, and activation of endogenous EphA2 or EphA3 triggers transient, rapid deadhesion of prostate carcinoma or malignant melanoma cells, respectively.

# V. SUMMARY

The coordinated movement of cells and cell layers is an essential process involved in shaping the vertebrate body during normal development, whereas aberrant cell migration and adhesion in adult organisms are hallmarks of metastatic cancer progression. Ephrins and their Eph receptors act as guidance cues and the molecular mechanisms of their interactions are tailored to facilitate and direct the movement of cells and cell layers during various developmental processes. Emerging evidence suggests that these molecules may fulfill similar functions during tumor invasion and metastasis.

## Glossary

**avidity** The functional combining strength of the contact between a protein and an interaction partner (or protein complex, e.g., antibody or receptor, containing multiple binding sites for the interaction partner, e.g., antigen or ligand). Avidity is governed by the affinity of the individual reaction, the number of binding epitopes (valency), and sterical hindrance of the interacting components.

**axon projection** Long extensions of neurons that conduct nerve impulses to terminals distant from the nerve cell body. The axon projection is the defined path and destination point of the axon.

**clustering** The occurrence of crowded groups of receptors or ligands on the cell membrane.

**contact repulsion** Guidance force acting on the leading part (growth cone) of an axon; the force is relayed by the substratum on direct contact rather than by chemical diffusion.

**expression gradient** Graded expression of a molecule (protein, RNA) in a defined compartment of the organism.

**forward and reverse signaling** Traditionally, the signal transduction mechanism originating from activated receptors is referred to as forward signaling. Reverse signaling, a signaling cascade within the ligand-expressing cell, is triggered on receptor binding to membrane-bound ligands.

**plasticity** Ability of a cell, cell body, or organelle (i.e., generally a system) to adapt to a changed environment.

**receptor tyrosine kinase** Transmembrane receptor protein; harbors an enzyme domain with protein tyrosine kinase activity as part of its cytoplasmic portion. Many growth factor receptors are receptor tyrosine kinases.

**SH2 domain** Src homology domain; a protein domain common to many cytoplasmic signal transduction molecules; interacts specifically with a linear epitope containing a phosphorylated tyrosine residue as part of defined consensus sequences.

## Further Reading

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# Epidermal Growth Factor (EGF) Family

Antony W. Burgess
*Ludwig Institute for Cancer Research, Australia*

I. INTRODUCTION
II. EGF RECEPTOR DISCOVERY AND CANCER
III. STRUCTURES OF EGF/TGF-α
IV. BIOSYNTHESIS
V. PHYSIOLOGY OF EGF FAMILY MEMBERS
VI. PATHOLOGY AND TUMORIGENESIS: EGF AND EGF RECEPTOR FAMILY

**The biosynthesis and release of different epidermal growth factor (EGF) family members are important in many physiological and pathological situations. Recent discoveries have linked one of the EGF family members, transforming growth factor-α (TGF-α), and the EGF receptor to the determination of mammalian circadian rhythms. Several hormones that act via G-protein-coupled receptors are known to stimulate the expression of a metalloproteinase that releases (and activates) heparin-binding EGF on the cell surface. Many tumors secrete TGF-α and stimulate tumor cell proliferation and survival by activating the EGF receptors on the same cell. Antagonists of TGF-α action and/or inhibitors of the EGF receptor kinase are potential antitumor agents.**

## I. INTRODUCTION

Modern growth factor research was initiated in the late 1940s when Victor Hamburger reported that a chicken tumor was innervated. He hypothesized that many tumors secreted substances that attract host neurites to the tumor. These concepts led Stanley Cohen to discover epidermal growth factor as a contaminant of nerve growth factor purified from mouse salivary glands. Cohen identified EGF as the protein that accelerated incisor eruption and eyelid opening in newborn mice. Although it took several years to purify the EGF protein, it quickly became obvious that EGF was a potent peptide mitogen for tissue culture cells. The human form of EGF (called urogastrone) was actually isolated from human urine by Harry Gregory in 1975 on the basis of its ability to inhibit the secretion of gastric acid in the stomachs of dogs. Human and mouse EGFs are clearly related, the homology being almost 70% (Fig. 1).

The disulfide bonds are conserved in all EGF family members (suggesting that the fold of the peptide is likely to be similar).

## II. EGF RECEPTOR DISCOVERY AND CANCER

The search for the cell surface receptor for EGF led to some of the first glimpses of growth factor receptor biology and the first direct connection between growth factors and the transforming processes associated with cancer. As soon as Cohen and his colleagues detected the EGF receptor (by the binding of the radiolabeled ligand), it was clear that the ligand-bound receptor was activated (i.e., the tyrosine kinase was stimulated) and internalized quickly.

**SH2 domain** Src homology domain; a protein domain common to many cytoplasmic signal transduction molecules; interacts specifically with a linear epitope containing a phosphorylated tyrosine residue as part of defined consensus sequences.

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```
Human EGF      NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR

Mouse EGF      NSYPGCPSSYDGYCLNGGVCMHIESLDSYTCNCVIGYSGDRCQTRDLRWWELR

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**FIGURE 1** Comparison of the amino acid sequences of human and mouse EGF. As indicated by the asterisks, the two are nearly 70% identical.

When studying the binding of radioiodinated EGF to normal and cancer cells, it became clear that the cell surface EGF receptors were missing from the cancer cells. When oncogenic viruses transform mouse fibroblasts, the EGF receptors disappear from the cell surface. In a set of remarkable observations, Stanley Cohen (the discoverer of EGF and a Nobel Prize winner) and his colleagues linked the induction of receptor disappearance by oncogenic viruses to the induction of a secreted protein that bound to the EGF receptor. At first, this transforming protein was thought to be a unique product of cancer cells [and as a consequence it was named sarcoma growth factor (SGF)], but subsequently, the biological activities attributed to SGF were shown to be due to two separate proteins: transforming growth factor-α and transforming growth factor-β (TGF-β). TGF-α binds to the EGF receptor and initiates activation, down-regulation, and intracellular signaling in the usual way. TGF-β binds to a separate class of cell surface receptors. The signaling from the TGF-β receptors is complex, sometimes synergizing with TGF-α, sometimes inhibiting cell division.

These studies opened the pathway to an understanding of some of the most important biochemical events associated with mitogenic signaling in both normal and cancer cells. As Cohen and his colleagues were investigating the EGF receptor, other cancer biologists were studying the oncogenic proteins associated with the acute transforming viruses of animal cells, such as v-src. It was shown that v-src was actually a constitutively active form of a cellular tyrosine kinase enzyme (c-src). Although serine/threonine kinases had been studied intensely, tyrosine kinases had hardly been recognized, and indeed they are a minor component of the total protein kinase activity of most cells. The EGF receptor was shown to be a ligand-dependent tyrosine kinase. Biological and biochemical studies on the EGF/EGF receptor system have led the way to a profound change in our understanding of the intracellular signaling processes that are important for regulating cell differentiation, division, and survival.

## III. STRUCTURES OF EGF/TGF-α

It required the advent of molecular biological cloning and sequencing techniques to determine the amino acid sequence for TGF-α. Clearly, TGF-α is related to EGF (Fig. 2).

Despite the fact that only 40% of the TGF-α residues are homologous to EGF, all of the disulfide bonds are conserved and the peptides compete for the same high-affinity EGF receptor binding. Although in low abundance, the broad biological distribution and potency of TGF-α suggest that in many tissues it is more likely than EGF to be a physiological ligand of the EGF receptor. Both EGF and TGF-α are synthesized as cell surface transmembrane proteins. Although the cell surface forms are capable of activating receptors, the mitogenic peptides are released from the cell surface by metalloproteases. Until recently, our understanding of the regulation of TGF-α secretion, degradation, and physiology has been extremely limited. In a fascinating study on circadian rhythms, a case has been made that the release, action, and degradation of TGF-α are responsible for both the underlying rhythm and the light responses.

```
Human TGF-α  VVSHFNDCPDSHTQFCFH-GTCRFLVQEDKPACVCHSGYVGARCEHADLLA

Human EGF      NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR


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**FIGURE 2** Amino acid sequence of human TGF-α and EGF, which shows 41% identity.

Once EGF and TGF-α had been cloned, a considerable effort was made to determine their three-dimensional structures by X-ray crystallography; however, both peptides proved difficult to crystallize. Nuclear magnetic resonance (NMR) studies were more successful, and despite the sequence differences and difficulties in achieving high resolution, it was clear that the three-dimensional structures of the different family members were closely related. These structural studies led to an immediate reevaluation of the domain structure of the peptide backbone. Although the primary structure emphasized a three-loop structure based around the disulfide bond assignment, the three-dimensional structures indicated that both EGF and TGF-α have two distinct regions, the N- and C-terminal domains, and that there is only minimal contact between the domains. The N-terminal domain contains a significant length of antiparallel β-sheet. The family of mammalian peptides that bind with high affinity to the EGF receptor has been extended to include heparin-binding EGF (HB-EGF) and betacellulin (Fig. 3).

Leaving aside the disulfide residues, only seven residues are common to all four peptides; the three glycine residues are presumably involved in forming the EGF-like fold. His-16 is not conserved in other mammalian EGFs. Arg-41 and Leu-47 appear to be critical determinants of receptor binding. The three-dimensional structure of the EGF receptor/TGF-α complex has been solved and although many of the TGF-α residues are in contact with the receptor, the residues equivalent to Arg-41 and Leu-47 in EGF clearly form strong interactions with distinct receptor pockets. Crystal structures for EGF, HB-EGF, and TGF-α have now been solved; although there is overall similarity to the NMR structures, some of the details are quite different. Strong interactions with the receptors, which may induce a structural change in the ligand, appear to occur at the N- and C-termini. Overall, the $C^{\alpha}$ atom homology between TGF-α bound to its receptor, EGF free in solution, or HB-EGF bound to diphtheria toxin is extraordinary: the average deviation between these coordinate sets is less than 1 nm.

## IV. BIOSYNTHESIS

All members of the EGF/TGF-α are synthesized as larger precursors. The mitogenic fragments are released after proteolytic processing. The membrane-bound forms of the ligand appear to be important both biologically and as regulatory elements in several tissue systems. Receptor cross talk between G-protein systems and the EGF receptor system can occur when G-protein signaling induces the production of the metalloproteinase, which processes the membrane precursor of HB-EGF. The 160-amino-acid precursor pro-TGF-α is processed at residue 40 to yield the mitogenic TGF-α peptide. The EGF mitogenic peptide is synthesized and stored in the mouse salivary gland; it is associated with a binding protein and the protease that is responsible for cleaving the mitogenic 53-residue form of the growth factor from its 1207-residue precursor. HB-EGF is processed to at least five forms, mostly with different N-terminal cleavage patterns, but there also appear to be several differentially glycosylated forms of HB-EGF. The differential glycosylation may alter the pharmacokinetic properties of this growth factor, facilitating a prolonged action of the factor in different tissues.

## V. PHYSIOLOGY OF EGF FAMILY MEMBERS

Even after 50 years of intense research, the physiology of EGF is still obscure. The situation is not much better for the more recently identified family members such as TGF-α, betacellulin, amphiregulin (AR), Schwannoma-derived growth factor (SDGF), or

```
Human EGF       NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR

HB-EGF          KKRDPCLRKYKDFCIH-GECKYVKELRAPSCICHPGYHGERCHGLSLPVE

Betacellulin    THFSRCPKQYKHYCIH-GRCRFVVDEQTPSCICEKGYFGARCERVDLFY

Human TGF-α   VVSHFNDCPDSHTQFCFH-GTCRFLVQEDKPACVCHSGYVGARCEHADLLA

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**FIGURE 3** Members of the EGF family that bind with high affinity to the EGF receptor. HB-EGF, heparin-binding EGF.

HB-EGF. EGF was isolated originally from the salivary glands of male mice, which have extraordinary concentrations of the growth factor; in contrast, female mice have barely detectable levels of EGF in their salivary glands. Attempts to detect EGF in human and mouse tissues are made difficult by the low levels, although the precursor form (prepro-EGF) is detectable in the kidneys of male mice. Most tissues contain considerably more TGF-α than EGF. Gradually, a paradigm has emerged that in epithelial tissues, e.g., skin and the intestines, where there is a continuous production of cells, the levels of TGF-α control receptor activation and, consequently, the proliferation, differentiation, survival, and movement of cells in these tissues. However, when the TGF-α gene is removed from mice, cell production and differentiation in the self-renewing epithelial tissues are unaffected. In the absence of TGF-α, cell production continues unabated in both the skin and intestines. It is still not clear whether the other EGF family members replace the TGF-α or whether epithelial cell production in these tissues is regulated primarily by other cytokines or hormone systems. Mice that lack three EGF family members, namely EGF, TGF-α and amphiregulin, have been produced; although these triple-knockout mice are smaller than normal, there are no obvious differences in the skin or intestines. They do have impaired responses to injury: for example, when cysteamine is used to induce ulcers in mice, the lesions are more severe in the triple-knockout mice.

The biological actions of particular family members appear to depend on tissue-specific factors and/or the differentiation state of the target cells. AR and SDGF bind weakly to the EGF receptor on both NIH 3T3 and A-431 cells. The mitogenic action of both regulators on these cells is barely detectable (more than 1000-fold less potent), but on PC12 cells, at 100 n*M*, SDGF induces neurite outgrowth. Although AR is not as potent as SDGF or EGF for inducing neurite outgrowth, at 100 n*M*, AR induces differentiation of PC12 cells. Clearly, some of the regulators appear to be more potent as differentiation agents than as proliferative agents.

Interestingly, even when EGF and TGF-α stimulate cells via the EGF receptor, they can induce different effects. It has been determined that after ligand binding, the EGF receptor internalizes and partitions to the endosomes, where the vesicle acidifies. For the TGF-α: EGF receptor complex, acidification is sufficient to dissociate the complex, reduce tyrosine kinase signaling, and facilitate receptor recycling. In contrast, the EGF:EGF receptor complex is resistant to acidification and the ligand–receptor kinase continues to signal in the endosome, leading to the formation of multivesiculate bodies and degradation of both the ligand and receptor. Discovery of some EGF mutants that dissociate from the EGF receptor complex in the acidified endosome has revealed that these mutants block degradation, permit receptor recycling, and prolong signaling, i.e., they appear to be superagonists.

## VI. PATHOLOGY AND TUMORIGENESIS: EGF AND EGF RECEPTOR FAMILY

From the earliest descriptions of growth factor biochemistry, the EGF family members have been linked with particular tissue pathologies and tumorigenesis. Indeed, it was the search for the tumor-derived factors responsible for the innervation of a chicken sarcoma that led directly to the discovery of EGF. During the initial search for EGF receptors, it quickly became evident that many tumors secrete an EGF-like growth factor. Activation of EGF receptors is a critical process in many malignancies. Many of the acute oncogenic retroviruses induce the secretion of TGF-α, which maintains the viability and proliferative state of transformed cells. Inhibitors that interfere with the action of the EGF receptor and/or erbB-2 have promising antitumor effects in animals.

TGF-α is normally secreted at low levels in the epidermis, but there is also a strong association between the overexpression of TGF-α and the development of psoriasis, a chronic skin disease characterized by scaly patches. Excessive levels of TGF-α have also been associated with poor prognosis of some carcinomas, e.g., bladder tumors. Analysis of bladder tumor mRNA for the presence of EGF, amphiregulin, HB-EGF, betacellulin, epiregulin, the EGF receptor, and erbB-2 detected all of the messages except that for EGF. Interestingly, there was an extraordinarily strong correlation between a good prognosis and the presence of epiregulin mRNA (Fig. 4).

A high proportion of many carcinomas express activated, overexpressed, or mutated members of the erbB family of receptors. Indeed all advanced glioblastomas are associated with amplified EGF receptors and more than half of all head and neck cancers overexpress the EGF receptor. Except for the hair follicle, normal tissues do not express high levels of the EGF receptor nor is there any evidence that the EGF receptor is activated in unstimulated normal tissues. In contrast, tumor cells that overexpress the EGF receptor usually contain tyrosine

```
Human EGF            NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR

Epiregulin        VAQVSITKCSSMNGYCLH-GQCIYLVDMSQNYCRCEVGYTGVRCEHFFLTVHQ

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**FIGURE 4** Comparison of the amino acid sequences of human EGF and epiregulin, a member of the EGF family.

phosphorylated receptors, an indication that the receptor is activated and is providing the cells with proliferative and/or survival signals. These observations have prompted the search for therapeutic agents that inhibit the EGF receptor kinase or prevent the receptor from being activated. There is now considerable evidence from mouse xenograft studies that these inhibitors and/or antagonists can reduce the growth of many human tumors. EGF receptor kinase inhibitors and neutralizing antibodies are in early-phase clinical trials, both as single agents and in conjunction with other antitumor cytotoxic drugs. Most recently, there have been reports of lung tumor regressions in patients being treated with the EGF receptor kinase inhibitors. Similarly, some breast cancer patients who overexpress erbB-2 respond to treatment with antibodies (herceptin), which bind to this receptor. As we learn to combine signaling therapeutics, such as the EGF receptor inhibitors, with the conventional anticancer therapies, more effective cancer treatments will be developed.

Several cancers involve the erbB family members indirectly; in particular, tumors with the activated *ras* oncogene secrete ligands for the EGF receptor (e.g., TGF-α). Consequently, inhibitors of the EGF receptor kinase and erbB-2 block the proliferation of many *ras*-associated tumors. Other opportunities to block the action of the EGF receptor in tumor cells are available: for example, there are inhibitors that block kinases [e.g., p21-activated kinase (PAK)] downstream of the EGF receptor. When several points in tumorigenic signal transduction are blocked, the tumor cells are likely to die a rapid apoptotic death. Signaling therapeutics based on the molecular etiology of the tumorigenic processes are more likely to produce better outcomes for cancer patients. When it becomes possible to treat oncogenic lesions or the tumor suppressor lesions in humans, more effective treatments will be developed.

## Glossary

**growth factor** Small peptide that regulates cell production and differentiation in the tissue in which it is produced; often produced in excess by tumor cells.

**knockout mice** Species in which particular genes have been removed by homologous recombination in embryonic stem cells, by blastocyst injection, by transplantation, and by subsequent breeding programs.

**receptor** Cell surface protein in which the extracellular portion binds a growth factor. After formation of the growth factor:receptor complex, the intracellular portion becomes an active enzyme capable of initiating cytoplasmic signaling processes.

**tyrosine kinase** Enzyme with an activity associated with the epidermal growth factor receptor family; using ATP as the phosphate donor, catalyzes the addition of phosphate groups to specific tyrosine residues on protein substrates.

## See Also the Following Articles

**Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor B (VEGF-B)**

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# Epidermal Growth Factor (EGF) Receptor Signaling

Oleg Tikhomirov and Graham Carpenter

*Vanderbilt University*

I. EPIDERMAL GROWTH FACTOR RECEPTOR LIGANDS
II. EGF RECEPTOR STRUCTURE AND ACTIVATION
III. ADAPTOR PROTEINS
IV. Ras/MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY
V. SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION PATHWAY
VI. PHOSPHOINOSITIDE SIGNALS
VII. TRANSACTIVATION
VIII. ENDOCYTOSIS
IX. SUMMARY

**The epidermal growth factor (EGF) receptor is a signal transducer belonging to the tyrosine kinase family of proteins that control cell functions such as growth, migration, and survival. The EGF receptor system, including its ligands and downstream effector molecules, has been well conserved throughout evolution, as genetic studies in flies and worms have demonstrated. The clinical significance of this receptor is evidenced by the development of reagents (antibodies, kinase inhibitors) that block EGF receptor activity in cancer patients. The EGF receptor, also referred to as HER-1 or ErbB-1, is the prototype for a small family of molecules known as the ErbB receptors. This article will concentrate on the EGF receptor in terms of its known ligands, structure, activation mechanism, signal transduction pathways, endocytosis, and transmodulation.**

## I. EPIDERMAL GROWTH FACTOR RECEPTOR LIGANDS

Activation of the epidermal growth factor (EGF) receptor in mammalian cells depends on the presence of one of its specific ligands: EGF, transforming growth factor α, amphiregulin, betacellulin, epiregulin, heparin-binding growth factor (HB-EGF), or epigen (Fig. 1). Each ligand is the product of an independent gene and the major distinction among these growth factors is found in their individual patterns of expression. All members of the EGF ligand family are characterized by six conserved and similarly spaced cysteine residues that form three intramolecular disulfides. All ligands bind to the EGF receptor with approximately equivalent affinity and qualitatively provoke the same biological responses. Of these ligands, HB-EGF, betacellulin, and epiregulin are reported to also bind to ErbB-4, another receptor within the ErbB family (Fig. 1). Also, each ligand is initially expressed at the plasma membrane as a glycosylated membrane-anchored precursor, which is proteolytically cleaved from the cell surface to release the soluble growth factor that acts as an autocrine or a paracrine signal.

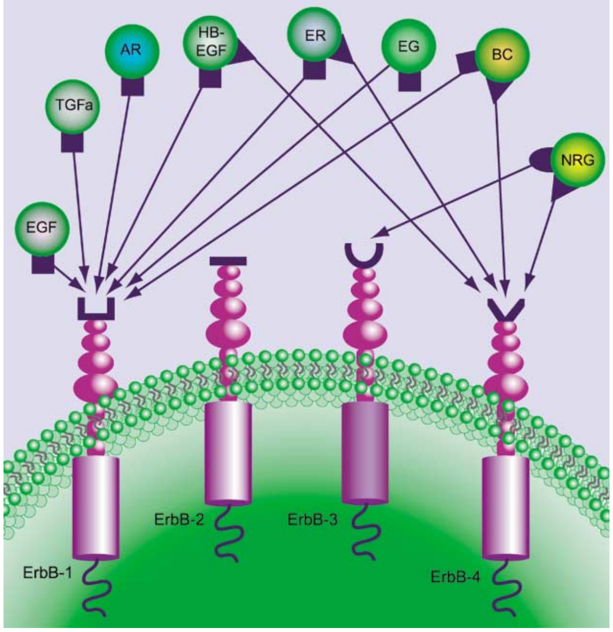

FIGURE 1 The family of ErbB receptors and ligands. The EGF receptor can bind seven different ligands with high affinity. ErbB-2 has no known ligand, ErbB-3 is a receptor with impaired kinase function and can bind neuregulins, and ErbB-4 can bind neuregulin, betacellulin, and HB-EGF. EGF, epidermal growth factor; TGFα, transforming growth factor α; AR, amphiregulin; ER, epiregulin; EG, epigen; HB-EGF, heparin-binding EGF; BC, betacellulin; NRG, neuregulin.

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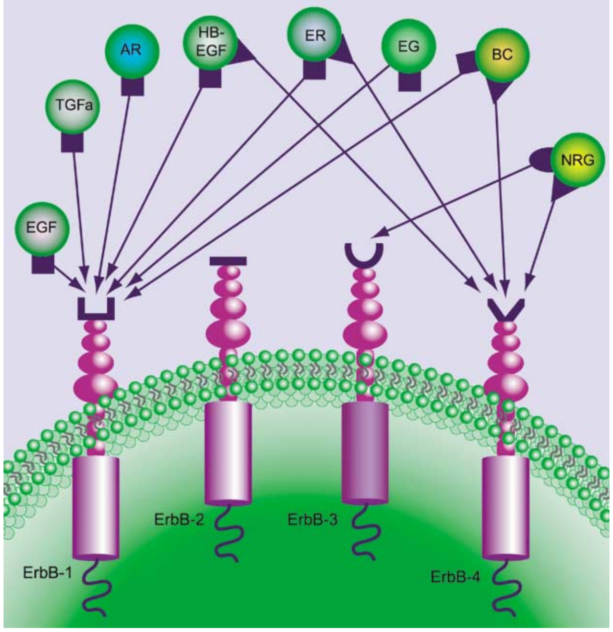

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## II. EGF RECEPTOR STRUCTURE AND ACTIVATION

In mammals, the EGF receptor family comprises four members: EGF receptor (ErbB-1/HER-1), ErbB-2 (HER-2/c-neu), ErbB-3 (HER-3), and ErbB-4 (HER-4) (Fig. 1). All members of the EGF receptor family have similar structural features: each is a Type I transmembrane receptor tyrosine kinase with two cysteine-rich extracellular domains, a single transmembrane domain, an uninterrupted kinase domain, and multiple autophosphorylation sites in a carboxy-terminal domain. Sequence homology is greatest within the kinase domains and least within the carboxy-terminal domains.

Ligand binding to the receptor extracellular domain results in activation of tyrosine kinase function in the cytoplasmic domain. The receptor's kinase activity is essential for biologic responses to cognate ligands because inactivation of the kinase by mutagenesis prevents growth factor activation of signaling pathways and cellular responses. The growth factor neuregulin (also known as heregulin) specifically binds to the ErbB-3 and ErbB-4 receptors (Fig. 1). A ligand for the ErbB-2 receptor has not been identified.

Following ligand binding, the ErbB-1 and ErbB-4 receptors are activated by dimerization between two identical receptors (homodimerization) or between two different ErbB receptors (heterodimerization). Dimerization mediates activation of the kinase domains by facilitating transphosphorylation between receptor monomers. However, the molecular mechanism that promotes dimerization of ErbB receptors is unknown. Although each ligand-binding member of the EGF receptor family (ErbB-1, ErbB-3, and ErbB-4) can participate in homo- or heterodimer formation, ErbB-2 is the preferred heterodimerization partner for each receptor. Therefore, ErbB-2 is considered to be a co-receptor. Heterodimerization between ErbB-2 and other ErbB receptors is thought to explain how ErbB-2 participates in growth responses. As a co-receptor, it may decrease ligand dissociation from a receptor heterodimer, thereby enhancing and/or prolonging the activation of signaling pathways. Alternatively, interaction with ErbB-2 may allow other ErbB receptors to provoke qualitatively unique signaling pathways. Heterodimers containing ErbB-2 usually induce stronger biological activity than do homodimers. In the case of ErbB-3, which has an enzymatically deficient kinase domain, dimerization with ErbB-2 is an essential event for signal production and biological responses.

The EGF receptor extracellular region is composed of four domains. Domains II and IV contain many cysteine residues in the form of disulfide bonds, approximately 25 in total. Domain III is thought to bind directly to EGF, whereas Domain I is secondarily involved in ligand binding.

Many glioblastomas have amplified EGF receptor genes or structural alterations within the gene, such as in-frame deletions. The most prevalent mutation in glioblastomas contains an in-frame deletion resulting in the removal of residues 6 through 273 in the extracellular domain of the EGF receptor. This mutant, designated EGFRvIII/δEGFR, lacks Domain I and a part of Domain II and cannot bind EGF. However, this receptor mutant can form stable dimers in the absence of a ligand and has constitutively enhanced tyrosine kinase activity.

The EGF receptor extracellular domain contains 12 potential sites for N-linked glycosylation. If the EGF receptor is synthesized in the presence of a glycosylation inhibitor, it cannot bind ligands and activate signaling pathways. If N-linked oligosaccharide chains are enzymatically removed from the mature EGF receptor, the altered receptor can bind ligand and activate its kinase. Therefore, glycosylation is important during biosynthesis for proper folding of the receptor within the endoplasmic reticulum to generate a ligand-binding competent conformation.

Ligand-induced receptor dimerization results in tyrosine kinase activation and receptor autophosphorylation at six residues in the carboxy-terminal domain (Tyr992, Tyr 1045, Tyr1068, Tyr1086, Tyr1148, and Tyr1173). However, the stoichiometry of tyrosine phosphorylation is unknown as are the relative levels of phosphorylation at each of the six tyrosine residues. The EGF receptor also contains numerous phosphorylated threonine and serine residues, whose functions are not entirely clear. Unlike many other receptor tyrosine kinases, autophosphorylation sites within the tyrosine kinase or juxtamembrane domains of the EGF receptor have not been described. Interestingly, other tyrosine residues in the EGF receptor are phosphorylated by the Src tyrosine kinase in a cell-specific manner, e.g., Tyr845, Tyr 891, Tyr 920, and Tyr 1101. Phosphorylation of Tyr845 is potentially important due to its location within a putative activation loop of the EGF receptor tyrosine kinase domain.

## III. ADAPTOR PROTEINS

Ligand-induced receptor dimerization and autophosphorylation create phosphotyrosine residues that serve as docking sites for various signal transducing proteins. The key to understanding how these sites initiate postreceptor signaling involves specialized protein motifs known as Src homology 2 (SH2) domains. SH2 domains were first identified as conserved sequences within the noncatalytic region of Src tyrosine kinases and subsequently have been found in a diverse range of proteins. Each SH2 domain is composed of approximately 100 residues and recognizes a phosphotyrosine residue together with adjacent C-terminal residues. This recognition determines the specificity of associations between individual SH2 domains and particular autophosphorylation sites.

Subsequent to the identification of SH2 domains, a second protein domain capable of recognizing phosphotyrosine residues, the phosphotyrosine-binding or PTB domain, was reported. PTB domain recognition of phosphotyrosine, in contrast to SH2 domains, depends on adjacent residues N-terminal to a phosphotyrosine residue. Cytoplasmic mediators that bind to EGF receptor phosphotyrosine residues through SH2 or PTB domains may be either adaptor proteins or enzymes. Adaptors, such as Shc, Grb2, or Crk, have a modular structure consisting of multiple protein-protein and/or protein-phospholipid interaction domains and function as signaling platforms to connect activated receptors with signaling pathways. The adaptor proteins may or may not be tyrosine phosphorylated. In some receptor tyrosine kinases, such as the platelet-derived growth factor receptor, each autophosphorylation site is dedicated to one or two associating proteins through their SH2 or PTB domains. However, in the EGF receptor, this is not the case, as mutagenesis of any single autophosphorylation site fails to abrogate interactions with any SH2/PTB domain-containing protein.

## IV. Ras/MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

Mitogen-activated protein (MAP) kinases have a central role in the control of cell responses to growth factors. Several MAP kinases have been identified as targets of the EGF receptor, among them the extracellular signal-regulated kinases Erk1 and Erk2, the Jun kinase (JNK), the stress-activated p38 kinase, and Erk5. EGF-induced activation of these serine/threonine kinases, particularly Erk1 and 2, serves as a model for signal transmission from the cell surface to the nucleus (Fig. 2).

Following Shc and Grb2 recruitment to the tyrosine phosphorylated EGF receptor, the guanine nucleotide exchange factor Sos, which binds constitutively to Grb2, is recruited to the receptor. Receptor association locates Sos at the cytoplasmic face of the plasma membrane and thereby facilitates its activation of Ras, a constitutive membrane protein, by facilitating the exchange of Ras·GDP to Ras·GTP. The GTP form of Ras interacts with Raf, a serine/threonine protein kinase, and Raf, in turn, activates the dual-specificity kinase MEK. The activation of this kinase results in the phosphorylation of Erk1 and 2 on threonine and tyrosine residues, producing the activated species of Erk1 and 2. MAP kinases phosphorylate substrates on serine/threonine residues in both the cytoplasm and the nucleus. EGF receptor ligands activate Erk1 and 2 very rapidly but transiently. The mechanism by which EGF receptor activation promotes activation of other MAP kinase family members is less well characterized.

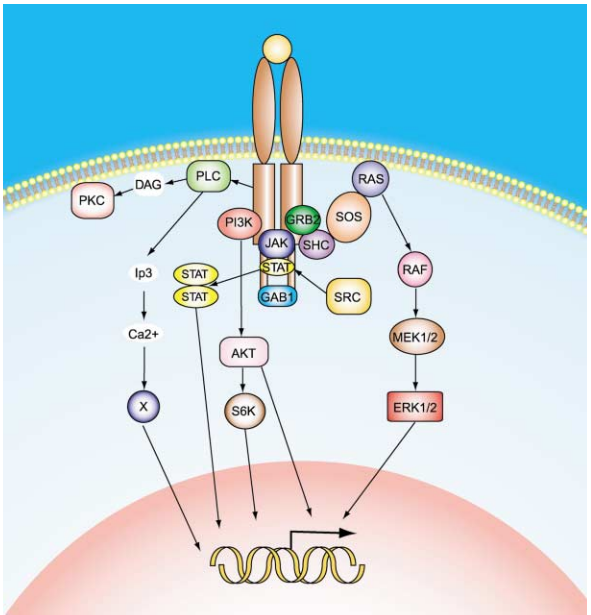

**FIGURE 2** EGF receptor signaling network. EGF receptor dimerization stimulates kinase activation and phosphorylation of numerous substrates. The EGF receptor autophosphorylation provides docking sites within EGFR intracellular region for adaptor proteins, such as Grb2, Shc, and Gab1, plus enzymes, such as PI-3 kinase or PLCγ1, which, in turn, activate different signaling pathways and influence nuclear functions.

Erk1 and 2 substrates include (1) transcription factors, (2) downstream serine/threonine kinases, and (3) other regulatory molecules, such as phospholipase A2. A critical feature of Erk1 and 2 function is the capacity of the activated molecules to translocate to the nucleus and thereby participate in the control of the expression of immediate-early genes by growth factors. For example, a nuclear target of Erk1 and 2 is the transcription factor Elk-1, which participates in the promoter-dependent transcription of c-*fos*.

The Ras/MAP kinase pathway, therefore, represents a multicomponent mechanism whereby a signal generated by EGF at the plasma membrane is received in the nucleus. Most studies indicate that stimulation of the Ras/MAP kinase pathway is essential for EGF-dependent mitogenesis. It is important to keep in mind that EGF receptor activation of Ras is not dedicated to the activation of Erk1 and 2 only. Ras·GTP does communicate with other signaling pathways as well, exemplifying the fact that signal transduction is a network of interlocking pathways.

## V. SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION PATHWAY

STAT (signal transducers and activators of transcription) proteins are a family of transcription factors that are activated by phosphorylation of a single tyrosine residue in response to extracellular ligands (Fig. 2). An active STAT dimer is formed through interactions between the SH2 domain of one monomer and the phosphotyrosine residue of the other monomer. Subsequently, the dimers translocate into the nucleus, recognize specific DNA elements in the promoters of genes, and thereby regulate transcription. In contrast to the Ras/MAP kinase pathway, the STAT mechanism represents a one-component pathway to transfer a signal from the EGF receptor at the plasma membrane to the nucleus. In many cell types, multiple STAT family members are expressed and phosphorylated by EGF. Since activated STATs can heterodimerize as well as homodimerize, the ultimate response is potentially diversified depending on promoter recognition of various STAT dimers.

The tyrosine kinase activity of the EGF receptor is required for STAT phosphorylation and activation. The EGF receptor, at least in A-431 cells, is associated with STAT1, STAT3, and STAT5 in the absence of EGF. Purified EGF receptor has been shown to tyrosine phosphorylate STATs, suggesting that the EGF receptor directly phosphorylates STAT molecules. However, EGF-induced STAT activation is also dependent on the tyrosine kinase Src. This suggests that following EGF stimulation, Src is rapidly recruited to STAT-EGF receptor complexes and the Src kinase then phosphorylates STAT. Pharmacologic inhibitors of Src kinase or a dominant-negative Src construct were shown to abrogate STAT tyrosine phosphorylation stimulated by EGF.

Although the exact role of STATs in the EGF-dependent cell responses is not entirely clear, there is evidence in some cell systems that certain STATs may mediate growth inhibition through the induction of $p21^{waf1}$, a cyclin-dependent kinase inhibitor. In other systems, STATs have been proposed to be necessary for EGF-induced cell proliferation or cell survival.

## VI. PHOSPHOINOSITIDE SIGNALS

A major signaling pathway for the EGF receptor involves the metabolism of phosphoinositide 4,5-bisphosphate (PI4,5-$P_2$). Phosphoinositides, minor phospholipid components of the plasma membrane, are composed of a diacylglycerol group, usually 1-stearyl-2-arachidonylglycerol, linked at the 3-position by a phosphodiester bond to a phosphorylated inositol group. PI4,5-$P_2$ is formed at the cytoplasmic leaflet of the plasma membrane bilayer by the sequential phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate to PI4,5-$P_2$. EGF can affect the fate of PI4,5-$P_2$ in two ways, which are described in the following paragraphs.

Phosphoinositide-specific phospholipase C (PLC) activity catalyzes the hydrolysis of PI4,5-$P_2$ to produce two second messengers, inositol 1,4,5-trisphosphate (Ins$P_3$) and diacylglycerol. $IP_3$ provokes the mobilization of intracellular $Ca^{2+}$, and diacylglycerol activates protein kinase C (Fig. 2). Mammalian cells express members of multiple PLC families designated $\beta$, $\gamma$, $\delta$, and $\epsilon$. Receptor tyrosine kinases, such as the EGF receptor, communicate with PLC-$\gamma$ isoforms, especially PLC$\gamma$1. Three tyrosine residues in PLC$\gamma$1 have been identified as sites of tyrosine phosphorylation. Tyr783 and Tyr1254 are essential for maximal Ins$P_3$ formation, but phosphorylation of Tyr771 seems dispensable. Following ligand stimulation, the EGF receptor is activated and the resulting autophosphorylation sites are recognized by the SH2 domains of PLC$\gamma$1. In contrast to the platelet-derived growth factor receptor, no single autophosphorylation site in the EGF receptor mediates this association with the two SH2 domains of PLC$\gamma$1, and both SH2 domains of PLC$\gamma$1 participate in its association with the EGF receptor, which is a prerequisite for tyrosine phosphorylation of this enzyme.

EGF receptor stimulation induces the rapid, but transient, translocation of PLCγ1 from the cytoplasm to the plasma membrane and especially to a membrane microdomain termed caveolae. The mechanism of membrane association for PLCγ1 is not entirely defined, but the N-terminal pleckstrin homology (PH) domain of PLCγ1, which recognizes phosphorylated phosphoinositides, such as PI3,4,5-$P_3$, may provide a means to localize PLCγ1 at the cytoplasmic face of the plasma membrane. This translocation step is critical, as the substrate for PLC enzyme activity is membrane localized. Although in some cells, such as T cells, it is clear that PLCγ1 activation influences gene expression, this has not yet been demonstrated for EGF-treated cells. However, evidence has been presented to implicate PLCγ1 in EGF-dependent cell migration.

Multiple phosphoinositide-3 (P{I-3) kinases have been identified, which preferentially phosphorylate PI, PI4-P, or PI4,5-$P_2$ at the D-3 position of the inositol ring (Fig. 2). PI-3 kinase (PI-3K) consists of a regulatory subunit, p85, which interacts with activated receptor tyrosine kinases through two SH2 domains, and a catalytic subunit, p110. Although PI-3K can phosphorylate several phosphoinositides, the most significant substrate for signal transduction appears to be PI4,5-$P_2$, the phosphorylation of which generates the second messenger PI3,4,5-$P_3$. This novel phosphoinositide cannot be hydrolyzed by any known phospholipase. Instead, its removal depends on lipid phosphatases, particularly the tumor suppressor PTEN.

PI3,4,5-$P_3$ rapidly accumulates in growth factor-treated cells. In the case of EGF, the activation of PI-3 kinase appears to be cell type-dependent. In some cells, EGF does not provoke detectable increases in PI3,4,5-$P_3$, whereas in other cell types, such as PC-12 cells, it does so rapidly. In the latter case, EGF-dependent activation of PI-3 kinase seems to require the participation of ErbB-3 probably as a consequence of the formation of ErbB-1/ErbB-3 heterodimers. Apparently, ligand-dependent activation of the EGF receptor tyrosine kinase does not produce a phosphotyrosine docking site in the EGF receptor that is recognized by the p85 subunit of PI-3 kinase. However, ErbB-3 has such a docking site, which is produced by transphosphorylation in the context of a dimer with the activated EGF receptor. Alternatively, it is possible that the adaptor protein Gab1 may, when tyrosine phosphorylated by the EGF receptor, mediate the activation of PI-3 kinase.

An important downstream target of PI3,4,5-$P_3$ is protein kinase B (PKB)/Akt, which is phosphorylated and activated after membrane recruitment by both PKB/Akt and the serine/threonine kinase PDK1. Both PKB/Akt and PDK1 use PH domains to facilitate recognition of PI3,4,5-$P_3$ and translocation to the plasma membrane. Activation of PKB stimulates cell survival or anti-apoptotic pathways. The EGF receptor vIII (EGFRvIII) mutation is frequently found in human tumors and has enhanced tumorigenicity and constitutive kinase activity. This EGF receptor mutant produces a low level of activation of the Ras-MAP kinase pathway, yet a high level of PI-3 kinase activity is constitutively present in EGFRvIII-transformed cells. This suggests an important role for PI3,4,5-$P_3$ in EGFRvIII-mediated neoplastic transformation.

## VII. TRANSACTIVATION

In a classical model, the EGF receptor is activated by the binding of its specific ligands. It has now become clear that receptor activation also can be achieved by a wide variety of stimuli that do not bind to the EGF receptor. These stimuli include hormones (e.g., angiotensin, thrombin, and bombesin) that bind to and activate various G-protein-coupled receptors, membrane depolarization (particularly $Ca^{2+}$ fluxes in neuronal cells), adhesion receptors (integrins), cytokine receptors (e.g., growth hormone), and stress agents (e.g., arsenite, UV radiation, γ-irradiation, and oxidants). There seem to be two mechanisms by which these agents activate the EGF receptor. These are described in the following paragraphs, but it should be understood that the mechanisms are not mutually exclusive.

In one mechanism, activation of the EGF receptor is achieved by agents that regulate the production of soluble ligands for the EGF receptor. All EGF receptor ligands are produced as transmembrane precursors located on the cell surface so that the EGF-like domain is within the extracellular domain of the precursor. The available evidence indicates that transmembrane metalloproteases are able to cleave these precursor molecules within the extracellular domain close to the transmembrane domain and thereby produce soluble ligand. Hence, in this mechanism, the point of action for heterologous agents is not the EGF receptor, but rather the proteolytic system that converts growth factor precursors to soluble agonists. Whether this involves signaling pathways that influence the precursor molecules, the protease, or both is not yet clear.

A second mechanism does not involve liberation of soluble ligand but instead depends on the activation of a nonreceptor tyrosine kinase, such as Src or JAK, which in turn phosphorylates the EGF receptor. Novel Src-dependent tyrosine phosphorylation sites on the EGF receptor have been reported and in many cases these lie within the kinase domain of the EGF receptor. This phosphorylation may increase the catalytic activity of the EGF receptor kinase domain and/or create docking sites for SH2/PTB domain-containing adaptors and enzymes as previously described.

Regardless of the exact mechanism, transactivation of the EGF receptor seems to be a mechanism by which a variety of external agents or stimuli influence mitogenic pathways. To date, nearly all transactivation studies have been performed in cultured cells and it remains to be determined whether this represents a significant biological process in the animal.

## VIII. ENDOCYTOSIS

Following ligand addition, the EGF receptor is rapidly endocytosed (Fig. 3). The most likely function of endocytosis is to remove activated ligand-receptor complexes from the plasma membrane and thereby attenuate the propagation of signaling pathways. Although internalization does not immediately deactivate the EGF receptor, these complexes are ultimately processed to the lysosome, where both receptor and growth factor are degraded. The net result of this receptor trafficking pathway is to substantially decrease the number of receptors present on the plasma membrane, a process termed down-regulation.

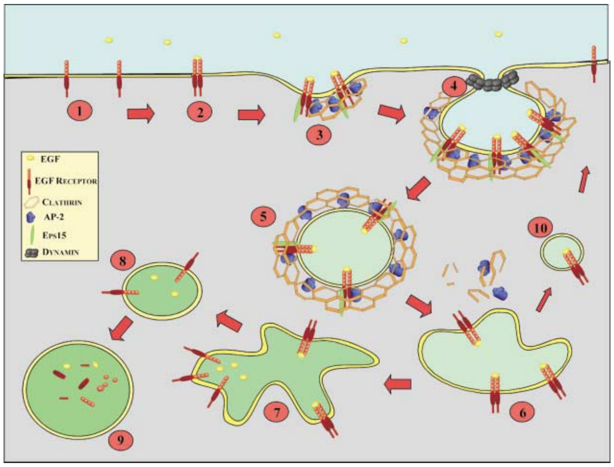

**FIGURE 3** Model of EGF receptor endocytosis. In response to ligand binding (1), EGF receptor molecules dimerize (2) and bind Eps15, AP-2, and other proteins that localize the EGF receptor to clathrin-coated pits (3). It is not clear whether the EGF receptor is targeted to preformed coated pits or whether components of the coated pit are assembled around receptor dimers. Dynamin vesiculates the coated pit (4), which results in clathrin-coated vesicle formation (5). These vesicles mature into early endosomes, which involves removal of clathrin and other components (6). Early endosomes mature to multivesicular bodies (7). Although a minor fraction of internalized EGF receptors are recycled to the cell surface (10), most receptors degraded as late endosomes (8) fuse with lysosomes (9).

The EGF receptor is internalized by a mechanism that requires receptor kinase activity and ill-defined sequence motifs in the carboxy-terminal domain of the receptor. Certain tyrosine phosphorylation substrates, such as Eps15, are thought to be factors that recruit EGF receptors to the endocytic pathway. Eps15 is rapidly phosphorylated by the activated EGF receptor and is required for receptor internalization through clathrin-coated pits. Following ligand binding, Eps15 translocates to the plasma membrane and localizes to the rim of coated pits, ubiquitous structures that mediate internalization of activated EGF receptors and a variety of other receptors, such as the transferrin receptor. Although routes of internalization other than coated pits are known for some receptors, there is no evidence for entry of EGF receptor into cells other than through coated pits.

The major components of coated pits are clathrin, AP-2, and dynamin, plus the numerous proteins that associate with these components (Fig. 3). Activated receptors, having appropriate sorting signals for entry into and retention by coated pits, are then concentrated in coated pits, and following pit invagination, they are delivered into the cytoplasm in the form of clathrin-coated vesicles, termed endosomes. However, the specific mechanism by which activated EGF receptors are sorted to coated pits is unknown, but seems to involve association with AP-2 and the tyrosine phosphorylation of Eps15. The latter process seems to be relatively specific for the EGF receptor, as Eps15 mutants that cannot be tyrosine phosphorylated block internalization of EGF receptors but not transferrin receptors.

The GTPase dynamin is required for the formation of coated vesicles from coated pits. Dynamin binds to membrane-embedded phosphoinositides in coated pits through its PH domain to form helical tubes. Dynamin then constricts and fragments membrane tubules. Expression of dominant-negative dynamin mutants blocks EGF receptor internalization.

Following internalization through coated pits, the EGF receptor is sorted into early and late endosomes prior to lysosomal delivery. Once it reaches the late endosomes, the EGF receptor is either targeted to lysosomes or recycled to the cell surface. In normal circumstances, the majority of EGF receptors are targeted to lysosomes, with a minor fraction recycled. Sorting between endosomal compartments appears to be independent of receptor kinase activity; rather, occupancy-induced conformational changes in the receptor are thought to be required.

Studies with native and chimeric ErbB receptors have revealed that although the ligand-occupied EGF receptor is rapidly internalized and degraded, all other members of this family are only slowly internalized at a rate equivalent to a kinase-negative EGF receptor. Overexpression of ErbB-2, which is commonly observed in many human cancers, retards lysosomal targeting and down-regulation of the EGF receptor. This appears to be due to the fact that heterodimers are inefficiently recognized by either the coated pit sorting proteins or subsequent intracellular sorting machinery. Dimerization of the EGF receptor with ErbB-2, therefore, may enhance signaling through the EGF receptor pathway by reducing endocytosis and down-regulation.

It remains an open question as to whether endocytosis is also a mechanism to initiate or propagate signaling pathways that influence cellular responses to EGF receptor ligands. The bulk of the evidence suggests that although postreceptor signaling components are found in endosomes together with internalized EGF receptors, these localizations are more related to ending signaling events than to initiating these events.

## IX. SUMMARY

The EGF receptor is specifically activated by the binding of cognate ligand at the cell surface. Ligand binding promotes receptor dimerization and activation of receptor tyrosine kinase activity. As a consequence, receptor autophosphorylation creates docking sites that mediate tyrosine phosphorylation of cellular proteins and activation of signal transduction pathways, which relay biochemical information from the plasma membrane to the nucleus to provoke changes in cellular growth.

## Glossary

**adaptors** Proteins composed of protein-protein and/or protein-phospholipid interaction domains, but without an enzymatic activity.

**dimerization** The process by which two related proteins become physically associated.

**endocytosis** The process by which molecules on the surface of cells are brought into the cell and then degraded or recycled back to the cell surface.

**epidermal growth factor receptor** A cell surface transmembrane protein that specifically binds cognate ligands and transduces a biologic response, usually related to the control of cell proliferation.

**G-protein** A low-molecular-weight protein that binds GTP in its active state and GDP in its inactive state and affects the activation of other proteins.

**growth factors** Polypeptides that act to stimulate cell proliferation by binding to a receptor.

**phosphoinositides** Plasma membrane-localized phospholipids that have a regulatory function as opposed to a structural function in the membrane.

**phospholipase C** An enzyme that hydrolyzes a particular polyphosphoinoside to produce two second-messenger molecules.

**protein kinase** An enzyme that transfers a phosphate group from ATP to the side chain of a tyrosine or serine/-threonine residues in a protein.

**transcription factor** A protein that regulates the transcription of a gene by interacting with DNA regulatory elements, directly or indirectly.

## See Also the Following Articles

**Angiotensins • Crosstalk of Nuclear Receptors with STAT Factors • Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • Heterotrimeric G-Proteins • Protein Kinases**

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# Eplerenone, Pharmacological Effects of

Amy E. Rudolph, Ricardo Rocha, and
Ellen G. McMahon

*Pharmacia Corporation, Peapack, New Jersey and
St. Louis, Missouri*

I. INTRODUCTION
II. MINERALOCORTICOID RECEPTOR SELECTIVITY
III. ANTIHYPERTENSIVE EFFECTS
IV. END-ORGAN PROTECTION
V. POTENTIAL MECHANISM OF ACTION
VI. SUMMARY

**For many years it has been acknowledged that activation of the renin-angiotensin-aldosterone system (RAAS) is associated with deleterious outcomes in patients with congestive heart failure and hypertension. Because RAAS is the fundamental mechanism in the body responsible for blood pressure and volume homeostasis, a number of commonly prescribed medications for hypertension and heart failure target this system. Angiotensin-converting enzyme inhibitors block conversion of inactive angiotensin I peptide to the active vasoconstrictor molecule angiotensin II. Angiotensin receptor blockers block angiotensin II type 1 receptors. An additional mechanism for RAAS modulation has emerged based on blockade of aldosterone binding to its cognate receptor, the mineralocorticoid receptor.**

## I. INTRODUCTION

The classical mineralocorticoid effect of aldosterone on unidirectional transepithelial sodium transport in the kidney was long thought to be the predominant cardiorenal effect of this hormone. However, there is now convincing evidence that aldosterone mediates significant deleterious cardiovascular effects via activation of mineralocorticoid receptors (MR) localized outside the kidney-namely, in the heart, vasculature, and brain. It is now postulated that many of the detrimental effects of aldosterone are mediated through MR activation in these nonclassical target tissues.

The most direct and compelling evidence that aldosterone can negatively impact the cardiovascular system was provided by the Randomized Aldactone Evaluation Study (RALES) trial in heart-failure patients (New York Heart Association Class III/IV). In this study, when the nonselective mineralocorticoid receptor antagonist spironolactone was added to standard therapy for congestive heart failure [in most patients angiotensin-converting enzyme inhibitor (ACE-I), diuretic/digitalis], a 30% mortality benefit was achieved. Although the mortality benefit with spironolactone is clear, clinically significant endocrine side effects such as impotence, gynecomastia, menstrual irregularities, and breast pain as a result of limited MR selectivity associated with spironolactone prohibit widespread use of this agent.

Eplerenone (other names include SC-66110, CGP-30 083, and epoxymexrenone) is the first agent to block the mineralocorticoid receptor with a high degree of selectivity and is under development for human therapeutic use in the treatment of hypertension and heart failure postmyocardial infarction. Clinical and preclinical studies have linked elevated aldosterone to high blood pressure, cardiac hypertrophy, cardiac and vascular fibrosis, and increased risk of mortality in patients with congestive heart failure. Preclinical studies indicate that eplerenone is a selective MR antagonist that effectively attenuates the deleterious effects of aldosterone, displays antihypertensive properties, and provides end-organ protective effects in experimental models of hypertension and heart failure independent of blood pressure lowering.

## II. MINERALOCORTICOID RECEPTOR SELECTIVITY

The affinity of eplerenone for human steroid receptors was quantitated *in vitro* using a transcriptional transactivation assay in which the ability of eplerenone to antagonize the aldosterone transcriptional response at human MR was evaluated. Eplerenone

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AMY E. RUDOLPH, RICARDO ROCHA, AND
ELLEN G. MCMAHON

*Pharmacia Corporation, Peapack, New Jersey and
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## II. MINERALOCORTICOID RECEPTOR SELECTIVITY

The affinity of eplerenone for human steroid receptors was quantitated *in vitro* using a transcriptional transactivation assay in which the ability of eplerenone to antagonize the aldosterone transcriptional response at human MR was evaluated. Eplerenone inhibits human MR transcriptional activation by aldosterone in a concentration-dependent manner with a calculated $IC_{50}$ of 291 n*M*. In addition to binding the MR, the binding of eplerenone to other steroid receptors (androgen, glucocorticoid, and progesterone) isolated from appropriate tissues was also measured using [$^3$H]methyltrienolone for androgen receptors isolated from rat ventral prostate, [$^3$H]dexamethasone for glucocorticoid receptors isolated from rat kidney, and [$^3$H]progesterone for progesterone receptors isolated from rabbit uteri. Binding of eplerenone to these steroid receptors was compared to the nonselective mineralocorticoid receptor antagonist, spironolactone. As shown in Table 1, eplerenone binds MR with approximately 20-fold lower affinity compared to spironolactone. However, compared to spironolactone, eplerenone is devoid of appreciable binding to androgen or progesterone receptors and binds with lower affinity to glucocorticoid receptors. Despite the reduced binding affinity of eplerenone for MR compared to spironolactone, eplerenone has approximately 2-fold greater oral potency ($ED_{50} = 0.8$ vs 1.7 mg/kg) as an aldosterone antagonist at the renal mineralocorticoid receptor in adrenalectomized rats. These disparate findings may be attributed to the reduced plasma protein binding of eplerenone as compared to spironolactone. *In vitro* binding studies demonstrated that the addition of 5 or 20% rat plasma did not alter the $IC_{50}$ calculated for eplerenone binding to isolated rat colon MR. In contrast, the $IC_{50}$ value calculated for spironolactone increased by 2.5-fold in the presence of 20% rat plasma. Thus, eplerenone selectively binds MR and exhibits similar *in vivo* potency compared to spironolactone.

**TABLE 1 Relative Binding Affinities of Eplerenone and Spironolactone for Steroid Receptors *In Vitro*[a]**

| Receptor (standard) | Eplerenone | Spironolactone |
|---|---|---|
| Mineralocorticoid (aldosterone) | 0.005 ± 6.5%<br>($n = 7$) | 0.11 ± 36.4%<br>($n = 7$) |
| Androgen (methyltrienolone) | 0.0000076 ± 26.3%<br>($n = 6$) | 0.009 ± 16.5%<br>($n = 7$) |
| Glucocorticoid (dexamethasone) | 0.00018 ± 16.1%<br>($n = 4$) | 0.0018 ± 22.2%<br>($n = 4$) |
| Progesterone (progesterone) | <0.00005<br>($n = 5$) | 0.007 ± 11.0%<br>($n = 5$) |

[a] Affinities expressed as a fraction of the binding affinity of the standard ligand at each receptor. Binding affinity of the standard ligand is set at 1. Standard error mean reflects the percentage of the mean. Data reproduced from de Gasparo *et al.* (1987), with permission.

## III. ANTIHYPERTENSIVE EFFECTS

*In vivo*, circulating glucocorticoids are in vast excess of mineralocorticoids (100- to 1000-fold); however, both hormones bind MR with similar affinity. The enzyme 11-β-hydroxysteroid dehydrogenase 2 (11-βHSD2) confers MR specificity for aldosterone by metabolizing endogenous glucocorticoids to their inactive 11-dehydro derivatives. Glycyrrhizic acid (GA), an established inhibitor of 11-βHSD2 found in licorice, enables endogenous glucocorticoids to activate MR, resulting in excessive sodium retention and severe hypertension. The antihypertensive properties of eplerenone were evaluated in rats with elevated blood pressure resulting from 21 days of GA administration [control systolic blood pressure (SBP), 142 ± 8 mmHg; GA SBP, 185 ± 9 mmHg). Two weeks of treatment with eplerenone lowered blood pressure to normal levels (143 ± 9 mmHg), restored impaired endothelial-dependent relaxation in response to acetylcholine (control, 73 ± 6%; eplerenone, 97 ± 3%), and normalized the 50% decrease in vascular nitrate levels induced by GA and the 2.5-fold elevation of aortic endothelin-1 levels associated with this form of hypertension. Thus, eplerenone normalizes blood pressure and improves endothelial function in a model of hypertension produced by overstimulation of MR with corticosteroids.

The antihypertensive effect of eplerenone exhibited in experimental models has also been demonstrated in clinical studies. A dose-dependent reduction in systolic and diastolic blood pressure (DBP) has been demonstrated in mildly and moderately hypertensive patients [DBP, $\geq 95$ mmHg and $< 114$ mmHg by cuff, and mean 24-h ambulatory blood pressure monitoring (ABPM) DBP $\geq 85$ mmHg] without accompanying endocrine side effects. A group of 417 patients was randomized to receive placebo, eplerenone (either 25, 50, or 200 mg twice daily or 50, 100, or 400 mg daily), or spironolactone (50 mg twice daily). Within the eplerenone-treated groups, adjusted mean changes from baseline (SBP/DBP) at week 8 were as follows: seated, $-4.4$ to $-15.0/-4.4$ to $-8.9$ mmHg; 24-h ABPM, $-6.2$ to $-16.1/-4.1$ to $-9.0$ mmHg. Within the spironolactone-treated group, adjusted mean changes from baseline at week 8 were as follows: seated, $-16.7/-9.5$ mmHg; 24-h ABPM, $-15.8/-8.7$ mmHg. At daily doses of 50 to 400 mg, eplerenone dose-dependently reduced blood pressure compared to placebo ($P \leq 0.01$) and to a similar extent compared to spironolactone.

## IV. END-ORGAN PROTECTION

Clinical studies have clearly demonstrated a positive correlation between plasma aldosterone levels and stroke, renal dysfunction, left ventricular hypertrophy, and decreased vascular compliance. The detrimental action of aldosterone on these target organs has been further elucidated using genetic and experimental models of hypertension showing that inappropriately high levels of aldosterone induce severe vascular injury in the brain, kidney, and heart. Preclinical studies have demonstrated that pharmacological antagonism of aldosterone or adrenalectomy markedly attenuates tissue and vascular damage and associated inflammation.

### A. Brain

The cerebrovascular protective effects of eplerenone were evaluated in stroke-prone spontaneously hypertensive (SHRSP) rats. Saline-drinking SHRSP rats receiving vehicle developed stroke signs and died by 18 weeks of age. In contrast, eplerenone-treated littermates exhibited no signs of stroke until 18 weeks of age, at which point only one of the seven eplerenone-treated animals developed stroke and died. Histopathologic analysis of cerebral tissue from vehicle-treated animals revealed severe cerebrovascular injury with hallmark features of stroke, including liquefaction necrosis, fibrinoid necrosis of cerebral vessels, and focal hemorrhages. Eplerenone-treated rats, however, demonstrated a marked attenuation of cerebrovascular lesions accompanied by stroke prevention and reduced mortality, which was not associated with decreased arterial blood pressure. Cerebral protection was evidenced using semiquantitative injury scoring (vehicle, 3.1 ± 0.4; eplerenone, 0.8 ± 0.5; $P < 0.05$, scored from 0 to 4). These findings are consistent with the previously demonstrated cerebral protective effects of spironolactone and suggest a role for endogenous aldosterone in the development of stroke in rats with severe spontaneous hypertension.

### B. Kidney

The impact of eplerenone on hypertension-induced renal vascular damage was evaluated in experimental rat models of spontaneous and induced hypertension. In saline-drinking, hypertensive SHRSP rats, eplerenone prevented the development of proteinuria (vehicle, 85 ± 11 mg/day; eplerenone, 16 ± 3 mg/day) and renal arteriopathy (vehicle, 34.2 ± 4.1%; eplerenone, 1.3 ± 0.6%; calculated as the number of arteries and arterioles with lesions per 100 glomeruli) without appreciably influencing blood pressure (SBP vehicle, 227 ± 4 mmHg; eplerenone, 219 ± 6 mmHg). Therefore, eplerenone protects renal vasculature via mechanisms independent of blood pressure reduction.

Angiotensin II has long been identified as the key mediator of hypertension-induced renal tissue and vascular injury, whereas the deleterious effects of aldosterone have not been comprehensively characterized. Therefore, the role of angiotensin II (AII) and aldosterone in the renal damage that accompanies hypertension in SHRSP rats was evaluated. Saline-drinking SHRSP rats were administered the ACE inhibitor captopril, captopril in the presence of AII, or captopril in the presence of AII and eplerenone. Captopril afforded marked protection against glomerular and renal vascular damage and proteinuria (Table 2). AII infusion in captopril-treated rats increased plasma aldosterone and, as expected, reversed the protection from ACE inhibition. In contrast, the pathology induced by AII infusion in captopril-treated rats was substantially attenuated by treatment with eplerenone in the absence of

**TABLE 2 Renal Lesion Scores for Captopril-treated, AII-Infused, Saline-Drinking SHRSP Treated with Vehicle or Eplerenone**[a]

| Treatment | Glomerular damage (%) | Renal arteriopathy (% of glomeruli) |
|---|---|---|
| Captopril ($n = 10$) | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Captopril + AII ($n = 7$) | 14.9 ± 2.7[b] | 15.9 ± 2.2[b] |
| Captopril + AII + eplerenone ($n = 7$) | 3.2 ± 0.7[b,c] | 3.6 ± 1.1[b,c] |

[a] AII, Angiotensin II; SHRSP, stroke-prone spontaneously hypertensive rats. Values are mean ± standard error mean. Glomerular lesions were expressed as a percentage of the total number of glomeruli present per midcoronal section examined. Vascular damage was expressed as the number of arteries and arterioles with lesions per 100 glomeruli. Captopril dose was 50 mg/kg/day; AII dose was 25 ng/min; eplerenone dose was 100 mg/kg/day. Data from Rocha *et al.* (1998b).

[b] Significantly different from captopril treatment.

[c] Significantly different from captopril + AII treatment.

antihypertensive effects. These findings demonstrate that hypertension-induced renal pathology in this model is mediated through the renin-angiotensin-aldosterone system, and aldosterone, in addition to AII, appears to be a primary mediator of renal tissue damage.

The mechanisms of aldosterone-driven renal vascular injury were evaluated in uninephrectomized rats receiving salt and chronic aldosterone treatment in the presence or absence of eplerenone. Aldosterone/salt-treated rats developed severe hypertension, albuminuria, vascular damage, and renal lesions compared to rats receiving vehicle. Severe renal arteriopathy was accompanied by glomerular and tubular damage, marked perivascular inflammation, and elevated message levels for the cytokines osteopontin (OPN, ~18-fold), monocyte chemoattractant protein-1 (MCP-1, ~3-fold), interleukin-1β (IL-1β, ~2-fold), and interleukin-6 (IL-6, ~7-fold). Eplerenone significantly attenuated, although did not completely normalize, systolic blood pressure in rats treated with aldosterone/salt for 28 days (vehicle, 131 ± 4 mmHg; aldosterone/salt, 220 ± 4 mmHg; eplerenone, 187 ± 7 mmHg) and dramatically reduced renal vascular injury (vehicle, 0 ± 0; aldosterone/salt, 3.7 ± 0.2; eplerenone, 0.5 ± 0.3; scored from 0 to 4) and the concomitant inflammatory response. Thus, elevated aldosterone in combination with salt may be an important mechanism involved in the onset and development of hypertension-induced renal vascular disease.

## C. Heart

Activation of the local renin-angiotensin-aldosterone system is known to occur during myocardial ischemia/reperfusion and elevated levels of aldosterone may negatively impact myocardial function under these conditions. The effect of acute exposure to aldosterone on functional recovery from an ischemia/reperfusion challenge and the ability of eplerenone to block the negative effects of aldosterone exposure were examined in a Langendorff isolated rat heart preparation. Hearts were perfused with vehicle, aldosterone, or aldosterone in the presence of eplerenone, and contractile recovery following ischemia/reperfusion was quantitated by monitoring left ventricular developed pressure (LVDP). Exposure to 1.39 n*M* aldosterone reduced the magnitude of recovery following ischemia/reperfusion challenge from 41.8 ± 3.1% of preischemia LVDP in vehicle-treated animals to 28.1 ± 3.4% in aldosterone-treated rats. However, the recovery response of hearts perfused with aldosterone in the presence of 1.13 μ*M* eplerenone was similar to that of vehicle-treated hearts (39.0 ± 5.9%, Fig. 1). Thus, eplerenone effectively blocks the negative effects of exogenous aldosterone following myocardial ischemia/reperfusion in the isolated rat heart.

In addition to the functional benefit afforded by eplerenone in isolated rat hearts exposed to aldosterone, myocardial protective properties of eplerenone have also been demonstrated *in vivo*. Myocardial infarction initiates adaptive tissue remodeling, which is initially reparative but evolves into a maladaptive sequela of events exemplified by reactive myocardial fibrosis and left ventricular dilatation. The effect of MR antagonism on these processes was evaluated in rats administered eplerenone or vehicle immediately following permanent ligation of the left coronary artery. Infarct healing and left ventricular remodeling were evaluated at 3, 7, and 28 days postmyocardial infarction (post-MI).

Eplerenone did not affect scar collagen deposition or infarct expansion as evidenced by a similar collagen volume fraction (CVF) in the infarcted myocardium and thinning ratio, respectively. At 7 days post-MI, the CVF was calculated at 47.1 ± 4.4 for vehicle-treated animals and 48.3 ± 4.6 for rats administered eplerenone; the thinning ratio was 0.42 ± 0.04 for vehicle-treated animals versus 0.50 ± 0.06 in eplerenone-treated rats. Moreover, a protective effect of eplerenone was demonstrated at 28 days post-MI; i.e., reactive fibrosis in the viable myocardium was elevated in vehicle-treated animals, but not in eplerenone-treated animals compared to

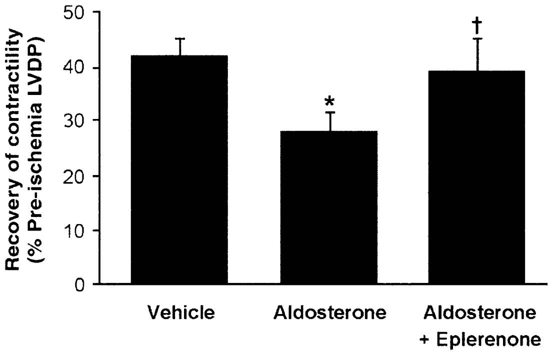

FIGURE 1 Effects of aldosterone and aldosterone plus eplerenone on the recovery of myocardial contractility measured 40 min after initiation of reperfusion and expressed as percent recovery from baseline of left ventricular developed pressure (LVDP) in an isolated heart model of ischemia and reperfusion. *, $p < 0.05$ vs vehicle. †, $p < 0.05$ vs eplerenone + aldosterone.

sham rats (CVF, vehicle, 3.2 ± 0.4%; eplerenone, 2.1 ± 0.4%; sham, 1.65 ± 0.2%). Thus, mineralocorticoid receptor antagonism with eplerenone does not retard infarct healing when initiated within 24 h of infarction in the rat. In addition, eplerenone administration protects against reactive fibrosis in the left ventricular viable myocardium.

The temporal relationship between molecular changes and myocardial injury in response to aldosterone/salt treatment was evaluated in rat cardiac tissue following 1, 2, or 4 weeks of exposure to exogenous aldosterone and salt. No significant increases in interstitial collagen fraction were detected throughout the study. However, histopathologic analysis in coronary arteries revealed severe vascular inflammatory lesions associated with leukocyte infiltration. Coronary injury was evident starting at 2 weeks and extended occasionally to the surrounding myocardium, resulting in focal ischemic/necrotic changes. Vascular lesions were preceded by the progressive up-regulation of cyclooxygenase 2 (COX-2, up to 4-fold), macrophage MCP-1 (up to 4-fold), and OPN (up to 13-fold) mRNA. Eplerenone reduced focal necrosis, myocardial vascular inflammation, and attenuated cardiac cytokine expression.

## V. POTENTIAL MECHANISM OF ACTION

In addition to the antihypertensive effects of eplerenone demonstrated in preclinical and clinical studies, substantial end-organ protection has been observed in experimental models. Even in the absence of blood pressure reduction, eplerenone attenuates the progressive development of vascular inflammation and subsequent maladaptive remodeling in end organs, resulting in protection of the brain, kidney, and heart. Indeed, selective aldosterone blockade by eplerenone attenuates both the inflammatory cellular infiltration and the elevated cytokine expression that exemplify vascular inflammation in these target organs. Thus, current dogma is evolving from the classic paradigm of aldosterone modulation of sodium and water homeostasis to include a role for this steroid hormone as a catalyst for vascular inflammation and subsequent progressive tissue damage.

## VI. SUMMARY

Eplerenone represents the first selective aldosterone blocker with proven antihypertensive efficacy in preclinical and clinical settings. End-organ protection has also been clearly demonstrated in experimental models, potentially via attenuation of the initial vascular inflammatory response and subsequent pathophysiology. Although animal models are invaluable tools for the dissection and understanding of eplerenone pharmacology, they represent only approximations of the complexity of human pathophysiology. The question remains if these protective effects translate to human disease states, a postulate that is now being carefully evaluated in clinical studies.

## Glossary

**aldosterone** The principal mineralocorticoid; produced by the zona glomerulosa of the adrenal cortex, aldosterone facilitates potassium exchange for sodium in the distal renal tubule, causing sodium reabsorption and potassium and hydrogen loss.

**angiotensin II** A potent vasoconstrictor peptide that stimulates aldosterone synthesis and secretion from the adrenal zona glomerulosa and participates in the regulation of local and systemic hemodynamic regulation.

**mineralocorticoid receptor** A member of the steroid/thyroid/retinoid/orphan receptor family of nuclear transactivating factors.

**proteinuria** Elevated urinary protein as a result of renal dysfunction.

**renin–angiotensin–aldosterone system** The primary mechanism by which blood pressure and volume are maintained; prolonged activation of this system is associated with deleterious outcomes in patients with congestive heart failure and hypertension.

**stroke-prone spontaneously hypertensive rats** A genetic model of spontaneous hypertension accompanied by end-organ damage, including cardiac hypertrophy and renal dysfunction, with mortality primarily resulting from stroke.

## See Also the Following Articles

**Angiotensins • Angiotensin II Receptor Signaling • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoids and Hypertension • Mineralocorticoid Receptor, Natural Mutations of**

## Further Reading

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# ER

See ***Estrogen Receptor***

# Erythropoietin, Biochemistry of

Kelly Wong, Peter Tsai, Armin Jegalian,
Nathalie Kertesz, Robert Lee, and Hong Wu
*University of California, Los Angeles*

I. INTRODUCTION
II. BIOCHEMICAL PROPERTIES OF EPO
III. EPO LEVELS AND ITS REGULATION
IV. EPO AND EPOR: SIGNAL TRANSDUCTION
V. ANIMAL MODELS FOR EPO FUNCTION
VI. CLINICAL APPLICATIONS
VII. EPO FUNCTION IN OTHER LINEAGES
VIII. SUMMARY

**Erythropoeitin (EPO) is a cytokine that stimulates erythropoiesis, the formation of red blood cells. The mature EPO protein consists of 166 amino acids; its predicted tertiary structure is an anti-parallel bundle of four α-helices. The primary site of EPO production is the kidney. In addition to tissue-specific control, regulation of EPO production occurs mainly at the transcriptional level, and the production of EPO can be induced under anemic and hypoxic conditions.**

## I. INTRODUCTION

Oxygen is essential for life. The oxygen consumption of the human body varies based on an individual's metabolic demand and can be limited by the environmental oxygen concentration. Early observations provided links between symptoms such as fatigue, shortness of breath, and cardiac palpitations and either low oxygen tension or anemia. Further studies showed that low oxygen tension resulted in an increased number of red blood cells, the cells responsible for the delivery of oxygen to systemic organs and tissues.

The red blood cell was the first identified blood cell type, and its oxygen-carrying function by hemoglobin was observed by Hoppe-Seyler in the 19th century. However, the regulation of red blood cell formation, or erythropoiesis, remained unknown until 1906 when Carnot and Deflandre first introduced the idea of a humoral mediator for erythropoiesis. Almost 50 years later, Erslev and Reissmann formally tested this hypothesis experimentally by injecting serum from anemic rabbits into normal rabbits and noting a significant increase of red blood cells in the recipient animals. They further proved this hypothesis by using a parabiotic model in rats to demonstrate a circulating erythropoiesis-stimulating factor, now known as erythropoietin (EPO).

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Biochemical characterization of EPO became possible only after Goldwasser and colleagues obtained

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## II. BIOCHEMICAL PROPERTIES OF EPO

Biochemical characterization of EPO became possible only after Goldwasser and colleagues obtained partially purified protein from the urine of anemic patients in 1977. The molecular characterization of EPO was further advanced when Lin *et al*. and Jacobs *et al*. cloned the cDNA encoding human EPO in 1985. The human protein consists of a 193-amino-acid sequence with a 27-residue signal leader sequence, resulting in a 166-amino-acid mature protein (Fig. 1). The protein contains two disulfide linkages and multiple glycosylation sites. Sequence analysis indicates that EPO belongs to a family of cytokines, including growth hormone, prolactin, colony-stimulating factor, and the interleukins. The predicted EPO tertiary structure is an anti-parallel bundle of four α-helices, structurally analogous to the growth hormone molecule. The molecular weight of mature EPO is approximately 30.4 kDa with 40% of its mass derived from carbohydrates. As shown in Fig. 1, the mature protein contains one O-linked glycosylation site (Ser-126) and three N-linked glycosylation sites (Asn-24, Asn-38, and Asn-83). Different carbohydrate structures can be found on the same glycosylation site: e.g., the N-linked chains may contain two, three, or four branches, each ending with a sialic acid molecule. The O-linked site may contain zero to two sialic acid molecules. This posttranslational modification gives rise to heterogeneous EPO molecules with different numbers of sialic acid molecules attached, thereby varying the net negative charge. In order to study the effects of this microheterogeneity on EPO's biological activity, different isoforms of EPO were isolated and injected into animals. Isoforms with higher sialic acid content have greater *in vivo* biological activity and a longer serum half-life. In contrast, higher sialic acid content leads to lower *in vitro* receptor-binding affinity. These results suggest that the serum half-life and clearance rate have stronger effects on EPO's biological activity *in vivo* than does receptor-binding affinity.

## III. EPO LEVELS AND ITS REGULATION

Although hypoxia clearly stimulates EPO production, how and where this is accomplished was unclear until Jacobsen demonstrated that nephrectomized animals

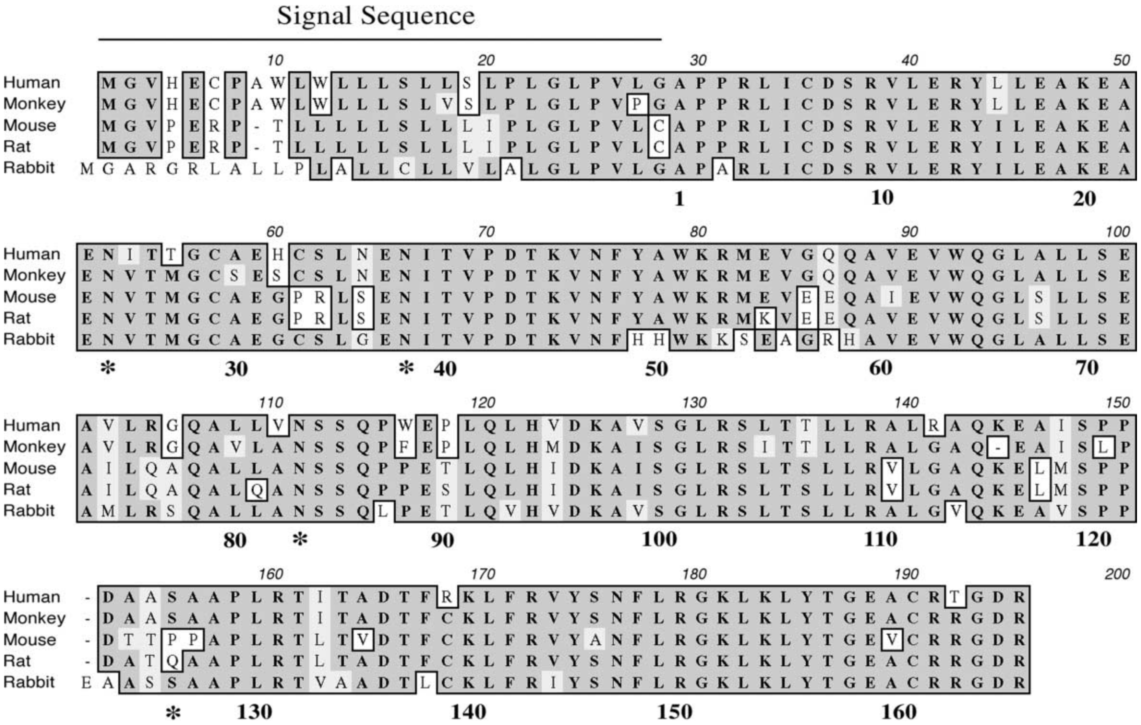

**FIGURE 1** Alignment of the EPO amino acid sequence across human, monkey, mouse, rabbit, and rat showing a high degree of homology. Signal sequence comprises approximately the first 27 residues depending on the species. Glycosylation sites are marked with an asterisk below the asparagine (N) or serine (S) residues in relation to the human sequence. Boldface numbers below the alignment represent numbering of the mature sequence, and numbers at the top of the alignment represent numbering from the start of the signal sequence. Dark shaded residues indicate identity and lightly shaded residues indicate similarity.

failed to mount an EPO response to hypoxia, suggesting that the kidney was the primary site of EPO production in adult animals. Koury and Lacombe in 1988 localized erythropoietin expression to interstitial cells close to the proximal tubular cells of the kidney by *in situ* hybridization. The kidney is the primary site for EPO production, at least in part due to its function in sensing oxygen tension and extracellular volume. By regulating red cell mass through EPO production and plasma volume through excretion of salt and water, the kidney sets the hematocrit at a normal value of 45%. The adult liver is also capable of producing approximately 10–15% of the circulating EPO in anemic rats. These levels, however, are not sufficient to sustain a normal level of erythropoiesis in the absence of renal EPO production.

EPO released into the blood circulation travels to major erythropoietic organs, such as the bone marrow and the spleen (in rodent), and binds to its cell surface receptor (EPOR) on erythroid-specific precursors to stimulate red blood cell production and increase the peripheral red cell mass. In the normal healthy individual, the serum EPO level is very low, ranging from 10 to 30 mU/ml. No sex-related differences in EPO levels have been observed and EPO levels are not affected by the menstrual cycle.

In addition to tissue-specific control, regulation of EPO production occurs mainly at the level of transcription and can be induced under anemic and hypoxic conditions. *EPO* mRNA levels increase 200- to 1000-fold in mice with bleeding-induced anemia or severe hypoxia in order to compensate for the blood loss and reduced oxygen-carrying capacity. *EPO* expression is also carefully regulated to prevent the overproduction of red blood cells. In anemic mice, the level of EPO induction is proportional to the level of anemia induced. Abnormally high red cell mass or hematocrit, clinically defined as erythrocytosis, can increase blood viscosity and cause hypertension. A considerable amount of research has been invested in determining how the body is able to sense the changes in oxygen tension and how that translates into EPO production. Interestingly, EPO is found only in placental mammals. An 80–82% amino acid identity exists between the human sequence and that of monkey, mouse, rat, and rabbit (Fig. 1). Studies on the *EPO* locus itself have uncovered several transcriptional regulatory elements. The *EPO* gene consists of five exons and four introns. In addition to a high level of conservation within the *EPO* coding sequence across mammalian species, regions in the promoter, the first intron, and a small region $3'$ to the polyadenylation site are also highly conserved. These similarities suggest the existence of conserved transcriptional regulatory mechanisms. Studies using transgenic mice carrying different regions of the putative regulatory sequences demonstrated that (1) *cis*-acting elements for tissue-specific and developmentally regulated *EPO* expression lie outside the 400 and 700 bp of $5'$- and $3'$-flanking sequences, respectively, and (2) *cis*-acting sequences within the 400-bp $5'$ and 700 bp $3'$-flanking regions are capable of mediating an oxygen-related response. Subsequent studies with larger regions of the $5'$-promoter regions identified a region 0.4 to 6 kb upstream of the *EPO* gene that may possess binding sites for functionally relevant repressors. Further upstream, kidney-specific inducible elements were found between 9.5 and 14 kb upstream of the *EPO* gene. Study of the conserved region $3'$ to the polyadenylation site (Fig. 2) also provided information crucial for *EPO* regulation. A hypoxia-inducible enhancer was discovered in a region 120 bp $3'$ to the polyadenylation site. Its activity is independent of orientation or distance from the promoter. Fine-mapping of the $3'$-enhancer sequence reveals three sites critical for regulation of the *EPO* gene by hypoxia (Fig. 2). The first site on the $5'$-end has the sequence TACGTGCT, which is the consensus binding site for the transcription factor hypoxia-inducible factor-1a (HIF-1a) and the aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimeric complex. This binding site is essential for HIF-1 dependent and hypoxia-induced *EPO* expression. A second site lies only 7 bp $3'$ of the HIF-1 binding site with the sequence CACA. Although no protein is known to bind to this sequence, mutating this site leads to loss of the hypoxia-inducible function of the enhancer. The third site is located 10 bp further downstream from the CACA site and consists of two consensus binding sites for steroid hormone receptor. Screening for nuclear hormone receptors capable of binding to this site led to the identification of HNF-4. Using Hep3B, a liver cell line capable of producing EPO in response to hypoxia, HNF-4 is shown to be required for the response to low oxygen levels. Introducing dominant negative HNF-4 into Hep3B cells abolished their ability to respond to hypoxia by inducing *EPO* expression. Moreover, like *EPO*, HNF-4 is also expressed in the renal cortex of the kidney and the liver.

The aforementioned *cis*-acting element in the $5'$-promoter region also has a role in the response to hypoxia, maintaining a 10-fold induction in response to hypoxia in the absence of the $3'$-enhancer. In the

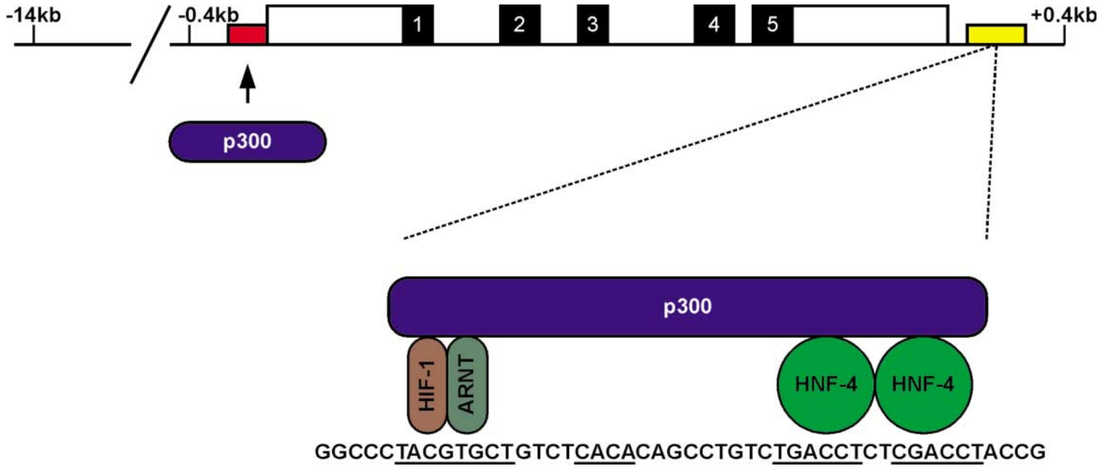

**FIGURE 2** *EPO* gene regulation in adults. Coding regions of the five exons are shown in black. The 5′- and 3′-untranslated regions are shown as boxes. The 3′-enhancer (small box in upper right-hand side) is expanded. p300 is capable of binding and activating the minimal promoter (small box in upper left-hand side) and can act synergistically with the 3′-enhancer to increase *EPO* transcription in response to hypoxia. Consensus DNA sequences for hypoxia-inducible expression are underlined. Adapted from Ebert, B. L., and Bunn, H. F. Regulation of the erythropoietin gene. *Blood* 1999; 94: 1864–1877. Copyright American Society of Hematology. Used with permission.

presence of the 3′-enhancer, the 5′ *cis*- acting element synergistically activates *EPO* expression by 40-fold in response to hypoxia. Taken together, the 5′-promoter and 3′-enhancer of the *EPO* gene constitute a potent transcriptional control unit, which regulates EPO production in response to hypoxic conditions or anemic states.

## IV. EPO AND EPOR: SIGNAL TRANSDUCTION

Given the thoroughness of our understanding of EPO signaling and the structural and functional similarities between members of the cytokine receptor superfamily, EPO signaling can serve as a model for understanding cytokine signal transduction in general. EPO signals through its cognate receptor, the EPOR, a protein expressed on the surface of appropriate target cells. Because the EPOR lacks any known catalytic function, it relies on the activity of associated Janus kinase 2 (JAK2). Upon EPO binding to dimerized EPORs, JAK2 molecules are brought into close proximity, phosphorylating and thereby activating one another. Activated JAK2 then phosphorylates individual tyrosine residues within the cytosolic region of EPOR, thereby creating docking sites for recruiting SH2 domain-containing signaling molecules, including (1) signal transducer and activator of transcription 5 (STAT5), molecules of which, once phosphorylated, dimerize and translocate into the nucleus, where they initiate transcription of target genes; and (2) the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which activates other downstream signaling molecules, such as serine/threonine kinase Akt; and (3) Grb2, an adapter molecule that mediates activation of the Ras-mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 3). Ultimately, these and other signaling cascades produce alterations in the patterns of gene expression. Such “target genes” in erythrocytes include those involved in cellular proliferation (e.g., c-myc and c-fos) and survival (e.g., Bcl-$X_L$), as well as cell type-specific genes, such as those encoding globins (the protein component of hemoglobin), the transferrin receptor (which mediates entry of iron into cells), and certain membrane structural proteins (which contribute to the characteristic biconcave structure of erythrocytes).

Although it has long been presumed that cytokine signaling must be negatively regulated with respect to both duration and intensity, only in recent years has a better understanding of this process been achieved. To generate more precise control over cytokine signaling, several mechanisms of negative regulation exist. First of all, the hematopoietic protein tyrosine phosphatase SH-PTP1 (also called HCP and PTP1C) associates through its SH2 domain with a specific phosphotyrosine residue of the EPOR and is responsible for dephosphorylating and thus inactivating JAK2. In fact, mutating this tyrosine to phenylalanine renders cells that express the mutant receptor hyperresponsive to EPO stimulation.

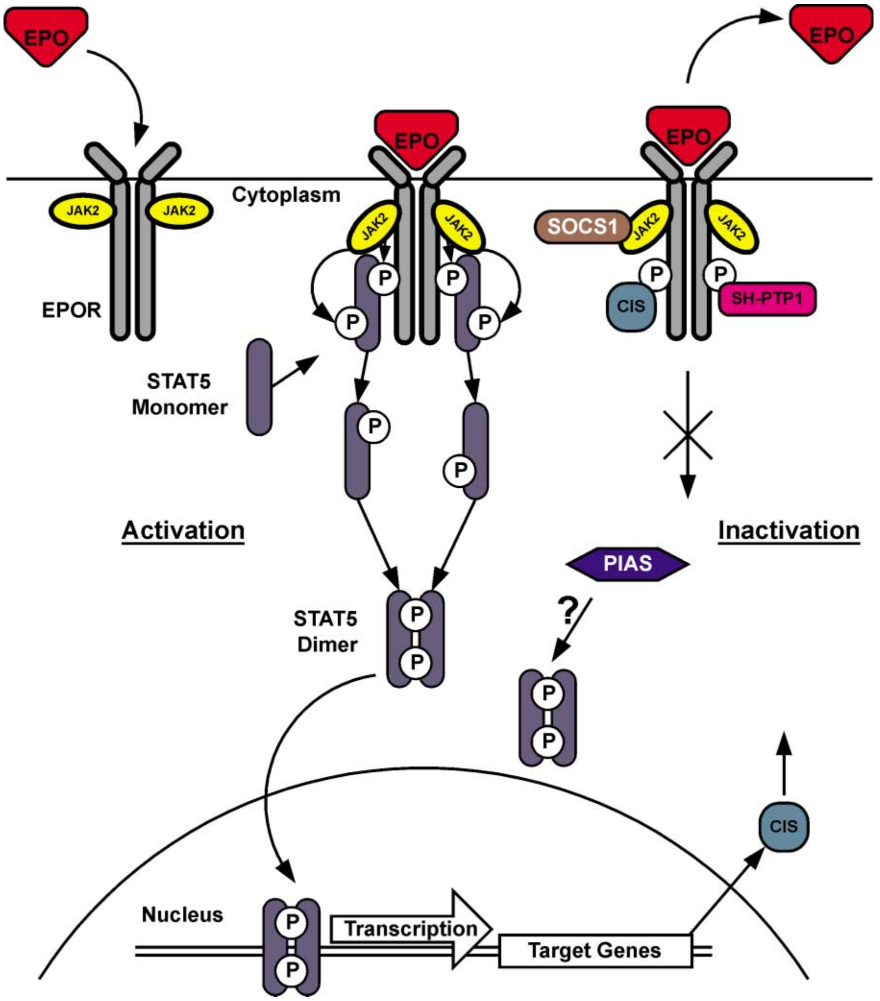

**FIGURE 3** EPO/EPOR signal transduction. Activation: EPO binds the EPOR and activates JAK2. JAK2 then phosphorylates the tyrosine residues of the EPOR cytoplasmic tail, which provides a docking site for recruiting the SH2 domain-containing signaling molecules, such as STAT5. STAT5 is subsequently tyrosine phosphorylated, dimerizes, and translocates to the nucleus to activate the transcription of target genes. Inactivation of the receptor occurs through various means, including binding of the receptor and JAK2 molecule by various inhibitors, such as CIS and SOCS1, and dephosphorylation of JAK2 by SH-PTP1 phosphatase. It is unclear whether PIAS molecules participate in negative regulation of EPOR signaling by modulating the function of STAT5.

Naturally occurring variants of the human *EPOR* lacking the C-terminal negative regulatory domain, including this tyrosine residue, may give rise to familial erythrocytosis.

Also contributing to the attenuation of cytokine signaling are the protein families PIAS (protein inhibitors of activated STATs, with four mammalian family members identified) and SOCS (suppressor of cytokine signaling, with eight known mammalian family members). Although the role of PIAS family members in EPO signaling has not been addressed, multiple SOCS family members appear to counteract EPO signaling by acting on different targets. Whereas SOCS1 appears to bind to JAK2 to inhibit its activity, CIS, another SOCS family member, binds to the EPOR itself (1) to compete with STAT5 for binding to specific phosphotyrosine residues and (2) possibly to deliver active signaling complexes to the proteasome for degradation.

Finally, EPO signaling may be modulated by cell surface expression of the EPOR and EPO–EPOR complex internalization and degradation. The mechanisms controlling these events are poorly understood.

## V. ANIMAL MODELS FOR EPO FUNCTION

Whereas biochemical analysis has helped identify key players involved in EPO signaling, genetic analysis, involving either the fruit fly, *Drosophila melanogaster*, or the house mouse, *Mus musculus*, has provided insight into their functional relevance. The hemolymph of insects is analogous to mammalian blood, in that it carries nutrients and cells involved in immunity. *D. melanogaster* does not need red blood cells as an oxygen carrier and sequencing information derived from the fly genome project shows no homologue to either *EPO* or *EPOR*. Nevertheless, signaling molecules known to be important for EPOR signaling do exist in the fly. In fact, gain-of-function mutations of the JAK homologue *hopscotch* lead to the overproliferation and premature differentiation of larval blood cells.

Although *D. melanogaster* permits powerful genetic analysis due to its low maintenance cost and short generation time, the mouse is physiologically more relevant to humans. A powerful technique for studying the role of a particular gene in mice is achieved through homologous recombination in embryonic stem cells. Mice lacking *EPO* or *EPOR* die with severe anemia at approximately embryonic day 13 (mouse gestation lasts for 18–21 days, depending on strain, diet, and other factors). Interestingly, *EPO* and *EPOR* are unnecessary for erythroid commitment, but instead are required for their terminal differentiation into mature red blood cells. Thus, as shown in Fig. 4, whereas normal fetal livers contain cells at various differentiation stages within the erythroid lineage, livers lacking *EPO* or *EPOR* are completely pale and fail to produce mature erythrocytes. Furthermore, "primitive" erythropoiesis, which occurs very early in development in the yolk sac compartment, appears to be intact in either knockout. This result would suggest that *EPO* and *EPOR* are necessary only for the production of adult-type or "definitive" red blood cells, which are generated at midgestation throughout adulthood. Finally, the fact that both deficient mouse strains exhibit very similar, if not identical, phenotypes suggests that only one receptor exists for EPO and that no other naturally occurring ligands or receptors can replace either protein.

*JAK2*-deficient mice are very reminiscent of *EPO*- and *EPOR*-deficient mice as they too die as embryos with anemia. However, the degree of anemia is greater in *JAK2* null mice, which is not surprising since JAK2 is implicated in signaling by many cytokines other than EPO, including thrombopoietin, interleukin-3, and granulocyte/macrophage-colony-stimulating

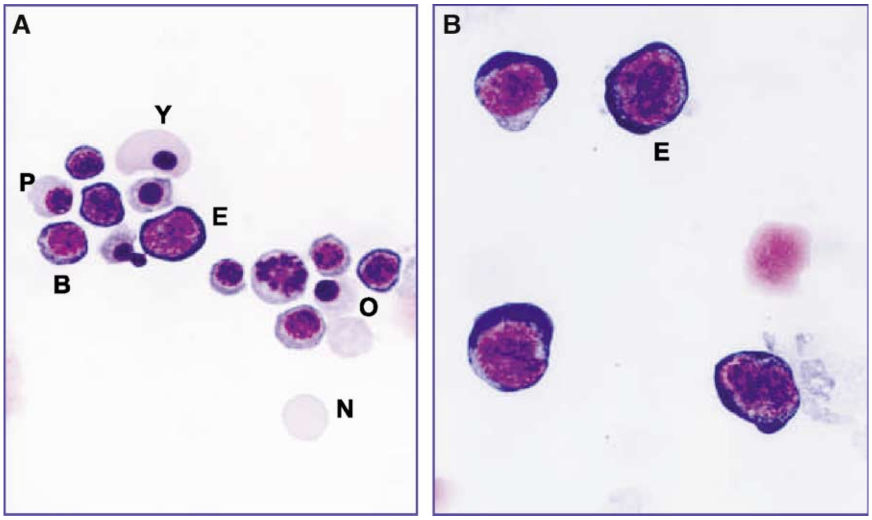

**FIGURE 4** EPO is essential for the differentiation of erythroid progenitor cells in the fetal liver. Cytospin preparation of liver from a wild-type (A) and a homozygous mutant (B) liver stained with Giemsa. Numerous erythropoietic cells at all stages of differentiation are seen in the WT fetal liver, including proerythroblasts (E), basophilic erythroblasts (P), orthochromatic erythroblasts (O), and nonnucleated erythrocytes (N). Yolk sac-derived nucleated erythrocytes (Y) are also seen. In the mutant liver preparation, proerythroblasts are prominent with a noticeable absence of cells at later stages of differentiation. Reprinted from *Cell* 83, Wu, H., Liu, X., Jaenisch, R. and Losish, H. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. 56–67, 1995. With permission from Elsevier Science.

factor, all of which can positively regulate erythropoiesis although they are dispensable for steady-state erythropoiesis. Thus, *JAK2*, like *EPO* and *EPOR*, is both a critical and nonredundant player in erythropoiesis.

Among seven mammalian STAT family members, STAT5A and STAT5B have been implicated to be involved in EPO signaling based on *in vitro* experiments. However, disrupting either or both genes in mice does not result in erythropoietic defects similar to that in *EPO*, *EPOR*, or *JAK2* mutant mice, although deficiencies exist in signaling by other cytokines, namely, prolactin and growth hormone. Possible explanations for the relatively subtle effects of STAT5 on erythropoiesis *in vivo* include redundancy with other components of EPO signaling that are downstream of JAK2 (e.g., MAPK or PI3K) or possibly the existence of a hitherto unidentified STAT or STAT-related protein that plays a role in EPO signaling *in vivo*.

Members of the SOCS family have also been deleted in mice. Disruption of *SOCS3* results in erythrocytosis so severe that mice die during embryogenesis. In contrast, *SOCS3* overexpression results in a severe anemia, much like what is seen in the *EPO*-, *EPOR*-, or *JAK2*-deficient mice.

## VI. CLINICAL APPLICATIONS

The pharmacological use of recombinant human EPO has been well established clinically in treating anemia associated with a variety of pathophysiological conditions. Anemia can be classified by hemoglobin (Hb) levels and subdivided into the following categories: mild (10–12 g/dl), moderate (8–10 g/dl), severe (6.5–7.9 g/dl), and life-threatening (<6.5 g/dl). Hb is contained mostly within erythrocytes, which function in binding, transporting, and delivering oxygen to the periphery. Loss of red blood cells results in a compensatory response by the body to maintain oxygen delivery to tissues. One such response is the induction of *EPO* expression, which subsequently results in an increase in red cell mass.

Anemia can present with a wide spectrum of symptoms involving virtually every organ system, including heart palpitations, pale and cold skin, dyspnea, headaches, dizziness, vertigo, decline in cognitive function, retinal damage, angina, heart failure, left ventricular hypertrophy, proteinuria, decreased libido, impotence, amenorrhea, irregular bowel movement, indigestion, malabsorption, tachypnea, pulmonary edema, generalized edema, and renal failure. Because of their many detrimental effects on quality of life, anemias can pose significant health problems.

Although they can result from a variety of causes, a large number of anemias in the clinical setting result from a decreased production of red cells. The most promising method in treating this type of anemia has been recombinant EPO. Recombinant human EPO (or Epoetin α) has been approved by the Food and Drug Administration for treatment of anemia associated with chronic renal failure. EPO has also proved to be beneficial for treatment of anemia associated with Zidovudine therapy for patients infected with human immunodeficiency virus, anemia associated with chemotherapy in cancer patients, and anemia associated with surgery. Recent studies have also suggested potential therapeutic uses for EPO in the treatment of disorders such as myelodysplastic syndrome, anemias of prematurity, and congestive heart failure.

Because of its relative safety and efficacy, athletes attempting to improve their oxygen-carrying capacity have frequently abused recombinant EPO. Given their similarity, recombinant EPO cannot be distinguished from endogenous EPO by currently available immunoassays. However, an electrophoresis method can distinguish endogenous EPO and synthetic EPO because of subtle discrepancies in posttranslational modifications that introduce charge differences between the EPO isoforms. In fact, when this technique was employed, urine samples of 28 of 102 tested cyclists of the 1998 Tour de France were found to have electrophoresis patterns indicative of recombinant EPO.

## VII. EPO FUNCTION IN OTHER LINEAGES

The role of erythropoietin in stimulating red blood cell formation has been clearly defined. However, recent evidence suggests that EPO may have more diverse roles as well. For instance, *EPOR* expression has been detected in human umbilical cord and placental endothelial cell lines, and EPO was capable of stimulating endothelial cell proliferation *in vitro*. In addition, EPO is known to induce a pro-angiogenic phenotype in cultured endothelial cells and stimulated neovascularization in the chick chorioallantoic membrane. EPO also plays an important role in myoblast proliferation as well as in cardiac morphogenesis.

*EPOR* expression has also been found in PC12 cells (a cell line with neuronal-like properties), hippocampal and cortical neurons, and astrocytes. EPO-binding sites can be localized in both murine and human fetal brains. Similar to EPO production in the kidney, endogenous EPO expression in neurons and astrocytes is controlled by oxygen-sensing mechanisms that involve the redox state of the brain. In animal models, EPO has been shown to protect neurons from glutamate-induced as well as ischemia-induced apoptotic cell death. Thus, research toward understanding all the *in vivo* functions of EPO can lead to new and improved therapeutic regimens and indications beyond the treatment of anemias.

## VIII. SUMMARY

The study of EPO has gone from the concept that a hormone-like molecule exists and is capable of stimulating red blood cell formation to the cloning of the gene and its biochemical and molecular characterizations. Through decades of study on EPO, research that focused on its biological properties has led to the development of a highly successful therapeutic drug that has significantly improved the quality of human life. Further studies with elegant genetic animal models have proven its *in vivo* biological function, and the dissection of its signaling pathway has progressed to the level of not just exploring what EPO turns on but also what turns off the signal from the EPOR. In an attempt to improve on Mother Nature, researchers have begun to isolate small peptides or to identify small chemical molecules that can mimic EPO function by activating the EPOR. Even though an enormous amount of information has been generated, more remains to be learned, and researchers will strive to better understand the functions of this essential hormone in regulating red blood cell formation and its role in other lineages.

## Glossary

**anemia** A significant reduction in red cell mass and a corresponding decrease in the oxygen-carrying capacity of the blood.

**cytokine** A member of a large family of secreted proteins, defined by structural similarities in their receptors, which regulate fundamental biological processes, such as hematopoiesis (the formation of blood cells), immunity, and wound healing.

**enhancer** A *cis*-acting sequence that increases the utilization of eukaryotic promoters and can function in either orientation and in any location (either upstream or downstream), relative to the promoter.

**erythrocyte** A mature red blood cell that circulates within the body to deliver oxygen to tissues and, in exchange, remove carbon dioxide and hydrogen ion from tissues.

**erythrocytosis** An abnormal increase in red blood cells in the circulating blood. This elevation is usually accompanied by corresponding increases in the quantity of hemoglobin and in the hematocrit.

**glycosylation** The conjugation of carbohydrate residues, which for proteins can occur through the side-chain oxygen atom of serine or threonine residues by O-glycosidic linkages or to the side-chain nitrogen of asparagine residues by N-glycosidic linkages.

**hypoxia** A condition in which oxygen tension is lower than the normal level.

**kinase** Any protein that catalyzes phosphorylation (the transfer of a phosphate group from a donor to a substrate molecule).

## See Also the Following Articles

**Angiogenesis • *Drosophila* Neuropeptides • Flt3 Ligand • Protein Kinases**

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# Erythropoietin, Genetics of

Eugene Goldwasser
*The University of Chicago*

I. HISTORY OF ERYTHROPOIETIN
II. THE ERYTHROPOIETIN GENE
III. THE ERYTHROPOIETIN PROTEIN
IV. SITE OF ORIGIN
V. ERYTHROPOIETIN GENE EXPRESSION
VI. CLINICAL APPLICATION

**Erythropoietin is both a hormone and a growth factor. It fits the classical definition of a hormone being made in one organ, the mammalian kidney, being secreted into the bloodstream, and acting at another site, the blood-forming system, i.e., the bone marrow in adults. As a growth factor, it is required for red blood cell formation from more primitive cells of the hematopoietic system. In adult human beings, it regulates normal erythropoiesis, stimulating the production of approximately 2.5 million red cells per second.**

## I. HISTORY OF ERYTHROPOIETIN

The existence of erythropoietin (epo) was first suggested by Carnot and Deflandre in 1906. They described it (in current terminology) as a feedback regulator: when the number of red cells was below the normal setpoint, the mechanism to accelerate red cell formation was activated by secretion of epo; when the setpoint was regained, the mechanism was thought to turn off, keeping the number of red cells relatively constant. It is now known that normal red cell formation is regulated by continuous secretion of epo so that the formation of new red cells matches the loss of old cells. The system, however, can respond to a need for red cells, for example, due to blood loss or hypoxia, by increased secretion of epo and the consequent increased formation of red cells. The experimental evidence published by Carnot and Deflandre was neither convincing nor reproducible and for almost 50 years the question of whether they had demonstrated that there was such a substance was in serious doubt. Its existence is now unquestionable.

The contemporary era of epo research began with the purification of human urinary epo and, 8 years later, the cloning of its gene. These advancements set the stage for a great increase in research and publications on epo: in the period from 1906 to 1976, there was an average of 16 papers on the subject published per year; from 1976 to 2000, this number was 237 per year. Of course, the greater part of this increase was due to the advent of recombinant DNA technology, making it possible not only to clone the gene and study its expression, but to produce epo in cells engineered to carry the transgene, harvest epo, and purify it in industrial amounts for clinical use. Much of the increase in the number of publications was associated with molecular biology studies and clinical application.

## II. THE ERYTHROPOIETIN GENE

The human epo gene is on chromosome 7 (7pter–q22) and is a 2144 bp stretch of DNA. The structural gene consists of four introns, comprising 1562 bp, and five exons, totaling 582 bp. There is an 81 bp sequence coding for a signal peptide immediately downstream of the ATG start site. For the sequence of the signal peptide, see Fig. 1. Upstream of the signal sequence there do not appear to be any canonical promoter sequences. Downstream of the termination site, there is an important sequence defining the enhancer, hypoxia-response element, coding for hypoxia-inducible factor-1. There is little if any homology between the epo gene and the sequences of other mammalian genes except for these regulatory elements in the 3′-flanking region.

## III. THE ERYTHROPOIETIN PROTEIN

The amino acid sequence of human epo (Table 1) shows it to consist of approximately 34% hydrophobic amino acids. There is little significant homology between the amino acid sequence of human epo and that of any other human hormone, cytokine, or growth factor.

$H_2NMGVHECPAWLWLLLSLLSLPLGLPVLGCOOH$

**FIGURE 1** Signal peptide sequence of human erythropoietin.

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$H_2$NMGVHECPAWLWLLLSLLSLPLGLPVLGCOOH

**FIGURE 1** Signal peptide sequence of human erythropoietin.

TABLE 1 Amino Acid Sequence of Human Erythropoietin

A**P**PR**LI**CDSR**V**LERY**L**LEAK
EAEN**I**TTGCAEHCS**L**NEN**I**T
**VP**DTK**V**N**F**YA**W**KR**M**E**V**GGQA
**V**E**VW**QG**L**A**LL**SEAVLRGQA**L**
**I**VNSSQ**PLQ**LH**V**DKA**V**S
G**L**RS**L**TTLLRA**L**GAQKEA**IS**
**PP**DAASAA**P**LRTITADT**FRK**
**LF**R**VY**SN**FL**RGKLK**LY**TGEA
CRTGD

*Note.* The hydrophobic residues are in boldface type.

TABLE 2 Active Site Residues Human Erythropoietin

| Receptor site 1 | Receptor site 2 |
|---|---|
| Lys-20 A helix | Val-11 A helix |
| Thr-44 A-B loop | Tyr-15 A helix |
| Lys-45 A-B loop | Val-99 C helix |
| Val-46 A-B loop | Ser-100 C helix |
| Phe-48 $B^1$ helix | Arg-103 C helix |
| Arg-143 D helix | Ser-104 C helix |
| Asn-147 D helix | Leu-108 C helix |
| Arg-150 D helix | Arg-110 C helix |
| Gly-151 D helix | |
| Leu-155 D helix | |

There are 166 codons in the gene but the circulating protein has only 165 amino acid residues; the C-terminal arginine is removed posttranscriptionally. There are two disulfide bonds, one linking cysteine 7 near the N-terminus with cysteine 161 near the C-terminus and the other linking residues 29 and 33. There are four glycosylation sites; three are N-linked at asparagine residues 23, 28, and 83. The fourth is an O-linked site at serine 126. The carbohydrate content of human epo is 30–40% depending on the source. Recombinant human epo has a molecular weight of 30.4 kDa by hydrodynamic measurements. As estimated by denaturing gel electrophoresis, its apparent molecular weight is 34–37 kDa. When size-exclusion chromatography (gel filtration) is used, the apparent molecular weight is approximately 60 kDa. These differences reflect the effect of the large amount of carbohydrate on the apparent molecular volume of the hormone.

Studies of the "active site" of human epo by chemical methods and by site-directed mutagenesis indicate that the following single amino acid residues are important for its biological activity: Val-11, Arg-14, Tyr-15, Arg-103, Ser-104, Leu-105, and Gly-151. The Cys-7 to Cys-161 disulfide bridge is also required for activity. These findings were verified by X-ray diffraction and solution of the three-dimensional structure of recombinant aglyco-epo complexed to the dimerized exo domain of its specific receptor. Aglyco-epo consists of a four-helix bundle with helices A and B plus $B^1$ in the up configuration and helices C, $C^1$, and D in the down configuration. The connecting loops have variable lengths: A–B has 20 residues, B–C has 7, and C–D has 16. Helices A and C are interrupted by small nonhelical regions (Table 2).

The dimerized receptor has asymmetric sites for binding its ligand. Site 1 on one of the monomers is a high-affinity site with a $K_d$ of less than 1 nM. It interacts with residues on helices A, $B^1$, and D and part of the A–B loop. Site 2, with a $K_d$ of approximately 1 μM, interacts with the A and C helices. The regions of epo involved in the more biologically important interactions with receptors, based on analysis of intermolecular contact areas, are summarized in Table 3.

The four branched oligosaccharide chains in naturally occurring recombinant epo all have multiple sialic acid termini and are clustered at the region of the molecule away from the region that interacts with the receptor. Structures of some of the oligosaccharides have been published. The carbohydrate is required for biological activity when tested *in vivo*, but for activity on cells in culture, the sugars are not required. The carbohydrate acts in the secretion process, stabilizes the hormone, increases its solubility in water, keeps it monomeric, and prevents rapid clearance from the circulation. Epo devoid of sialic acid termini has no activity *in vivo* because of its rapid uptake by a liver receptor that recognizes terminal galactose residues and removes the asialo-epo from

TABLE 3 Helical Structure of Human Epo Sequence

| | |
|---|---|
| $H^2$ N APPRLICD | |
| **SRVLERYLLEAKEAENIT** | A helix |
| TGCATEHCSLNENITVDTKV | A-B loop |
| **NFYAWK** | $B^1$ helix |
| RME | |
| **VGQGAVEVWQGLALLSEAVLRGQALLV** | B helix |
| NSSQPWE | B-C loop |
| **PLQLHVDKAVSGLRSLTTLLRAL** | C helix |
| **GAQKEAISP** | C-D loop |
| PDAASAAPLRTITADT | C-D loop |
| **FRKLFRVYSNFLRGKLKLYTGEAC** | D helix |
| RTGD COOH | |

*Note.* Boldface indicate helices.

the circulation. The activity of asialo-epo *in vitro*, however, is greater than that of native epo and it binds to cellular receptors with a $K_d$ approximately one-third that of sialylated epo. Whether of natural or recombinant origin, epo has a high degree of microheterogeneity; i.e., each glycosylated site has a number of different oligosaccharides associated with it.

A new epo derivative engineered to contain two additional N-linked oligosaccharide chains at amino acid positions 30 and 88 has been developed by recombinant methods. In comparison with native epo, it is more active, is more stable, and is cleared from the circulation with a half-time approximately three times greater than that for native epo containing four carbohydrate chains. Paradoxically, the new epo-like glycoprotein shows an inverse relationship between binding affinity and biological activity. Even though the affinity for the receptor seems to be decreased, the longer half-life results in greater biological activity *in vivo*.

There is ample evidence that epo action, prior to internalization, involves dimerization of the receptor. The studies cited above of the epo receptor–exo domain complex yielded information about the features of the epo molecule involved in the dimerization. Based on this information, peptides smaller than epo and nonpeptide mimetics of epo have been developed. Some of these are epo antagonists and others are agonists. Thus far, none of the epo agonists have proved to be as potent as natural epo in either *in vitro* or *in vivo* assays. The concept, however, is a valid one and it is likely that further research will result in the production of synthetic compounds capable of replacing epo as an inducer of red cell formation.

## IV. SITE OF ORIGIN

Epo is normally formed by the adult mammalian kidney. Under an anemic or hypoxic stress, the system is capable of a several thousand-fold increase in synthesis and secretion. Under severe conditions, the adult liver is recruited to produce epo. Without such stimuli, the adult mammalian kidney secretes epo at a rate sufficient to maintain the steady state concentration in human plasma at approximately 5 pM (150 pg/ml). Early in fetal development, epo is made in the liver, which also contains epo-responsive cells. At around the time of birth, epo formation switches to the kidney, but the mechanism regulating this shift is unknown. The source of epo is still a point of experimental disagreement. One group of studies puts the source in the peritubular, interstitial cells of the renal cortex. Another group locates the source in renal tubular cells. Perhaps both are correct. Further experimentation is needed to resolve the question.

There is also some evidence showing epo and/or its mRNA to be present in some cells of the blood-forming system, suggesting that epo may have a role in some intracellular or autocrine processes in addition to its action as an inducer of red cell differentiation via external cellular receptors. Evidence is also accumulating for a role of epo and its receptor in neural tissue. Epo and the receptor are expressed in the brain as a result of hypoxic damage and appear to be involved in neuroprotection. This effect is probably not a function of its role in red cell formation, since any effect on the marrow would require days or weeks to result in increased red cell mass. The evidence suggests that epo can act on cell proliferation of some nonhematopoietic cells.

Epo is also made and regulated by hypoxia, in some transformed (notably hepatocarcinoma) that can be grown or studied *in vitro*. These systems have been greatly important in the study of epo gene expression.

## V. ERYTHROPOIETIN GENE EXPRESSION

Whether by renal cells, brain cells, or some transformed cells (hepatocellular carcinoma), epo gene expression, primarily at the level of transcription, can be stimulated by hypoxia or by divalent metal cations, especially cobalt. The mechanisms by which these two different stimuli act on epo-producing cells may not be the same. This brief account will treat only the response to hypoxia.

In whole animals, the renal response is regulated by "available" oxygen; i.e., when the tissue's need for oxygen exceeds the capacity of the red cell mass to deliver oxygen, epo expression is accelerated. In kidney, the increase in epo expression is due to an increase in the number of cells with epo mRNA detectable by *in situ* hybridization rather than to an increase in epo mRNA per cell. In transformed cells, it is not yet clear which of the two possible alternatives is true. When oxygen supply is greater than the metabolic need for it, epo gene expression in whole animals is reduced. Despite the enormous effort devoted to identifying the nature of the intracellular element that can sense the level of ambient oxygen and compare it to the required oxygen level, the problem has not yet been solved.

The response to hypoxia in not limited to epo gene expression; several other genes are also activated by reducing available oxygen, and the mechanism seems to be the same for all of them. Whatever the oxygen sensor may be in molecular terms, the transcription of hypoxia-responsive genes has a common early pathway. This pathway involves hypoxia-inducible factor-1 (HIF-1), which is a dimer of HIF-1α and the other subunit, HIF-1β, or aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α under normoxic conditions is degraded very rapidly by the ubiquitin–proteasome pathway. The signal for HIF-1α ubiquitination is a specific hydroxylation of proline residue 564. Hydroxylated HIF-1α then binds to the tumor suppressor protein pVHL and is hydrolyzed. In the presence of oxygen levels that are lower than needed, HIF-1α is stabilized by complexing with HIF-1β and the heterodimer is translocated to the nucleus, where it acts as a transcriptional activator for a number of genes, including epo.

## VI. CLINICAL APPLICATION

As might be expected from its source in the kidney, patients with chronic renal failure and those on dialysis are deficient in epo. This results in debilitating anemia, which now can be corrected by administration of human recombinant epo. The patients are then freed of the need for transfusions with all its attendant difficulties and have significantly improved lives. Anemias due to chemotherapy are also treatable with epo. It is also useful in building up a supply of blood to be used for autologous transfusions.

For much of the earlier research on epo, quantitative studies depended on assays *in vivo*. These methods were insensitive, lengthy, and expensive. When pure epo became generally available, assay methods based on the interaction of epo with highly specific antibodies were developed. Immuno-based assays are sensitive, rapid, and relatively inexpensive. Because of the potentially dangerous misuse of epo by athletes who try to build up their red cell mass prior to competition, newer methods capable of distinguishing between recombinant epo and natural, circulating epo would be very useful. Progress in developing such techniques is being made.

## Glossary

**aglyco-epo** Erythropoietin containing no carbohydrate chains.

**erythropoietin** The hormone or growth factor that regulates red blood cell formation.

**exon** A coding sequence of DNA.

**hypoxia** A deficiency of oxygen reaching the body tissues.

**intron** Intervening segment of DNA separating sequences that code for proteins; introns carry no protein-coding information.

**$K_d$** Dissociation constant, generally applied to ligand-to-receptor binding.

**kilodalton** Unit of molecular weight.

## See Also the Following Articles

**Angiogenesis • Stem Cell Factor**

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# Estrogen and Progesterone Receptors in Breast Cancer

Torsten A. Hopp and Suzanne A.W. Fuqua
*Baylor College of Medicine*

I. INTRODUCTION
II. NORMAL DEVELOPMENTAL PATTERN OF THE BREAST
III. ER IN BREAST CANCER DEVELOPMENT AND PROGRESSION
IV. ER AS A PROGNOSTIC AND PREDICTIVE FACTOR IN BREAST CANCER
V. ER IN BREAST CANCER PREVENTION
VI. ER VARIANTS IN BREAST CANCER
VII. PR IN BREAST CANCER DEVELOPMENT AND PROGRESSION
VIII. PR AS A PROGNOSTIC AND PREDICTIVE FACTOR IN BREAST CANCER
IX. CONCLUSIONS

**Breast cancer is a classical hormone-dependent neoplasm, and a large of body of epidemiological evidence suggests that increased hormonal exposure, such as early menarche, late menopause, postmenopausal obesity, and hormone replacement therapy, is associated with increased breast cancer risk.**

## I. INTRODUCTION

Common to the risk factors for breast cancer is the lifetime exposure of breast tissue to the ovarian hormones estrogen and progestin. On the other hand, these steroid hormones and their nuclear receptors, estrogen receptor-α (ER-α) and ER-β and progesterone receptor-A (PR-A) and PR-B, are essential for the proper development and maintenance of the mammary gland, and it is still uncertain how the receptors contribute to breast tumorigenesis.

The majority of breast cancers are at least initially hormone-dependent, and two-thirds of these tumors express ER-α and PR-A and/or PR-B. Expression of these hormone receptors is not only associated with a lower risk of relapse and prolonged overall survival, but is also a strong predictor for response to growth inhibition by anti-estrogens, such as tamoxifen. However, correlations between receptor content and response to anti-estrogen treatment are not perfect, and tumors often develop resistance to these treatment modalities. Even though both ER and PR measurements have been used for the clinical management of breast cancer for more than 20 years, the molecular mechanisms underlying how these receptor isoforms impact on tumor development and progression are still largely unclear. This article will relate the current knowledge about the role of ER and PR isoforms to breast cancer development, treatment, and progression.

## II. NORMAL DEVELOPMENTAL PATTERN OF THE BREAST

The human breast is a highly dynamic organ comprising glandular tissue and surrounding stroma, both of which undergo cycles of proliferation, differentiation, and apoptosis during a female's lifetime. Growth usually occurs from the distal end of ducts at a club-shaped morphological structure, the terminal end bud, which bifurcates into two smaller but morphologically more developed ductules. The mammary gland is hierarchically organized with clusters of, on average, 11 ductules forming lobule type 1 (Lob1). Through a gradual process of new duct formation and continuous differentiation, Lob1 grows in size, resulting in lobule types 2 and 3, with the latter being the most differentiated structure, found mainly in premenopausal parous women. The mammary gland fully differentiates during pregnancy into lobule type 4. The proliferative activity of the breast depends on the degree of differentiation and is highest in the undifferentiated Lob1 present in young nulliparous women. Maximal proliferation occurs during the luteal phase when progesterone reaches its highest levels, not during the follicular phase when estrogen levels are highest.

Estrogens and their receptors, ER-α and ER-β, are considered to play a major role in promoting cell proliferation and ductal outgrowth in the mammary gland. Although there is a clear connection between estrogen and proliferation, ER-α-positive epithelial cells, which constitute only approximately 10–20% of the overall cell population in the normal breast, are not the cells that are actively dividing in the normal situation. Furthermore, recent studies have determined that the more widely expressed ER-β protein is expressed mostly in myoepithelial cells, ruling out the possibility that this receptor form is directly mediating proliferation in the normal breast. These observations have led to the current model of normal growth in the mammary gland where estrogen stimulates ER-positive cells to produce a growth factor that in turn results in proliferation of the neighboring ER-negative cells.

PR-A and PR-B expression is observed mostly in cells that also contain ER-α and is often viewed as a downstream marker for the proper functioning of ER-α. However, mice depleted of PR demonstrate that this hormone receptor functions in mammary gland development in a manner clearly different from that of the ER. These knockout mice show that PR is essential for lobulo-alveolar development but not ductal morphogenesis. Unlike ER-α and ER-β, PR-A and PR-B are co-expressed at similar levels within the same epithelial cells in the normal breast.

Although expression of ER-α and PR-A and PR-B in the normal breast is directly proportional to the rate of cell proliferation, only a very few epithelial cells expressing the hormone receptors progress through the cell cycle, as determined by the lack of markers associated with proliferation, such as Ki67 and cyclin D1 in proliferating cells. However, the indirect regulation of proliferation by estrogens and progestins seen in normal breast tissues is dramatically altered during tumorigenesis, when ER and PR are frequently found in the proliferating cells and thus are directly related to cell proliferation.

## III. ER IN BREAST CANCER DEVELOPMENT AND PROGRESSION

Epidemiological studies suggest that invasive breast cancers arise from a series of putative precursor lesions, including typical ductal hyperplasia, atypical ductal hyperplasia, and ductal carcinoma *in situ*. Women with these lesions show approximately 2-, 4-, and 10-fold increased relative risk, respectively, of later developing invasive breast cancer. Moreover, it has been shown that these proliferative, premalignant lesions share their loss of heterozygosity pattern with more advanced lesions from the same breast, suggesting that invasive breast tumors indeed arise from these premalignant precursors.

Since premalignant lesions in the breast are most often characterized by elevated ER-α expression as well as intense proliferation, it has been hypothesized in several epidemiological studies that these ER-expressing premaligant lesions have a selective growth advantage in the presence of estrogen and may play a role in the progression of these premalignant lesions to cancer. ER-β expression appears to be inverse to ER-α expression. ER-β expression is lower in these premalignant precursors than in normal epithelium, leading some researchers to suggest that ER-β may play a "suppressor" role in malignant progression, although there are limited data to support this view.

## IV. ER AS A PROGNOSTIC AND PREDICTIVE FACTOR IN BREAST CANCER

ER-α status as determined by ligand-binding assays, and more lately using immunohistochemistry, is one of only a few biological markers that are used to make treatment decisions in breast cancer. It is now well established that almost 70% of tumors in women contain ER-α, with older women being more likely to have ER-α-positive tumors. Furthermore, the presence of ER-α often correlates with low S-phase fraction, diploidy, well-differentiated histology, and the absence of mutations or amplification of oncogenes, such as p53, HER-2/neu, and epidermal growth factor receptor. For reasons that are currently unknown, metastases of ER-α-positive tumors are more likely to spread to soft tissue, bone, or reproductive organs, whereas metastases of ER-α-negative tumors tend to be commonly found in brain and liver. In addition, several clinical studies with long-term follow-up have established that patients with ER-α-positive tumors have a lower risk of developing a recurrence within 5 years than patients with ER-α-negative tumors. However, in studies with follow-up periods of longer than 5 years, the prognostic relevance of ER disappears, and patients with ER-positive tumors are as likely to relapse as patients with ER-negative tumors. The time-dependence of ER-α as a prognostic marker is not yet understood. Thus, ER is now thought to be a weak prognostic factor of the natural history of breast.

On the other hand, ER is a strong predictor for the likelihood of response to endocrine therapies such as estrogens, anti-estrogens, surgical and medical ovariectomy, aromatase inhibitors, or progesterones. Specifically, numerous large clinical studies over the past 30 years have revealed that approximately 60% of previously untreated patients with advanced, ER-positive tumors will receive benefit from treatment with the anti-estrogen tamoxifen, with low response rates of 5–10% in women with ER-negative breast cancer. It has also been observed that the higher the ER content (>100 fmol/mg protein as determined by ligand-binding assay) in breast tumors, the better the response rate to hormonal treatment. A major clinical use of the ER assay in breast cancer is to identify a group of patients who have little or no chance of benefiting from anti-estrogen treatment and who thus should receive other, more aggressive treatment modalities. In addition to its use as a predictive marker for the likelihood of response to first-line hormonal therapy, ER status is important in predicting benefit from subsequent hormonal treatment, but response rates of relapsing ER-positive patients are considerably lower than those for previously untreated patients.

## V. ER IN BREAST CANCER PREVENTION

Even though breast cancer treatment is successful in prolonging the survival of women afflicted by this malignancy, there are still more than 180,000 women newly diagnosed with breast cancer yearly in the United States alone. This represents a major public health issue, and research efforts are increasingly being focused on preventing breast cancer. Since ER-α levels are most often high in premalignant as well as malignant lesions of the breast, this receptor and its signaling pathway provide a rational target for prevention treatment strategies.

One of the first large-scale randomized clinical trials to test the effect of an anti-estrogen (tamoxifen) on breast cancer prevention was the NSABP P1 breast cancer trial, which enrolled more than 13,000 healthy women at high risk of developing this malignancy. This trial reported a 49% reduction in the incidence of invasive breast cancer in women taking tamoxifen for 5 years. Another anti-estrogen, raloxifene, which was originally developed for the treatment of osteoporosis and clinically tested in the Multiple Outcomes of Raloxifene Evaluation trial on more than 7500 postmenopausal women, also decreased the risk of invasive breast cancer in treated women. However, treatment with tamoxifen or raloxifene increased the relative risk of developing thromboembolic disease, but only tamoxifen treatment resulted in a significant increase in endometrial cancers. In 1999, a clinical trial, the Study of Tamoxifen and Raloxifene, was designed to directly compare both anti-estrogens in the prevention of breast cancer; results are anticipated to be released in 2006.

## VI. ER VARIANTS IN BREAST CANCER

Both of the ER genes undergo excessive alternative splicing in normal and neoplastic estrogen-responsive tissues. Alternative splicing results in ER-α and ER-β mRNA variants with single or multiple exons being skipped, and these variants are usually co-expressed along with the wild-type receptors. It is still unclear whether any of the ER splicing variants are expressed at the protein level and to what extent the formation of heterodimers of these splice variants with ER-α and ER-β perturb the ER signaling pathway. In addition, very little is currently known about different ER-β splice variants, so this section will focus on four relatively commonly ER-α splice variants.

The most frequently observed ER-α mRNA splice variants are those lacking either exon 4 or exon 7. Deletion of exon 4 results in the removal of parts of the DNA-binding and hinge domains, as well as the amino-terminal region of the ligand-binding domain, whereas deletion of exon 7 removes part of the hormone-binding domain including the hormone-dependent transcriptional activation function AF-2. Both receptor splice variants act as dominant-negative inhibitors of normal ER-α function in a cell type-specific manner. Furthermore, expression of the exon 4-deleted variant in breast tumors has shown significant positive correlation with high histological grade and PR-positive levels, both of which are markers for good clinical outcomes in breast cancers.

Another dominant-negative inhibitor of normal ER-α function is the exon 3-deleted mRNA splice variant, which is missing part of the DNA-binding domain. This receptor variant is found in both normal and primary breast cancers, but the ratio of wild-type ER-α to the exon 3-deleted variant is approximately 30-fold reduced in breast cancer and breast cancer cell lines compared to normal tissue. Furthermore, stable expression of this variant in MCF-7 breast cancer cells reduced the expression of ER-regulated genes and also reduced invasiveness and estrogen-stimulated anchorage-independent growth. The reduced expression of the exon 3-deleted variant in breast cancer supports the emerging concept that the expression of ER-α mRNA splice variants is altered and may possibly play a role in tumorigenesis.

One of the best studied mRNA splice variants is the exon 5-deleted variant, which is also the only one demonstrated to be stably translated into protein in breast cancer cell lines and breast tumors. The variant encodes a truncated 40 kDa protein that lacks most of the ligand-binding domain but still contains the AF-1 transactivation function and the DNA-binding domain and demonstrates variable strengths of ligand-independent transcriptional activity. This variant was first detected in ER-negative/PR-positive tumors, and it was recently shown that expression of this variant is significantly increased in ER-positive breast cancers from relapsing patients.

In contrast to the abundant expression of ER-α mRNA splice variants in both normal and neoplastic tissue, mutations of the ER-α gene have not been frequently reported in primary breast cancer. Polymorphisms and missense mutations of the ER-α gene have been found in primary and metastatic breast cancers, but these changes do not appear to correlate with clinical parameters, such as tumor size or tumor histology. In addition, mutations in the receptor that potentially affect its normal function and that might alter the downstream estrogen signaling pathway have been demonstrated; an example is the ER-α mutant with a tyrosine to asparagine substitution at amino acid 537. This mutant receptor was isolated from a metastatic breast cancer, and it exhibits a potent, estradiol-independent transcriptional activity that is only weakly affected by estrogens and antiestrogens. Tyrosine 537 in ER-α is a phosphorylated residue, and its phosphorylation may be required for efficient estrogen binding. The substitution of the tyrosine residue with an asparagine may produce a conformational change in ER-α that mimics hormone binding, resulting in constitutive receptor co-activator binding. Further work is required to determine whether other metastatic lesions contain this mutation.

It has also been discovered that ~30% of premalignant breast lesions contain a somatic mutation in ER-α that results in the replacement of lysine 303 with an arginine (K303R ER-α); this mutation confers an increased sensitivity to estrogen and causes cells to maximally proliferate in response to very low levels of estrogen. Thus, the presence of this hypersensitive mutation in many premalignant lesions with high levels of ER-α expression may serve to promote progression to cancer. The K303R ER-α mutation is thus a gain-of-function change, and interestingly, it occurs at the major acetylation site of the receptor. Similarly, mutations in the β-catenin protein acetylation site are frequent in cancers and represent a gain-of-function alteration, suggesting an important role for acetylation in key regulatory proteins.

## VII. PR IN BREAST CANCER DEVELOPMENT AND PROGRESSION

The PR is a positively regulated estrogen-responsive gene, and its expression is thought to indicate that functional ER is present. However, it is becoming increasingly clear that PR has its own clinical and biological significance in breast cancer. Much of the current understanding of the expression of the two PR isoforms comes from studies using rodent mammary glands, in which PR-A predominates over PR-B at a ratio of 3:1. Since PR-A and PR-B possess different and sometimes opposing transcriptional activities, it is believed that the regulated expression of both receptors is critical for appropriate mammary responsiveness to progesterone. Indeed, in transgenic mice expressing additional PR-A, mammary gland development is characterized by excessive lateral ductal branching, whereas transgenic mice expressing additional PR-B show inappropriate alveolar growth. Clearly, these observations show that the balance of the two receptor isoforms is critical in the control of progesterone responses in normal breast tissue.

Unlike the rodent mammary gland, normal human breast co-expresses PR-A and PR-B at similar levels within the same cells throughout the menstrual cycle. It is thought that coordinate expression of PR-A and PR-B may be required for normal progesterone response of the mammary gland and that changes in this ratio occur early in tumorigenesis. Indeed, one recent study showed that loss of coordinate receptor isoform expression is found in 30% of ADH and more than 50% of DCIS and IBC. In addition, several *in vitro* and *in vivo* studies have shown that overexpression of PR-A is associated with the loss of adherent properties in T47D breast cancer cells and disruption of the basement membrane in the mouse mammary gland—both properties of cells with increased invasive potential. In contrast, PR-B overexpression results in premature growth arrest in the mouse mammary gland, suggesting that this receptor isoform might be involved in the down-regulation of cell growth. These data clearly demonstrate that coordinate expression of the PR isoforms is crucial for normal development and that an imbalance in their expression patterns can alter normal growth in the breast.

## VIII. PR AS A PROGNOSTIC AND PREDICTIVE FACTOR IN BREAST CANCER

In breast tumor samples, PR (as measured by ligand-binding assay) has many of the same prognostic and predictive implications as ER. Approximately 50% of primary breast tumors are positive for both PR and ER, whereas only 4% are negative for ER but still positive for PR. In addition, well-differentiated tumors are more likely to be PR-positive than are poorly differentiated tumors. Several clinical studies have confirmed that elevated PR levels correlate with an increased probability of response to tamoxifen, longer time to treatment failure, and longer overall survival.

The classical methods for determining PR levels in clinical samples are various ligand-binding assays as well as receptor antibody-based assays such as immunohistochemistry. However, these assays cannot differentiate between the two PR isoforms, so that information about the expression and the potential separate roles of these two receptors as prognostic and/or predictive factors is lost. Only two papers have examined PR-A and PR-B levels in small datasets showing that expression of the PR isoforms is very heterogeneous. In addition, high PR-A levels have been associated with a less differentiated tumor phenotype. More studies examining the two forms separately are needed for definitive conclusions to be drawn.

## IX. CONCLUSIONS

Normal mammary cells contain a small but distinct population of ductal epithelial cells expressing ER and PR, and these hormone receptor expression patterns are significantly altered during breast carcinogenesis. Much of the breast cancer research in the past 30 years has focused on ER-α, but recently it has become clear that PR is not just a marker for a functional ER signaling pathway, but has its own role in breast cancer development and progression. Furthermore, ER and PR status is routinely used in the clinic to make treatment decisions. Receptor levels are only very weak prognostic indicators, and receptor-positive patients have a better outcome during the first 5 years of treatment. However, ER and PR status is most important in predicting benefit from hormone therapy. The role of different ER somatic mutations such as the K303R ER-α mutation or the role of ER-β in clinical applications is the subject of continued study.

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## Glossary

**anti-estrogens** Drugs such as tamoxifen and raloxifene that bind to the estrogen receptor and inhibit transcriptional activation of estrogen-regulated genes; they are commonly used for the treatment and prevention of breast cancer.

**breast cancer** A hormone-dependent neoplasm that is commonly derived from epithelial cells lining the terminal duct lobular unit of the mammary gland.

**estrogen receptor** Nuclear transcription factor that binds the steroid hormone estrogen and modulates estrogen-mediated transcription of hormone-regulated genes such as PS2 and cyclin D1.

**progesterone receptor** Nuclear transcription factor that binds the steroid hormone progesterone and modulates progesterone-mediated transcription of hormone-regulated genes such as STAT5A.

## See Also the Following Articles

**Aromatase and Estrogen Insufficiency • Estrogen and Spermatogenesis • Estrogen in the Male: Nature, Sources, and Biological Effects • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Biology and Lessons from Knockout Mice • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Phytoestrogens • SERMs (Selective Estrogen Receptor Modulators)**

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# Estrogen and Spermatogenesis

Liza O'Donnell

*Prince Henry's Institute of Medical Research, Clayton, Victoria, Australia*

I. INTRODUCTION
II. BRIEF OVERVIEW OF SPERMATOGENESIS
III. BIOSYNTHESIS AND ACTION OF ESTROGEN—GENERAL CONSIDERATIONS
IV. AROMATASE AND ESTROGEN RECEPTORS IN THE TESTIS AND EPIDIDYMIS
V. EFFECTS OF ESTROGEN ADMINISTRATION ON SPERMATOGENESIS—IDENTIFICATION OF ESTROGEN-RESPONSIVE SITES
VI. ESTROGEN AND SPERMATOGENESIS—LESSONS FROM TRANSGENIC MICE
VII. CONCLUSIONS

**Male fertility is dependent on sperm production, the process of spermatogenesis. Although this process is well known to be regulated by gonadotropins and androgens, it is now apparent that spermatogenesis and male fertility are dependent on estrogen action and can be disrupted by inappropriate exposure to estrogenic substances.**

## I. INTRODUCTION

Although androgens are considered "male" hormones and are produced in high concentrations by the testis, it is well known that the testis has the capacity to synthesize estrogen from testosterone in a reaction catalyzed by the aromatase enzyme. The testis also has the capacity to respond to estrogens because estrogen receptors (ERs) are present within the various cell types of the testis, although the cellular distribution of ER subtypes is still somewhat unclear.

It has been known for many years that administration of estradiol, usually in supraphysiological doses, or of potent estrogenic ligands, such as diethyl stilbestrol (DES), has deleterious effects on male fertility in rodent models. Such models of exogenous estrogen administration have been important for identifying sites in spermatogenesis that are responsive to estrogen, and provide support to the proposition that inappropriate exposure to environmental estrogens may harm male fertility. This proposition has received considerable interest due to reported decreases in sperm counts in humans over the past 50 years, although there is controversy as to the whether sperm counts have actually declined and whether exposure of humans to the relatively low levels of estrogens in the environment would cause significant health problems.

Definitive evidence of a physiological role for estrogen in spermatogenesis and male fertility, and of the key sites that are estrogen-dependent, has only recently been revealed by transgenic mouse models, in which either the ability to produce or to respond to estrogen is impaired. The characterization of defects to the process of spermatogenesis in these mouse models provides valuable clues as to where and how estrogen acts in the male reproductive system. The fact that estrogen overexposure or deficiency impairs sperm production and male fertility, together with the demonstration of estrogen production and action within the testis and epididymis, suggests that estrogen can now be considered a hormone important for spermatogenesis and for male fertility.

## II. BRIEF OVERVIEW OF SPERMATOGENESIS

### A. Germ Cell Development

Spermatogenesis is the process of male germ cell development. Immature spermatogonia within the testis proliferate and differentiate to form meiotic spermatocytes in which DNA replication and pairing

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# Estrogen and Spermatogenesis

Liza O'Donnell

*Prince Henry's Institute of Medical Research, Clayton, Victoria, Australia*

I. INTRODUCTION
II. BRIEF OVERVIEW OF SPERMATOGENESIS
III. BIOSYNTHESIS AND ACTION OF ESTROGEN—GENERAL CONSIDERATIONS
IV. AROMATASE AND ESTROGEN RECEPTORS IN THE TESTIS AND EPIDIDYMIS
V. EFFECTS OF ESTROGEN ADMINISTRATION ON SPERMATOGENESIS—IDENTIFICATION OF ESTROGEN-RESPONSIVE SITES
VI. ESTROGEN AND SPERMATOGENESIS—LESSONS FROM TRANSGENIC MICE
VII. CONCLUSIONS

**Male fertility is dependent on sperm production, the process of spermatogenesis. Although this process is well known to be regulated by gonadotropins and androgens, it is now apparent that spermatogenesis and male fertility are dependent on estrogen action and can be disrupted by inappropriate exposure to estrogenic substances.**

## I. INTRODUCTION

Although androgens are considered "male" hormones and are produced in high concentrations by the testis, it is well known that the testis has the capacity to synthesize estrogen from testosterone in a reaction catalyzed by the aromatase enzyme. The testis also has the capacity to respond to estrogens because estrogen receptors (ERs) are present within the various cell types of the testis, although the cellular distribution of ER subtypes is still somewhat unclear.

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## II. BRIEF OVERVIEW OF SPERMATOGENESIS

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Spermatogenesis is the process of male germ cell development. Immature spermatogonia within the testis proliferate and differentiate to form meiotic spermatocytes in which DNA replication and pairing of homologous chromosomes occur. Spermatocytes then undergo two meiotic divisions to yield haploid, round spermatids. Round spermatids differentiate, with no further division, into highly specialized mature, elongated spermatids in a process known as spermiogenesis, in which acrosome formation, DNA compaction, nuclear shaping, flagellum formation, and cytoplasm condensation and elimination occur. The mature spermatid is then released from the epithelium prior to its passage to the epididymis. The full fertilizing potential of the released spermatozoan is achieved during the progression and maturation of sperm through the excurrent duct system (efferent ductules) and epididymis.

### B. Organization of the Testis

Spermatogenesis takes place within the seminiferous tubules of the testis. The series of convoluted tubules end in a "reservoir" known as the rete testis. Sperm exit the testis via the rete testis, which is connected to a series of extratesticular ductules, known as the efferent ductules, that connect the rete testis to the initial segment at the head of the epididymis. The efferent ductules resorb a majority of the fluid arising from the seminiferous tubules so that spermatozoa become concentrated as they enter the epididymis, thus ensuring that a large number of spermatozoa are released on ejaculation. Sperm then traverse the ducts of the initial segment, caput, corpus, and cauda epididymis during their final maturation phase, attaining motility.

Germ cells are supported and nurtured during their development in the seminiferous tubules by the somatic Sertoli cells. These morphologically complex epithelial cells have an extensive cytoplasm with numerous cup-shaped processes encompassing the various germ cell types. Sertoli cells reside on a basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells, which surround the seminiferous tubules. The interstitial space between the seminiferous tubules contains the steroidogenic Leydig cells, which are primarily responsible for androgen production, and the blood and lymphatic vessels, which are essential for the movement of hormones and nutrients into and out of the testis.

### C. Regulation of Spermatogenesis

Germ cell development relies on a highly coordinated interaction with the Sertoli cell, involving communication either directly via ligand/receptor-mediated interactions or via the release of paracrine factors. Spermatogenesis is absolutely dependent on gonadotropins and androgens. Luteinizing hormone (LH) from the pituitary acts on receptors within Leydig cells to stimulate the production of testosterone, which in turn acts on androgen receptors within Sertoli cells to promote spermatogenesis. Follicle-stimulating hormone (FSH) is not essential for spermatogenesis, although quantitatively normal sperm production and function require the action of FSH. Sertoli cells contain receptors for both androgen and FSH but germ cells apparently do not; thus the Sertoli cell receives endocrine signals from the interstitium and circulation and subsequently synthesizes factors that modulate germ cell development. Leydig and Sertoli cells can also be considered endocrine cells because they produce hormones that are secreted into the circulation and act on other tissues; Leydig cells secrete into the circulation high concentrations of testosterone, which has a negative feedback effect on pituitary gonadotropin secretion, whereas Sertoli cells produce hormones such as inhibin and activin, which regulate pituitary FSH release.

## III. BIOSYNTHESIS AND ACTION OF ESTROGEN—GENERAL CONSIDERATIONS

### A. Biosynthesis

The biosynthesis of estrogen from testosterone is catalyzed, in an irreversible reaction, by a microsomal member of the cytochrome P450 superfamily, namely, aromatase cytochrome P450 (P450arom, the product of the *CYP19* gene). This heme protein is responsible for binding the $C_{19}$ androgenic steroid substrate and catalyzing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens. The reducing equivalents for this reaction are supplied from NADPH via a ubiquitous microsomal flavoprotein, NADPH-cytochrome P450 reductase. Aromatase P450 is found in many tissues, including the testis, and is regulated in a cell- and tissue-specific manner via the use of alternative promoters and alternative exons I, which are spliced into the 5′-untranslated region of the aromatase transcript in a tissue-specific fashion.

### B. Action

Estrogen receptors are members of the ligand-activated nuclear receptor family, which includes receptors for other steroid hormones. The first ER gene (encoding ERα) was cloned in the 1980s and for several years it was thought that only one form of ER existed. However a second ER gene (encoding ERβ) was cloned in the mid-1990s. Both receptors have a high affinity for estradiol but exhibit various functional differences, including different affinities for other estrogenic ligands as well as cell and tissue specificity. The distinct properties exhibited by ERα and ERβ have been exploited for therapeutic use by the development of selective estrogen receptor modulators (SERMs). Estrogenic ligands can act on their receptors in a classic steroid receptor pathway involving ligand binding, receptor activation and dimerization (homo- or heterodimers), nuclear translocation and the subsequent binding of the ligand-receptor complex to estrogen-responsive elements within the promoter regions of target genes, recruitment of cofactors, and regulation of transcription. Ligand-bound ERs can also modulate transcription independently of direct DNA binding, by binding to proteins within a preformed transcriptional complex. Ligand-independent transcriptional activation of ERs by various growth factors, for example, is also possible.

Estrogen can also act in a nongenomic fashion, inducing rapid (within seconds to minutes) increases in the concentration of calcium or cyclic adenosine monophosphate (cAMP) second messengers, presumably via receptors on the plasma membrane. These receptors appear to be related to classic ERs in some, but not all, cases. There is also evidence to show that rapid plasma membrane-dependent actions of estrogen act in concert with the classic ER-mediated genomic pathway.

## IV. AROMATASE AND ESTROGEN RECEPTORS IN THE TESTIS AND EPIDIDYMIS

It has been known for decades that the testis can produce estrogen, and the concentration of estrogen in the testis and rete testis fluid far exceeds circulating levels. The particular cell types within the testis that can produce and/or respond to estrogen (Fig. 1) has received considerable attention in recent years due to the increased interest in estrogen's role in male fertility.

### A. Aromatase

Aromatase is found in the testis from the fetal stage to adulthood. During pubertal development, when germ cell development is in the early stages, aromatase expression is thought to be primarily in the Sertoli and Leydig cells. Estrogen production by Sertoli cells is particularly high during the brief postnatal period of Sertoli cell division and is stimulated by FSH. In the adult testis, the Leydig cells express a high level of aromatase, but Sertoli cell expression has markedly decreased. Germ cells, which are the predominant cell type in the adult testis, are now known to express aromatase and synthesize estrogen. Along with Leydig cells, germ cells are a primary source of estrogen in the adult testis.

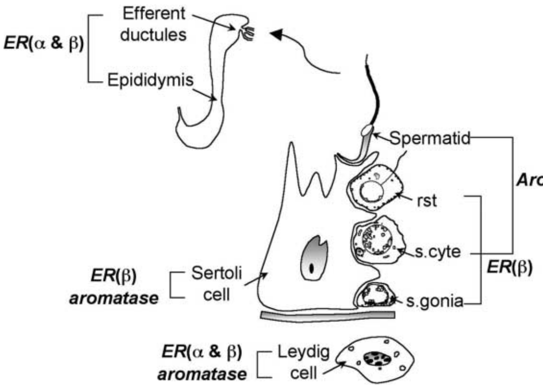

FIGURE 1 Diagram of the likely cellular localization of aromatase and estrogen receptors (ERs) in the testis. Efferent ductules and epididymis are also shown because these tissues are regulated by estrogen and have an impact on spermatogenesis. The likely ER subtype (α or β) is shown in parentheses, although controversy still exists as to the exact subtype present in each cell population. Abbreviations: rst, round spermatid; s.cyte, spermatocyte; s.gonia, spermatogonia.

The ability of germ cells to produce estrogen is first apparent in the meiotic pachytene spermatocyte phase, when the expression of aromatase is first detected. Postmeiotic round spermatids also express aromatase and produce estrogen. The capacity to produce estrogen is retained throughout spermatid elongation and although most of the cytoplasm of the spermatid is shed prior to its release from the seminiferous epithelium, there is evidence to suggest that the remaining portion of cytoplasm, the cytoplasmic droplet that is attached to the flagella, can synthesize estrogen. The production of estrogen by germ cells and mature sperm has been hypothesized to be the reason why rete testis fluid in many species contains high concentrations of estrogen.

### B. Estrogen Receptors

Leydig cells, Sertoli cells, and germ cells have the capacity to respond to estrogen by the expression of ERs. Thus actions of estrogen in the testis can conceivably be endocrine, paracrine, or autocrine. There is controversy to date as to the cellular localization of the estrogen receptor subtypes within the testis, and a considerable amount of conflicting data. There is good evidence for both ERα and ERβ in the Leydig cells from various species and thus it seems likely that this cell type is capable of estrogen action via ERα- and ERβ-mediated actions. There are numerous reports of ERβ in Sertoli cells, but only limited studies demonstrating ERα. This also seems to be the case in germ cells, with numerous descriptions of ERβ, but more limited evidence for ERα in the various germ cell populations. The cell-specific expression of ER in the various germ cell populations is controversial, but there is good evidence in various species for ERβ expression in spermatogonia, some spermatocytes, and some spermatids.

Another important consideration is ER expression by the efferent ductules and epididymis, because these tissues are a target for estrogen action. ERα expression is particularly high in efferent ductules of rodents, but ERβ is also present in various cell types. Localization of ER subtypes within the various cells of the epididymis is again controversial, although it is likely that this tissue contains both subtypes.

## V. EFFECTS OF ESTROGEN ADMINISTRATION ON SPERMATOGENESIS—IDENTIFICATION OF ESTROGEN-RESPONSIVE SITES

Experiments involving exogenous estrogen administration, usually to rodents, have been important for identifying the cells and processes in spermatogenesis (Table 1) that are responsive to estrogen.

### A. Hypothalamo–Pituitary–Testis Axis

Initiation of spermatogenesis during neonatal development and maintenance during adulthood require the secretion of gonadotropins from the pituitary and thus are dependent on the balance of the hypothalamo–pituitary–testis axis. A major component of the negative feedback action of androgens on gonadotropin secretion is mediated via aromatization to estrogen. Administration of supraphysiological doses of estrogen during neonatal development or adulthood therefore suppresses gonadotropin secretion, leading to a secondary impairment of spermatogenesis. Neonatal estrogen exposure can have long-term effects on the hypothalamo–pituitary–testis axis and can permanently impair testicular function, as demonstrated in rodent models.

### B. Testicular Descent

Testicular descent is also sensitive to estrogen, because exposure to high levels of estrogens *in utero* causes cryptorchidism (failure of testicular descent) in rodents, and perhaps in humans. The mechanism of estrogen action on testicular descent may be in part dependent on the regulation of the insulin-3 (*Insl3*) gene in fetal Leydig cells, because this gene appears to be down-regulated by estrogen overexposure and transgenic mice lacking this gene are cryptorchid.

### C. Leydig Cells

Leydig cell precursors undergo rapid proliferation early in postnatal development and then differentiate into adult-type Leydig cells. Neonatal estrogen exposure can interfere with Leydig cell development and proliferation during puberty, perhaps causing permanent changes to Leydig cell function. Estrogen administration *in vivo* or *in vitro* appears to inhibit Leydig cell steroidogenic enzymes and decrease androgen production.

**TABLE 1 Summary of Proposed Roles of Estrogen in Spermatogenesis Utilizing Estrogen Administration Models and Transgenic Mice**

| Model | Administration[a] | Deficiency[b] |
|---|---|---|
| Leydig cells | Interferes with proliferation, differentiation, and function | Hyperplasia in ArKO mice |
| Sertoli cells | Impairs maturation | Reduced fluid secretion by tubules in ERαKO |
| Germ cells | Promotes proliferation and survival *in vitro* | Apoptosis in ArKO; defects in ERαKO a consequence of efferent ductule dysfunction |
| Efferent ductules[c] | Impairs development and function | Impaired development and function in ERαKO |
| Epididymis[c] | Impairs development | Various morphological abnormalities in ERαKO |

[a] Exposure to physiological or supraphysiological estrogen or estrogenic ligands *in vivo* or *in vitro*.

[b] Congenital deficiency due to targeted disruption of ER or aromatase.

[c] These tissues have been included because their function is important for normal sperm function, and efferent ductule dysfunction has a secondary impact on spermatogenesis.

### D. Sertoli Cells

In rodents, Sertoli cells proliferate during the late fetal and early postnatal periods. This period of Sertoli cell proliferation, and the postproliferative differentiation and maturation of these cells, are essential for the full spermatogenic potential of the adult. The fact that proliferative Sertoli cells secrete high levels of estrogen, but nonproliferative Sertoli cells have decreased estrogen production, has led to the suggestion that estrogen is stimulatory for Sertoli cell proliferation and inhibitory for differentiation. Consistent with this, neonatal exposure to DES impairs Sertoli cell maturation, independent of its effects on gonadotropin secretion, causing permanent defects to spermatogenesis in adulthood.

### E. Germ Cells

The localization of aromatase and ERs in various germ cell populations suggests a role for estrogen in germ cell development. Information on the effects of estrogen administration on germ cells has been gleaned from *in vitro* studies, thus effects on other hormones and cell populations do not confound the results. Estrogen administration stimulates proliferation of prespermatogonial cells, or gonocytes, and prevents germ cell apoptosis in seminiferous tubules *in vitro*. Thus, it is possible that endogenous estrogen, along with other hormones, controls germ cell proliferation and survival. Estrogen can also regulate the function of mature spermatozoa in an apparent nongenomic mechanism.

### F. Efferent Ductules and Epididymis

Exposure to high doses of estrogen *in vivo* in rodents can impair the resorptive function of the efferent ductules. Exposure to high doses of estrogens during the first few days of life causes dilation of the rete testis and an accumulation of fluid in the testis, which then causes secondary defects in the seminiferous epithelium and spermatogenesis. The mechanism of this effect on the efferent ductules may be via changes in the expression of ERs and of genes important in fluid resorption, as well as changes in cellular morphology. Exposure to high doses of estrogens *in utero* causes epididymal granulomas, whereas exposure during the neonatal period causes a variety of defects in epididymal development.

## VI. ESTROGEN AND SPERMATOGENESIS—LESSONS FROM TRANSGENIC MICE

In general, two important observations have been made in transgenic mouse models regarding estrogen and spermatogenesis. First, exogenous estrogen can stimulate spermatogenesis in a transgenic mouse that is congenitally deficient in gonadotropins (the *hpg* mouse). Second, transgenic mice that lack functional genes for aromatase (aromatase knockout, ArKO), ERα (ERαKO), and ERα + ERβ (ERαβKO) exhibit disruptions to spermatogenesis and infertility. These latter models have provided much information as to the requirement for estrogen in the development and maintenance of spermatogenesis and fertility (Table 1).

### A. ERαKO

This mouse model was the first to show definitive roles for estrogen action in many physiological processes, including spermatogenesis. ERαKO mice are infertile, with disrupted germ cell development in the seminiferous epithelium. This disruption to spermatogenesis is now known to be secondary to abnormal development and function of the efferent ductules. The congenital absence of ERα causes marked disturbances in the ability of the efferent ductules to resorb fluid leaving the testis, causing a buildup of fluid in the rete testis and seminiferous tubules, which then interferes with normal germ cell development. Thus, efferent ductule dysfunction is thought to be a primary cause of infertility in these animals. Germ cell transplantation studies show that germ cells do not require ERα for development. ERαKO mice show some defects in cremaster muscle development, which is involved in testicular descent.

### B. ERβKO (ERβ Null Mice)

Despite the widespread localization of ERβ in the testis and seminiferous epithelium, ERβKO male mice are fully fertile, and no defects in spermatogenesis have been reported. These observations perhaps suggest compensation by ERα, although such a proposition has not been tested.

### C. ERαβKO

Breeding of mice lacking ERα or ERβ results in the generation of mice deficient in both ERs. The spermatogenic phenotype in ERαβKO mice is apparently similar to ERαKO mice, because ERαβKO mice also exhibit infertility, reduced sperm numbers in the epididymis, and apparent dilation of seminiferous tubule lumens.

### D. ArKO

Several independent lines of ArKO mice have been generated, all of which exhibit varying degrees of infertility. ArKO mice are initially fertile, with apparently normal testicular development. The lack of enlarged seminiferous tubule lumens at any age suggests that efferent ductule function is normal and fluid accumulation does not occur. In one line of ArKO mice, at the round spermatid stage a marked loss of germ cells by apoptosis was described, suggesting that endogenous estrogen is important for germ cell development. Leydig cell hyperplasia and defective acrosome biosynthesis were also observed in these animals.

### E. Aromatase Overexpression

Mice overexpressing aromatase have been generated and provide complementary evidence to exogenous estrogen administration models. These mice have high circulating levels of estrogen and show Leydig cell hyperplasia and/or Leydig cell tumors. Like *in utero* exposure to high levels of estrogen, mice overexpressing aromatase have failed testicular descent; this cryptorchidism may at least in part explain the failure of spermatogenesis and infertility in these mice.

## VII. CONCLUSIONS

Taking together the results from studies localizing aromatase and ERs in the testis and epididymis, and from estrogen administration and transgenic mice models, it is apparent that spermatogenesis is dependent on estrogen action. Various processes important for spermatogenesis are impaired by inappropriate exposure to estrogens during fetal and pubertal development, as well as in adulthood, highlighting the importance of a normal balance between androgens and estrogens for male fertility. Spermatogenesis is also impaired by a congenital deficiency of ERα or aromatase, providing clues as to the physiological roles for estrogen action. There are enough discrepancies between the phenotypes of ER- and aromatase-null mice to speculate on the existence of (1) undiscovered aromatase or ER genes, (2) estradiol-independent ER actions, and/or (3) estrogen actions independent of ERα or ERβ in the testis and male reproductive tract. Nevertheless, it is clear that normal spermatogenesis requires estrogen, and that the timing, amount, and duration of estrogen action must be tightly coordinated for the development and maintenance of normal sperm production.

## Glossary

| | |
|---|---|
| **aromatase** | Enzyme that catalyzes the conversion of androgens to estrogens. A member of the cytochrome P450 superfamily, namely, aromatase cytochrome P450 (P450arom, product of the *CYP19* gene). In common with other microsomal P450 enzymes, aromatase requires NADPH-cytochrome P450 reductase to provide reducing equivalents. |
| **efferent ductules** | Series of ductules connecting the testis to the head of the epididymis; their primary function is to resorb fluid leaving the testis in order to concentrate sperm on entry to the epididymis. |
| **estrogen** | Substance that promotes development of female secondary sexual characteristics. The "traditional" estrogen is 17β-estradiol, although numerous substances have estrogenic activity. |
| **estrogen receptor** *α* | The first identified receptor for estrogen; estrogen receptors are members of the nuclear receptor family. |
| **estrogen receptor** *β* | A second receptor for estrogen that was identified more recently; encoded by a separate gene. |
| **Leydig cells** | Steroidogenic cells of the testis; their main function is to secrete androgens. |
| **Sertoli cells** | The "nurse" cells of the seminiferous epithelium; these cells provide nutritional and structural support for developing germ cells. |
| **spermatids** | Haploid germ cells that undergo differentiation from an early, rounded form to a mature, elongated form. |
| **spermatocytes** | Tetraploid germ cells undergoing meiosis. |
| **spermatogenesis** | Process by which immature germ cells divide and differentiate into mature spermatozoa. |
| **spermatogonia** | Proliferative and differentiating diploid cells that are the most immature germ cell type. |

## See Also the Following Articles

**Aromatase and Estrogen Insufficiency • Estrogen and the Male: Nature, Sources and Biological Effects • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Biology and Lessons from Knockout Mice • Phytoestrogens • Spermatogenesis, Hormonal Control of**

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# Estrogen in the Male: Nature, Sources, and Biological Effects

Hans-Udo Schweikert
*University of Bonn*

I. INTRODUCTION
II. CHARACTERISTIC FEATURES OF ANDROGEN METABOLISM AND ACTION IN MALES
III. MUTATIONS THAT IMPAIR ANDROGEN ACTION IN THE HUMAN
IV. STEROID PATHWAYS IN PERIPHERAL TISSUES
V. ESTROGEN SYNTHESIS IN PERIPHERAL TISSUES
VI. ORIGIN OF ESTROGENS IN MALES
VII. REGULATION OF PERIPHERAL ESTROGEN PRODUCTION
VIII. INCREASED ESTROGEN PRODUCTION IN MALES
IX. SERUM ESTROGEN LEVELS IN MALES
X. BIOLOGICAL ACTIONS OF ESTROGEN IN MALES
XI. MUTATIONS THAT IMPAIR THE FUNCTION OF THE ESTROGEN RECEPTOR OR THE FORMATION OF THE ENZYME AROMATASE IN HUMAN MALES
XII. MUTATIONS THAT IMPAIR THE FUNCTION OF THE ESTROGEN RECEPTOR OR THE FORMATION OF THE ENZYME AROMATASE IN RODENTS
XIII. SUMMARY

**Estrogen is part of the male endocrine system. In healthy males the main source of estrogens is the conversion of circulating androgens in peripheral tissues. Data obtained from human mutations impairing either estrogen action or formation, and from corresponding knockout mouse models, demonstrate that in the male, estrogen plays an important biological role in the maturation and homeostasis of the skeleton. At present the impact of estrogen on other tissues or functions in the male, including the cardiovascular system, fertility, or glucose and lipid metabolism, is less clear.**

## I. INTRODUCTION

Recognition of estrogens as "male" hormones dates to 1934, when Zondek reported that the urine of stallions contained large quantities of estrogen, whereas the urine of mares contained lower quantities of estrogen. Zondek concluded from the observation that "the conversion of the male into the female hormone appeared to be quite possible." Three years later, Steinach and Kun provided supporting evidence for this assumption when they found that urinary excretion of estrogen increased after the administration of testosterone propionate to men. In 1979, MacDonald and co-workers demonstrated that the conversion of circulating testosterone and androstenedione by the enzyme aromatase in peripheral tissues is quantitatively the major source of estrogen in men. However, the biological role of estrogen in males remained largely unknown until 1994, when complete estrogen insensitivity (estrogen resistance) due to a nonfunctional mutated estrogen receptor (ER) had been recognized in a man who had a distinctive phenotype, mainly eunuchoid habitus and severe osteopenia, despite normal serum androgen and elevated estrogen levels. Thereafter, the importance of estrogen in males was further

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# Estrogen in the Male: Nature, Sources, and Biological Effects

Hans-Udo Schweikert
*University of Bonn*

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XIII. SUMMARY

**Estrogen is part of the male endocrine system. In healthy males the main source of estrogens is the conversion of circulating androgens in peripheral tissues. Data obtained from human mutations impairing either estrogen action or formation, and from corresponding knockout mouse models, demonstrate that in the male, estrogen plays an important biological role in the maturation and homeostasis of the skeleton. At present the impact of estrogen on other tissues or functions in the male, including the cardiovascular system, fertility, or glucose and lipid metabolism, is less clear.**

## I. INTRODUCTION

Recognition of estrogens as "male" hormones dates to 1934, when Zondek reported that the urine of stallions contained large quantities of estrogen, whereas the urine of mares contained lower quantities of estrogen. Zondek concluded from the observation that "the conversion of the male into the female hormone appeared to be quite possible." Three years later, Steinach and Kun provided supporting evidence for this assumption when they found that urinary excretion of estrogen increased after the administration of testosterone propionate to men. In 1979, MacDonald and co-workers demonstrated that the conversion of circulating testosterone and androstenedione by the enzyme aromatase in peripheral tissues is quantitatively the major source of estrogen in men. However, the biological role of estrogen in males remained largely unknown until 1994, when complete estrogen insensitivity (estrogen resistance) due to a nonfunctional mutated estrogen receptor (ER) had been recognized in a man who had a distinctive phenotype, mainly eunuchoid habitus and severe osteopenia, despite normal serum androgen and elevated estrogen levels. Thereafter, the importance of estrogen in males was further substantiated by the description of males with complete estrogen deficiency due to mutations of the aromatase gene. The two men with aromatase deficiency reported hitherto had a similar phenotype as in estrogen resistance but in contrast to the former showed significant bone maturation with estrogen therapy. These observations document the important role of normal estrogen formation and action in the male, notably bone. Two other findings have provided further insight into the complex mechanism(s) of action of estrogen in males. In 1996, the cloning of a second estrogen receptor, ER-β (as opposed to the classical ER, which is now designated ER-α), was reported. Moreover, knockout mouse models with disruption of the genes encoding the estrogen receptors (αERKO, βERKO, and αβERKO double knockout mice) and the enzyme aromatase (ARKO mouse) have been developed.

## II. CHARACTERISTIC FEATURES OF ANDROGEN METABOLISM AND ACTION IN MALES

Although androgens act at the molecular level like other steroid hormones, i.e., by combining with the androgen receptor (AR), their mechanism of action is characterized by the fact that in many androgen target tissues, testosterone is converted to 5α-dihydrotestosterone (DHT) by steroid 5α-reductase (5α-R) activities before binding to the AR. Two 5α-R isozymes, designated types 1 and 2, have been identified and originate from different genes. 5α-R type 1, the predominant isozyme, has been identified in a variety of human tissues such as nongenital skin, kidney, and brain. In genital tissues such as the prostate and the external genitalia, 5α-R type 2 appears to be the predominant isozyme. DHT is biologically more potent than testosterone as an androgen because DHT binds more tightly to the androgen receptor and the DHT–receptor complex is more readily transferred to the DNA-binding state and activates a receptor reporter gene more efficiently than testosterone. Consequently, DHT serves as a local androgen amplification mechanism of testosterone and may also regulate specific genes.

Because of their chemical structure, testosterone and androstenedione (which is secreted mainly by the adrenal glands) not only can be reduced in a variety of peripheral tissues to 5α-reduced metabolites, to produce or lead to the formation of the biologically important DHT, but can also serve as substrates for aromatization to estrogen (to estradiol from testosterone and to estrone from androstenedione; Fig. 1). Both the 5α-reduction and the aromatization of testosterone and androstenedione are irreversible reactions under biological conditions.

The property of circulating testosterone and androstenedione serving as precursors for the synthesis of estrogen and for the formation of DHT makes it difficult to study the androgenic effects in their target organs, since every androgenic effect observed must be investigated with regard to the extent to which it is induced by either testosterone, DHT, or estradiol or, alternatively, by their synergistic action.

## III. MUTATIONS THAT IMPAIR ANDROGEN ACTION IN THE HUMAN

Insight into the specific action of both testosterone and DHT has been obtained from two different single gene mutations in the human, namely, male pseudohermaphroditism due to complete resistance to androgens (the complete androgen insensitivity syndrome, CAIS; also known as testicular feminization) and that due to deficient 5α-reductase activity (5α-reductase deficiency). Both disorders impair androgen action and are natural models for studying the role of testosterone and DHT in males.

### A. Complete Androgen Insensitivity Syndrome

A 46,XY karyotype, testes and an unambiguously female habitus and psychosexual orientation characterize individuals with CAIS. Axillary and pubic hair are sparse or absent (hairless women) and the external genitalia are female. The female internal genitalia, the uterus and the fallopian tubes, are absent; the vagina is thus blind-ending. The testes are located in the abdomen, the inguinal canal, or in the labia majora. They contain normal Leydig cells and seminiferous tubules without spermatogenesis. In the adult, gonadotropin secretion and testosterone production are in the upper normal range for men or are elevated and estrogen production is increased. Adults tend to be tall, and bone age is normal. Bone mass is within the female range, which indicates that estrogens alone do not fully compensate for the loss of androgen receptor function. The underlying defects of the disorder are various mutations in the androgen receptor gene.

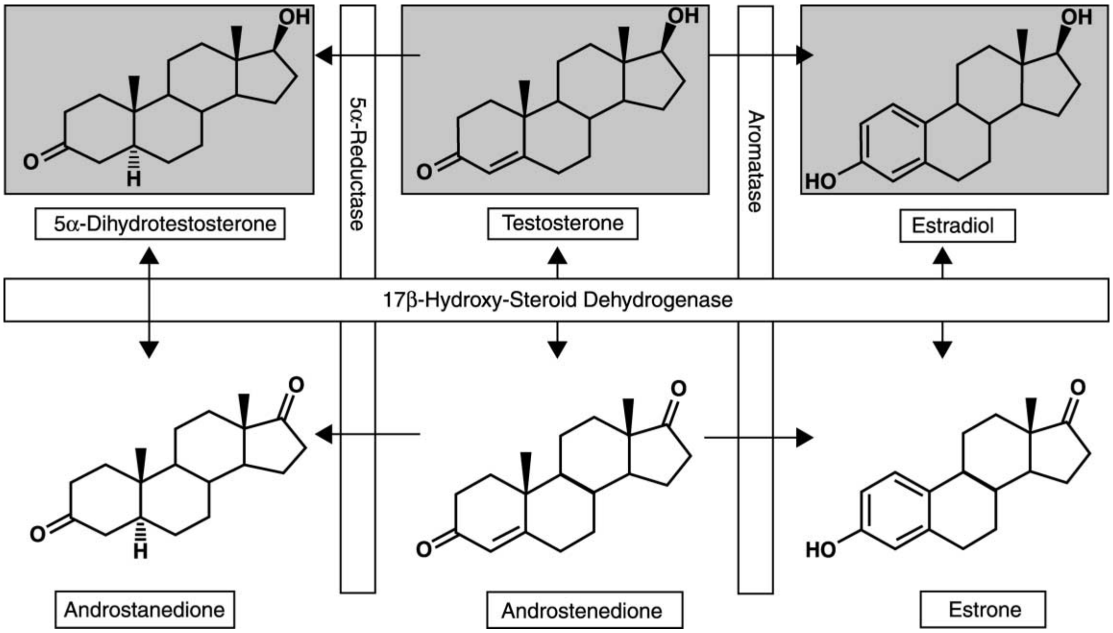

**FIGURE 1** Major pathways for the synthesis of active androgens and estradiol (shaded boxes) in peripheral human tissues. Two isozymes of the 5α-reductase and six isozymes of the 17β-hydroxysteroid dehydrogenase family, of which isozyme types 1, 3, 5, and 7 catalyze the reduction and types 2 and 4 catalyze the oxidation of steroids, have been determined to be expressed in human peripheral tissues. Small amounts of androstenedione can also be synthesized from adrenal androgens such as DHEA and its sulfate via the sequence DHEAS → DHEA → androstenedione. The conversion is mediated by the sequential action of the enzymes steroid sulfatase and 3β-hydroxysteroid dehydrogenase (two isozymes).

## B. 5α-Reductase Deficiency

This disorder is caused by mutations in the 5α-R type 2 gene. Affected individuals have a normal male karyotype, 46,XY, and testes, but incomplete virilization. The infant usually presents with undescended testes, ambiguous genitalia, such as a bifid scrotum, a severely hypoplastic penis, and hypospadias. However, the Wolffian duct derivates, the epidymidis, the vas deferens, and the seminal vesicle, are normally developed and open into a blind vaginal pouch. Female internal genitalia are absent. Virilization is observed at puberty (probably due to the conversion of testosterone to DHT by the 5α-R type 1) with phallic enlargement, descent of the testes, and deepening of the voice. Gynecomastia does not develop. Bone mineral content is in the low range for men. However, male-type facial and body hair commonly is scanty and the prostate remains rudimentary. Spermatogenesis is absent or impaired. Although most patients have been raised as girls, gender orientation in adulthood is usually unambiguously male. In the adult, serum gonadotropin levels are within the normal range or slightly elevated and testosterone production and estrogen production are those of normal men. Serum DHT levels are either subnormal or in the low normal range.

Since virilization in the disorder is defective only in the external genitalia and the prostate, where the 5α-R type 2 is normally expressed, it can be deduced that testosterone is the androgen responsible for the differentiation of the Wolffian duct into the epidymidis, the vas deferens, and the seminal vesicle, and it is DHT that mediates masculinization of the prostate and the external genitalia.

Taken together, the findings in individuals with single gene mutations that impair either DHT formation or testosterone and DHT action demonstrate that major effects of testosterone and DHT action include the development of the male sexual phenotype during embryogenesis (development of the internal and external genitalia as well as the prostate), the promotion of virilization at the time of puberty and its maintenance during adulthood, and gender orientation. In addition, the action of androgens is involved in the regulation of gonadotropin secretion, in the initiation and maintenance of spermatogenesis, and at least to some extent in the accumulation of male bone mineral density.

## IV. STEROID PATHWAYS IN PERIPHERAL TISSUES

The enzymes 5α-reductase, 17β-hydroxysteroid dehydrogenase (17β-HSD), and aromatase are the major metabolizing enzymes necessary for the formation of biologically active androgens (testosterone and DHT) and estrogens, mainly estradiol, from circulating precursors. Their activities are expressed to various extents in extraglandular tissues (Fig. 1). Androstenedione can also be formed from dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), which are secretion products of the adrenal. The complexity of this system in human peripheral tissues is highlighted by the presence of two 5α-reductase isozymes and at least six 17β-HSD isozymes. The isozymes of the 17β-HSD family catalyze either the reduction or the oxidation of sex steroids and therefore represent important control elements for their activation (by reduction) or inactivation (by oxidation). The enzyme sulfatase mediates the conversion of DHEAS to DHEA, and the latter can be transformed to androstenedione by means of the 3β-hydroxysteroid dehydrogenase, of which two isozymes are expressed in peripheral tissues. It is clear that, by means of this enzymatic machinery, peripheral cells and tissues can modulate, i.e., activate or inactivate, the biological potencies of circulating steroids. The potent androgens and estrogen formed from biologically inactive circulating precursors in peripheral tissues can be utilized to act directly at the site of their biosynthesis, e.g., by binding to their specific receptors (intracrine mechanism); alternatively, the steroids can be released to act on neighboring cells (paracrine mechanism) or can be secreted into the circulation to act at distal sites (endocrine function).

## V. ESTROGEN SYNTHESIS IN PERIPHERAL TISSUES

A variety of peripheral tissues have been identified to produce estrogen from circulating androstenedione and testosterone such as adipose tissue, brain, liver, skin (fibroblasts), hair roots, skeletal muscle, prostate, and bone.

### A. Enzyme Aromatase

Estrogen formation from androstenedione and testosterone in peripheral and testicular tissues is synthesized by the catalytic action of the membrane-bound enzyme aromatase, a cytochrome P450-dependent enzyme. NADPH is the necessary coenzyme. Aromatase action involves oxidation, removal of the methyl group at the carbon 19 position of testosterone or androstenedione, and removal of the hydrogen at the carbon 1β-position (Fig. 1). For aromatase activity, three molecules of oxygen are required per molecule of steroid. Aromatase is encoded by the CYP19 gene, which encodes a protein that in many species consists of approximately 500 amino acids. The coding region of the gene spans nine exons beginning with exon II, and expression of the gene is determined by tissue-specific promoters, which lead to transcripts that are always the same regardless of the site of expression.

The overall rates of estrogen formation in the testis (estrogens are not formed in the adrenals) and in the peripheral tissues are much lower than those for other steroid hormones, a relationship that is reflected by the greater potency, lower secretory rates, and lower blood levels of estrogen (Table 1).

## VI. ORIGIN OF ESTROGENS IN MALES

MacDonald and co-workers, using isotope infusion techniques, have assessed the daily production rate of estrogens and androgens in young healthy men. The mean daily plasma production rate of testosterone

**TABLE I** **Serum Levels, Daily Production Rates, and Origin of Androgens and Estrogens in Men**

| Steroid | Serum levels | Daily production rates | Origin |
|---|---|---|---|
| Testosterone | 3–10 ng/ml | 5–10 mg | >95% secreted by the testes |
| Androstenedione | 0.5–3.5 ng/ml | 2–4 mg | Approximately 60% secreted by the adrenals, rest formed from DHEAS and testosterone in peripheral tissues |
| Estradiol | 20–40 pg/ml | 40–60 μg | Approximately 85% from peripheral conversion of testosterone, androstenedione, and estrone, 15% from testicular secretion |
| Estrone | 20–40 pg/ml | 50–70 μg | 95–100% from peripheral conversion of androstenedione, testosterone, and estradiol |

was approximately 6 mg and that of androstenedione was 3.0 mg. The mean daily production rate of estrone was approximately 60 μg and that of estradiol was 45 μg. Estrone formation was derived totally by peripheral aromatization of circulating androstenedione and testosterone, whereas of the 45 μg of estradiol produced, only approximately 10 μg was secreted by the testes. Weinstein and co-workers have obtained similar results; testicular daily estradiol secretion was approximately 10 μg, or approximately 25% of the total estradiol production, whereas estrone secretion accounted for approximately 5%. Taken together, these studies demonstrate that in healthy men approximately 95 to 100% of the total daily production of estrone and 85% of estradiol are derived from peripheral aromatization of circulating serum androstenedione and testosterone, whereas the testes secrete only small amounts of estrogen (Table 1).

## VII. REGULATION OF PERIPHERAL ESTROGEN PRODUCTION

The factors that regulate estrogen formation are still not fully understood. Short-term administration of human chorionic gonadotropin in men before surgery resulted in a simultaneous increase of estradiol, estrone, and testosterone into spermatic vein blood. The rate of estrogen production is not influenced by castration or adrenalectomy, but is lowered due to the decreased concentrations of circulating androgen precursors, which serve as a substrate for aromatization.

## VIII. INCREASED ESTROGEN PRODUCTION IN MALES

Estrogen formation is enhanced with increasing body weight and with age. With slowly decreasing testosterone levels with advancing age and in the presence of obesity, the development of signs of feminization, especially gynecomastia, is more likely since there is an increased conversion of the remaining precursors to their respective estrogenic products. A markedly increased conversion of androstenedione to estrone is observed in liver disease and in adrenal tumors secreting androgen precursors. An increase in estrogen production that results ultimately in a decreased testosterone:estrogen production ratio is also present in a number of clinical entities associated with gynecomastia. In cirrhosis, due to decreased clearance of testosterone by the liver, the extrahepatic conversion of testosterone to androstenedione is increased. Since androstenedione is a better substrate for peripheral aromatization than testosterone, its decreased hepatic clearance provides an increased testosterone availability for its oxidation to androstenedione in peripheral tissues. Because the hepatic clearance of androstenedione is also decreased, the availability of both androstenedione and testosterone is increased, which again favors peripheral estrogen formation.

Furthermore, increased testicular estrogen secretion occurs in men with disorders that are characterized by increased gonadotropin secretion such as in Klinefelter's syndrome (males with an additional X chromosome resulting in the 47,XXY karyotype), CAIS (see above), and hypergonadotropic hypogonadism.

## IX. SERUM ESTROGEN LEVELS IN MALES

### A. Childhood

When serum estradiol levels are determined with conventional immunoassays, levels are close to the detection limit of these assays and are similar in boys and girls. However, significant sex differences have been discovered with newly developed ultrasensitive recombinant cell bioassays. Mean serum estradiol levels (picograms per milliliter) in prepubertal children in one study were 0.08 in boys and 0.6 in girls (assay detection limit 0.02 pg/ml) and in a second study were somewhat higher (1.4 pg/ml in boys and 3.5 pg/ml in girls; assay detection limit $<1.0$ pg).

### B. Puberty

Utilizing an ultrasensitive estradiol detection assay, it has been demonstrated that as normally growing boys progress into puberty, a significant rise in estradiol levels occurs simultaneously with the peak growth velocity, an average of 3 years after the onset of puberty. Thereafter, estrogen levels continue to increase until the end of puberty, and by that time reach an average serum level of approximately 12 pg/ml.

### C. Adulthood

Estradiol and estrone levels as measured by immunoassays span a range from approximately 20 to 40 pg/ml (Table 1) and on average tend to increase with advancing age. However, because of increased sex hormone-binding globulin, free estradiol levels appear to be largely unchanged in aging.

## X. BIOLOGICAL ACTIONS OF ESTROGEN IN MALES

The role of estrogen in development, growth, and other physiological functions in the human male has been thought to be relatively unimportant. Recent findings have challenged this view and have advanced our knowledge on the biological action of estrogen in males.

### A. Estrogen Receptors

The biological effects of estradiol are predominantly mediated by two distinct intracellular receptors, the "classical" estrogen receptor (now designated ER-α), which was cloned approximately 15 years ago, and a second estrogen receptor, ER-β, which was cloned in the mid-1990s. Two different genes encode both ERs. They are ligand-inducible transcription factors that belong to the nuclear hormone superfamily. Since the DNA- and ligand-binding domains of both receptors exhibit a high degree of homology, both receptors can interact with the identical DNA-response elements and display similar *in vitro* binding profiles for endogenous and synthetic estrogens. In addition, there may be an interaction between the two ERs. In males, ER-α is predominantly expressed in the testis, pituitary, liver, heart, kidney, skeletal muscle, and osteoblasts, whereas ER-β transcripts predominate in the prostate. Transcripts for both receptors are present in approximately similar amounts in the epididymis, adrenals, bone, thyroid, and various regions of the brain.

## XI. MUTATIONS THAT IMPAIR THE FUNCTION OF THE ESTROGEN RECEPTOR OR THE FORMATION OF THE ENZYME AROMATASE IN HUMAN MALES

In contrast to gene mutations that impair androgen action, only few cases of naturally occurring mutations of the genes encoding either the estrogen receptor (ER-α) or the enzyme aromatase have been identified.

### A. Estrogen Resistance Syndrome

Resistance to estrogen action has been reported in a single case of a 28-year-old man with a premature stop codon, which resulted in a truncated, nonfunctional ER-α protein. The patient was tall (204 cm, >95 percentile; weight 127 kg) and had a bone age of 15 years with evidence of slow and continued linear growth during adulthood. The man had a normal onset of puberty, was normally virilized, and reported no history of gender identity disorder. He had normal male genitalia, normal testes, and a normal-sized prostate. Semen analysis revealed a normal sperm count with decreased viability. His serum estradiol and estrone, serum follicle-stimulating hormone (FSH), and serum luteinizing hormone (LH) concentrations were elevated, whereas serum testosterone concentrations were normal and serum DHT was borderline low. The patient did not respond to high-dose estrogen therapy; e.g., he did not develop gynecomastia or impotency. Neither gonadotropins nor estrogen-dependent proteins, such as sex hormone-binding globulin or serum prolactin, changed with treatment. Bone age and bone mineral content remained unaltered during estrogen administration. These observations demonstrate that estrogen plays an important biological role in the maturation and homeostasis of the male skeleton and indicate that estrogen action on bone is mediated predominantly by ER-α. A follow-up study of the patient at the age of 31 years provided evidence of premature coronary artery disease, and serum glucose and lipid analyses revealed abnormalities; however, at present it is unclear whether these disorders are related to the nonfunctional ER-α.

### B. Aromatase Deficiency

Despite the size and complexity of the CYP19 gene, aromatase deficiency has been reported in only nine cases. Three of the cases were male (two men and one infant), and all had normal male genitalia at birth. The two adults had an unremarkable childhood, normal pubertal development, and normal gender identity. In one man, extremely low estrogen levels and markedly elevated serum testosterone, FSH, and LH concentrations were found. In the second man, whose CYP19 gene mutation was associated with a lesser degree of aromatase deficiency, serum estradiol was low, serum testosterone was normal, and the gonadotropin levels were slightly elevated. Testis size in the two men differed considerably, being large in one (25 ml volume) and subnormal (8 ml) in the other. The men were extremely tall (>3 standard deviations) with continued growth into adulthood and unfused epiphyses in their midtwenties. Both had osteopenia, and estrogen treatment resulted in epiphyseal closure and an increase in bone mass. These observations again support an essential role of estrogen in the maturation of the skeleton and the accrual of bone mass.

## XII. MUTATIONS THAT IMPAIR THE FUNCTION OF THE ESTROGEN RECEPTOR OR THE FORMATION OF THE ENZYME AROMATASE IN RODENTS

### A. Estrogen Receptor Knockout Mice

αERKO mice and αβERKO mice have reduced femoral length and significantly reduced bone mineralization. αERKO mice have cortical osteopenia and increased bone turnover, which is more pronounced in the male than in the female. In contrast, βERKO mice have a skeletal phenotype that is indistinguishable from that of wild-type males. The most striking phenotype in the male αERKO and αβERKO mice includes testicular degeneration and epididymal dysfunction combined with deficits of sexual behavior and infertility. In comparison, βERKO males are fertile and exhibit normal sexual behavior.

### B. Aromatase Knockout Mice

Like αERKO mice, ARKO mice display significantly reduced femur length and have osteopenia. Other major phenotypic differences between wild-type and ARKO males are manifested mainly in the reproductive tissues. They cause infertility in aging males and severe deficits in sexual behavior.

Taken together, the knockout mouse models demonstrate that estrogen is important for bone mass accrual in the male. For hitherto unknown reasons, the knockout mice show less pronounced skeletal phenotypic differences than humans with the corresponding mutations. Furthermore, important species-related differences exist; for example, in the human, in contrast to mice, estrogen deficiency or estrogen resistance does not appear to affect male psychosexual development.

## XIII. SUMMARY

The conversion of circulating testosterone and androstenedione by the enzyme aromatase in peripheral tissues is quantitatively the major source of estrogen in men. The current data obtained from human mutations impairing either estrogen action or formation and from corresponding knockout mouse models indicate that estrogen deficiency is not lethal for a conceptus (either male or female). They demonstrate that in the male, estrogen plays a major role in the control of bone growth as well as in the accumulation and maintenance of bone mass. Less clear at present is the impact of estrogen on other tissues or functions in the male, including the cardiovascular system, fertility, and glucose and lipid metabolism.

## Glossary

**androgen or estrogen resistance** Unresponsiveness or insensitivity of tissues toward the action of androgens or estrogens, caused by a functional defective androgen or estrogen receptor.

**knockout mouse** Animal model involving targeted gene(s) disruption.

**male pseudo-hermaphroditism** Disorder of phenotypic sex, caused by defective masculinization of the male embryo. Can result from defects of testicular androgen synthesis or defects in androgen action (see androgen resistance).

**peripheral tissues** Extraglandular tissues, i.e., extratesticular and extra-adrenal tissues.

## See Also the Following Articles

**Androgen Receptor-Related Pathology • Androgen Receptor Structure and Function • Aromatase and Estrogen Insufficiency • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Estrogen and Spermatogenesis • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Biology and Lessons from Knockout Mice • Estrogen Receptor Crosstalk with Cellular Signaling Pathways**

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# Estrogen Insufficiency

See ***Aromatase and Estrogen Insufficiency***

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# Estrogen Receptor (ER) Actions through Other Transcription Factor Sites

Stephen Safe
*Texas A&M University*

I. INTRODUCTION
II. OTHER ER-DEPENDENT GENOMIC PATHWAYS OF ESTROGEN ACTION
III. ER-DEPENDENT NONGENOMIC PATHWAYS ACTIVATED BY E2
IV. SUMMARY

**Estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) are members of the nuclear receptor family of ligand-activated transcription factors that mediate tissue-specific responses to 17β-estradiol and other steroidal and nonsteroidal estrogens. Classical activation of genes through consensus (or nonconsensus) estrogen response elements requires interactions with ER-α or ER-β homo- or heterodimers and a host of other nuclear cofactors that are essential for ligand-activated gene expression. Nuclear estrogen receptors also activate genes through other DNA-dependent and -independent pathways, and there is increasing evidence that estrogen receptor interactions with other transcription factors and proteins may be important.**

## I. INTRODUCTION

The classical mechanism of estrogen action involves ligand-dependent formation of nuclear estrogen receptor (ER) homo- or heterodimers and interaction with consensus palindromic estrogen response elements (EREs) [i.e., $-$GGTCA(N)$_3$TGACC$-$] in target 17β-estradiol (E2)-responsive gene promoters (Fig. 1). The subsequent recruitment of other nuclear co-activator and coregulatory proteins and interactions with the basal transcription machinery result in transactivation. This DNA-dependent mechanism of ER-mediated responses has been characterized for several genes that contain consensus or nonconsensus EREs; however, it has also been shown that hormonal activation of many other genes may involve genomic (nuclear) pathways that are DNA dependent or independent and other mechanisms that do not require nuclear ERs (i.e., nongenomic).

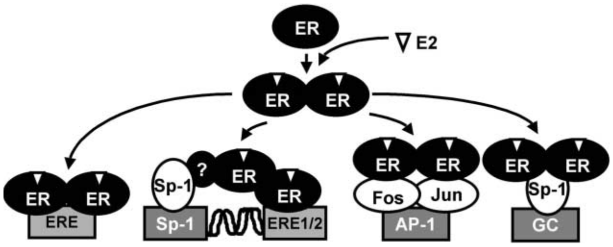

**FIGURE 1** Pathways for nuclear estrogen receptor (ER) action through genomic DNA-dependent and -independent interactions. Estrogens and other ER agonists bind the receptor and activate genes through binding response elements or by direct interactions with other DNA-bound transcription factors. ERE, Estrogen response element; E2, 17β-estradiol; AP-1, activator protein 1.

ER subtype-selective ligand synthesized by the same group showed full ER-α agonism but pure ER-β antagonism.

The discovery of a second estrogen receptor will require that we revisit many biological and clinical studies and reinterpret them in the light of the presence of two receptors. Such studies are now being performed, generating interesting and sometimes unexpected findings.

## Acknowledgments

This work was made possible due to the generous support of the Swedish Cancer Society and Karo Bio AB.

## Glossary

**BERKO** Mice with inactivated, knocked-out versions of estrogen receptor-β.

**estrogen receptor-α (ER-α)** The first cloned estrogen receptor, which displays differences compared to ER-β with regard to structure, ligand binding, expression profile, and physiological effects.

**estrogen receptor-β (ER-β)** A protein that, after binding its ligand, estrogen, activates the transcription of genes, resulting in specific physiological effects.

**estrogen-response element** The specific estrogen receptor-binding DNA sequence in the promoter region of genes that are regulated by estrogen.

## See Also the Following Articles

**Estrogen Receptor (ER) Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • SERMs (Selective Estrogen Receptor Modulators)**

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# Estrogen Receptor Biology and Lessons from Knockout Mice

Deborah L. Swope and Kenneth S. Korach
*National Institute of Environmental Health Sciences, North Carolina*

I. ESTROGEN RECEPTORS: STRUCTURE AND MECHANISM OF ACTION
II. ESTROGEN RECEPTOR KNOCKOUT MODELS (α, β, AND α/β)
III. ESTROGEN RECEPTOR BIOLOGY

**The steroid hormone estrogen induces a wide variety of physiological responses in mammalian tissues. Estrogen is found in both females and males, although at higher levels in females. Estrogen causes its multitude of effects through estrogen receptors that mediate the transcription of a number of genes involved in reproduction, metabolism, and growth and differentiation. The current understanding of estrogen action is rooted in a model in which the estrogen receptor is bound by ligand, homodimerizes, and interacts with high-affinity binding sites for estrogen receptors found in the promoters of target genes. The bound receptor activates transcription of the target gene. Estrogen receptors also modulate expression of genes that do not contain these binding sites, can be activated in a ligand-independent manner by growth factors, and can also function as repressors of transcription.**

# I. ESTROGEN RECEPTORS: STRUCTURE AND MECHANISM OF ACTION

Estrogen receptors (ERs) belong to the nuclear hormone receptor (NHR) superfamily of proteins, which includes over 200 members that share several homologous domains and functions. ERs are class I members of this superfamily. Class I receptors function as transcription factors; their activities are mediated by a variety of small ligands that interact with the ligand-binding domain (LBD) of the receptor to activate transcription. A majority of the receptors in this family bind as dimers through the DNA-binding domain (DBD) to specific sequences called hormone response elements (HREs); the HREs are found in the promoters of target genes, and through their interactions regulate the expression of target genes.

## A. Structure of ERs

Two ER proteins have been identified: ER-α and ER-β. Each is a product of a separate gene located on separate chromosomes, although the proteins are highly related, suggesting a common evolutionary ancestry. Numerous splice variants of both ER-α and ER-β have been observed in normal tissues, in tumors, and in transformed cell lines, but Western blotting studies have identified the predominantly expressed 599-amino-acid protein for ER-α (mouse) and the 530-amino-acid protein for ER-β. ERs, like other NHRs, contain a variable amino-terminal A/B domain, which contains a ligand-independent activation function (AF-1); a highly conserved C domain, which contains two zinc fingers and is required for DNA binding; the hinge, or D domain, which contains nuclear localization signals; and a large hydrophobic E domain, which contains the dimerization domain, binds to ligands, and also contains the major ligand-dependent activation function, AF-2 (Fig. 1). ERs also contain a unique F domain at the C-terminus; domain F is not seen in other NHRs and is poorly conserved between ER-α and ER-β. The function of domain F is unclear, but deletion and mutagenesis of the F domain have suggested a role in influencing transcriptional activity of the ER.

The greatest degree of homology between receptors in the NHR superfamily lies in the DNA-binding domain, which is composed of two zinc fingers that bind DNA with high affinity. Due to the high degree of homology between the DBDs of ER-α and ER-β (97%; Fig. 1), these two receptors bind to the same estrogen response elements (EREs). The consensus ERE is a 13-base-pair (bp) palindromic sequence (GGTCAnnnTGACC) consisting of two 5-bp inverted repeats with a 3-bp spacer; most genes contain nonconsensus EREs in their promoters. Each ER molecule in the dimer binds to one 5-bp repeat (also known as a half-site). *In vitro* studies have shown that the two estrogen receptors can homodimerize (α/α or β/β) or heterodimerize (α/β). However, only cells that express both receptors would be capable of forming heterodimers; to date, co-expression has been observed in a limited number of cell types *in vivo*, and it remains unknown whether heterodimers form productive complexes in these cells. The ERs also share a moderate degree of homology in the LBD (60%; Fig. 1), which likely accounts for the similar binding affinities of ER-α and ER-β for estradiol and other ligands, including diethyl stilbestrol (DES) and 4-hydroxytamoxifen.

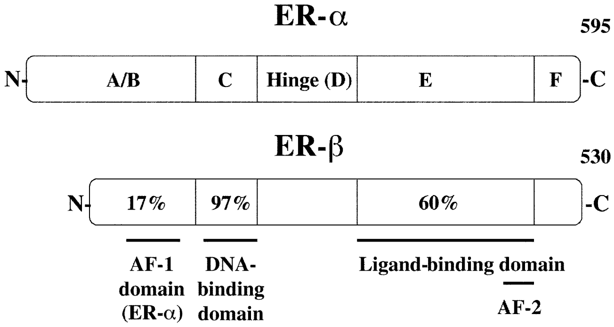

FIGURE 1 Major functional domains of the mouse estrogen receptors (ERs). This schematic depicts the six functional domains (A through F) found in ERs. Homology between ER-α and ER-β structural domains is also shown. Note that the greatest degree of homology lies between the DNA-binding domain (C domain) and the ligand-binding domain (E domain).

## B. Transcriptional Activation by ERs

ERs activate transcription of target genes in response to a number of stimuli, including estrogens, xenoestrogens, and growth factors. The classical mechanism by which the ER activates transcription is a ligand-dependent response mediated by a conformational change in the ER induced when estrogen (or another agonist) binds to the LBD, which promotes dimerization (Fig. 2). This conformational change also alters the position of helix 12, an α-helix that contains the AF-2 activation domain. This hydrophobic AF-2 motif interacts with LXXLL motifs [known as nuclear receptor (NR) boxes] found in co-activator/cofactor molecules such as steroid receptor co-activator-1 (SRC-1), glutamate receptor-interacting

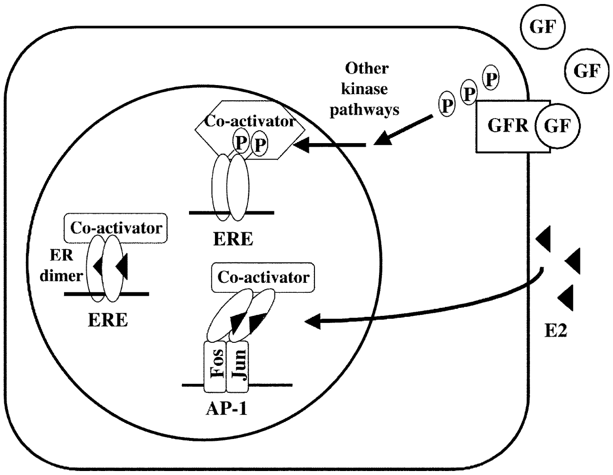

FIGURE 2 Mechanisms of estrogen receptor (ER) transcriptional activation. This diagram details several mechanisms by which the ERs activate transcription in response to a variety of signals on different types of promoters. Transcription regulated by estrogen response elements (EREs) is initiated following binding of estradiol (E2) to the ER, dimerization, and binding to the ERE. Activation from an ERE can also result from activation via the activation function-1 domain by growth factor (GF) signaling. Additionally, non-ERE-mediated activation can occur via an activator protein 1 (AP-1) site (or Sp-1 site; not shown). GFR, Growth factor receptor; P, progesterone.

protein 1 (GRIP1), and $Ca^{2+}$/cAMP response element-binding protein (CBP)/p300, as well as others. The repositioning of helix 12 forms a co-activator-binding surface on the ER that enables the AF-2 domain to interact with these molecules, enhancing the formation of a productive transcriptional complex.

Additionally, ERs activate transcription through ligand-independent pathways that involve signaling in the absence of estrogen (Fig. 2). A number of signaling pathways can modulate ER activation, including growth factors, protein kinase A (PKA), and protein kinase C (PKC). Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) can induce activation of genes regulated by EREs; this activity requires ERs, as shown by disruption of induction in *in vitro* studies as well as in studies in ER-α knockout mice or in studies involving treatment with the ER antagonist ICI 182,780. Activation requires the AF-1 domain of human ER-α, which can be phosphorylated at Ser-118 by the mitogen-activated protein kinase (MAPK) pathway following stimulation by EGF or IGF-I. This event potentiates interaction with the p68 RNA helicase, which functions as an AF-1-specific co-activator, and enhances expression of genes regulated by the ERE. In contrast, ER-β does not appear to have a functional AF-1 domain, although there are reports of phosphorylation events potentiating interaction of the A/B domain with the co-activator SRC-1 in the absence of ligand. These alternative mechanisms for activating the ERs may represent another means for mediating or enhancing transcription via the ERs in environments with low hormone levels.

ERs can also regulate gene expression in the absence of EREs. Expression of genes with AP-1 sites in their promoters can be stimulated by ER-α indirectly interacting with the AP-1 response element via the Fos and Jun heterodimer (Fig. 2). ER-α and ER-β display opposite effects at this promoter: ER-α can activate transcription from the AP-1 element under the influence of estradiol, but ER-β is unable to do so. However, ER-β can activate from AP-1 sites when exposed to antiestrogens. This effect can be explained by the differences in AF-1 function seen between ER-α and ER-β, because activation of the AP-1 element by ERs requires both AF-1 and AF-2. Likewise, ER-α can activate transcription via the GC-rich Sp-1 binding site [found, for example, in the retinoic acid receptor α (RAR α) gene promoter] indirectly by interacting with the Sp-1 transcription factor in the absence of ligand, or by binding as an ER-Sp-1 complex to ERE half-sites located near the Sp-1 binding site in gene promoters.

There are also many reports of ER-mediated rapid cytosolic responses that are nongenomic actions, i.e., they do not involve regulation of transcription. Membrane-associated estrogen-binding proteins have been described, and several studies suggest that the nuclear and membrane ERs are similar, if not the same. The rapid responses mediated by these proteins appear to be G-protein-mediated events, resulting in the activation of PKC, release of calcium, and other signaling pathways. ER-α also has been observed to associate with a number of cytosolic proteins involved in cell signaling, including SRC and Akt, a serine/threonine kinase. Future studies of these membrane-associated events will continue to broaden our understanding of the nongenomic roles of the ERs.

## II. ESTROGEN RECEPTOR KNOCKOUT MODELS (α, β, AND α/β)

Until recently, mutations of ERs were unknown in humans, and so knockout models of the various estrogen receptors were developed in mice in order to facilitate studies of estrogen action *in vivo*. Much of the early progress made in determining the role of estrogen and estrogen receptor was gained from pharmacological or surgical (castration) manipulations of laboratory animals, from cell culture models, and from *in vitro* studies. However, much of the current information is derived from studies conducted on the recently developed knockout models, which have provided invaluable insights into a number of aspects of the ERs in normal and disease states.

### A. ER-α Knockout Mice

Disruption of the ER-α gene in mice using gene targeting techniques has resulted in the ER-α knockout (αERKO). This line of mice displays a complex phenotype detailed in Table 1. Development of the reproductive tracts of males and females appears normal in these knockouts, and the mice are healthy and have a normal life span. However, at the age of maturity a number of reproductive phenotypes become evident. The homozygous αERKO females are completely infertile; cannot ovulate, have numerous hemorrhagic ovarian cysts, and exhibit a "trapped follicle" phenotype after superovulation. No corpora lutea are evident. The αERKO female has an increased level of circulating luteinizing hormone (LH); if treated with gonadotropin-releasing hormone (GnRH) antagonists, the hemorrhagic cysts characteristic of αERKO ovaries do not develop, suggesting that the elevated level of LH is responsible for this ovarian phenotype. The αERKOs not only lack the ability to ovulate, but implantation studies using donor embryos have revealed that they also cannot support a pregnancy. They also do not show the classical responses to the administration of estradiol, i.e., there is no increase in uterine wet weight or in the expression of progesterone receptor (PR), and therefore the uterus is unable to prepare properly for pregnancy. αERKO females also do not undergo normal mammary gland development; no alveoli are evident and the ducts do not develop past the epithelial rudiment. Additionally, αERKO females have a high level of serum estradiol, most likely a result of the lack of negative feedback through ER-α that is required to regulate the levels of estradiol.

αERKO males are likewise infertile; prenatal development is normal, although testes are slightly smaller than those of wild-type males. Young males produce viable sperm, but this ability decreases with age. This may be due to atrophy of the epithelium of the seminiferous tubules and the severe dilation of the efferent ductules in the testes that causes a decreased ability to resorb fluid. However, studies have shown that αERKO sperm can develop normally if transplanted to a wild-type male, suggesting that expression of ER-α in the environment, but not in the germ cells, is required for proper sperm development. αERKO males, like the females, show an increased level of circulating LH.

### B. ER-β Knockout Mice

As with the αERKO mice, the βERKO mice reproductive tracts appear to undergo normal development. βERKO males have normal fertility and appear to have no testicular phenotype. However, the female βERKO is subfertile, but the phenotype is distinct from that observed in the αERKO. A "trapped follicle" phenotype is also evident following superovulation as with the αERKO, but where complete anovulation is seen in the αERKO, βERKOs do spontaneously ovulate, but with decreased frequency, resulting in small litters. There is no increase in LH as seen in the αERKO mice; the hemorrhagic cysts that characterize the αERKO ovary due to increased LH are not seen in the βERKO ovary. Normal uterine responses to estradiol treatment (increase in uterine weight, normal implantation, and increased expression of PR) are present in the βERKO mice. The females have normal mammary gland development, lactate normally, and successfully nurse pups.

### C. "Double" Knockout Mice

The double knockout displays an overt phenotype that resembles the αERKO mice in many ways (see Table 1). Both sexes have normal prenatal development of the reproductive tracts. A notable difference between the αERKO and α/βERKO lines is the progressive appearance in the adult ovary of abnormal follicles that resemble seminiferous tubules. Three types of follicles are observed: normal follicles, "intermediate" follicles that contain degenerating oocytes and granulosa cells, and follicles that have lost the germ cell and have no granulosa cells. Instead, cells lining the lumen of both of these abnormal follicle types resemble Sertoli cells; the "intermediate" follicle type contains both granulosa cells and these Sertoli-type cells. Markers of Sertoli cells, including Müllerian inhibiting substance (MIS) and Sox9, are detected in both of the abnormal follicle

**TABLE 1 Phenotypes of ER Knockout Mouse Models**

| Tissue/system | αERKO | βERKO | α/βERKO |
|---|---|---|---|
| Fertility | Females and males infertile | Females subfertile, decreased litter size; males fertile | Both sexes infertile (resembles αERKO phenotype) |
| Female reproductive system | | | |
| Uterus | Hypoplastic uterus; no uterotropic response to estradiol; no implantation | Normal responses to estradiol; can support normal pregnancies | Resembles αERKO |
| Ovary | No ovulation; immature follicles and appearance of hemorrhagic cysts at puberty; "trapped follicle" phenotype after superovulation; elevated levels of estrogen and testosterone | Normal appearance; reduced ovulation | Granulosa cells undergo sex reversal, become Sertoli-like cells |
| Male reproductive system | | | |
| Testes | Normal development; testes decrease in weight with age; fluid resorption in efferent ducts decreases with age; sperm have poor motility, cannot fertilize oocytes *in vitro* | Normal | Resembles αERKO phenotype |
| Mammary gland | Immature; only a ductal rudiment present (development not stimulated by estradiol) | Normal development; normal lactation | Immature; resembles αERKO phenotype |
| Bone | Female: decreased bone diameter; male: decreased density; both sexes are shorter than wild type | Normal in males; increase in bone density in females | Both sexes shorter |
| Cardiovascular | Estradiol protection normal; decreased basal NO activity | Normal estradiol protection | Estradiol does not protect against increases in vascular medial area, but still protects against proliferation after injury |
| Gonadotropin/ hormone levels | Females and males: elevated levels of LH, estradiol, and testosterone; females: reduced prolactin levels | Normal | Elevated LH |
| Mating behavior | Decreased aggression; deficient mating behaviors | Normal sexual behavior | Males display no mounting behavior |

types, suggesting that the granulosa cells have "redifferentiated" to a Sertoli cell type; this phenotype is most likely due to the loss of germ cells.

Other physiological systems are also affected by loss of one or both of the ERs. Effects have been noted in bone, the cardiovascular system, and behavior, all systems in which estrogen has been implicated for normal function. These effects are detailed in Table 1. All of the ERKO models demonstrate some defects in bone: αERKOs and α/βERKOs are shorter. However, there are some gender differences; the αERKO males have lower bone density and the females have smaller diameter bones. There is no apparent defect in the bones of βERKO males, but the females show an increased bone density. It is uncertain whether these effects are developmentally related or if the phenotypes are a result of the effects of abnormal steroid responses in the adults.

Estrogen has long been implicated in gender differences observed in the rates of heart disease seen in humans. Studies have indicated that ER-α is the main ER expressed in the aorta, but that ER-β is also expressed in the vasculature and has been linked with hypertension. Interestingly, the αERKO and βERKO models do not show a loss of estrogen protection from vascular injury, but the α/βERKO model shows an attenuated response, suggesting that the two ER types might compensate for one another or that another pathway may be involved in this effect. However, an effect on nitric oxide (NO) synthesis in male αERKOs has been noted; these mice have an impaired ability to synthesize basal levels of nitric oxide, but can respond normally to the effects of NO.

Mating behavior, not surprisingly, is also affected by disruption of ER expression. Disruption of ER-α expression severely affects mating behaviors in the αERKO males and females. Adult female αERKOs are not sexually receptive in the presence of males and may be attacked by males; both behaviors may reflect elevated levels of testosterone in αERKO females. The αERKO males display somewhat normal behavior toward females in that they will attempt to mount a female, although the frequency of this behavior is reduced. However, intromission and ejaculation are nearly completely absent in these males, suggesting that ER-α is critical to the consummation of mating. In contrast, βERKO males and females appear to have normal mating behaviors. Surprisingly, α/βERKO males lose all mounting responses, suggesting that either individual ER can compensate.

## III. ESTROGEN RECEPTOR BIOLOGY

To date, only one mutation of the coding region of the ER-α gene has been identified in humans; prior to the identification of the patient in whom the gene was identified, it was assumed that mutations in ER-α were lethal. A 28-year-old adult male was observed to have tall stature with continued slow growth, delayed bone maturation, insulin resistance, and increased levels of luteinizing hormone and estradiol. Estrogen therapy had no effect on the patient's symptoms, and it was determined that he carried a mutation on both alleles of ER-α that resulted in a premature stop codon. Similar phenotypes have been described in other patients with defects in estrogen synthesis, many due to defects in aromatase, which is required for the synthesis of endogenous estrogens. However, the limited number of patients exhibiting such syndromes and difficulty in obtaining samples from them has made it difficult to study many aspects of estrogen action in humans. The difficulty in identifying humans with estrogen receptor mutations has led many researchers to postulate that ER-α mutations might be lethal. Successful generation of the different ERKO lines and the subsequent identification of the patient homozygous for a disrupting mutation disproved this theory and also demonstrated that ER-α was not required for proper prenatal development.

Development of mouse lines lacking either or both of the ERs has therefore given us invaluable tools for the *in vivo* study of the roles of estrogen and estrogen receptors. Many of the phenotypes observed in the ERKOs are consistent with those seen in the aromatase-deficient and the estrogen-mutant patient, suggesting that the various mouse lines represent good models for the study of estrogen action in mammals. Surprisingly, neither of the ERs is required for prenatal development of the reproductive tracts in males or females, as demonstrated by the normal development of these systems in all of the knockout models. However, ERs are required for normal reproductive function, as established by the various phenotypes that emerge at puberty in the ERKO models. Development of the double (α/βERKO) knockout line has allowed investigators to confirm the roles of ER-α and ER-β by studying the individual genes for these receptors. Continuing studies of the estrogen receptor knockout mice and generation of new mouse lines with modified estrogen receptors will allow investigators to add more details to the complex role of estrogen in normal physiology.

## Glossary

**activation function-1** The ligand-independent domain found in the N-terminus of ER-α that can be activated by non-estrogen-dependent signaling. ER-β lacks this function.

**activation function-2** This major activation domain of ERs is located in the C-terminus of the proteins. It lies at the C-terminal end of the ligand-binding domain and is characterized by a hydrophobic α-helical structure with the invariant amino acid motif &phis;&phis; XE&phis;&phis; (where &phis; is any hydrophobic amino acid, X is any amino acid, and E is glutamic acid). This helix interacts with LXXLL motifs found in co-activators (L is leucine).

**co-activator** Accessory protein that interacts with nuclear receptors when the receptor is in an active conformation, enhancing transcriptional activation. Co-activators function as a "bridge" from the receptor to the general transcriptional apparatus; interact with receptors via LXXLL motifs.

**DNA-binding domains** Portions of the receptor proteins containing a double zinc finger motif that binds to the DNA sequence.

**estrogen receptor** Protein target for the steroid hormone estrogen. Two distinct forms of this nuclear receptor have been identified, ER-α and ER-β.

**estrogen receptor knockouts** Mouse lines developed using gene targeting strategies to disrupt expression of endogenous genes encoding ER-α (αERKO) or ER-β (αERKO).

**estrogen response element** Specific binding sequence for the estrogen receptor; located in the promoter of estrogen target genes. The consensus estrogen response element is a 13-base-pair inverted palindrome containing a 3-base-pair spacer (GGTCAnnnTGACC).

**ligand-binding domains** Large C-terminal domains that are well conserved among members of the nuclear hormone receptor superfamily. Composed of several α-helices, these domains are involved in hormone binding, dimerization, and activation of hormone-dependent transcription.

## See Also the Following Articles

**Apoptosis Gene Knockouts • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Knockout of Gonadotropins and Their Receptor Genes**

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# Estrogen Receptor Crosstalk with Cellular Signaling Pathways

Carolyn L. Smith
*Baylor College of Medicine*

I. INTRODUCTION
II. ESTROGEN RECEPTOR PHOSPHORYLATION AND TRANSCRIPTIONAL ACTIVITY
III. ESTROGEN RECEPTOR CROSSTALK WITH THE CYCLIC AMP SIGNALING PATHWAY
IV. ESTROGEN RECEPTOR CROSSTALK WITH THE MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY

## Glossary

**activation function-1** The ligand-independent domain found in the N-terminus of ER-α that can be activated by non-estrogen-dependent signaling. ER-β lacks this function.

**activation function-2** This major activation domain of ERs is located in the C-terminus of the proteins. It lies at the C-terminal end of the ligand-binding domain and is characterized by a hydrophobic α-helical structure with the invariant amino acid motif &phis;&phis; XE&phis;&phis; (where &phis; is any hydrophobic amino acid, X is any amino acid, and E is glutamic acid). This helix interacts with LXXLL motifs found in co-activators (L is leucine).

**co-activator** Accessory protein that interacts with nuclear receptors when the receptor is in an active conformation, enhancing transcriptional activation. Co-activators function as a "bridge" from the receptor to the general transcriptional apparatus; interact with receptors via LXXLL motifs.

**DNA-binding domains** Portions of the receptor proteins containing a double zinc finger motif that binds to the DNA sequence.

**estrogen receptor** Protein target for the steroid hormone estrogen. Two distinct forms of this nuclear receptor have been identified, ER-α and ER-β.

**estrogen receptor knockouts** Mouse lines developed using gene targeting strategies to disrupt expression of endogenous genes encoding ER-α (αERKO) or ER-β (αERKO).

**estrogen response element** Specific binding sequence for the estrogen receptor; located in the promoter of estrogen target genes. The consensus estrogen response element is a 13-base-pair inverted palindrome containing a 3-base-pair spacer (GGTCAnnnTGACC).

**ligand-binding domains** Large C-terminal domains that are well conserved among members of the nuclear hormone receptor superfamily. Composed of several α-helices, these domains are involved in hormone binding, dimerization, and activation of hormone-dependent transcription.

## See Also the Following Articles

**Apoptosis Gene Knockouts • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Knockout of Gonadotropins and Their Receptor Genes**

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# Estrogen Receptor Crosstalk with Cellular Signaling Pathways

CAROLYN L. SMITH
*Baylor College of Medicine*

I. INTRODUCTION
II. ESTROGEN RECEPTOR PHOSPHORYLATION AND TRANSCRIPTIONAL ACTIVITY
III. ESTROGEN RECEPTOR CROSSTALK WITH THE CYCLIC AMP SIGNALING PATHWAY
IV. ESTROGEN RECEPTOR CROSSTALK WITH THE MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY

V. ESTROGEN RECEPTOR CROSSTALK WITH THE PROTEIN KINASE C SIGNALING PATHWAY
VI. ESTROGEN RECEPTOR CROSSTALK WITH THE AKT SIGNALING PATHWAY

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**Estrogen receptors respond to a number of nonsteroidal molecules through the process of signal transduction pathway crosstalk. In this process, the signal transduction pathways affect the transcriptional activity of the receptor, which is typically regulated by other agents (e.g., estrogens). The activity of the estrogen receptor can be influenced by pathway crosstalk in the presence or absence of the receptor's cognate ligand.**

## I. INTRODUCTION

The two estrogen receptors, ER-α and ER-β, are predominantly nuclear proteins that mediate that biological effects of estrogens in their role as ligand-regulated transcription factors. Estrogens enter the cell by passive diffusion and bind to their nuclear receptors, whereupon the receptors dimerize, tightly bind specific DNA sequences termed estrogen-response elements, and undergo a conformational change that promotes their interaction with co-activator proteins. Although in this classical model, an extracellular signal (i.e., estrogen) enters the cell and thereby regulates ER function, there are a number of extracellular agents that still possess the ability to affect ER transcriptional activity. Indeed, communication between the extracellular environment and the nucleus is important for the regulation of gene expression and cellular function. Information can be transmitted between the two compartments via intracellular signal transduction pathways that utilize a variety of second messengers, protein kinases, and/or molecules able to translocate from the cytoplasm to the nucleus. Thus, in addition to the relatively well-characterized steroid-dependent activation of transcription, ERs are responsive to a variety of nonsteroidal molecules through a process of signal transduction pathway cross talk with the receptors and/or their associated proteins that can regulate the receptor's transcriptional activity (e.g., co-activators, co-repressors, and heat shock proteins). Pathway crosstalk can alter the activity of the receptor in the absence or presence of its cognate ligand.

## II. ESTROGEN RECEPTOR PHOSPHORYLATION AND TRANSCRIPTIONAL ACTIVITY

Stimulation of ER transcriptional activity by its ligand, estrogen, is accompanied by increases in receptor phosphorylation, suggesting a correlation between receptor phosphorylation and transcriptional activity. To date, five *in vivo* phosphorylation sites (Ser-104, Ser-106, Ser-118, Ser-167, and Tyr-537) have been mapped for ER-α (see Fig. 1). The ER-α is hyperphosphorylated in response to hormone, and different studies suggest that either Ser-118 or Ser-167 is the major estrogen-induced phosphorylation site, perhaps reflecting cell type-specific differences (COS-1 versus MCF-7 cells) in ER phosphorylation or resulting from differences in the techniques used to map these residues. Detailed studies mapping *in vivo* estrogen-induced ER-β phosphorylation sites have not been reported, but the A/B/C/D region of the murine receptor is phosphorylated, and alanine mutation of Ser-106 and Ser-124 reduces the phosphorylation of this region of ER-β in response to epidermal growth factor (EGF) treatment.

Mutation of three of the amino-terminal phosphorylation sites of ER-α (Ser-104, Ser-106, and Ser-118) to alanine residues significantly reduces the estrogen-induced transcriptional activity of this receptor and highlights the importance of these amino acids for full receptor function. Although the basis by which these amino acids affect ER transcriptional activity has not been fully established, there is evidence that phosphorylation of these sites positively influences receptor interaction with the p68/p72 co-activators. Similarly, the putative phosphorylation sites in the A/B region of ER-β affect its interaction with steroid receptor co-activator-1 (SRC-1). The extent to which mutation of these phosphorylation sites affects estrogen-induced ER-α transcriptional activity varies with both cell type and promoter context. A likely explanation for these differences is that the relationships between phosphorylation and co-activator and possibly co-repressor interaction with ER vary in a cell- and/or promoter-specific manner.

## III. ESTROGEN RECEPTOR CROSSTALK WITH THE CYCLIC AMP SIGNALING PATHWAY

The relationship between ER phosphorylation and transcriptional activity is consistent with the ability of several pharmacological agents able to increase

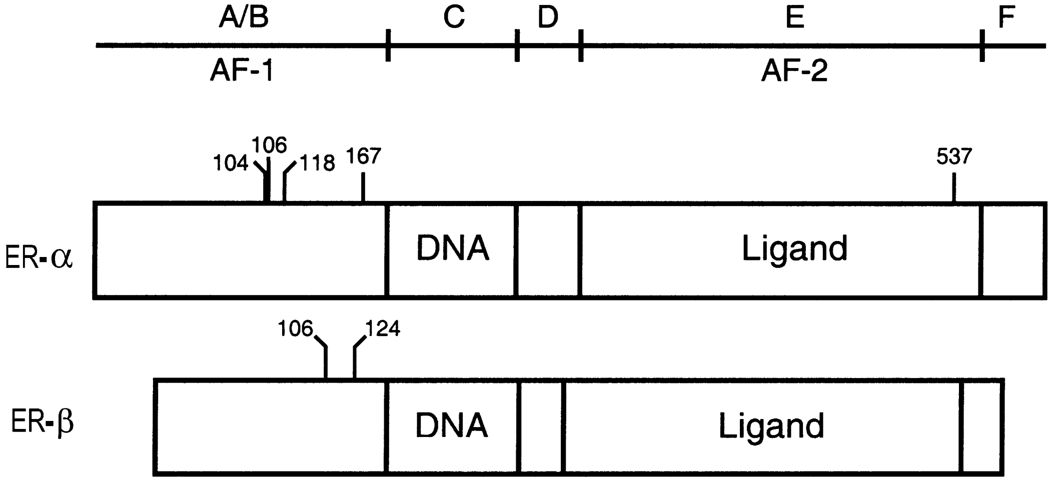

FIGURE 1 Schematic representation of ER-α and ER-β. The DNA- and ligand-binding domains are indicated for each receptor, and the numbers indicate the location of phosphorylation sites. The boundaries of regions A thru F and the positions of the two activation functions (AF-1 and AF-2) are shown at the top.

intracellular cyclic AMP (cAMP) levels and protein kinase A (PKA) activity to induce receptor phosphorylation and stimulate ER-α transcriptional activity. For instance, cholera toxin (CT; a G-protein activator) in combination with 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor) increases intracellular cAMP and thus activates PKA. Treatment of cultured uterine cells with these pharmacological agents results in increased progesterone receptor (PR) expression, a known ER target gene. Similarly, 8-bromo-cyclic AMP (8Br-cAMP), a cell-permeable cAMP analogue, induces PR gene expression, and the pure anti-estrogen ICI 164,384 was able to block this stimulation, thereby demonstrating that this response was ER-dependent. In addition, the neurotransmitter dopamine, apparently by activating a cAMP/PKA-dependent signaling pathway, has been shown to stimulate ER-α-dependent gene expression. Although CT/IBMX treatment results in increased phosphorylation of the receptor, transient transfection studies indicated that cAMP activation of ER-α does not require phosphorylation of any of the potential cAMP-dependent PKA phosphorylation sites located in the C-terminus of the receptor, even though it is this region that becomes phosphorylated in response to CT/IBMX treatment. Another study, which examined the ability of CT/IBMX to stimulate the transcription of several synthetic ER target genes in CHO and 3T3 cells, revealed that activation also depends on cell and promoter context and suggests that other factors (e.g., other transcription factors or co-activators) contribute to the ability of cAMP signaling pathways to regulate ER-dependent gene expression. In addition, the ability of some cAMP/PKA-mediated responses to be blocked by cycloheximide indicates that *de novo* synthesis of other cellular factors contributes to determining cell and promoter dependence. It should be noted that cAMP/PKA and estrogen signaling pathways can synergistically stimulate ER-α transcriptional activity, suggesting that these two pathways activate receptor function through distinct mechanisms.

The ability of the cAMP-dependent signaling pathway(s) to activate another member of the steroid receptor superfamily, the chicken progesterone receptor, without stimulating its phosphorylation suggested that receptor-associated proteins that contribute to nuclear receptor-dependent transcription might themselves be targets of signal transduction pathways. Indeed, it has been demonstrated that 8Br-cAMP enhances phosphorylation of the SRC-1 co-activator on amino acids Thr-1179 and Ser-1185, and mutating these residues to nonphosphorylatable alanines reduces co-activator-dependent enhancement of PR activity induced by either progesterone or 8Br-cAMP. These residues are thought to play a part in stabilizing SRC-1's interaction with another co-activator protein, pCAF (p300/CBP-associated factor). Similarly, a PKA-dependent signaling pathway enhances the intrinsic transcriptional activity of another ER co-activator, CBP (CREB-binding protein). Thus, cAMP signaling may increase ER-dependent gene expression by increasing the transcriptional activity of co-activators that bind to this receptor. In addition, the cell cycle regulator cyclin D1 has been demonstrated to enhance ER-dependent transcription, possibly by enhancing SRC-1 recruitment to ER-α. The cAMP-mediated signaling pathway increases cyclin D1 association with ER, suggesting that the receptor's enhanced activity may also be the result of improved interaction with other co-activators. Thus, cAMP-mediated receptor-dependent transcription may depend on protein-protein interactions and also modulation of the intrinsic activity of co-activators.

## IV. ESTROGEN RECEPTOR CROSSTALK WITH THE MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY

Treatment of cells with either EGF or insulin-like growth factor-1 (IGF-1) results in the stimulation of ER-α and ER-β transcriptional activity. Importantly, these responses are blocked by the anti-estrogens ICI 164,384 or EM652, demonstrating that they are ER-dependent. In contrast to the activation function-2 (AF-2) domains of the ERs, the activity of the AF-1 domains is constitutive and in many contexts is dependent on serine phosphorylation. Moreover, growth factor activation of ER-α leads to phosphorylation of Ser-118, whereas ER-β appears to become phosphorylated at Ser-106 and Ser-124. Consequently, mutation of these residues to alanines results in the loss of EGF-induced activation of ER-α and ER-β. The phosphorylation of ER-α is likely mediated by mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK) since overexpression of constitutively activated ras or constitutively active MAPK kinase (both are upstream activators of MAPK) results in enhanced AF-1 activity. Conversely, a dominant negative form of MAPK kinase decreases activity. Inhibition of EGF-induced ER-β transcriptional activity by the MEK1 (MAPK kinase-1) inhibitor PD98059 also indicates that EGF activation of ER-β is achieved via a MAPK signal transduction pathway. Taken together, EGF activation of both receptors appears to be mediated through MAPK/ERK. However, other studies suggest that EGF-mediated activation of ER-α might also occur through phosphorylation of Ser-167 by pp90$^{rsk1}$ or Akt (see below). It is unclear what determines the type of pathway that growth factors utilize to cross-talk with ERs. Signaling by the Src/JNK and p38 pathways, two other types of MAPKs, also stimulates ER-α transcriptional activity, although these kinases do not appear to require Ser-118 phosphorylation and JNK is unable to phosphorylate ER-α. Thus, multiple factors may mediate growth factor-dependent signaling to ER-α.

The ability of growth factors to activate ERs may arise in part by crosstalk with co-activators. The SRC/p160 family co-activators AIB1 (amplied in breast cancer-1) and GRIP1 (glucocorticoid receptor-interacting protein-1) can be phosphorylated by MAPK (ERK2) *in vitro*. Moreover, constitutively active MEK1 stimulated the intrinsic transcriptional activity of AIB1. In addition, the p300 general co-activator could be co-immunoprecipitated with AIB1 in a MEK1-dependent manner, suggesting that this signaling pathway enhanced p300-AIB1 protein–protein interaction. Similarly, the GRIP1 co-activator is phosphorylated by ERK2 on Ser-736 *in vitro* and mutating this residue to an alanine blunts the ability of EGF to induce the intrinsic transcriptional activity of GRIP1. Furthermore, the S736A mutant's ability to enhance the transcriptional activity of the CBP general co-activator was substantially impaired relative to the wild-type GRIP1. Taken together, these results suggest that MAPK phosphorylation of p160 co-activator family members serves to enhance their interactions with p300/CBP and can therefore lead to indirect regulation of receptor-dependent transcription.

Several *in vivo* EGF and IGF-1 responses have been attributed to the ability of these growth factors to stimulate ER activity. For instance, in ER-α knockout (ERKO) mice, EGF induction of PR gene expression is absent. Importantly, EGF receptor expression, autophosphorylation, and ability to stimulate c-fos activity in response to EGF stimulation were not altered in ERKO mice compared to wild type mice, indicating that the EGF signaling pathway is intact. Recently, it was demonstrated that EGF-mediated activation of ER-α is also involved in the lordosis behavioral response in rodents.

## V. ESTROGEN RECEPTOR CROSSTALK WITH THE PROTEIN KINASE C SIGNALING PATHWAY

ER-α-dependent transcriptional responses can also be influenced by agents that stimulate the intracellular protein kinase C (PKC) signaling pathway. Interestingly, the gonadotropin-releasing hormone activation of ER reporter genes can be blocked by GF109203X, a PKC inhibitor. The phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which activates PKC, increases phosphorylation of the ER-α A/B domain, and this is at least partially attributed to Ser-118 phosphorylation. ER-α may be activated by TPA alone or in a synergistic manner with TPA plus estrogen, but ER-α-dependent responses can also be inhibited by TPA in a promoter-dependent and cell type-dependent manner. However, TPA can also reduce ER-α expression levels and receptor binding to its estrogen-response element *in vitro*. The implications of these TPA-mediated effects relative to ER-α transcriptional activity are unclear.

## VI. ESTROGEN RECEPTOR CROSSTALK WITH THE AKT SIGNALING PATHWAY

Although growth factors can signal to ER-α via MAPKs, they may also stimulate receptor transcriptional activity via the serine/threonine kinase Akt (also known as protein B kinase). Activation of Akt kinase activity is dependent on phosphatidylinositol 3-kinase (PI3-K), and the specific PI3-K inhibitor, LY294002, blocks activation of ER-α transcriptional activity in cells treated with either EGF or IGF-1. In addition, expression of a dominant negative form of Akt blocks growth factor activation of ER-α, whereas a constitutively active form is able to mimic growth factor activation of ER-α transcriptional activity and induction of PR gene expression. The ICI 182,780 anti-estrogen blocks Akt activation of ER-α. Moreover, ER-α can be phosphorylated by Akt *in vitro* and *in vivo*, and mutation of Ser-167 to an alanine blocks Akt stimulation of ER-α transcriptional activity, indicating that Akt signaling cross-talks directly with ER-α.

## Glossary

**co-activators** Molecules, generally proteins, that stimulate the activity of transcription factors, such as the estrogen receptors.

**crosstalk** The ability of signal transduction pathways to influence the activity of molecules, such as the estrogen receptors, that are typically regulated by other agents.

**signal transduction pathway** A group of molecules that enable signals initiated at remote locations to communicate to the final target through intermediary steps.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Crosstalk of Nuclear Receptors with STAT Factors • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Ethylene

PAUL B. LARSEN
*University of California, Riverside*

I. INTRODUCTION
II. PHYSIOLOGICAL AND AGRICULTURAL RELEVANCE
III. BIOSYNTHESIS
IV. PERCEPTION
V. CONTROL
VI. SUMMARY

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# Estrogen Insufficiency

See *Aromatase and Estrogen Insufficiency*

# Estrogen Receptor (ER) Actions through Other Transcription Factor Sites

Stephen Safe
*Texas A&M University*

I. INTRODUCTION
II. OTHER ER-DEPENDENT GENOMIC PATHWAYS OF ESTROGEN ACTION
III. ER-DEPENDENT NONGENOMIC PATHWAYS ACTIVATED BY E2
IV. SUMMARY

**Estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) are members of the nuclear receptor family of ligand-activated transcription factors that mediate tissue-specific responses to 17β-estradiol and other steroidal and nonsteroidal estrogens. Classical activation of genes through consensus (or nonconsensus) estrogen response elements requires interactions with ER-α or ER-β homo- or heterodimers and a host of other nuclear cofactors that are essential for ligand-activated gene expression. Nuclear estrogen receptors also activate genes through other DNA-dependent and -independent pathways, and there is increasing evidence that estrogen receptor interactions with other transcription factors and proteins may be important.**

## I. INTRODUCTION

The classical mechanism of estrogen action involves ligand-dependent formation of nuclear estrogen receptor (ER) homo- or heterodimers and interaction with consensus palindromic estrogen response elements (EREs) [i.e., –GGTCA(N)$_3$TGACC–] in target 17β-estradiol (E2)-responsive gene promoters (Fig. 1). The subsequent recruitment of other nuclear co-activator and coregulatory proteins and interactions with the basal transcription machinery result in transactivation. This DNA-dependent mechanism of ER-mediated responses has been characterized for several genes that contain consensus or nonconsensus EREs; however, it has also been shown that hormonal activation of many other genes may involve genomic (nuclear) pathways that are DNA dependent or independent and other mechanisms that do not require nuclear ERs (i.e., nongenomic).

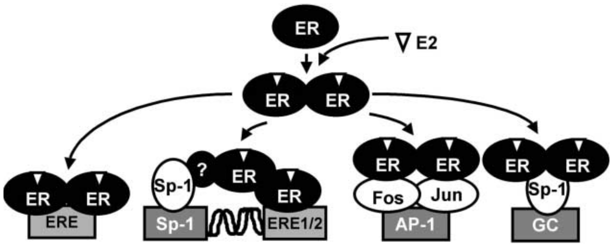

**FIGURE 1** Pathways for nuclear estrogen receptor (ER) action through genomic DNA-dependent and -independent interactions. Estrogens and other ER agonists bind the receptor and activate genes through binding response elements or by direct interactions with other DNA-bound transcription factors. ERE, Estrogen response element; E2, 17β-estradiol; AP-1, activator protein 1.

## II. OTHER ER-DEPENDENT GENOMIC PATHWAYS OF ESTROGEN ACTION

Transcriptional activation of E2-responsive genes through ER–ERE binding also requires interactions of ERs with co-activator/coregulatory proteins as well as other DNA-bound transcription factors. The importance of these interacting proteins as enhancers or inhibitors of E2-dependent gene expression is dependent on cell context, and this can be a critical factor that determines which ER-mediated pathways are functional. Several studies have identified E2-responsive gene promoters containing one or more ERE half-sites (ERE1/2) and GC-rich Sp protein binding sites, and research in our laboratory has characterized E2-responsive ERE1/2$(N)_x$Sp motifs (Fig. 1) in the cathepsin D, heat-shock protein 27, and transforming growth factor-α gene promoters, where $N = 23$, 10, and 31, respectively. Both GC-rich and ERE1/2 sites are required for transactivation, and formation of ER/Sp-1–DNA complexes is also dependent on other proteins that have not been identified (Fig. 1).

There is increasing evidence that ligand-dependent activation of many E2-responsive genes is promoter DNA independent and involves ER interactions with other DNA-bound transcription factors (Fig. 1). Estrogens down-regulate several erythroid cell-specific genes, and at least one pathway for this response involves ER-α interaction with GATA-1, an erythroid transcription factor. Interactions between ER-α and ER-β with the activator protein 1 (AP-1) complex has demonstrated a potentially important DNA-independent genomic pathway that modulates AP-1 promoters and the response is dependent on ER subtype, ligand structure, and cell/tissue context. For example, in HeLa cells transfected with an AP-1 promoter–reporter construct, both estrogens and antiestrogens (tamoxifen/ICI 182780) activate ER-α/AP-1, whereas antiestrogens, but not estrogens, activate ER-β/AP-1.

DNA-dependent interactions of ER-α/Sp-1 with ERE1/2$(N)_x$Sp-1 have been characterized in some gene promoters in breast cancer cell lines; however, more recent studies have demonstrated ER-α/Sp-1 interactions with GC-rich promoter elements may be a critical DNA-independent pathway for regulating hormone-responsive genes in breast cancer cell lines. For example, results of promoter analyses suggest that the following E2-responsive genes are up-regulated through ER-α/Sp-1 in breast cancer cells: c-*fos* and genes for retinoic acid receptor α1, insulin-like growth factor binding protein 4, thymidylate synthase, cyclin D1, E2F1, adenosine deaminase, cathepsin D, DNA polymerase α, and several other genes involved in hormone-induced DNA synthesis and cell cycle progression. In other cell lines, ER-α/Sp-1 activates genes for the vitamin D receptor, progesterone receptor, epidermal growth factor receptor, low-density lipoprotein receptor, and the receptor for advanced glycation end products, and a recent study suggests that ER-α/Sp-1 down-regulates insulin-like growth factor receptor in rat aortic smooth muscle cells. Sp-1 is only one member of an expanding family of zinc finger nuclear transcription factors and there is evidence that other Sp proteins bind ERs to modulate cell-specific expression of genes. For example, we have shown that ER-α/Sp-3 is necessary for E2-dependent decreased vascular endothelial growth factor (VEGF) expression in HEC1A endometrial cancer cells, whereas both ER-α/Sp-1 and ER-α/Sp-3 play a role in hormonal activation of VEGF in ZR-75 breast cancer cells. Thus, interactions of ER-α and possibly ER-β with Sp family proteins can modulate cell-specific expression of E2-regulated genes, and there are reports that other ligand-activated or orphan nuclear receptors also affect gene expression through binding Sp proteins.

## III. ER-DEPENDENT NONGENOMIC PATHWAYS ACTIVATED BY E2

Research carried out in primary and transformed cell lines from multiple tissues/organs indicate that E2

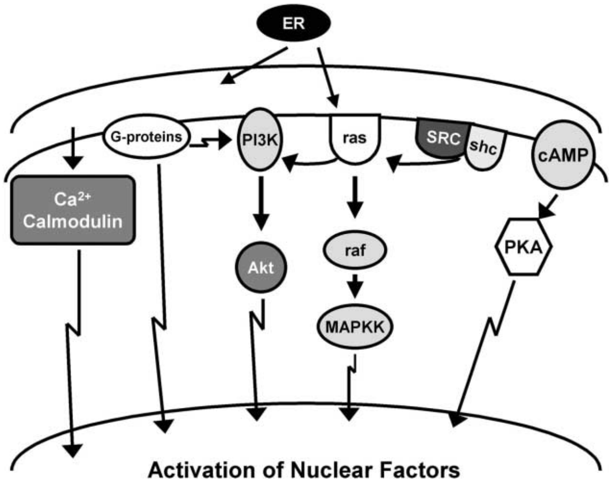

**FIGURE 2** Estrogen receptor (ER)-dependent activation of multiple kinases by E2 through nongenomic pathways. Estrogen activates multiple kinase pathways through interactions with a membrane-bound or -associated ERs; multiple kinase activities can be induced and these are cell context dependent. PI3K, Phosphoinositide 3-kinase; MAPKK, mitogen-activated protein kinase kinase; PKA, protein kinase A.

induces rapid responses (<5 min) that cannot be mediated by the nuclear ERs. Many of these nongenomic responses up-regulated by E2 resemble those induced by growth factors/cytokines through their corresponding cell membrane receptors, and this includes rapid activation of intracellular kinases and increased phosphorylation/activation of their downstream nuclear transcription factor substrates. For example, a recent study in this laboratory has reported that within 5–10 min after treatment of MCF-7 breast cancer cells with E2, there was a significant increase in phosphorylation of mitogen-activated protein kinase 1/2 (MAPK1/2), increased phosphorylation of the transcription factor Elk-1, and transactivation of a construct containing a serum response element from the c-*fos* gene linked to a luciferase reporter gene. Similar results were obtained after treatment of MCF-7 cells with insulin-like growth factor-I (IGF-I). Studies in several laboratories show that E2 rapidly activates multiple intracellular kinase pathways (Fig. 2), including SRC, ras, MAPK, phosphoinositide 3-kinase (PI3K), cAMP–protein kinase A (PKA), calcium ion/calmodulin, protein kinase C, and G-protein-coupled pathways (Fig. 2).

The ER has been identified in the cell membrane, and activation of kinases by E2 in ER-negative cell lines can be observed after transfection with ER-α or ER-β and incorporation of these proteins into the cell membrane. At present, the precise location, structure, and function of membrane ERs are unclear in most cell lines, and factors that control their cell context-dependent activation of one or more kinase pathways have not been determined. A further complication in understanding the diversity of ER-dependent nongenomic pathways has been the direct association of ER-α with multiple membrane-associated intracellular regulatory proteins. For example, ER-α binds directly to the SH2 domain of Src; ER-α also interacts with the PI3K p85 regulatory unit (but not the SH2 or SH3 domains of p85), the IGF-IR, calmodulin, and the $G_{\alpha i}$ subunit of the membrane G-protein complex (Fig. 3). The contributions of membrane or intracellular ERs on cell-context dependent interactions with these regulatory proteins and their activation of kinase pathways are unknown.

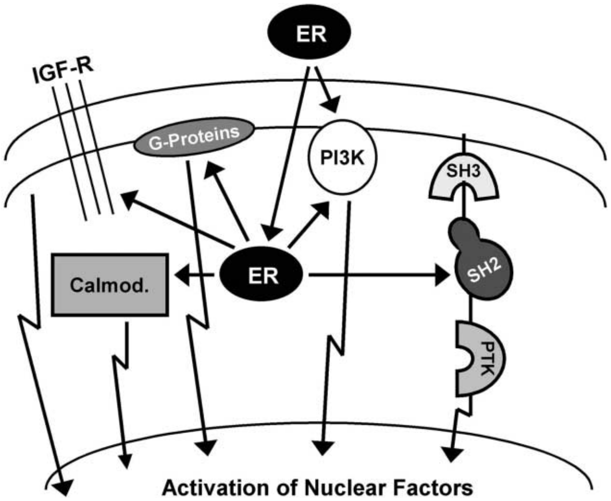

FIGURE 3 Examples of direct interactions of estrogen receptor-α (ER) with key intracellular proteins that regulate kinase pathways. There is evidence that ligand-bound ER-α also binds specific domains of intracellular kinases; however, the functional consequences of these interactions are dependent on cell context. PI3K, Phosphoinositide 3-kinase; IGF-R, insulin-like growth factor receptor; Calmod., calmodulin.

## IV. SUMMARY

Regulation of E2-responsive genes is critical for normal physiology in multiple tissue/cell types; estrogens also contribute to various tissue-specific adverse responses, including breast cancer and other hormone-dependent cancers. Not surprisingly, our understanding of transcriptional modulation (increased/decreased) of E2-responsive genes has become increasingly complex. It is now understood that classical activation of genes through consensus (or nonconsensus) EREs requires interactions with ER-α or ER-β homo- or heterodimers, and a host of other nuclear cofactors that are essential for ligand-activated gene expression. Nuclear ERs also activate genes through other DNA-dependent and -independent pathways, and there is increasing evidence that ER interactions with AP-1, Sp family transcription factors, GATA, NF-κB, and other proteins may also be important for regulation of E2-responsive genes. Nongenomic ER-α-dependent activation of kinases by E2 has also been characterized in several cell types. The increasing complexity and multiplicity of E2-dependent regulatory pathways is consistent with the diverse functions of this hormone, which plays an essential role not only in development of the female and male reproductive tract but in many other organs/tissues, including the vascular system, bone, and brain.

## Acknowledgments

This work was supported by grants CA76636, ES09253, and ES09106 from the National Institutes of Health.

## Glossary

**co-activators/coregulators** Nuclear factors that directly or indirectly interact with estrogen receptors and other receptors to modulate transcriptional activation of target genes.

**genomic** Refers to nuclear pathways for estrogen receptor action.

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## See Also the Following Articles

**Estrogen and Progesterone Receptors in Breast Cancer • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Biology and Lessons from Knockout Mice • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Vascular Endothelial Growth Factor B (VEGF-B)**

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Chen, J. D. (2001). Steroid/nuclear receptor coactivators. *Vitam. Horm.* **58**, 391–447.

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# Estrogen Receptor-α Structure and Function

Ann M. Nardulli
*University of Illinois, Urbana-Champaign*

I. OVERALL ESTROGEN RECEPTOR-α STRUCTURE
II. STRUCTURE OF THE DNA-BINDING DOMAIN
III. STRUCTURE OF THE LIGAND-BINDING DOMAIN
IV. ASSOCIATION OF ESTROGEN RECEPTOR-α WITH COREGULATORY PROTEINS
V. MECHANISM OF ACTION
VI. EFFECTS OF ESTROGENS
VII. EFFECTS OF ANTIESTROGENS
VIII. SUMMARY

**Estrogen receptor-α is a member of a large family of transcription factors that are activated by hormone binding and that interact directly with DNA to bring about changes in transcription. Estrogen receptor-α mediates the effects of estrogens and antiestrogens in target cells and is found in target cell nuclei. The activity of estrogen receptor-α is modulated by different ligands, estrogen response elements, transcription factors, co-activators, and corepressors, conferring versatility in hormone signal response and cell regulation.**

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Estrogen receptor-α (ER-α) is a member of a large superfamily of ligand-activated transcription factors that includes steroid, thyroid, and retinoid receptors and numerous orphan receptors with ligands that

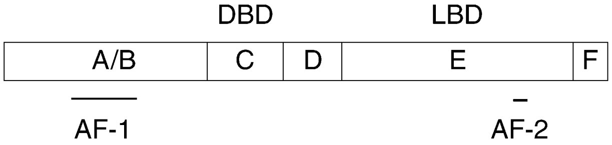

**FIGURE 1** ER-α domain structure. ER-α is composed of six domains (A through F). The DNA-binding domain (DBD) is responsible for interacting specifically with the estrogen response element. The ligand-binding domain (LBD) interacts with numerous agonistic and antagonistic hormones. The positions of the amino-terminal (A/B), carboxy-terminal (F), and hinge (D) regions of the receptor are shown as well as the transcription activation functions AF-1 and AF-2.

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Estrogen receptor-α (ER-α) is a member of a large superfamily of ligand-activated transcription factors that includes steroid, thyroid, and retinoid receptors and numerous orphan receptors with ligands that

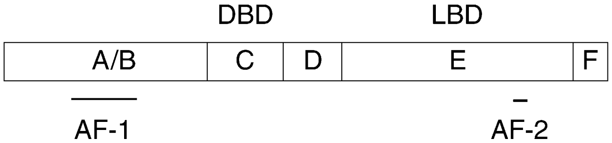

FIGURE 1 ER-α domain structure. ER-α is composed of six domains (A through F). The DNA-binding domain (DBD) is responsible for interacting specifically with the estrogen response element. The ligand-binding domain (LBD) interacts with numerous agonistic and antagonistic hormones. The positions of the amino-terminal (A/B), carboxy-terminal (F), and hinge (D) regions of the receptor are shown as well as the transcription activation functions AF-1 and AF-2.

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have not been defined. Molecular and biochemical analyses have demonstrated that, like other members of the nuclear receptor superfamily, ER-α contains six domains (A through F; Fig. 1) that are structurally and functionally conserved. When the ER-α sequence from different species is compared, the most highly conserved region of the receptor is domain C, the DNA-binding domain (DBD), which contains two $Cys_2Cys_2$ zinc finger motifs. The DNA-binding domain is necessary and sufficient for binding of ER-α to a specific nucleotide sequence, the estrogen response element (ERE), which resides in estrogen-responsive genes. Domain E, the ligand-binding domain (LBD), is also highly conserved and is responsible for interaction of the receptor with various estrogens and antiestrogens. In addition to these two highly conserved domains are regions with considerable variation in amino acid sequence, including the amino-terminal A/B domain, the carboxy-terminal F domain, and the centrally located hinge region, domain D. Within receptor domains A–F are discrete amino acid sequences that are important in maintaining receptor function. Sequence analysis and functional studies of wild-type and mutant ER-α proteins have identified two receptor regions that are important in enhancing transcription of estrogen-responsive genes. Transcription activation function-1 (AF-1) is localized in the amino-terminal A/B domain of the receptor and serves as a ligand-independent transcription activation domain. Activation function-2 (AF-2) resides in the hormone-binding domain and acts as a ligand-inducible activation domain.

## II. STRUCTURE OF THE DNA-BINDING DOMAIN

ER-α binds with high affinity and specificity to the ERE, which is typically located in the 5′ flanking region of estrogen-responsive genes. The consensus ERE comprises two inverted, palindromic half-sites separated by three intervening nucleotides that vary in nucleotide sequence (GGTCAnnnTGACC). Although it has not been possible at this point to examine the interaction of full-length ER-α with the ERE, X-ray crystallographic analysis has been used to examine the interaction of the ERE with the region of the receptor responsible for the specificity of this interaction, the DBD. These studies have demonstrated that the DBD binds to the two ERE half-sites as a homodimer (Fig. 2). Each of the DBD monomers is composed of two amphipathic α-helices that interact at their midpoints and are oriented at right angles. The "recognition helix" of each monomer is seated in the major groove of the DNA helix and forms a network of hydrogen bonds with specific nucleotides in the ERE half-site. The second α-helix forms multiple hydrophobic interactions with the recognition helix, which helps to stabilize DBD structure. The two DBD monomers interact with each other through a dimerization domain, which is a discrete region located between the two α-helices. Together, these protein–protein and protein–DNA interactions contribute to stabilization of the DBD–ERE complex.

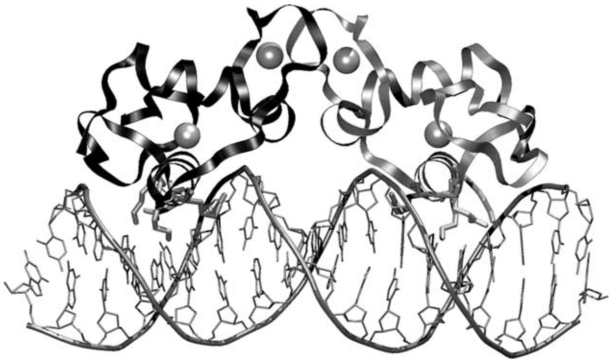

**FIGURE 2** Structure of ER-α DBD bound to the consensus ERE. Two DBD monomers bind as a dimer to the major groove of the DNA helix, which contains the palindromic consensus ERE. Amino acids in the "recognition helix" of each DBD contact specific nucleotides in each ERE half-site. The two zinc atoms that coordinate four cysteine residues in each DBD monomer are shown as spheres. Figure provided by Dorina Kosztin and Klaus Schulten, University of Illinois, Urbana-Champaign.

## III. STRUCTURE OF THE LIGAND-BINDING DOMAIN

The ER-α LBD is a discrete multifunctional domain located near the carboxy terminus of the receptor (Fig. 1) and is the portion of the receptor responsible for interacting specifically with a variety of naturally occurring and synthetic estrogens and antiestrogens. The LBD is composed of 12 α-helices that form a wedge-shaped structure (Fig. 3). Three of the α-helices (H5/6, H9, and H10) form an antiparallel core that is sandwiched between two α-helical layers (H1-4 and H7, H8, and H11). Located in the narrow end of the wedge is a hydrophobic cavity that is excluded from the external environment and forms the ligand-binding pocket. Because of the relatively large size of

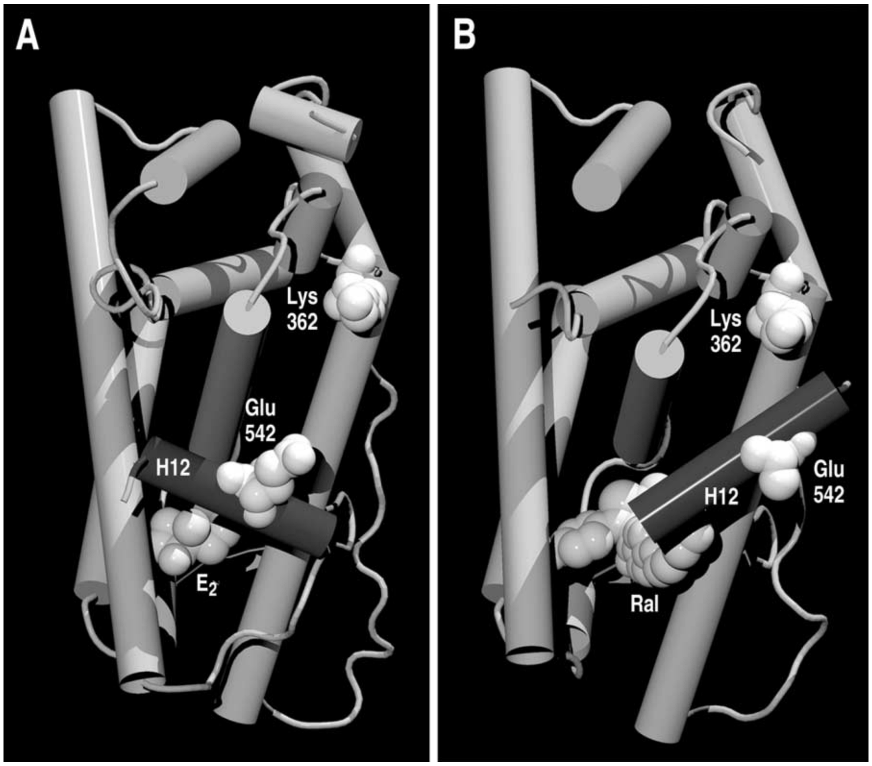

**FIGURE 3** Structure of the ER-α LBD bound to estrogen or raloxifene. α-Helices 1–11 (light gray cylinders); and α-helix 12 (dark gray cylinder) comprise the wedge-shaped ER-α LBD. Binding of the LBD to 17β-estradiol (A) or raloxifene (B) causes a dramatic change in the position of helix 12 (H12). Lys-362 in helix 3, Glu-542 in helix 12, and the ligands 17β-estradiol (E2) and raloxifene (Ral) are represented as light-colored spheres. Figure provided by Dorina Kosztin and Klaus Schulten, University of Illinois, Urbana-Champaign.

this hydrophobic cavity, the ER-α LBD can accommodate a diverse range of estrogenic and antiestrogenic ligands.

Although biochemical studies have suggested for some time that ER-α conformation is different when bound to estrogens and antiestrogens, these differences have been most clearly delineated in X-ray crystallographic studies carried out with the ER-α LBD. Binding of an estrogen such as 17β-estradiol or diethyl stilbestrol to the ER-α LBD results in the positioning of helix 12 over the ligand-binding pocket (Fig. 3A). In contrast, when the LBD is bound to an antiestrogen such as raloxifene or 4-hydroxytamoxifen, the bulky side chains of these antiestrogens protrude out of the ligand-binding pocket and require the repositioning of helix 12 (Fig. 3B). Helix 12 is rotated 130° in the raloxifene-bound LBD compared to the estrogen-bound LBD. Thus, the conformation of the LBD is different when bound to estrogens and antiestrogens, most notably in the positioning of helix 12.

## IV. ASSOCIATION OF ESTROGEN RECEPTOR-α WITH COREGULATORY PROTEINS

It has become increasingly clear that ER-α does not function in isolation, but that it requires the participation of coregulatory proteins to modulate transcription of estrogen-responsive genes effectively. A number of co-activator proteins that interact with ER-α and enhance estrogen-mediated transactivation have been identified, including steroid receptor co-activator-1 (SRC-1), transcription intermediary factor-2 (TIF2), and amplified in breast cancer 1 (AIB1). These co-activators contain receptor interaction domains with the amino acid sequence LXXLL, where L is a leucine and X is any amino acid. These co-activator LXXLL motifs bind to a shallow, hydrophobic groove that is formed in the LBD when it is bound to estrogen. A glutamic acid in helix 12 (Glu-542) and a lysine in helix 3 (Lys-362) of the ER-α LBD form a “charge clamp” that marks the boundaries of the co-activator binding surface and that helps to anchor the co-activator LXXLL motif in the hydrophobic groove of the estrogen-bound receptor (Fig. 3A). When the LBD is occupied by an antiestrogen, helix 12 is reoriented and binds along the co-activator binding surface, thereby inhibiting contact between the receptor and the co-activator LXXLL motif (Fig. 3B). Corepressors, which bind to the antiestrogen-occupied ER-α and inhibit transcription, have also been identified. The recruitment of co-activator and corepressor proteins with histone acetylase and deacetylase activities, respectively, appears to be important in modulating chromatin structure and the accessibility of transcription factors to their respective DNA binding sites. Whereas acetylation of histones is associated with more open chromatin structure, increased accessibility of transcription factor binding sites, and increased transactivation, deacetylation of histones is associated with more compact chromatin structure, decreased accessibility of transcription factor binding sites, and decreased transactivation. The transcription of estrogen-responsive genes is most likely subject to the combined effects of co-activator and corepressor proteins.

## V. MECHANISM OF ACTION

The most clearly defined mechanism by which ER-α alters cell function is through direct interaction with DNA. Circulating estrogen enters a cell and binds to the intracellular ER-α, which is present in the nucleus of target cells (Fig. 4). On binding hormone, ER-α undergoes a change in conformation and the estrogen-occupied receptor dimerizes and interacts with EREs residing in estrogen-responsive genes. Interaction of the estrogen-occupied ER-α with the ERE modulates transcription of ERE-containing genes and alters the levels of specific mRNA transcripts. Nuclear mRNA is processed and exported to the cytoplasm, where it is translated into protein. Thus, the ER-α–ERE interaction is a critical link in the chain of events that leads to changes in cellular function. Although the consensus ERE has been the most widely studied ER-α binding site, most of the estrogen-responsive genes identified to date have EREs that deviate from the consensus sequence by one or more nucleotides. This variation in nucleotide sequence influences receptor binding, transcription activation, and recruitment of co-activator proteins.

A number of genes have been identified that do not possess an ERE sequence but are clearly estrogen responsive. Recent evidence suggests that ER-α regulates transcription of some of these estrogen-responsive genes by interacting with DNA-bound proteins such as Sp-1 and activator protein 1 (AP-1) rather than binding directly to DNA. There is also accumulating evidence that nongenomic events, which include posttranslational modifications such as phosphorylation, may be involved in regulating rapid responses to hormone.

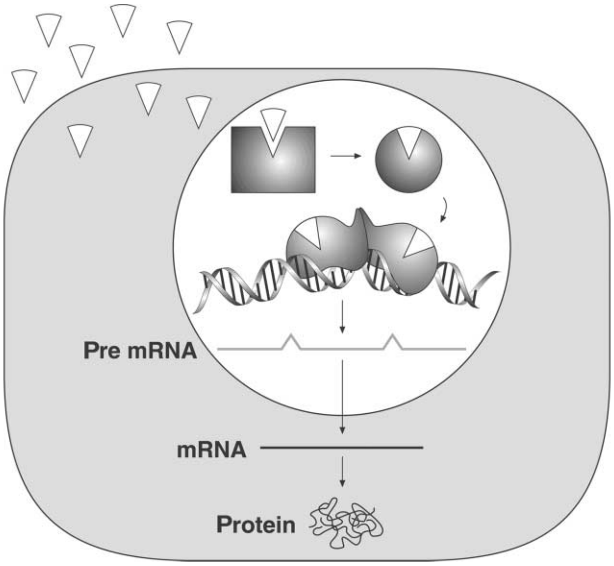

FIGURE 4 Mechanism of estrogen action. Circulating estrogen enters the cell and binds to ER-α located in the nucleus of target cells. Binding of hormone induces a conformational change in receptor structure and causes ER-α to dimerize and bind to estrogen response elements residing in estrogen-responsive genes. Interaction of ER-α with the estrogen response element alters transcription of estrogen-responsive genes. The newly synthesized mRNA transcripts are processed in the nucleus and transported to the cytoplasm, where they are translated into proteins.

## VI. EFFECTS OF ESTROGENS

Estrogen treatment of target cells alters transcription of a wide variety of genes. Estrogen is a hormone of critical importance in the development and maintenance of reproductive function. Although circulating estrogen typically inhibits gonadotropin secretion from the anterior pituitary, the rising estrogen levels at midcycle initiate a luteinizing hormone (LH) surge from the anterior pituitary, which in turn induces ovulation. Thus, estrogen supplementation has been extensively used to regulate ovulation and fertility. Estrogen acts on the uterus to increase cell proliferation and is required for mammary gland development. Estrogen also appears to play a role in maintaining bone mineral density and neural and cardiovascular physiology. Interestingly, it is now clear that estrogen plays a crucial role in the male reproductive tract and is required for concentration of sperm. Studies in ER-α-deficient mice indicate that estrogen may also influence behavior in both males and females.

## VII. EFFECTS OF ANTIESTROGENS

Antiestrogens bind to ER-α and inhibit the actions of estrogens in target cells. Tamoxifen inhibits estrogen-induced tumor cell proliferation and has been the most widely used antiestrogen for breast cancer treatment and prevention. In contrast to its antagonistic effects in breast cancer cells, tamoxifen has agonistic effects in other tissues and, like estrogen, helps to maintain bone mineral density, improve lipid profiles, and stimulate uterine endometrial cell growth. Tamoxifen's agonistic effects in the uterus result in an increased incidence of endometrial cancer. Thus, tamoxifen acts as an antagonist in mammary tissue, but as an agonist in uterus and bone. On the basis of its mixed agonist/antagonist activity in different tissues, tamoxifen has been reclassified as a selective estrogen receptor modulator (SERM). Another SERM, raloxifene, functions as an antagonist in mammary tissue and decreases the incidence of breast cancer in women who are at increased risk of developing this disease. Unlike tamoxifen, raloxifene functions as an antagonist in the uterus and is not associated with an increased incidence of endometrial cancer. The development of new SERMs that function as agonists in bone, cardiovascular, and neural cells, but as antagonists in mammary and uterine cells, could provide new therapies for breast cancer treatment and prevention and for the treatment of postmenopausal symptoms.

## VIII. SUMMARY

The estrogen receptor is a ligand-activated transcription factor that resides in the nucleus of target cells. Those regions of the receptor responsible for interacting with DNA and hormones have been defined in detail and have provided a greater understanding of how the receptor functions in target cells. The ability of different ligands, EREs, transcription factors, co-activators, and corepressors to interact with ER-α and to modulate its activity provides target cells with tremendous versatility in responding to hormonal signals and regulating cellular function.

## Glossary

| | |
|---|---|
| **agonist** | Naturally occurring or synthetic compound that binds to a receptor and mimics a specific effect. |
| **antagonist** | Naturally occurring or synthetic compound that binds to a receptor and opposes the actions of an agonist. |
| **antiestrogen** | Steroidal or nonsteroidal compound that binds to the estrogen receptor and opposes the actions of estrogen when administered in combination with estrogen, but may also elicit estrogenic effects when administered alone. |
| **co-activator** | Protein that interacts with the estrogen receptor and enhances transcription of estrogen-responsive genes. |
| **corepressor** | Protein that interacts with the estrogen receptor and inhibits transcription of estrogen-responsive genes. |
| **estrogen** | Naturally occurring or synthetic steroid that binds to the estrogen receptor and stimulates the development of female secondary sex characteristics and promotes growth and maintenance of the female reproductive system. |
| **estrogen response element** | Specific nucleotide sequence located in an estrogen-responsive gene; the estrogen receptor binds to the sequence and thereby alters expression of the gene. |
| **selective estrogen receptor modulator** | Ligand that binds to the estrogen receptor and has agonistic or antagonistic effects that are cell and promoter specific. |

## See Also the Following Articles

**Estrogen Receptor (ER) Actions through Other Transcription Factor Sites • Estrogen Receptor-β Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • SERMs (Selective Estrogen Receptor Modulators)**

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# Estrogen Receptor-β Structure and Function

Karin Dahlman-Wright, Konrad Koehler*, and Jan-Åke Gustaffson

*Karolinska Institute, Sweden • *Karo Bio AB, Sweden*

I. INTRODUCTION
II. ER-β GENE STRUCTURE
III. ER-β PROTEIN STRUCTURE AND INTERACTING MOLECULES
IV. TRANSCRIPTIONAL ACTIVATION
V. ANIMAL MODELS
VI. ER-β IN PHYSIOLOGY
VII. CONCLUSIONS

**For a long time estrogen receptor-α (ER-α) was thought to be the single mediator of the physiological effects of estrogen. However, in 1996, a novel ER was discovered in the rat prostate. This receptor was named ER-β, to distinguish it from the previously identified ER-α. ER-β homologues have subsequently been cloned from human, mouse and various other species including cow and goldfish. The discovery of ER-β has forced us to re-evaluate the biology of estrogen. ER-β is abundant in the male reproductive tract and considerable attention has been paid to the role of ER-β in the male reproductive tract.**

## I. INTRODUCTION

ER-β belongs to a family of ligand-activated transcription factors, the nuclear receptor family. Binding of ligand results in association with hormone-responsive DNA elements in the regulatory regions of target genes. Physical interaction of the DNA-bound receptor with co-activator proteins then leads to changes in the transcriptional rate of regulated genes.

The structure of the ER-β gene reveals how several transcripts and proteins with potentially important biological roles may be generated. The three-dimensional structure of the ER-β ligand-binding domain provides insight into how different ligands may lead to different biological end-points. Animals with eliminated expression of ER-β are instrumental in defining the role of this receptor in physiology and have already provided important information. Although the focus of this article is on ER-β, it is necessary to also discuss ER-α, since it is the coordinated and integrated activities of these two receptors that bring about the physiological effects of estrogen.

## II. ER-β GENE STRUCTURE

The human ER-β gene is localized on chromosome 14q23–q24. The 59 kb gene includes eight coding exons (Fig. 1). In humans, there is an alternative last exon designated CX (Fig. 1).

ER-β has the same general primary structure as all nuclear receptors (NRs) (Fig. 2a). This structure includes an N-terminal domain termed the A/B domain. This domain harbors a transcriptional activation function (AF-1). Next to the A/B domain is the DNA-binding domain (DBD), also termed the C-domain. This is the most conserved region of the receptor protein, including two zinc-finger motifs, where eight strictly conserved cysteine residues coordinate two zinc atoms. This domain also includes

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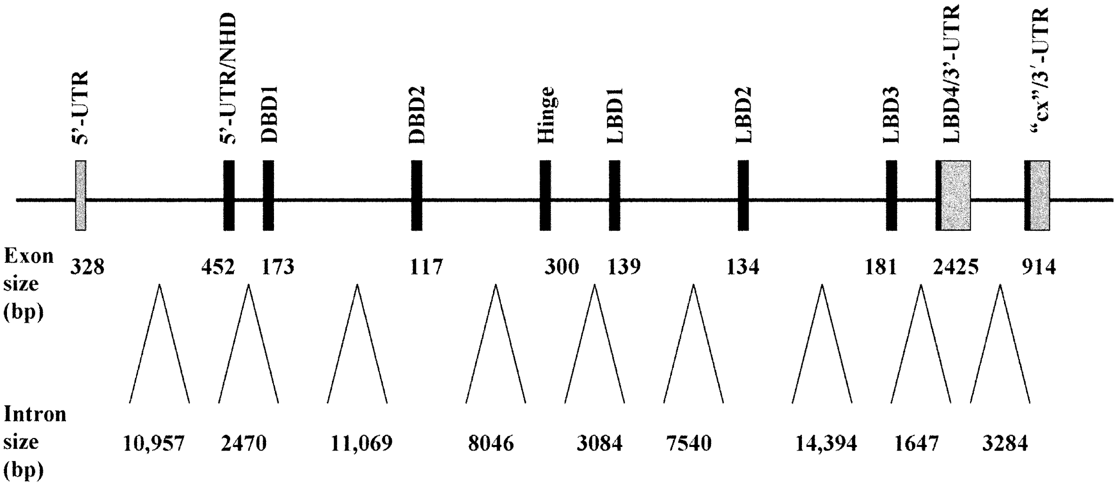


**FIGURE 1** The gene structure of ER-β. Protein coding sequences are shown in black; untranslated regions (UTRs) are shown in gray.

a dimerization region. The DBD is linked to the ligand-binding domain (LBD) by the hinge domain (domain D). Domain D is poorly conserved. The LBD or the E/F domain includes multiple functions including binding of agonists or antagonists, dimerization, binding of co-factors, as well as transcriptional activation. The transactivation function at the C-terminus (AF-2) is in most cases dependent on the binding of ligand for activity.

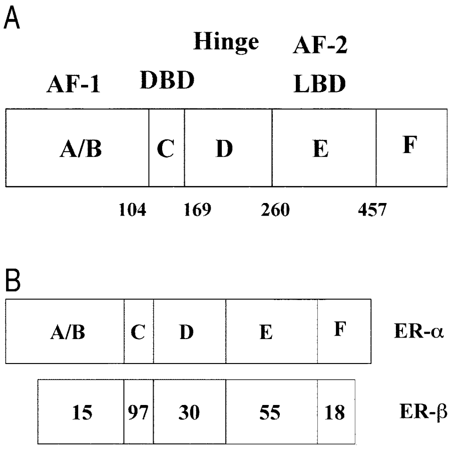


**FIGURE 2** (A) Overall structural organization of the ER-β protein. Numbers below the schematic representation of the ER-β protein refer to the first and last amino acids of the human DBD and LBD, respectively. A/B, Transcriptional activation function; C, DNA-binding domain and dimerization region; E/F, ligand-binding domain, transcriptional activation function, and dimerization region. (B) Homology between ER-α and ER-β (%).

The amino acid sequence homology between ER-α and ER-β is approximately 97% in the DBD and approximately 55% in the LBD. Regions that are directly involved in ligand binding and transcriptional activation show a higher degree of conservation (Fig. 2b). Human ER-β has approximately 89% identity with rat ER-β and 88% identity with mouse ER-β.

## A. ER-β Isoforms

### 1. *Alternative Translational Initiation*

Alternative translational initiation gives rise to proteins containing 530 and 548 amino acids, respectively, in addition to the 485-amino-acid protein originally cloned. Both of the longer receptor isoforms have been identified in rodents. Only the 530-amino-acid form has been observed in human. These isoforms have not yet been characterized with regard to specific physiological functions.

### 2. *ER-βins*

ER-βins, also called ER-β2, contains an extra 54 bp insertion in the reading frame, causing an 18-amino-acid insertion in the LBD between exons 5 and 6 (Fig. 3). This insertion occurs through alternative splicing. ER-βins shows severely impaired ligand binding and transcriptional activation. However, ER-βins binds to an estrogen-response element (ERE) and can heterodimerize with ER-β variants and ER-α. ER-βins acts as a dominant negative regulator of ER-β and ER-α and causes a dose-dependent inhibition of ER-β and ER-α transcriptional activity. ER-βins has been described for mouse and rat.

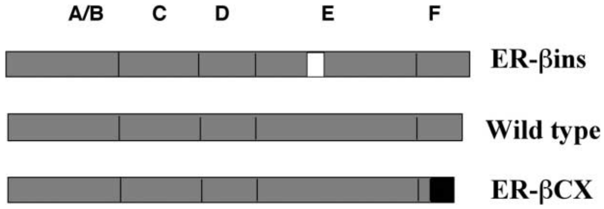


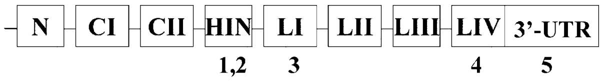
**FIGURE 3** ER-β isoforms. The ins domain found in the variant ER-βins in rodents is indicated in white. The CX domain found in the variant ER-βCX in humans is indicated in black.

### 3. *ER-βCX*

Another splice variant occurring in the human, ER-βCX, has an alternative last exon and C-terminus (Fig. 3). The last 61 amino acids encoding parts of helix 11 and helix 12 in wild-type ER-β is replaced by 26 novel amino acids to produce ER-βCX. This isoform does not bind ligand or DNA. DNA binding by ER-α is inhibited by ER-βCX. This finding is in contrast to the lack of an influence on ER-β DNA complexes. When ER-βCX is co-transfected with ER-α or ER-β, it inhibits ligand-induced transactivation by ER-α but does not influence ER-β-mediated transactivation. Other ER-β splice variants have also been cloned. Interestingly, it has been shown that expression of ER-βCX is increased in several forms of cancer, including cancer of the breast, ovary, and prostate. Other isoforms have also been shown to be associated with disease.

## B. ER-β Polymorphism

Analysis of a German population identified two common polymorphisms in the ER-β gene, one silent change in exon L1 and one change in the 3′-untranslated region (Fig. 4). Polymorphism refers to the existence of different base pairs at a given nucleotide position. It is evident that the ER-β gene contains very few polymorphic nucleotide positions. Several less common polymorphisms were also identified in the German population (Fig. 4). These polymorphisms change the amino acid sequence in the hinge region. The functional consequences of these mutations remain to be determined.



| | | | Allele frequency |
|---|---|---|---|
| 1 | 809 (del 21) | Δ Q238 to K244 | < 0.01 |
| 2 | 846 G → A | G-250-S | < 0.01 |
| 3 | 1082 G → A | Silent | 0.04 |
| 4 | 1421 T → C | Silent | < 0.01 |
| 5 | 1730 A → G | 3'-UTR | 0.60 |

**FIGURE 4** ER-β genetic variants. Data from Rosenkranz, K., *et al.* (1998) *J. Clin. Endocrinol. Metab.* **83**, 4524.

Recently, a polymorphic dinucleotide CA repeat in the noncoding 3′-portion of the gene was identified. It has been suggested to be associated with bone mineral density in women. An additional study showed an association between the length of the CA repeat and androgen levels in women. Women with a short CA repeat region displayed higher levels of total and free testosterone than women with many CA repeats. However, the molecular mechanism by which the length of the CA repeat leads to changes in androgen levels remains obscure. It is unclear whether the CA repeat affects the activity and/or expression level of the ER-β protein. However, there are reasons to believe that repeat nucleotide sequences (micro/minisatellites), even when situated in untranslated regions, may influence the expression of a gene.

## C. The ER-β Promoter

The promoters of the mouse and human ER-β genes have been cloned and preliminary functional analyses have been reported. A 2.1 kb fragment of the human promoter exhibited functional promoter activity in ER-β-positive cells but not in ER-β-negative cells, suggesting that it maintained the cell-restricted expression of the intact ER-β gene. Two transcription start sites were observed, which are located about 200 bp apart approximately 400 bp upstream from the ATG. The proximal promoter contains both TATA box and initiator (Inr) elements and is highly GC-rich. The Inr sequence is highly homologous to the preferred Inr sequence. Serial deletion analysis revealed that a 293 bp region encompassing the TATA box and Inr element possesses basal promoter activity. An *Alu* repeat sequence exists between −1416 and −1703. This *Alu* element belongs to a new class of *Alu* DNA repeats, which contain an imperfect ERE and function as ER-dependent transcriptional enhancers. The sequence of this DNA element is GGTCAnnnTGGTC. This element might be responsible for the observed 30-to 40-fold estradiol induction of ER-β expression in T47D cells. Consistent with this observation, deletion of this *Alu* element in the ER-β promoter resulted in decreased promoter activity in reporter assays. Several binding sites for transcription factors including SP-1, AP-1, Oct-1, and N-myc were noted. Deletion analysis of the ER-β promoter using appropriate reporter assays identified enhancer and silencer elements. A negative regulatory element was identified at −425 using this analysis. This element has the sequence AGCCTCTCT and is referred to as the negative regulatory element. This sequence is the consensus sequence for a ubiquitous transcriptional silencer that represses transcription in the absence of steroids. The mouse ER-β promoter contains several putative *cis*-acting elements, many of which are also found in the mouse ER-α promoter. Therefore, the expression of both ERs may be partially regulated by a common mechanism.

## III. ER-β PROTEIN STRUCTURE AND INTERACTING MOLECULES

### A. The DNA-Binding Domain

The DNA-binding domain includes two so-called zinc fingers, in which four cysteines coordinate one zinc atom, and folds into a "finger-like" structure. The P-box refers to a sequence of three amino acids in the first zinc finger, which is involved in DNA sequence recognition. The D-box refers to a sequence of approximately 5 amino acids in the second zinc finger, which is responsible for protein–protein interactions in the dimer that binds to DNA.

The three-dimensional structure of the ER-β DBD has not been determined. However, since the identity between ER-α and ER-β is 97% in this domain, the structures must be very similar.

The three-dimensional structure of ER-α DBD bound to an ERE has been determined. The structure of the protein–DNA complex has revealed the exact mechanism of how amino acids in the so-called P-box recognize specific base pairs in the estrogen-response elements. The structure also revealed how the D-box is used for dimerization, thereby restricting the spacing between the two half-sites constituting the ERE.

### B. The Ligand-Binding Domain

Crystal structures for various NR LBDs with or without bound ligand have shown that the binding of ligand leads to a conformational change in the receptor protein, resulting in a transcriptionally active protein. Furthermore, the architecture of the domain is conserved with 12 α-helices. The three-dimensional structures for the ER-β LBD bound to raloxifene (a cell- and tissue-specific agonist–antagonist) and the isoflavonoid genistein (an ER-β-specific agonist), respectively, have been reported (Figs. 5a and 5b). Three-dimensional structures for ER-α bound to various agonists and antagonists have also been reported.

The ER-α agonist structures show a central layer of three helices, packed between two additional layers of helices creating a molecular scaffold with the ligand-binding pocket close to the surface of one end. Helix 12 (H12) is positioned across the ligand-binding pocket. This configuration facilitates interaction between the receptor and co-activators. When anti-estrogens are bound, H12 translocates to a position that masks the co-activator-binding site. In the crystal structure of genistein-bound ER-β LBD,

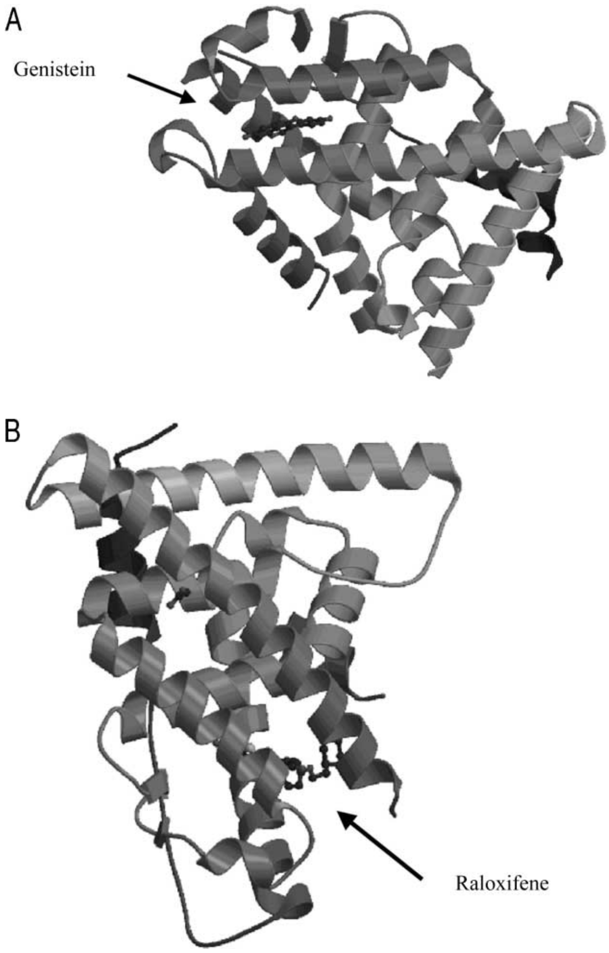


**FIGURE 5** (A) Crystallographic structure of ER-β LBD complexed with genistein. (B) Crystallographic structure of ER-β LBD complexed with raloxifene. These figures were produced using MOLSCRIPT [Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950] and Raster3D [Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* **277**, 505–524].

H12 does not adopt the typical agonist conformation but rather adopts the position of an antagonist–ER LBD complex. This particular positioning of H12 might explain why genistein is a partial agonist.

## C. Dimerization

ER-β dimerization is required for transcriptional activation. *In vitro*, ER-β dimerization is independent of ligand. However, whether this is also the case *in vivo* needs to be established. ER-β and ER-α form functional heterodimers *in vitro* and *in vivo*. ER-β and ER-α are co-expressed in various cell types, suggesting that heterodimerization occurs *in vivo*.

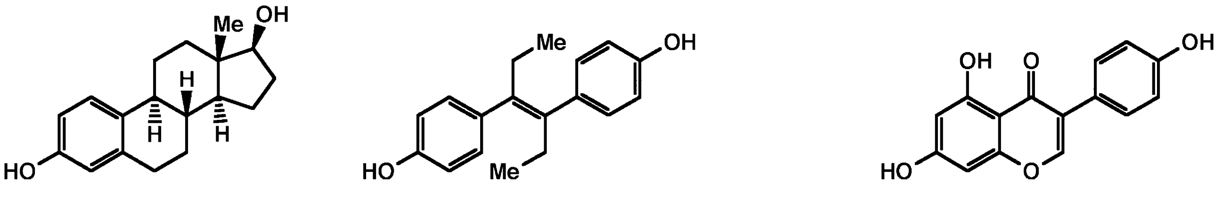
## D. Ligand Binding

ER-β and ER-α have similar ligand-binding specificities. The natural estrogen 17β-estradiol (E2) binds to both ER-α and ER-β with similar high affinity (dissociation constant, $K_d$, 0.1 nM for ER-α and 0.4 nM for ER-β). This is not surprising considering the particularly high sequence similarity in the region of the LBDs that is directly involved in ligand binding. However, there are major differences between the ER isoforms regarding affinities for several compounds as well as ligand-induced responses. For example, tamoxifen is a pure antagonist for ER-β but for ER-α its agonist and antagonist properties depend on the cellular context.

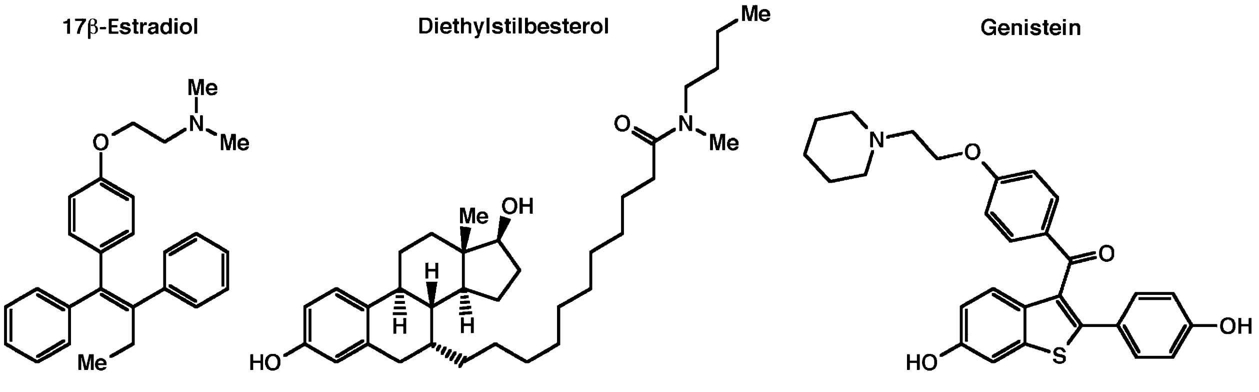
Phytoestrogens, like genistein, generally have higher affinities for ER-β than for ER-α. At low nanomolar concentrations, genistein-activated estrogen-specific transcription is ER-β specific. However, the maximal transcriptional activity achieved with ER-β is only half of the activity obtained with ER-α at higher concentrations of genistein. Fig. 6 shows the molecular structures of some agonists and antagonists with estrogenic activity. Several large pharmaceutical companies are involved in the development of ER-β-specific selective estrogen receptor modulators (SERMs). However, none of these drugs has yet reached the market. SERMs may have the potential to achieve the beneficial effects of estrogens but avoid the unwanted side effects. Indications for such compounds are menopausal symptoms, e.g., hot flashes, osteoporosis, and cardiovascular disease.

## E. Are Phytoestrogens Natural SERMs?

Phytoestrogens are nonsteroidal polyphenolic compounds present in several edible plants. Phytoestrogens can be divided into four subclasses based on chemical structure: isoflavonoids, flavonoids, coumestans, and lignans. The major dietary source of isoflavonoids (e.g., genistein) is soybean. Flavonoids are widely distributed in the plant kingdom and are



17β-Estradiol Diethylstilbesterol Genistein



Tamoxifene ICI-164,384 Raloxifene

FIGURE 6 Molecular structures of selected estrogen receptor agonists and antagonists.

present in several edible plants. Coumestans, present in alfalfa sprouts, for example, are not common in the human diet. Isoflavones and coumestrol interact with ER *in vitro* but the binding affinities of different compounds with similar structures differ remarkably. Some flavonoids show modest estrogenic activity and others are completely inactive. Lignans have very weak estrogenic activity and require millimolar concentrations to demonstrate any ER-mediated activity.

### F. Co-regulators

Co-regulators, i.e., co-activators and co-repressors, enhance or suppress the activity of NRs, respectively. These proteins usually exist in large complexes and some have enzymatic activities. Many co-regulators have the consensus amino acid sequence LXXLL for NR interaction. This sequence can be found in single or multiple copies in NR-interacting proteins. During the past 5 years, protein–protein interaction screenings and biochemical approaches have led to the identification of a large number of co-regulators that act at different functional levels of NR interaction. The majority of these co-regulators bind to the LBD. Co-regulators, in a simplified view, connect the ER with components of the basal transcriptional machinery and chromatin components.

Co-activators that have been shown to be relevant for ER function include the steroid receptor co-activator (SRC) family, CREB-binding protein (CBP)/p300, and the thyroid hormone receptor-associated protein (TRAP)/vitamin D-receptor interacting protein (DRIP) complex. SRC proteins are involved in recruiting chromatin and/or histone-modifying enzymatic activities to ligand-activated ERs bound at target genes. The histone acetylase activity of CBP has been shown to be required for efficient ER target gene activation *in vivo*. The TRAP/DRIP complex may mediate signals from ERs to the RNA polymerase multiprotein complex. A few differences between ER-α and ER-β with regard to co-activator recruitment have been reported. The interaction between ER-β and the TRAP/DRIP complex seems stronger than that of ER-α. On the other hand, ER-α interacts more strongly with SRC-3 than does ER-β.

Co-repressors that have been shown to affect ER function include N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid receptors). RIP140 (receptor-interacting protein-140) and SHP (short heterodimerization partner) bind to AF-2 of ERs and exhibit negative co-regulatory functions at least partially because they compete with SRC-1 binding and function. Interestingly, ER-β interaction with SRC-1 and SHP *in vitro* is ligand-independent, in contrast to the interaction of ER-α with these co-factors, which is ligand-dependent.

### G. Phosphorylation

Phosphorylation of Ser-106 and Ser-124 (nomenclature refers to the 548-amino-acid mouse ER-β) of ER by MAPK results in ligand-independent recruitment of SRC-1 and transcriptional activation. Co-expression of components of the MAPK pathway also enhances estrogen-induced gene activation.

A conserved tyrosine amino acid residue in ER-β LBD can be mutated to produce a constitutively active receptor. This residue is phosphorylated by the Src family of protein kinases. Mutated receptors appear to be in an active conformation as judged by proteolytic digestion (and show ligand-independent interaction with the co-activator SRC-1). This ligand-independent event can be inhibited by antagonists. In wild-type ER, phosphorylation of this Tyr residue might increase the affinity of the receptor protein for the ligand.

## IV. TRANSCRIPTIONAL ACTIVATION

Both ER-α and ER-β are capable of activating transcription from the classical EREs. However, ER-β demonstrates a weaker transactivating capacity and requires 10-fold higher concentrations of 17β-estradiol for full transactivating capacity in most cell types compared to ER-α (Fig. 7). In addition, as pointed out above, ER-β fails to show tamoxifen-induced agonist activity, due to a functional difference in the A/B domains of the ER isoforms. The isolated ER-β AF-1 domain fails to initiate transcription when fused to the GAL4 DBD. On conventional estrogen-responsive DNA elements, ER-β and ER-α show similar agonist and antagonistic profiles. However, this is not the case for all types of response elements. The estrogen agonists 17β-estradiol and diethylstilbestrol, in the presence of ER-β, inhibit transcription from an AP-1 element (Fig. 8). Moreover, the anti-estrogens raloxifene and ICI 164,384 induce transcription from an AP-1 element in the presence of ER-β in all cell systems tested (Fig. 8). Tamoxifen induces transcription from these elements in the presence of ER-α in some but not all cellular systems.

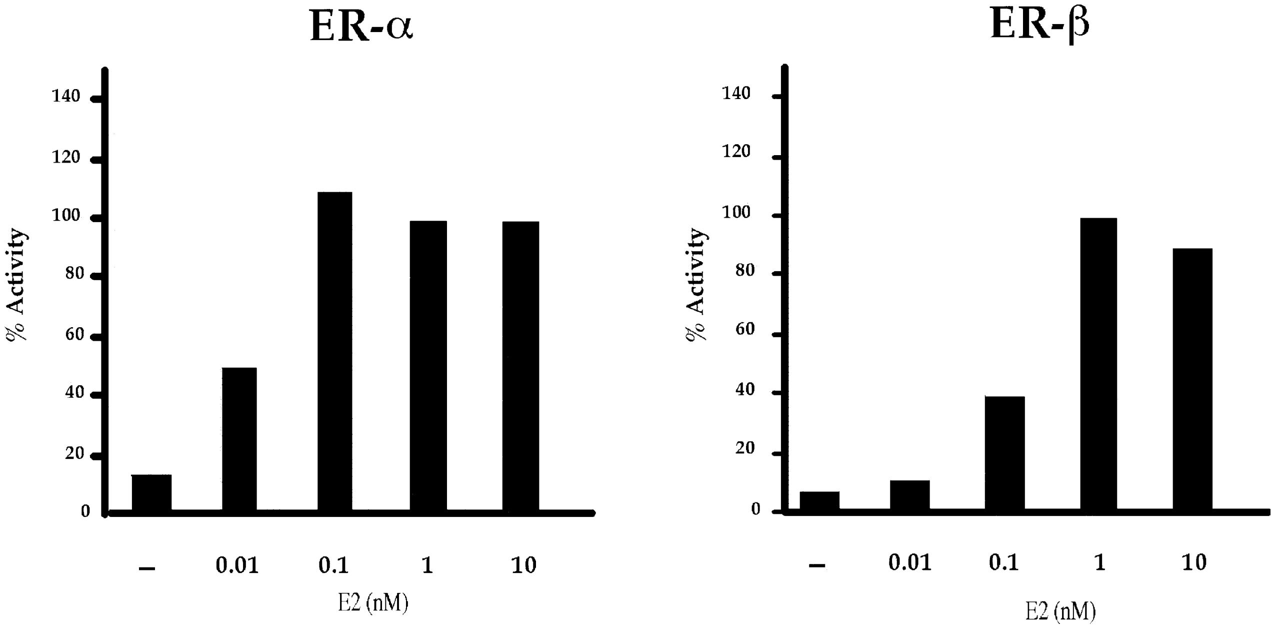


FIGURE 7 Estrogen-mediated transcriptional activity of ERE sequences. The ability of ER-α and ER-β to induce transcriptional activation from an ERE was tested in reporter assays. The concentration of E2 (17β-estradiol) is indicated. Reprinted from Pettersson, K., *et al.* (2000) *Oncogene* **19**, 4970–4978, with permission.

## V. ANIMAL MODELS

### A. ER-β Expression

Tissue-specific expression of ER-β is important to consider when one attempts to understand the phenotype of ER-β knockout animals. In rodents, the tissues with the highest level of expression of ER-β are prostate, ovary, and lungs. ER-β is also present in the mammary gland, bone, uterus, epididymis, ovary, kidney, bladder, intestine, central nervous system, and cardiovascular system.

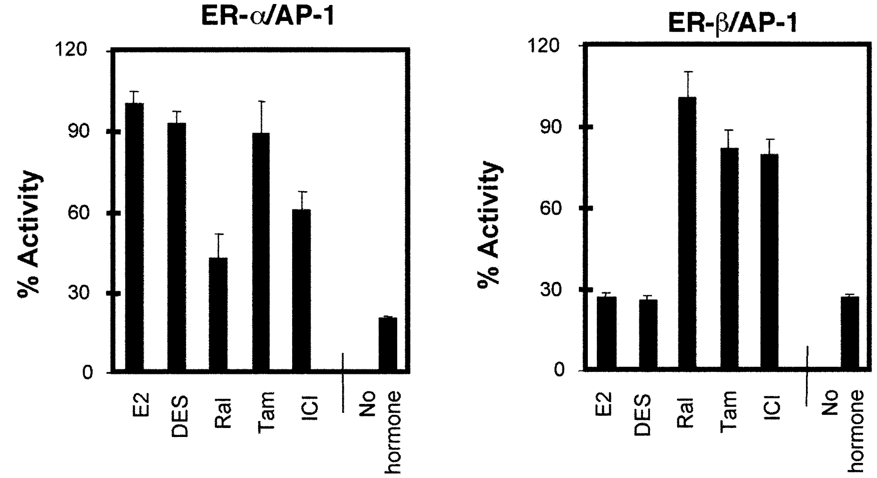


FIGURE 8 Estrogen-mediated transcriptional activity of AP-1 sequences. The ability of ER-α and ER-β to induce transcriptional activation from an AP-1 element was tested in a reporter assay. E2, 17β-estradiol; DES, diethylstilbestrol; Ral, raloxifene; Tam, tamoxifen; ICI, ICI 164,384. Reprinted from Paech *et al.* (1997) with permission.

## B. ER-β Knockout Animals

Knockout animals are those in which production of a particular protein has been eliminated due to deletion of parts of the gene that are necessary for the production of a functional protein. ER-β knockout animals (BERKO) are viable.

### *1. The Reproductive Tissues*

BERKO female mice have low fertility and disturbed ovarian function with almost no spontaneous ovulations and premature ovarian failure. There is a paucity of corpora lutea and an increase in the number of follicles with premature atresia. Follicles are actively recruited into the growth pool but they fail to mature. The oocytes are not released and they die within the preantral follicles. Uterine dysfunction probably contributes to the severe subfertility of BERKO female mice since fetuses are resorbed. The litter size is always small. ER-β knockout mice display abnormal epithelial growth in the breast and development of severe cystic breast disease. BERKO male mice show prostate hyperplasia and dysplasia reminiscent of what is sometimes seen in human. Studies have shown that decreased expression of ER-β is associated with increased expression of the androgen receptor and it is likely that this is relevant for the observed phenotype.

### *2. Bone*

BERKO mice show no alterations in bone phenotype before puberty. Adult female BERKO mice have increased limb length and increased cortical bone mineral content. Trabecular bone is normal. No abnormalities have been seen in the bones of male BERKO mice.

### *3. Central Nervous System*

We have found that the brains of BERKO mice show several morphological abnormalities. There is a regional neuronal hypocellularity in the brain, with a severe neuronal deficit in the somatosensory cortex and a remarkable proliferation of astroglial cells in the limbic system but not in the cortex. These abnormalities are evident at 2 months of age. As BERKO mice age, the neuronal deficit becomes more pronounced, and at 2 years of age, there is a degeneration of neuronal cell bodies throughout the brain.

### *4. Additional Phenotypes*

BERKO female and male mice have elevated blood pressure. BERKO male mice have a tendency for reduced weight of retroperitoneal fat pads, suggesting a role for ER-β in white adipose tissue. There are also abnormalities in the immune system.

## VI. ER-β IN PHYSIOLOGY

Below are some examples of the role of estrogen in physiology and what is known about the differing roles of the two estrogen receptors in these contexts. It is beyond the scope of this article to make a comprehensive review of the role of estrogen in physiology.

## A. The Reproductive Tissues

### *1. Prostate*

Estrogen has been widely used for the treatment of advanced prostatic cancer. However, the precise mechanisms of its effects on prostatic cancers remain uncertain. In addition to acting on the pituitary–testicular axis to effectively lower circulating levels of androgens and act as chemical castration agents, estrogens are believed to have direct growth inhibitory effects on prostatic cancer via induction of apoptosis or cell cycle arrest. Several investigations have demonstrated expression of ER-β in both normal and hyperplastic epithelia. In contrast, expression of the ER-α protein is restricted to stromal cells. These findings suggest that estrogens have direct effects on normal epithelium via ER-β signaling pathways.

In the human prostate, expression of ER-β was reported to be consistently expressed in normal prostate, but in many studies expression was decreased or absent in prostatic cancer, paralleling tumor grade. In contrast, one study demonstrated that the ER-β protein was expressed at a higher level in cancer than in normal epithelium. The expression of ER-β variants in prostate cancers was recently reported. It was shown that expression of the ER-βCX variant was inversely associated with wild-type ER-β expression. Furthermore, ER-βCX was expressed significantly more in the high-grade cancers than in the low-grade cancers, and expression of ER-βCX was a bad prognostic sign. These studies suggest that ER-β and ER-βCX may be good prognostic indicators for prostatic cancers and that ER-β-specific agonists will provide opportunities for improved therapeutic strategies.

### *2. Breast Tissue*

It is believed that the ER-α protein plays a central role in the development and progression of most breast cancers. This is the basis for the use of anti-estrogens, including tamoxifen, for the treatment of breast cancers. Moreover, the presence of ER-α protein in tumor tissue is an important prognostic factor that correlates with higher survival rates and lower risk of relapse.

Because ER-α-containing epithelial cells in the normal breast do not express proliferation markers, the mechanisms through which estrogen induces epithelial growth are not clear. The prevailing concept is that estrogen induces the secretion of growth factors from the stroma and that these agents stimulate epithelial cells to proliferate. ER-α has long been thought to be responsible for the secretion of growth factors in response to estradiol in the stroma. This appears to be the case, at least in rodents. In humans, several studies have failed to demonstrate the presence of ER-α in stroma from normal tissue and cancer tissue. Whether the distribution of ERs in different cellular compartments of breast tissue is different between rodents and humans or whether there are variations between individuals are questions that need further examination. In recent studies, ER-β was recently found to be the predominant ER in breast stroma. These findings indicate that ER-β may play a role in growth factor secretion.

Expression of ER-β mRNAs has been detected by reverse transcriptase-polymerase chain reaction in both normal and malignant human breast tissue, and ER-β protein has been identified in human breast tumors by Western blotting. It has been suggested that ER-β contributes to the initiation and progression of carcinogenesis, is expressed in breast cancers of higher grades, and is a marker for estrogen responsiveness.

We have recently investigated the correlation between estrogen receptors and proliferation markers in breast tissue samples. The presence of ER-α in breast cancer epithelium is associated with a decreased level of expression of the proliferation markers Ki67 and cyclin A, whereas ER-β expression is associated with elevated levels of these markers. The highest level of expression of either Ki67 or cyclin A is seen in cancers that are ER-α–ER-β. These results suggest that it might be ER-β and not ER-α that is related to proliferation in breast cancer.

In summary, much remains to be clarified regarding the role of ER-β in the breast.

## B. Effects of Estrogen on the Cardiovascular System

Many published clinical studies have shown that postmenopausal estrogen replacement is associated with a decreased incidence of cardiovascular disease even though this notion has recently been challenged. Several studies suggest that estrogen plays an important role in the cardiovascular system. However, the mechanism of action of estrogen on the cardiovascular system is poorly understood. For instance, few studies have addressed whether sex hormones (estrogen and testosterone) could affect cardiac remodeling or mass.

Both ERs are expressed in the myocardial cells of rats, guinea pigs, and humans. Which ER plays an important role in the heart is, however, unclear. ER-β knockout mice, in which the gene for ER-β has been disrupted, show elevated blood pressure with left ventricular wall thickness. These data suggest that ER-β may play an important role in blood pressure control and cardiac function. However, the mechanisms behind these observations remain unclear.

## C. Effects of Estrogen in the CNS

Estrogen exerts many functions in the central nervous system. It influences the development, plasticity, and survival of neurons with consequences for age-related neuronal degenerative diseases. Epidemiological studies suggest that E2 replacement therapy decreases the likelihood of developing Alzheimer's disease. However, estrogen replacement in older women did not affect the incidence or progression of Alzheimer's disease.

*In situ* hybridization and immunohistochemical studies in rodent and human brains have shown that there are neuronal populations that express both receptors and some that express only one of the two receptors. No morphological changes have been reported in mice lacking ER-α; however, as described above, striking morphological abnormalities have been observed in the brains of ER-β knockout mice.

## VII. CONCLUSIONS

The discovery of a second ER subtype, ER-β, has revitalized the search for improved drugs for menopausal hormone therapy, which would provide the benefits of estrogen replacement therapy but with fewer side effects than currently used drugs.

Several large pharmaceutical companies are engaged in the development of ER-α- and ER-β-selective drugs but as yet there is no information as to how far this work has progressed. However, synthetic ER subtype-selective ligands have been reported. The most ER-α-selective ligand showed 120-fold higher agonist potency for ER-α than for ER-β. Another

ER subtype-selective ligand synthesized by the same group showed full ER-α agonism but pure ER-β antagonism.

The discovery of a second estrogen receptor will require that we revisit many biological and clinical studies and reinterpret them in the light of the presence of two receptors. Such studies are now being performed, generating interesting and sometimes unexpected findings.

## Acknowledgments

This work was made possible due to the generous support of the Swedish Cancer Society and Karo Bio AB.

## Glossary

**BERKO** Mice with inactivated, knocked-out versions of estrogen receptor-β.

**estrogen receptor-α (ER-α)** The first cloned estrogen receptor, which displays differences compared to ER-β with regard to structure, ligand binding, expression profile, and physiological effects.

**estrogen receptor-β (ER-β)** A protein that, after binding its ligand, estrogen, activates the transcription of genes, resulting in specific physiological effects.

**estrogen-response element** The specific estrogen receptor-binding DNA sequence in the promoter region of genes that are regulated by estrogen.

## See Also the Following Articles

**Estrogen Receptor (ER) Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • SERMs (Selective Estrogen Receptor Modulators)**

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Encyclopedia of Hormones.

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# Estrogen Receptor Biology and Lessons from Knockout Mice

Deborah L. Swope and Kenneth S. Korach
*National Institute of Environmental Health Sciences, North Carolina*

I. ESTROGEN RECEPTORS: STRUCTURE AND MECHANISM OF ACTION
II. ESTROGEN RECEPTOR KNOCKOUT MODELS (α, β, AND α/β)
III. ESTROGEN RECEPTOR BIOLOGY

**The steroid hormone estrogen induces a wide variety of physiological responses in mammalian tissues. Estrogen is found in both females and males, although at higher levels in females. Estrogen causes its multitude of effects through estrogen receptors that mediate the transcription of a number of genes involved in reproduction, metabolism, and growth and differentiation. The current understanding of estrogen action is rooted in a model in which the estrogen receptor is bound by ligand, homodimerizes, and interacts with high-affinity binding sites for estrogen receptors found in the promoters of target genes. The bound receptor activates transcription of the target gene. Estrogen receptors also modulate expression of genes that do not contain these binding sites, can be activated in a ligand-independent manner by growth factors, and can also function as repressors of transcription.**

alone or in a synergistic manner with TPA plus estrogen, but ER-α-dependent responses can also be inhibited by TPA in a promoter-dependent and cell type-dependent manner. However, TPA can also reduce ER-α expression levels and receptor binding to its estrogen-response element *in vitro*. The implications of these TPA-mediated effects relative to ER-α transcriptional activity are unclear.

## VI. ESTROGEN RECEPTOR CROSSTALK WITH THE AKT SIGNALING PATHWAY

Although growth factors can signal to ER-α via MAPKs, they may also stimulate receptor transcriptional activity via the serine/threonine kinase Akt (also known as protein B kinase). Activation of Akt kinase activity is dependent on phosphatidylinositol 3-kinase (PI3-K), and the specific PI3-K inhibitor, LY294002, blocks activation of ER-α transcriptional activity in cells treated with either EGF or IGF-1. In addition, expression of a dominant negative form of Akt blocks growth factor activation of ER-α, whereas a constitutively active form is able to mimic growth factor activation of ER-α transcriptional activity and induction of PR gene expression. The ICI 182,780 anti-estrogen blocks Akt activation of ER-α. Moreover, ER-α can be phosphorylated by Akt *in vitro* and *in vivo*, and mutation of Ser-167 to an alanine blocks Akt stimulation of ER-α transcriptional activity, indicating that Akt signaling cross-talks directly with ER-α.

## Glossary

**co-activators** Molecules, generally proteins, that stimulate the activity of transcription factors, such as the estrogen receptors.

**crosstalk** The ability of signal transduction pathways to influence the activity of molecules, such as the estrogen receptors, that are typically regulated by other agents.

**signal transduction pathway** A group of molecules that enable signals initiated at remote locations to communicate to the final target through intermediary steps.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Crosstalk of Nuclear Receptors with STAT Factors • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Ethylene

Paul B. Larsen
*University of California, Riverside*

I. INTRODUCTION
II. PHYSIOLOGICAL AND AGRICULTURAL RELEVANCE
III. BIOSYNTHESIS
IV. PERCEPTION
V. CONTROL
VI. SUMMARY

**Ethylene is a simple gaseous hydrocarbon that is responsible for regulating physiological processes at all stages of plant growth and development. Ethylene plays a crucial role in a number of physiological processes, including seed germination, growth, adaptation to environmental stress, induction of pathogen defense responses, fruit ripening, and tissue senescence.**

## I. INTRODUCTION

Ethylene is considered to be one of the five classic plant hormones, with its biological activity being described as early as 1901 by Dimitry Neljubov, who reported that a component of illuminating gas, which he determined to be ethylene, had profound effects on the growth of pea seedlings. Subsequent physiological analysis has linked ethylene to processes ranging from seed germination and growth to fruit ripening and tissue senescence. Significant progress toward understanding the mechanisms underlying both its biosynthesis and its perception has been made.

## II. PHYSIOLOGICAL AND AGRICULTURAL RELEVANCE

Although ethylene plays a significant role in a wide range of physiological events, it is best known for mediating the induction of stress responses and senescence in plants. Ethylene has been implicated in processes throughout the life cycle of the plant, many of which have tremendous agricultural importance. These include seed germination and growth, adaptation to environmental stress, induction of pathogen defense responses, and promotion of both tissue senescence and fruit ripening.

The effects of ethylene on seed germination and seedling growth have been well documented. Ethylene promotes seedling emergence, giving the growing embryo the necessary mechanical strength to penetrate the seed coat. Perhaps the most scientifically important and at the same time agriculturally insignificant process is the seedling triple response. This response is limited to dark-grown seedlings (e.g., germinating seedlings in soil). Characteristics of the triple response include shortening and thickening of the hypocotyl and root, proliferation of root hairs, and most dramatically, formation of a pronounced apical hook. The triple-response adaptation provides the growing seedling with the mechanical strength necessary to penetrate the soil. Use of this response as a phenotype for identification of ethylene-related mutants has given the means to identify and characterize several components of the ethylene-signaling pathway, as will be reviewed in Section IV.

As mentioned, ethylene is responsible for mediating several adaptive responses in plants. Ethylene is critically important for controlling adaptation to growth in a low-oxygen environment (hypoxia), such as is found in a flooded field. Following flooding, ethylene production is stimulated in the roots, resulting in mechanisms that increase the availability of oxygen to root tissue. These mechanisms include formation of aerenchyma tissue, which consists of gas-filled channels in root tissue that develop from the senescence or separation of root cells. Aerenchyma tissue serves to create an internal gas exchange channel from the shoot, which is not in a hypoxic environment, to the root, thus supplying oxygen to the hypoxic root tissue. Persistence of low-oxygen conditions in root tissue promotes dramatic ethylene-mediated changes in leaves including triggering of epinasty (e.g., downward curvature) along with senescence and abscission, each of which is thought to reduce the level of stress imposed on the root.

Ethylene perception is an integral part of pathogen and pest responses in many plant species. Following infection or attack, ethylene production is greatly increased, resulting in the induction of a suite of genes responsible for defense and recovery. Genes include those that code for degradative enzymes such as glucanases and chitinases, both of which act to break down the cell walls of fungal invaders. In response to insect attack, ethylene induces the production of polypeptides that serve as proteinase inhibitors following ingestion. Ethylene is also responsible for stimulating the production of a class of compounds termed phytoalexins that have both antimicrobial and antifungal activity. Finally, ethylene acts to increase the total phenolic content in tissues following attack. This includes increased lignin biosynthesis, which is a cell wall component required for repair of damaged tissue.

Root development is dramatically affected by ethylene, including formation of root hairs and response to gravity. Ethylene is a positive regulator of root hair formation, causing proliferation of root hairs when it is present at high concentrations. Applications of compounds that block ethylene perception substantially reduce root hair number, which is consistent with ethylene's predicted role in stimulation of their production. In *Arabidopsis thaliana*, root hairs develop specifically from epidermal cells that overlap junctions between underlying cortical cells. This contextual development is believed to result from apoplastic translocation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) from the central portion of the root to the epidermal cell layer. Since epidermal cells that overlap the apoplastic junction between cortical cells receive higher levels of ACC, it is more likely that these cells will develop root hairs. Ethylene also plays a role in regulating the response of roots to gravity in some plant species, with ethylene treatment causing a 90° shift in the direction of growth and often causing roots to grow opposite to gravity.

In many monoecious plants (i.e., both male and female flowers on the same individual plant), flower sex is directly determined by ethylene. This is true for cucumbers, in which high ethylene production by the floral meristem results in female flowers. This is opposed by gibberellic acid, which promotes the production of male flowers. Ethylene is also critical for the death of the flower including abscission and senescence. Abscission is a controlled process that results in the breakdown of cells in an area called the abscission zone, leading to the detachment of leaves, fruit, and flowers from the body of the plant. Ethylene, working in conjunction with the plant hormone abscisic acid, causes increased expression of degradative enzymes that destroy this zone. Loss of cellular integrity in the region weakens the junction, causing the organ or tissue to fall off. In commercial orchards, application of ethylene is used as a method both to reduce fruit number (which allows for better development of the remaining fruit) and to loosen fruit prior to mechanical harvesting.

Of all the processes regulated by ethylene, senescence and fruit ripening are by far the most agriculturally significant. Senescence is a tightly controlled degradative process that leads to the death of leaves and flowers. Senescence represents the breakdown of tissue and remobilization of metabolites in response to environmental factors or as a result of tissue age. Senescence is promoted by ethylene and opposed by the plant hormone cytokinin. The progression of senescence is linked to substantial increases in ethylene production, which has a promotive effect on the rate of senescence, although elimination of ethylene perception does not prevent its onset. Senescence results from the induction of a tightly regulated subset of genes whose primary function is cellular degradation. Ultimately, senescence causes the breakdown of chlorophyll, proteins, DNA, RNA, and membranes as a means to free up nutrients for redistribution to other areas. A vivid example of senescence is the annual development of fall color found in deciduous forests, which represents the remobilization of nutrients from the senescing leaves to other nutrient sinks within the tree. Senescence is an important issue for the floral industry since ethylene generated following harvest causes the rapid senescence of many climacteric flowers including carnations. In general, flower senescence is commercially controlled by application of inhibitors of ethylene perception, which are supplied as additives dissolved in the vase solution.

Fruit ripening is probably the most commonly known response to ethylene, even having an old wives' tale associated with it. This tale, which happens to be true, asserts that if one places a ripe climacteric fruit (e.g., banana or avocado) into a paper bag with an immature climacteric fruit, the immature fruit will quickly be triggered to ripen. This is a function of ethylene perception and represents the stimulation of the ripening program by the high levels of ethylene released from the mature fruit. It should be noted that, although related, fruit ripening is distinct from senescence in that cellular integrity, including organellar structure, is maintained during the ripening process. As can be imagined, the process of fruit ripening has tremendous commercial relevance since this controls the development of the critical characteristics (e.g., texture, color, and taste) that consumers associate with fruit quality. Ethylene-dependent ripening is not a characteristic of all fruits but is found in many of the most commercially important fruits in the Western world. These include bananas, tomatoes, peaches, avocados, olives, pears, plums, and apples. In many cases, restriction of ethylene perception and/or production by these fruits results in incomplete or no ripening, which attests to the critical importance of ethylene in promoting this process. Ethylene-dependent ripening is correlated with the climacteric, which is a sustained burst in both aerobic respiration, presumably to produce the necessary energy for the progression of ripening, and ethylene production. As with ethylene production and perception, restriction of aerobic respiration by reducing the level of environmental oxygen or storage temperature is an effective means of limiting the progression of the ripening process.

Of the climacteric fruits, tomato has developed into the best-studied model system for the molecular and biochemical events that underlie the ripening process. One reason for this is the availability of several ripening-related tomato mutants that affect different aspects of this process including ethylene perception and ripening progression. Tomato fruit development can be divided into two distinct stages—the preclimacteric stage, which is a time of rapid cell division and fruit growth, and the climacteric stage, during which the ripening program is initiated and carried out. The onset of the climacteric stage is marked by the induction of several molecular and biochemical processes that promote ripening. These include production of a suite of enzymes, such as polygalacturonase and pectin methylesterase, that cause fruit softening through the breakdown of cell walls and the middle lamella, which is a pectin-rich area between the walls of adjoining cells. Additionally, the climacteric initiates changes in pigmentation of the tomato fruit, with chlorophyll being degraded at the same time that there is an increase in lycopene biosynthesis, which gives the tomato fruit its characteristic red coloration. Flavor attributes begin to develop at this point with the induction of several enzymes that are responsible for the breakdown of complex polysaccharides. Finally, the ripening program induces the expression of components of the ethylene biosynthesis pathway in order to sustain the high level of ethylene that ripening climacteric fruits evolve.

Modern agricultural practices have struggled with the balance between a fully ripened fruit and a marketable commodity. With the progression of ripening, fruits and vegetables become more difficult to transport due to tissue softening, increasing the incidence of damage and pathogen attack. Fruits such as tomatoes and bananas are currently harvested at an immature stage in order to have a fruit that can withstand the hazards of travel. Prior to delivery to market, these fruits are exposed to high levels of ethylene to stimulate the ripening process. In effect, the old wives' tale about ethylene has become the industry standard for the initiation of ripening in climacteric fruits.

## III. BIOSYNTHESIS

Ethylene is a simple unsaturated two-carbon molecule ($H_2C{=}CH_2$) that features a carbon–carbon double bond that is the basis for its biological activity. Ethylene biosynthesis was first elucidated in the late 1970s by Dr Shang Fa Yang at the University of California, Davis. The ethylene biosynthetic pathway consists of a simple three-step catalytic conversion of L-methionine into ethylene (see Fig. 1).

The first step is the combination of ATP and L-methionine into *S*-adenosylmethionine (SAM) by the enzyme methionine *S*-adenosyltransferase. Methionine *S*-adenosyltransferase is not considered to be the rate-limiting step of ethylene biosynthesis since SAM is required not only for ethylene production but also for the production of a diverse range of compounds including polyamines and lignin.

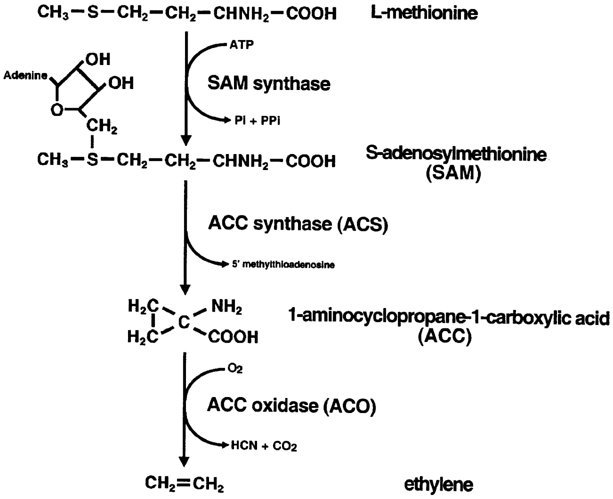

FIGURE 1 Ethylene biosynthesis pathway. Ethylene is produced through a simple two-step biochemical pathway that begins with the conversion of *S*-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. Subsequently, ACC is converted to ethylene by ACC oxidase. In general, ACC synthase is considered to be the key regulatory step of this pathway since expression and activity of this enzyme are both highly controlled.

SAM is subsequently converted to (ACC), which represents the first unique step of ethylene biosynthesis. This conversion is mediated by ACC synthase (ACS), which requires pyridoxal phosphate for activity. This step is highly regulated with increased ethylene biosynthesis generally being dependent on increased expression of ACS since it is a highly unstable enzyme. Overexpression or increased stability of ACS has been shown to result in greatly increased levels of ethylene production. Several inhibitors of ACS activity have been defined, with the most commonly used being L-α-(2-aminoethoxyvinyl)-glycine (AVG), which is generally inhibitory to pyridoxal phosphate-requiring enzymes.

The final step of ethylene biosynthesis is the formation of ethylene from ACC, which is catalyzed by the enzyme ACC oxidase (ACO). ACO requires $O_2$ for its activity and releases both cyanide and $CO_2$ as by-products. ACO is membrane localized and its activity is inhibited by membrane disruption. Inhibitors of ACO include $CoCl_2$ and α-aminoisobutyric acid. Generally, ACO is not considered to be the regulated step of ethylene production since elicitors of ethylene biosynthesis usually do not induce ACO expression.

Since ethylene is a gas, ethylene production rates by plants are easily measured using a gas chromatography system.

## IV. PERCEPTION

In the past 15 years, significant progress has been made toward elucidation of the molecular and biochemical mechanisms of ethylene perception. This progress has primarily been achieved through the use of *A. thaliana*, which has been adopted by plant science as a model system for molecular genetics and mutational analysis. By identifying mutants that have defects in ethylene perception, it has been possible to isolate many of the molecular components of the ethylene-signaling pathway, serving as a framework for plants in general since orthologues to these components have been found throughout the plant kingdom. This identification has been achieved through isolation of either mutants that cannot perceive ethylene (ethylene insensitive) or mutants that display ethylene responses even in the absence of added ethylene (constitutive ethylene response) (see Fig. 2).

The first ethylene-insensitive mutant was identified in 1986 when Drs Anthony Bleecker and Hans Kende at Michigan State University described an *Arabidopsis* mutant that did not display the seedling triple response following exposure to high levels of ethylene. This mutant, *etr1-1*, was the initial member of a group of *Arabidopsis* mutants with aberrant ethylene responses and was the first step toward defining this pathway. Subsequent efforts to identify the gene that was affected by this mutation led to the isolation and characterization of the first known ethylene receptor, *ETR1*. *ETR1* encodes a membrane-localized protein with three hydrophobic transmembrane regions along with a large cytoplasmic region (see Fig. 3). The transmembrane regions comprise an ethylene-binding pocket, which forms by dimerization of ETR1. Reconstitution of the transmembrane regions of ETR1 in a yeast system demonstrated that ETR1 is capable of binding ethylene, which is consistent with its role as an ethylene receptor.

It has long been speculated that a metal such as copper is required for ethylene binding, since copper is predicted to have an affinity for the carbon–carbon double bond of ethylene. Addition of copper to the yeast ethylene-binding system dramatically increased the efficiency of ethylene binding. Also, loss-of-function mutations in the *Arabidopsis* copper transporter *RAN1*, which block the delivery of copper to the ethylene receptors, disrupt normal receptor function and ethylene binding. Interestingly, one method of ethylene control commonly used in the cut-flower industry is to provide silver nitrate to the flower. Silver is capable of substituting for the copper ion in the ethylene-binding pocket. Although still capable of binding ethylene, silver causes some unknown change in the ethylene receptors that blocks normal function and inhibits ethylene signaling, thus blocking the initiation of ethylene-dependent programs such as flower senescence.

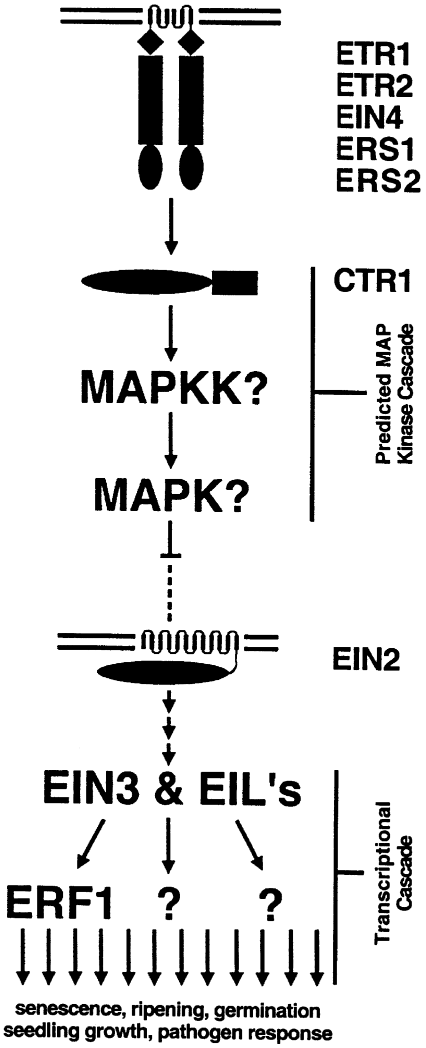
ETR1
ETR2
EIN4
ERS1
ERS2
CTR1
MAPKK?
MAPK?
Predicted MAP Kinase Cascade
EIN2
EIN3 & EIL's
ERF1
?
?
Transcriptional Cascade
senescence, ripening, germination
seedling growth, pathogen response

FIGURE 2 Schematic representation of the current understanding of the ethylene-signaling pathway. This includes a family of five ethylene receptors that regulate the activity of a predicted MAP kinase cascade. Ultimately, this pathway terminates in a transcriptional cascade that activates ethylene-dependent physiological changes.

ETR1 actually is a member of a family of five ethylene receptors in *Arabidopsis*, each with its own distinct structural characteristics (see Fig. 3). Families of ethylene receptors are also found in other

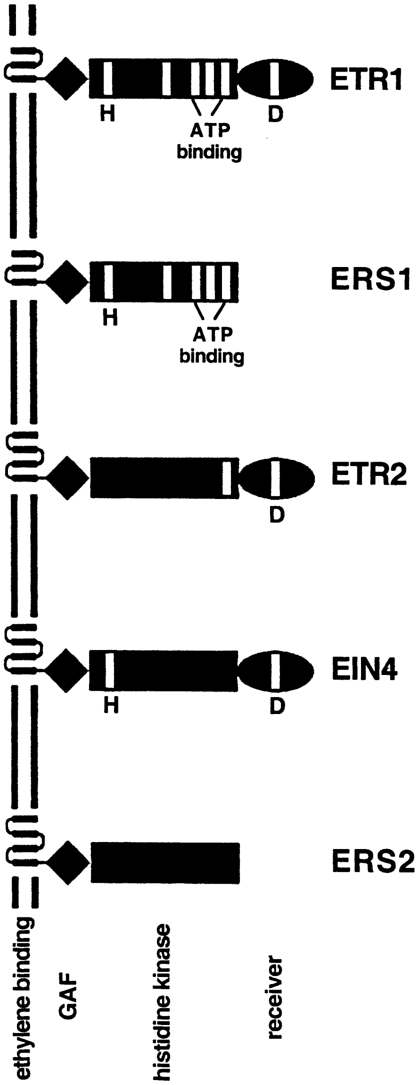

FIGURE 3 Architecture of the *Arabidopsis* ethylene receptor family. The *Arabidopsis* ethylene receptors can be divided into two classes based on whether or not they possess the requirements for histidine kinase activity. The subfamily 1 receptors ETR1 and ERS1 have all of the motifs (indicated as white bars) that are associated with functional bacterial two-component regulators. The subfamily 2 receptors (consisting of ETR2, EIN4, and ERS2) are missing some or all of these motifs, making it less clear as to their function in the regulation of downstream factors in ethylene signaling. All receptors have an ethylene-binding domain at their amino-terminus. Additionally, ETR1, ETR2, and EIN4 have a receiver domain at the carboxy-terminus. It is this domain that is predicted to directly control the activity of CTR1 or some other downstream factor.

plant species including tomato, carnation, melon, and maize. It is not known why there are multiple receptors in each species, but genetic evidence indicates that all five of the *Arabidopsis* receptors function to regulate the activity of a common downstream factor, CTR1. The ethylene receptors are related to a family of bacterial signaling proteins that are collectively called two-component regulators. Bacterial two-component regulators serve to "sniff" the environment, being responsible for detecting changes in the concentration of specific molecules and subsequently regulating gene expression to compensate for these changes. Two-component regulators generally consist of a combination of two proteins—a sensor protein and a response–regulator protein. The former has both an input domain, which is the point of binding for a specific ligand, and a histidine kinase domain, which initiates a signaling event in response to ligand binding. The response–regulator domain is most often composed of a receiver domain, which is the recipient of a phosphate from the histidine kinase, and an output domain, which regulates the activity of a downstream factor. In many cases, the bacterial output domain directly activates transcription. In general, ligand binding results in activation of the histidine kinase domain and autophosphorylation of a highly conserved histidine found in this domain. Subsequently, there is a phospho-transfer event, in which the phosphate attached to the histidine is transferred to a highly conserved aspartate in the receiver domain. It is the phosphorylation state of the receiver domain that determines the activity of the output domain.

The *Arabidopsis* ethylene receptors possess many of the features of bacterial two-component regulators. The family can be divided into two classes of receptors—one composed of *ETR1* and *ERS1* and the second consisting of *ETR2*, *EIN4*, and *ERS2*. Each receptor has a predicted ethylene-binding domain, which is characterized by a high level of conservation of the three hydrophobic transmembrane regions found in ETR1. Additionally, each protein has a soluble GAF domain of unknown function. Both ETR1 and ERS1 are different from the other receptors in that they possess all of the known requirements for having histidine kinase activity, with ETR1 exhibiting histidine autophosphorylation *in vitro*. In contrast, the subfamily 2 receptors possess histidine kinase-like domains but lack many or all of the motifs necessary for autophosphorylation. The ethylene receptors can also be divided based on whether or not they possess a receiver domain. Unlike many bacterial two-component regulator sensor proteins, ETR1, ETR2, and EIN4 all have receiver domains. In contrast, neither ERS1 nor ERS2 has this domain. In each case, the receiver domain possesses the conserved aspartate that serves as the phosphate acceptor in the bacterial two-component regulator scheme. This raises the interesting possibility that the ethylene receptors function in a manner similar to bacterial two-component regulators in that they regulate the activity of a downstream factor as a function of the phosphorylation state of the receiver. It is not clear why the different receptors have different aspects of the two-component machinery, but it suggests that the receptors work synergistically to regulate the ethylene-signaling pathway since ETR1 is the only receptor that has all of the requirements to complete a histidine to aspartate phospho-transfer.

CTR1, a mitogen-activated protein kinase kinase kinase (MAPKKK) similar to the mammalian MAPKKK Raf, acts downstream of the ethylene receptors (see Fig. 2). *CTR1* is a negative regulator of ethylene signaling, since loss-of-function mutations in this gene result in a constitutive ethylene-response phenotype (i.e., constitutive triple response). In general, MAPKKKs regulate a mitogen-activated protein (MAP) kinase cascade that propagates a signal through phosphorylation. Identification of a MAPKKK in the ethylene-signaling pathway suggests that a MAP kinase cascade may participate in ethylene signal transduction, but to date no MAP kinase kinase or MAP kinase has been associated with ethylene perception. Little is known about how CTR1 is regulated except that it interacts strongly with the cytoplasmic portions of the ethylene receptors ETR1 and ERS1, both in the yeast two-hybrid assay and *in vitro*. It has been argued that this association is part of the mechanism of CTR1 regulation and is consistent with the role of two-component regulators in other systems, most notably the *Saccharomyces cerevisiae* HOG1 pathway. In this system, the activity of a downstream MAPKKK is directly determined by the phosphorylation state of a response regulator. Interestingly, loss-of-function mutations in three or more ethylene receptors result in a constitutive ethylene-response phenotype, most likely due to the loss of activators of CTR1. This supports the hypothesis that the ethylene receptors function to regulate the activity of CTR1 and are required to maintain CTR1 in an active state in the absence of ethylene.

Although significant progress has been made toward identifying the components of the ethylene-signaling pathway, large gaps in our understanding remain. The steps that immediately follow *CTR1* remain unknown. Genetic analysis indicates that *EIN2* (i.e., *ethylene insensitive* 2) functions downstream of *CTR1* in the ethylene-signaling pathway, yet it is not clear how many steps separate the two. *EIN2* was identified as a recessive mutation that causes ethylene insensitivity, indicating that it functions as a positive regulator of ethylene signaling. *EIN2* can be divided into two domains—a region of 12 transmembrane loops and a cytoplasmic domain. It is not clear what role *EIN2* plays in ethylene signal transduction, but genetic evidence has shown that it is required for ethylene perception.

Ultimately, the ethylene-signaling pathway terminates in a transcriptional cascade. Several factors have been identified that act as transcriptional activators in response to ethylene, subsequently inducing the expression of ethylene-responsive genes. *EIN3* (i.e., ethylene insensitive 3) was the first ethylene-related transcription factor isolated, with loss-of-function mutations in this gene causing partial ethylene insensitivity. Analysis of the *Arabidopsis* genome has identified several EIL (EIN3-like) proteins that also function as transcriptional activators. Downstream of *EIN3* is a transcription factor, *ERF1* (i.e., ethylene-response factor 1), that is up-regulated by *EIN3* following ethylene exposure. *ERF1*, which is a member of a family of *ERF*-like genes in *Arabidopsis*, is responsible for activating the transcription of a subset of ethylene-regulated genes by binding to a common gene promoter element called the ethylene-response element. Ultimately, the transcriptional cascade headed by *EIN3* and the *EIL*s alters the transcription of a suite of genes that are responsible for the manifestation of the previously described ethylene-mediated physiological changes.

## V. CONTROL

As mentioned, there are several well-developed methods for controlling both ethylene biosynthesis and perception. Some of these have been adopted for commercial use to limit the progression of ethylene-dependent phenomena including senescence and ripening. Inhibitors have been identified for each of the unique steps of ethylene biosynthesis. These include AVG and (aminooxy)acetic acid, both of which inhibit the activity of ACC synthase. Although less effective, inhibitors of ACC oxidase have also been identified, including $CoCl_2$ and $\alpha$-aminoisobutyric acid. Of all the inhibitors of ethylene production, AVG is probably the most widely used commercially, including being applied in orchards to reduce the incidence of fruit abscission and to concomitantly increase fruit size.

Ethylene perception can be controlled with either competitive or noncompetitive inhibitors of ethylene binding. Silver nitrate is a noncompetitive inhibitor, working to displace copper from the ethylene-binding pocket. The biochemical mechanism of silver-mediated inhibition of ethylene perception is unclear since silver, like copper, has a high affinity for ethylene. It is likely that silver blocks some critical event that occurs between ethylene binding and initiation of signal transduction, forcing the receptor to maintain CTR1 in an active state regardless of the ethylene concentration in the environment. Silver is commercially important in the cut-flower industry but is not used in the fruit industry since it is a highly toxic heavy metal.

Competitive inhibitors vie with ethylene for binding to the copper moiety in the receptor pocket. All competitive inhibitors of ethylene binding have at least one carbon–carbon double bond, which is the point of association with the copper ion. Interestingly, some molecules that have this double bond actually can serve as ethylene agonists, in that they can stimulate weak ethylene responses. Propylene is an excellent example of an ethylene agonist, having three carbons with one carbon–carbon double bond. Propylene effectively competes with ethylene for binding and will elicit ethylene responses at a level that is 100–1000 times less effective than ethylene itself. With increasing molecular size, steric hindrance becomes an important issue, limiting the effectiveness of compounds as ethylene agonists but not necessarily their effectiveness as competitive inhibitors. This includes a class of cyclic carbon molecules, collectively referred to as cyclic olefins. These are large ring molecules that possess at least one carbon–carbon double bond. 2,5-Norbornadiene (NBD) and 1-methylcyclopropene (1-MCP) are the best competitive inhibitors described to date, with 1-MCP treatment causing irreversible inhibition of ethylene binding. Although there is great promise for these inhibitors, they have yet to be developed for commercial applications partly because of the noxious odor associated with many cyclic olefins, including NBD.

## VI. SUMMARY

In summary, ethylene has profound implications for plant growth and development at all stages of the life cycle. These range from mediating adaptive responses to an ever-changing environment to development of the qualities that consumers expect in a ripened fruit. The former is especially important for plant survival since plants are sedentary. For this reason, plants are incapable of avoiding environmental challenges and have been forced to evolve complex adaptive strategies to cope with these stresses. Because of its commercial importance, ethylene has received an intense level of scrutiny by plant scientists in the past century in order to not only understand its relevance to plant physiology but to also begin to unravel the fundamental mechanisms underlying its perception. Any advance in our knowledge of ethylene perception and its subsequent physiological effects furthers efforts to control processes that are critical for agriculture and food quality.

## Glossary

**agonist** A chemical compound capable of binding to a receptor and causing a reaction or activity.

**apical hook** A structure that forms at the uppermost portion of the hypocotyl that serves to reorient the cotyledons and apical meristem to prevent damage from soil-borne obstructions.

**climacteric** A substantial increase in aerobic respiration coupled with high levels of ethylene production associated with ripening of fruits such as tomatoes, avocados, and apples (which are therefore termed climacteric fruits).

**cyclic olefin** A member of a class of cyclic carbon structures that contain at least one carbon–carbon double bond that may competitively block ethylene binding.

**hypocotyl** The stem-like area between the cotyledons and primary root of the growing seedling.

**mitogen-activated protein (MAP) kinase cascade** A series of successive phosphorylation events responsible for the propagation of a signal; this cascade consists of three steps beginning with a MAP kinase kinase kinase that phosphorylates a MAP kinase kinase that subsequently phosphorylates a MAP kinase.

**seedling triple response** An ethylene-dependent phenotype found in dark-grown seedlings characterized by exaggerated apical hook formation, shortening and thickening of the hypocotyl and root, and proliferation of root hairs.

**two-component regulator** A protein–sensor system commonly found in bacteria, yeast, and plants that serves to detect changes in specific environmental stimuli.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Cytokinins • Gibberellins • Jasmonates • Salicylic Acid**

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# Extracellular Matrix and Follicle Development

R. J. Rodgers and H. F. Irving-Rodgers
*University of Adelaide, Australia*

I. INTRODUCTION
II. EXTRACELLULAR MATRIX IS IMPORTANT FOR FOLLICLE DEVELOPMENT
III. EXTRACELLULAR MATRIX OF THE MEMBRANA GRANULOSA
IV. FOLLICULAR FLUID
V. EXTRACELLULAR MATRIX OF THE OOCYTE AND CUMULUS CELLS
VI. EXTRACELLULAR MATRIX OF THE THECA
VII. FIBRONECTIN
VIII. SUMMARY

**The endocrine units within the adult ovary—follicles and corpora lutea—continually develop and regress, thus giving rise to the day-to-day variation in hormone secretion by the ovary. The formation of follicles and corpora lutea requires tissue remodeling and cellular replication and specialization. Considerable research effort has been directed at how hormones and growth factors regulate processes. Research efforts are now being directed at the composition (summarized in Table 1) and roles of the extracellular matrix in these processes.**

## I. INTRODUCTION

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## II. EXTRACELLULAR MATRIX IS IMPORTANT FOR FOLLICLE DEVELOPMENT

The extracellular matrix, more aptly termed the intercellular matrix, has many different roles. These include effects on cell behavior, such as migration, division, differentiation, cell death, and cell anchorage. Matrix can play a role in the fluid dynamics of a

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The extracellular matrix, more aptly termed the intercellular matrix, has many different roles. These include effects on cell behavior, such as migration, division, differentiation, cell death, and cell anchorage. Matrix can play a role in the fluid dynamics of a

TABLE I **Composition of Matrices in Ovarian Follicles**

| Matrix | Developmental stage | Laminins | Collagens | Other components |
|---|---|---|---|---|
| Follicular basal lamina | Primordial | α1, β2, γ1 | Type IV α1, α2, α3, α4, α5, α6 | |
| | Preantral | α1, β2, γ1 | Type IV α1, α2, α3, α4, α5, α6 | Nidogen, perlecan |
| | Antral | α1, β2, γ1 | Type IV α1, α2, | Nidogen, perlecan |
| | Atretic, antral | α1, β2, γ1, +/− α2 | Type IV α1, α2, | Nidogen, perlecan |
| Call-Enxer bodies | Preantal | α1, β2, γ1 | Type IV α1, α2, α3, α4, α5, α6 | Nidogen, perlecan |
| Theca interna in general | Antral | | Type VI | Fibronectin, versican |
| Thecal matrix | Antral | γ1 | Type IV α1, α2 | |
| Thecal sub endothelial basal lamina | Antral | β1, β2 | Type IV α1, α2 | |
| Thecal arteriole smooth muscle basal lamina | Antral | β2 | Type IV α1, α2 | |
| Theca externa matrix | Antral | | Types I and III | Versican, chrondroitin sulfate, dermatan sulfate |

tissue, either providing osmotic forces or filtering soluble materials as they pass through the matrix. Matrix can provide mechanical support of tissues, either rigid or elastic. In addition, nutrients, hormones, and other extracellular signals are often required to traverse the matrix in order to reach target cells. Matrix has the ability to bind growth factors. Binding can be direct or indirect via the specific binding proteins for the growth factors, ensuring that they act locally. In essence, extracellular matrix really defines or rather provides microenvironments, thus enabling cells to specialize in the matrix. It is not surprising then that extracellular matrix is generally a diverse mixture of components and that the composition of the matrix is important.

## III. EXTRACELLULAR MATRIX OF THE MEMBRANA GRANULOSA

In the ovary, the membrana granulosa of each ovarian follicle is enveloped by a follicular basal lamina, which separates it from the surrounding stromal elements in primordial follicles or from the theca interna in antral follicles. The follicular basal lamina is believed to play a role in influencing granulosa cell proliferation and differentiation. Additionally, in healthy follicles, it excludes capillaries, white blood cells, and nerve processes from the granulosa compartment until ovulation, at which time the basal lamina is degraded.

### A. Basal Laminas

Basal laminas are specialized sheets of extracellular matrix that in epithelia underlie the epithelial cells and separate them from the adjoining stroma. They influence epithelial cell proliferation and differentiation and can selectively retard the passage of molecules from one side of a basal lamina to the other. Basal laminas are composed of a lattice-type network of type IV collagen intertwined with a network of laminin. This structure is stabilized by the binding of entactin/nidogen to the collagen and laminin. The heparan sulfate proteoglycan perlecan and other molecules are associated with the type IV collagen-laminin backbone. Importantly, basal laminas in different regions of the body differ in the ratio of all these components. Furthermore, each "component" is really a class of several components. Thus, each type IV collagen molecule is composed of three α chains, but to date, six different types of α chain (α1 to α6) have been discovered. Potentially, any combination of these might be present. Similarly, each laminin molecule is composed of one α (A in the old nomenclature), one β (B1 in the old nomenclature), and one γ (B2 in the old nomenclature) chain, yet five different α chains, three β chains, and three γ chains have been discovered. It is believed that the unique composition of each basal lamina contributes to its specific functional properties.

### B. Selective Filtration by the Follicular Basal Lamina

Based upon the roles of basal laminas in other tissues and organs, it can be speculated that the follicular basal lamina is important for maintaining the polarity and the degree of specialization of granulosa cells that align the follicular basal lamina. The follicular basal lamina probably has a role in retarding the entry of higher molecular weight plasma proteins and molecules (e.g., low-density lipoproteins) into the follicular antrum. Conversely, if the flow of material in the other direction is similarly retarded, the follicular basal lamina may trap large molecules (e.g., some proteoglycans) synthesized by granulosa cells and oocytes in the follicular fluid. The molecular mass cut-off is calculated to be 100 to 850 kDa, based upon comparisons of the composition of follicular fluid with that of plasma. This does not necessarily indicate the cut-off imposed by the follicular basal lamina only, since plasma proteins also must traverse the vascular subendothelial basal lamina before reaching the follicular antrum.

Size may not be the only determinant of movement across the follicular basal lamina. It has been shown that the blood–follicle barrier to movement of inter-α-trypsin inhibitor is due to its negative charge, suggesting that the follicular basal lamina may also exclude material on this basis. In addition, the follicular basal lamina contains perlecan, a heparan sulfate proteoglycan. Heparan sulfate proteoglycans can bind a number of growth factors [e.g., fibroblast growth factor 2 (FGF2)] or their binding proteins [e.g., follistatin, insulin-like growth factor-binding protein 2 (IGF-BP2), and IGF-BP5]. Thus, it is possible that the follicular basal lamina may act as a barrier to the movement of growth factors.

## C. Matrix Interactions with Granulosa Cells

Studies of the effects of the components of basal laminas and other extracellular matrix components have been undertaken. One model that is ideal for studying the effects of the follicular basal lamina on granulosa cells is that of the chicken follicle. Methods for isolating the chicken follicle have been available since the mid-1970s but only recently has the chicken follicle been used for this purpose. In addition, other components of the basal lamina are available commercially: laminin 1, laminin 2, and even an assembled laminin 1 matrix (Matrigel); however, it is the components of laminin 3 that are present in the follicular basal lamina. Other matrix molecules (collagens, vitronectin, fibronectin, etc.) are available for culturing granulosa cells but none of these truly represent the appropriate matrix mimicing that found *in vivo*.

Cells interact with matrix via the cell surface adhesion receptors, the integrins. Integrins are heterodimeric glycoproteins composed of α- and β-subunits. Over 17 α- and 8 β-subunits are known, producing over 23 different heterodimeric combinations. Additional molecules within the cell associate with integrins in order for a cell–matrix junction to form. Thus, matrix provides the cells with anchorage. It is not simply a ligand–receptor interaction that determines the effects of the matrix on cells. Only a few integrins have been localized to granulosa cells in a number of species. These include α2, α3, α6, and α6β1. Tetraspanins interact with the integrins as part of the process by which the matrix links with intracellular signaling molecules, and indeed one of the most highly expressed proteins in granulosa cells from ovulating follicles was identified long ago (CD63, ME491).

## D. Changing Composition of the Follicular Basal Lamina

In follicles, the follicular basal lamina expands in surface area during follicular development and its composition changes as it does so. In bovine follicles, expression of type IV collagen α3 to α6 declines during growth, whereas α1 and α2 continue to be expressed. Laminin β1 is transiently expressed at the preantral stage, and α1, β2, and γ1 appear to be more highly expressed. Nidogen and perlecan are not present in primordial follicles but are expressed later in follicular development. Laminin α2 is very unusual. It is expressed in only a few healthy antral follicles.

## E. Cellular Origin of the Follicular Basal Lamina

Evidence is accumulating that the granulosa cells are the source of many components of the follicular basal lamina. The laminin γ1 chain is expressed by granulosa cells and other indirect evidence on the origins of follicular basal lamina stems from identifying the components of Call-Exner bodies (see below). These bodies are ultrastructurally similar to basal lamina and have been observed within the membrana granulosa of follicles *in vivo*, suggesting that they are synthesized by granulosa cells. Type IV collagen chains α1, α2, α3, α4, and α5, the laminin α1-, β2-, and γ1-subunits, perlecan, and nidogen/entactin have been localized to the Call-Exner bodies. Granulosa cells cultured under anchorage-independent conditions can also synthesize a basal lamina that contains at least type IV collagen and fibronectin. Whether there is a contribution from the theca in larger follicles, or indeed the stroma surrounding preantral follicles, to the production of the follicular basal lamina is not known. One candidate component is laminin α2. In other tissues where it is a component of an epithelial basal lamina, it appears to originate from the stroma. In bovine follicles, laminin α2 is expressed in only a very small proportion of healthy follicles but more commonly in atretic follicles. The expression pattern of this laminin chain is also different from that of the other laminin molecules examined, and hence a different cellular origin could explain this observation.

### F. Regulation of Synthesis of the Follicular Basal Lamina

Very few studies of the regulation of the synthesis of follicular basal lamina by follicles have been undertaken. With the advent of whole-follicle culture and the culture of granulosa cells without anchorage in which granulosa cells produce a basal lamina, opportunities now exist to study basal lamina regulation *in vitro*. Indeed, initial studies have identified ascorbate, known to be necessary for collagen synthesis, as being beneficial for follicle culture.

### G. Atresia and the Follicular Basal Lamina

The event of atresia that occurs in follicles is unusual in the body as it involves the complete destruction of the epithelial layer by death of all the epithelial cells. In other epithelia, such as lumenal gut epithelium or involuting glands, cells undergo apoptosis as they age, but this is regional and involves only a few cells at any one time. Certainly the stem cells survive to regenerate the populations of cells. However, the stem cells of the membrana granulosa do not survive follicle atresia. Thus, the question as to what happens to the follicular basal lamina during atresia is an interesting one.

Histochemical observations of bovine and ovine follicles undergoing atresia have identified laminin chains, perlecan, nidogen, and type IV collagen chains. In these studies, the components identified in atretic follicles were the same as those present in the class of follicles from which they were derived, except for laminin α2. Laminin α2 was found to be more commonly expressed in atretic follicles than in the healthy follicles from which they arose. However, the expression of laminin α2 did not correlate with the type or severity of atresia. As follicles shrink during atresia, folding of the basal lamina occurs. Thus, the follicular basal lamina survives the early events of atresia.

Even if the basal lamina is not completely destroyed early in atresia, macrophages, endothelial cells, and fibroblasts can breach the follicular basal lamina as they migrate from the thecal layer of atretic follicles. However, the movement of cells from the thecal side across the basal lamina is restricted while the basal lamina is aligned with healthy granulosa cells.

### H. Call-Exner Bodies

Call-Exner bodies of ovarian follicles have been described as "a ring of granulosa cells disposed radially around a central cavity filled with fluid and showing histochemical reactions similar to the liquor folliculi [follicular fluid]." They are present in follicles of many species and in ovarian tumors. The role, if any, of Call-Exner bodies is not known. The central extracellular region is lined by a basal lamina-like structure, which can be highly folded or unassembled. Bovine Call-Exner bodies of preantral follicles contain basal lamina components, including the α1 to α5 chains of type IV collagen, the α1, β2, and γ1 chains of laminin, perlecan, and nidogen, reflecting the composition of the follicular basal lamina at that stage of follicular development. On further follicular development, the basal lamina material of Call-Exner bodies changes in the apparent ratio of type IV collagen to laminin, similar to what occurs in the follicular basal lamina. In addition, laminin α2, which is believed to be derived from the thecal layer and not from granulosa cells, is not present in the Call-Exner bodies.

### I. Turnover and Degradation of the Extracellular Matrix

During follicle development, continual remodeling of the follicular wall occurs as the follicle enlarges. In particular, the structural collagens of the theca externa and in nearby stroma and tunica must be turned over to allow for expansion of the follicle. The follicular basal lamina must also be expanded. The vasculature continually expands within the theca interna and so too its associated matrix. These processes require the turnover of the matrix to be discrete since the final result is an extension or growth of the matrix, not a total degradation. The precise mechanism by which this occurs is not well understood, but clearly it involves the simultaneous degradation and synthesis of matrix. Enzymes involved in matrix degradation, including matrix metalloproteinases 1, 2, 9, and 13, tissue inhibitor of metalloproteinases 1 and 2, tissue plasminogen activator, urokinase plasminogen activator, and plasminogen activator inhibitor 1, have been studied in the ovary and were reviewed in 1999 by Smith and associates. Many of these enzymes have been studied for their role in ovulation, a process that involves dramatic changes in tissue structure as the follicle redevelops into a corpus luteum.

## IV. FOLLICULAR FLUID

Proteoglycans consist of a core protein with attached glycosaminoglycans. Follicular fluid has been analyzed for its composition of glycosaminoglycans, and the biochemical production of the glycosaminoglycans by granulosa cells has been studied by Yanagishita, Hascall, and colleagues. Recently, two proteoglycans, versican and perlecan, were identified in human "follicular fluid." These two proteoglycans and decorin were also identified in bovine follicles but were found to be components of the follicular wall, which readily contaminates follicular fluid on aspiration. Hyaluronan is a glycosaminoglycan that lacks a core protein. It has also been identified in follicular fluid surrounding the most antral layer of granulosa cells using link protein as the ligand. The roles, if any, of these proteoglycans in follicular fluid remain to be determined.

## V. EXTRACELLULAR MATRIX OF THE OOCYTE AND CUMULUS CELLS

The production of hyaluronan by cumulus cells has also been well studied for its role in expanding the cumulus–oocyte complex in ovulating follicles. Hyaluronan is assembled at the cell surface by a hyaluronan synthase. Three of these genes have been identified, and hyaluronan synthase 2 is expressed by cumulus cells. A number of hyaluronan-binding proteins have also been identified, which in follicles include versican, link protein, and members of the inter-α-trypsin inhibitor family. This family constitutes a complex set of molecules. Bikunin is produced in the liver by the proteolytic cleavage of a translational product that includes α2-microglobulin and bikunin. A number of additional proteins can attach via covalent bonds to the chondroitin sulfate side chain of bikunin. These include heavy chains 1, 2, or 3 (also known as SHAPs or serum-derived hyaluronan-associated proteins) and TNFα-stimulated gene-6 (TSG-6). These chains in turn can bind hyaluronan. These molecules are interesting as the SHAPs gain access to the follicular fluid of ovulating follicles from the circulation, whereas TSG-6 appears to be expressed by cumulus cells in response to the luteinizing hormone surge. The importance of these molecules is underscored by the loss of fertility associated with gene mutations.

The zona pellucida of the oocyte is a very specialized extracellular matrix composed of a number of oocyte-secreted glycoproteins [zona pellucida 1 (ZP1), ZP2, and ZP3]. The zona pellucida is important for fertilization and has been reviewed many times. Disruption of zona pellucida formation or its normal function either by gene "knockout" in mice or by immunization shows that the zona pellucida is required for normal follicular development and particularly for the formation of a proper cumulus complex.

## VI. EXTRACELLULAR MATRIX OF THE THECA

Laminin γ1 chain, laminin 1 components (α1, β1, or γ1), and type IV collagen α1 and α2 are present in bovine follicles throughout the theca interna and are not associated with any conventional basal laminas, such as those of blood vessels. At the electron microscopic level, fragments of basal lamina-like, electron-dense material have been observed and this matrix been designated the "thecal matrix." In other species, such as mouse and rat, in theca and in the interstitial tissue of developing gonads, a similar matrix has been observed by localizing laminin 1 components. The origins and functions, if any, of the thecal matrix are not known.

A number of basal laminas exist in the theca-subendothelial basal laminas and those surrounding the smooth muscle cells of arterioles. In bovine follicles, no laminin α1, α2, or γ1 is present but both β1 and β2 are in the subendothelial basal laminas of the blood vessels (arterioles, capillaries, and venules). The laminin β2 chain is also present in the smooth muscle layers surrounding the arterioles, which are located principally in the theca externa. This is consistent with studies in other tissues, in which the β1 chain has been reported to be limited to subendothelial basal laminas, whereas the β2 chain is present surrounding smooth muscle cells. Type IV collagen α1 and α2, but not α3, α4, α5, or α6, are present in the subendothelial basal lamina of arterioles, venules, and capillaries in the theca and in the basal laminas of arteriolar smooth muscle cells. The presence of multiple basal laminas throughout the thecal layers makes it difficult to study the expression of basal lamina components when relying solely on RNA analyses or Western blot analyses.

Since theca interna and externa are stromal layers, other types of matrix components are present. Structural collagens have been observed in the theca externa of many species, and collagens I and III have been identified by immunohistochemistry and by RNA analyses. Type VI collagen has also been identified in theca interna. One role of this molecule is in the organization of fibronectin, and fibronectin has been identified in the theca interna in some studies.

## VII. FIBRONECTIN

At least 20 different isoforms of fibronectin exist, due to alternative splicing of mRNA at three separate sites (the EDA, EDB, and V regions). A number of these splice variants are expressed in follicles; the EDA domain of fibronectin has been shown to be mitogenic for granulosa cells *in vitro*, and fibronectin synthesis by granulosa cells can be regulated by FGF2. There are a number of reports in which fibronectin has been localized in ovaries; often these studies used antibodies that recognize all forms of fibronectin. There does not appear to be a consistent pattern across species, with reports of fibronectin, usually of an undefined type, being localized to different follicle compartments. All of these reports may be accurate but given that a circulating form of fibronectin exists, which many of the antibodies used would react with, considerably more effort is required to identify the precise expression patterns of fibronectin isoforms during follicle development.

## VIII. SUMMARY

The study of the extracellular matrix is well advanced in other organs of the body. This has provided a vast body of knowledge and reagents with which to study the extracellular matrix in the ovary. The study of the extracellular matrix in the ovarian follicle is particularly interesting and challenging since the follicle has so many different and alternative phases of development. Recent years have seen a considerable increase in the knowledge of this subject and the next few years promise to produce results just as exciting.

## Glossary

**follicle** A small, narrow cavity or sac in an organ or tissue, such as those in the ovaries that contain developing eggs.

**luteinizing hormone** A gonadotropic glycoprotein hormone produced by the gonadotropes of the anterior pituitary gland.

**oocyte** A developing egg cell.

## See Also the Following Articles

**Corpus Luteum in Primates • Follicle Stimulating Hormone (FSH) • Folliculogenesis • Folliculogenesis, Early • Follitropin Receptor Signaling • Luteinizing Hormone (LH) • Oocyte Development and Maturation**

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# Fecundity Genes

K. P. McNatty*, J. L. Juengel*, T. Wilson†,
S. M. Galloway†, and G. H. Davis‡

*AgResearch Wallaceville Animal Research Centre,
New Zealand • †University of Otago, New Zealand •
‡AgResearch Invermay Agricultural Centre, New Zealand

I. INTRODUCTION
II. OVARIAN FOLLICULAR DEVELOPMENT
III. FecX$^{I}$ AND FecX$^{H}$
IV. FecB$^{B}$
V. FecX2$^{W}$
IV. FecI$^{I}$ AND FecC$^{C}$

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**Genetic mutations affecting ovulation rate are often referred to as fecundity genes (e.g., Fec), since the most noticeable effect is on the number of offspring produced by the animals that bear these mutations. Mechanisms by which animals with higher ovulation rates achieve these effects cannot be explained by currently understood interactions between the pituitary gland and ovary but recent results suggest that interactions within the ovary itself contribute significantly to this outcome.**

## I. INTRODUCTION

Ovulation rate in mammals is determined by a complex exchange of hormone signals between the pituitary gland and the ovary and by a localized exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells. Many mammals, such as humans, cattle, sheep, goats, and deer, normally have an ovulation rate of 1 egg/cycle, whereas other mammals, such as rats, mice, hamsters, cats, dogs, and pigs, have ovulation rates that vary between 4 and 15 eggs/cycle. Thus, when asking fundamental questions as to why some humans or farmed livestock (e.g., sheep) have a natural predisposition to produce two or three rather than one offspring, it is useful to examine the results from animals with a low-ovulation-rate phenotype.

The hormonal interactions between the pituitary gland and the ovary have been studied in some detail in both multiple and single ovulating species. The temporal interrelationships among the pituitary hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and the ovarian hormones, such as estradiol, progesterone, and inhibin are well understood. However, these interactions by themselves do not explain why some species always ovulate many eggs and other species ovulate mainly one egg. In contrast to our understanding of the pituitary–ovarian axis, there is a paucity of information regarding the hormonal interactions within the ovary and how these events might influence ovulation rate. In this regard, the inheritance patterns of several, naturally occurring genetic mutations that cause anovulation (e.g., sterility) or either modest (i.e., 0.2–0.5) or large (i.e., ≥ 0.8) increases in ovulation rate (Table 1) have been identified in sheep. When the mean inherited increase in ovulation rate is less than a whole number, this indicates that only some animals within a flock, at any moment in time, are expressing an increase above that observed in the control animals.

## II. OVARIAN FOLLICULAR DEVELOPMENT

A summary of the various stages of ovarian follicular growth in sheep, together with the stage of onset of expression of genes encoding hormones, hormone receptors, or binding proteins in oocytes, granulosa cells, and/or theca interna/externa is shown in Fig. 1. The list of genes expressed is by no means a complete summary of those currently known and many more are expected to be included in the future. Follicles may form as either a primordial (i.e., type 1) or a transitory (i.e., type 1a) structure. Oocytes of these so-called “nongrowing” follicles express genes encoding several hormone receptors [e.g., bone morphogenetic protein receptor type IB (BMPR-IB), also known as ALK6, BMPR-II, or c-kit] and also at least one known growth factor, growth differentiation factor 9. Although the essential growth-initiating signals are not known, ovarian follicles develop through the transitory and/or primary (i.e., type 2) stages without being receptive to the pituitary hormones (i.e., gonadotropins) FSH or LH. During the primary and preantral (i.e., types 3 and 4) stages of growth, an increasing number of hormones, hormone receptors, and/or binding proteins are synthesized by the oocyte, granulosa cells, or theca cells. Thus, the regulation of follicular development becomes increasingly complex. Current information indicates that many of the regulatory factors are members of the transforming growth factor-β (TGF-β) superfamily [e.g., bone morphogenetic

TABLE I **Sheep with Single Genetic Mutations or Putative Major Gene Mutations Affecting Fecundity**

| Name (Allele) | Chromosome | Phenotype (mean ovulation rate) | Phenotype (mean litter size) | Gene identification |
|---|---|---|---|---|
| Inverdale (FecX$^{I}$) | X | ++ (1.8) | ++ (1.6) | BMP15 |
| | | I + (2.9) | I + (2.3) | |
| | | II (0) | I (0) | |
| Hanna (FecX$^{H}$) | X | ++ (1.8) | ++ (1.6) | BMP15 |
| | | H + (2.9) | H + (2.2) | |
| | | HH (0) | HH (0) | |
| Booroola (FecB$^{B}$) | 6 | ++ (1.5) | ++ (1.3) | BMPR-IB |
| | | B + (2.8) | B + (2.2) | |
| | | BB (4.6) | BB (2.7) | |
| Woodlands (FecX2$^{W}$) | X | ++ (2.1) | ++ (1.9) | Unknown |
| | | W + (2.5) | W + (2.1) | |
| | | WW (2.5) | WW (unknown) | |
| Garole (FecB$^{B}$) | 6 | Unknown | BB (2.2 +) | BMPR-IB |
| Javanese (FecB$^{B}$) | 6 | ++ (1.4) | ++ (1.2) | BMPR-IB |
| | | B (2.7) | B + (1.9) | |
| | | BB (unknown) | BB (2.6) | |
| Thoka (FecI$^{I}$) | Unknown | ++ (2.2) | ++ (1.7) | Unknown |
| | | 1 + (3.4) | 1 + (2.4) | |
| Cambridge (FecC$^{C}$) | Unknown | ++ (1.9) | Unknown | Unknown |
| | | C + (3.2) | | |

proteins (BMPs), TGF-β$_{1,2,3}$, anti-Müllerian hormone, inhibins, and activins], receptors for this family (e.g., BMPR), or hormones that may act as binding proteins for some TGF-β family members (e.g., follistatin). Other prominent regulatory factors are c-kit and kit ligand, also known as stem cell factor, and members of the insulin-like growth factor family.

As follicles develop through the preantral and early antral (i.e., type 5) stages, granulosa cells become increasingly responsive to FSH and theca cells to LH. The roles that other pituitary hormones, such as prolactin and growth hormone, may play in the sheep is obscure. Nevertheless, throughout these stages of follicular growth, there is no absolute requirement for pituitary hormones. In sheep, some 17 or 18 doublings of the granulosa cell population occur as a type 1/1a follicle develops into a preovulatory follicle. During this developmental period, neither FSH nor LH is essential for the first 14–16 doublings of the granulosa cell populations or for the formation of the theca interna. It has therefore been deduced that early follicular growth is regulated, in large part, by a localized exchange of hormonal signals within the ovary and specifically within the follicle itself. Nevertheless, during the final stages of follicular development, FSH and LH are critically important for the synthesis and secretion of follicular hormones (e.g., steroids) and for determining the number of follicles that ovulate. For example, pituitary LH secretion is episodic and the frequency of these episodic concentrations in plasma regulates the theca-derived follicular secretions of the steroids androstenedione and testosterone. In contrast, the pituitary release of FSH is more "wave-like" and these more gradual variations in plasma FSH determine the number of antral follicles (i.e., large type 5 follicles) with granulosa cells capable of: (1) metabolizing theca-derived androstenedione or testosterone to estradiol; (2) synthesizing inhibin; and (3) acquiring LH receptors. In turn, and as a consequence of increasing follicular estradiol and inhibin output, the plasma concentration of FSH, but not the episodic frequency of LH release, is suppressed. This ensures that only the steroid-secreting, large follicles containing granulosa cells with LH receptors remain viable and capable of going on to ovulate. During the type 4 and 5 developmental stages, granulosa cells become increasingly sensitive to FSH in terms of their ability to synthesize the intracellular messenger cyclic adenosine monophosphate (cAMP). The ability of granulosa cells to synthesize estradiol and acquire LH receptors is influenced markedly by the plasma concentrations of FSH and the sensitivity of the cells per se to FSH. For example, in mammals, it is known that the administration of exogenous FSH increases ovulation rate in a dose-responsive manner. However, recent studies of naturally occurring genetic mutations in sheep demonstrate that, in addition to FSH, at least one member of the TGF-β superfamily,

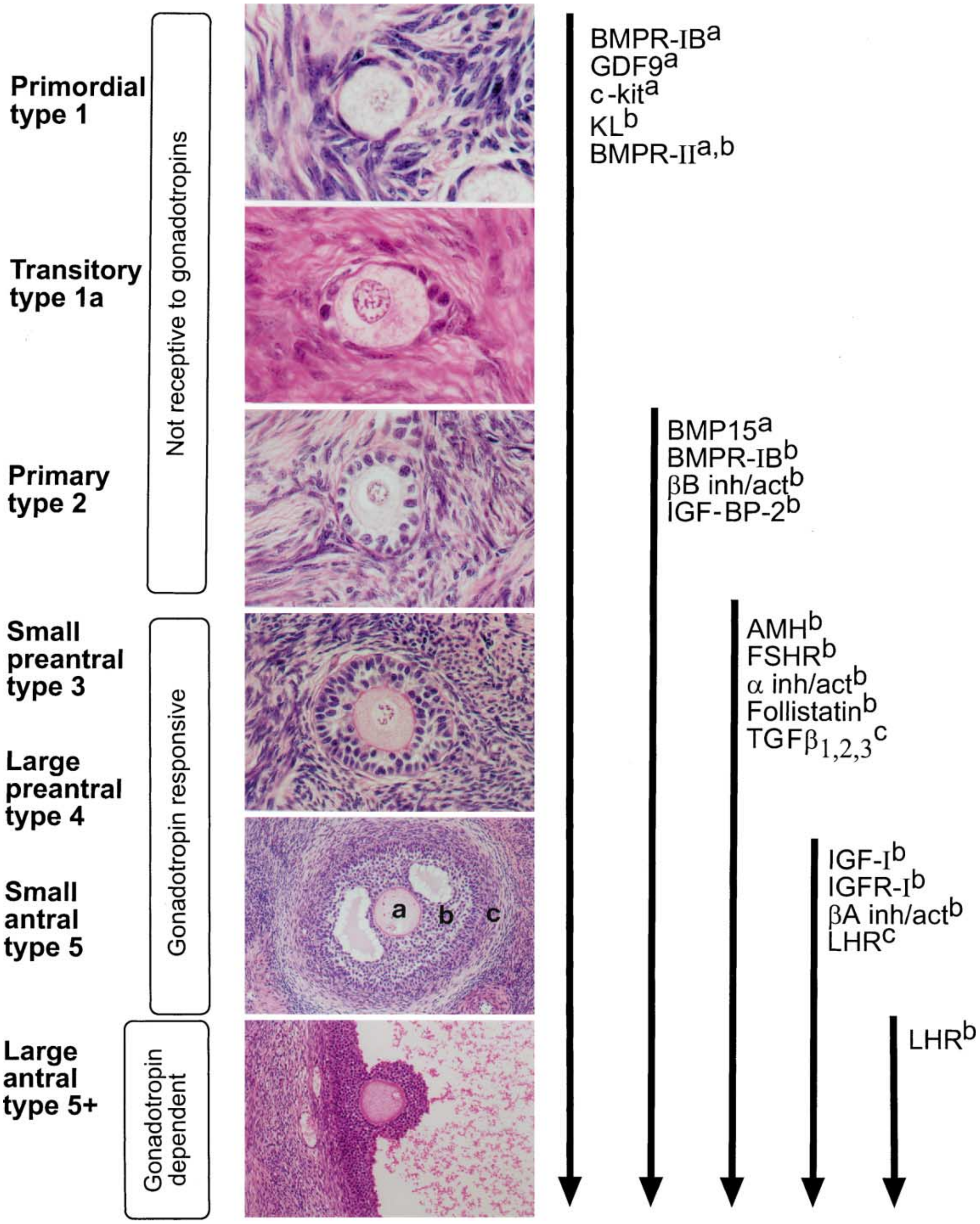
Primordial type 1
Transitory type 1a
Primary type 2
Small preantral type 3
Large preantral type 4
Small antral type 5
Large antral type 5+
Not receptive to gonadotropins
Gonadotropin responsive
Gonadotropin dependent
a
b
c
BMPR-IB$^a$
GDF9$^a$
c-kit$^a$
KL$^b$
BMPR-II$^{a,b}$
BMP15$^a$
BMPR-IB$^b$
βB inh/act$^b$
IGF-BP-2$^b$
AMH$^b$
FSHR$^b$
α inh/act$^b$
Follistatin$^b$
TGFβ$_{1,2,3}$$^c$
IGF-I$^b$
IGFR-I$^b$
βA inh/act$^b$
LHR$^c$
LHR$^b$

**FIGURE 1** The sequence of events during ovarian follicular development with respect to specific growth stage, gonadotropin sensitivity, and onset of expression of genes encoding hormones, hormone receptors, and/or binding proteins in the various cell types of the follicle. The symbols a, b, and c shown in the photomicrographs or as superscripts refer to the oocyte, granulosa cells, and theca interna/externa, respectively. BMPR-IB, bone morphogenetic protein receptor type IB (also known as ALK6); BMPR-II, bone morphogenetic protein receptor type II; GDF9, growth differentiation factor 9; KL, kit ligand; BMP15, bone morphogenetic protein 15 (sometimes referred to as GDF9B); βB inh/act, βB inhibin/activin subunit; AMH, anti-Müllerian hormone (sometimes referred to as Müllerian inhibiting substance); TGF-β$_{1,2,3}$ transforming growth factor-β$^{1,2,3}$; α inh/act, α inhibin/activin subunit; FSHR, FSH receptor; LHR, LH receptor; IGF, insulin-like growth factor-I; IGF-BP-2, IGF-binding protein type 2; IGFR-I, insulin-like growth factor receptor-I; βA inh/act, βA inhibin/activin subunit. Reprinted from Montgomery *et al.* (2001), with permission.

namely, BMP15, and also a receptor known as BMPR-IB have extremely important roles in determining ovulation rate.

## III. $\mathrm{FecX^I}$ AND $\mathrm{FecX^H}$

Both the Inverdale ($\mathrm{FecX^I}$) and the Hanna ($\mathrm{FecX^H}$) mutations have been mapped to the X chromosome and identified as separate point mutations in the coding region of BMP15 (Table 1). In $\mathrm{FecX^I}$ animals, a single T–A nucleotide substitution at nucleotide position 92 of the mature coding sequence results in an aspartic acid replacing a valine in a highly conserved region of the protein. In $\mathrm{FecX^H}$ animals, a single C–T nucleotide substitution at nucleotide position 67 results in a premature stop codon in place of glutamic acid at amino acid residue 23 of the mature protein. The effect of the Inverdale or Hanna mutation in heterozygous females is to increase ovulation rate by almost 1 egg/cycle, whereas homozygous females or females carrying one copy of Inverdale together with one copy of Hanna are sterile as follicular development is arrested at the primary (type 2) stage (Fig. 1). Gene expression studies of BMP15 in noncarriers, heterozygous carriers, and homozygous carriers indicate that within the ovary, only the oocytes in type 2 or larger follicles contain the mRNA for this gene. Studies using immunohistochemical techniques to localize BMP15 protein have identified its presence in the oocytes of noncarriers. Studies in rats with recombinant human BMP15 demonstrate that this factor can stimulate DNA synthesis in granulosa cells and therefore BMP15 can be considered a growth factor. At this time, the identity of the receptor for BMP15 in granulosa cells is unknown. Since the Inverdale, Hanna, and Inverdale × Hanna animals all display the same ovarian phenotype, it is clear that the presumptive modified BMP15 protein in Inverdale and the putative truncated BMP15 peptide in Hanna have little or no biological activity. Collectively, these findings establish that, in sheep, BMP15 is an oocyte-derived growth factor essential for stimulating the proliferation of granulosa cells from the type 2 stage of follicular development (Fig. 1). Of equal significance is the finding that animals that contain oocytes that produce half the amount of normal BMP15 have a higher ovulation rate. In heterozygous Inverdale animals, it is known that the plasma concentrations of FSH are not different from those in control (nonmutated) sheep. Thus, the question arises as to how an oocyte-derived growth factor can influence ovulation rate. It seems that the oocyte may achieve this effect by influencing the sensitivity of its adjacent granulosa cells to FSH. This interpretation is based on the following evidence from *in vitro* studies: (1) BMP15 inhibits FSH-induced differentiation of rat granulosa cells and (2) granulosa cells from heterozygous Inverdale ewes respond to FSH by producing higher levels of cAMP earlier in follicular development than cells from noncarrier ewes. Thus, the higher ovulation rate in heterozygous Inverdale or Hanna ewes is likely due to a higher proportion of follicles being more responsive to FSH than occurs in noncarrier animals.

## IV. $\mathrm{FecB^B}$

The mutation in Booroola ewes has been mapped to sheep chromosome 6 and was identified as a point mutation in the BMP type IB receptor (Table 1). A similar mutation has been identified in the highly fecund Garole and Javanese sheep (Table 1), suggesting that these animals have a common ancestry with Booroola sheep. In FecB animals, a single A to G substitution at nucleotide position 830 results in an arginine replacing a glutamine residue in a highly conserved region of this receptor. The ligand for this receptor is not known. The BMPR-IB mRNA, together with its companion BMPR-II mRNA, is present in oocytes from the type 1/1a stage of development. In granulosa cells, BMPR-II mRNA is present in type 1/1a follicles but BMPR-IB mRNA is not evident until the type 2 stage. BMPR-IB mRNA is also found in other tissues, such as the brain, pituitary gland, skeletal muscle, kidney, and uterus. Animals heterozygous for this mutation have a mean ovulation rate of approximately 3 eggs/cycle, whereas those that are homozygous have a mean ovulation rate of approximately 5 eggs/cycle (Table 1). The precise mechanism by which this mutation affects ovulation rate is not known. It seems likely that both the pituitary gland and the ovary are affected and that both interact to enhance ovulation rate. Homozygous carriers of $\mathrm{FecB^B}$ in Booroola ewes have higher plasma concentrations of FSH than noncarrier animals and have granulosa cells that become more responsive to FSH earlier in follicular development than noncarrier animals. In homozygous $\mathrm{FecB^B}$ females, both the oocyte and the granulosa cells differentiate and mature earlier in follicular development than in noncarrier animals. In $\mathrm{FecB^B}$ animals, the oocyte reaches its mature diameter in a type 4 follicle, whereas this does not occur in noncarriers until the type 5 stage of development.

In addition, granulosa cells in homozygous carriers are required to undergo only 16–17 doublings before ovulation instead of the 18–19 undertaken by noncarriers. This reduced number of doublings may be related to the earlier maturation of the oocyte, which in turn signals a greater proportion of granulosa cells to enter a differentiative pathway instead of a proliferative pathway. In addition to granulosa cells in $FecB^B$ carriers having a higher responsiveness to FSH earlier in development, these cells synthesize estradiol and acquire LH receptors at an earlier stage of follicular development than do noncarriers.

## V. $FecX2^W$

Recently, the inheritance patterns of ovulation rate in Woodlands ($Fec X2^W$) sheep indicate that it is on the X chromosome and likely to be a single or dominant set of mutations in a single gene. The $FecX2^W$ mutation has been shown not to be a mutation in BMP15. It is worth noting that $FecX2^W$ is maternally imprinted as only females inheriting the gene from their sire have increased ovulation rates. Moreover, the $FecX2^W$ mutation is expressed only on paternal inheritance from carrier males that are the progeny of dams in which the gene is silenced. The Woodlands gene is the first imprinted gene known to increase ovulation rate. The reproductive endocrinology of Woodlands sheep has not been reported.

## IV. $FecI^I$ AND $FecC^C$

Other sheep breeds with putative major genes are the Thoka ($FecI^I$) and Cambridge ($Fec^C$) breeds (Table 1). However, the chromosomal location, gene identity, and reproductive endocrinology of these animals are not known.

## Glossary

**allele** One of two or more alternative forms of a gene, each of which possesses a unique nucleotide sequence.

**fecundity** Offspring produced per female. Combines fertility (ability to produce offspring) and prolificacy (number of offspring).

**genotype** The entire array of genes carried by an individual or the particular allele at a specified locus in an individual.

**ovulation rate** Number of mature eggs released from the ovaries during one reproductive cycle.

**phenotype** The observable characteristics of an individual as determined by the interaction of its genotype and its environment.

## See Also the Following Articles

**Apoptosis Gene Knockouts • Follicle-Stimulating Hormone (FSH) • Folliculogenesis • Folliculogenesis, Early • Follitropin Receptor Signaling • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Placental Gene Expression**

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# Flt3 Ligand

SHULI ZHANG AND HAL E. BROXMEYER

*Indiana University School of Medicine and Walther Cancer Institute, Indianapolis*

I. INTRODUCTION
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**Flt3 ligand (FL) is a transmembrane protein that binds to Flt3, a fms-like tyrosine kinase 3 receptor, and triggers the intracellular signaling cascade that leads to the proliferation and development of hematopoietic stem cells and progenitor cells. For most activities, FL has modest effects by itself; however, FL manifests potent co-stimulating activity and actively synergizes with other cytokines for optimal biological effects.**

## I. INTRODUCTION

Blood cell production is a tightly controlled, kinetically active process. The mature blood cells in the body that are required to fight infection, provide humoral and cell-mediated immunity, allow us to breathe, and prevent bleeding are derived through a catenated system from immature subsets of rare cells, the hematopoietic stem and progenitor cells. In the adult, the stem and progenitor cells used for the formation of blood elements are produced and functional mainly in the bone marrow. However, ontologically, these cells are found first in the aorta–gonad–mesonephros region, in the yolk sac, and later in sequence in the fetal liver, fetal spleen, and fetal bone marrow. Adult blood has an extremely low frequency of these cells, unless they are inducibly mobilized from the marrow to blood through a variety of procedures, including the infusion of cytokines; in contrast, for as yet unknown reasons, the blood collected from the placenta and umbilical cord at the birth of a baby has a high frequency of hematopoietic stem and progenitor cells. For treatment of a number of malignant and nonmalignant disorders, the only hope for the patient is replacement of stem/progenitor cells from either allogeneic or autologous transplantation. For such transplants, stem and progenitor cells derived from adult bone marrow, placental and cord blood, and cytokine- and/or chemotherapy-mobilized peripheral blood have been used.

Under normal conditions, the production and differentiation of hematopoietic stem and progenitor cells are regulated by cell–cell and cytokine–cell interactions. In this context, cytokines can stimulate, augment, or suppress the growth of stem/progenitor cells. This article will focus on the growth factor and potent co-stimulating molecule Flt3 (fms-like tyrosine kinase 3) ligand (FL), which acts by binding to the Flt-3/Flk-2 tyrosine kinase receptor and triggering the intracellular signal transduction that determines the cellular actions regulating hematopoiesis.

## II. Flt3 LIGAND

Reports on the cloning of the Flt3 receptor were published in 1991, but the cloning of FL was not reported until 1993. The cloning of FL was accomplished from a cDNA clone isolated from an expression library of the murine T-cell line P7B-0.3A4 and from mouse thymic stromal cells. The FL cDNA encoded a type 1 transmembrane protein that was shown to stimulate the proliferation of cells transfected with Flt3. The soluble form of FL acted on the proliferation of phenotypically defined early subsets of blood cells, and it also enhanced the response of mouse stem cells and primitive subsets of human cells to other growth factors. A fragment of murine FL was used as a probe to clone human FL from a T-cell cDNA library. The human and murine forms of FL are 72% identical at the amino acid level, although the homology is greater in the extracellular region than in the cytoplasmic region. FL appears to be relatively non-species-specific in that the soluble forms of the human and murine FL are comparable in proliferation-inducing/co-stimulating activities on human and murine target cells. In many cases, the effects of FL have been documented in the presence and in the absence of serum in the culture medium and with purified cells, single isolated purified cells, and relatively unpure target cell populations. FL is widely expressed in many different tissues, yet the expression of its receptor, Flt3, is much more limited. The selected tissue expression of Flt3 will be covered in Section VI.

## III. BIOLOGICAL ACTIVITIES OF FL *IN VITRO*

Because tyrosine kinase receptors such as c-kit and c-fms have the respective ligands stem cell factor (SCF, also called c-kit ligand, steel factor, and mast cell growth factor) and macrophage colony-stimulating factor (M-CSF), which are potent but selective growth factors, and Flt-3 is expressed on primitive blood cells, it was assumed that once the ligand for Flt-3 was identified, it also would demonstrate growth factor activity.

### A. FL Effects on Proliferation of Myeloid Progenitor Cells

By itself, FL has relatively weak proliferation-stimulating activity on hematopoietic stem cells and progenitor cells. However, similar to the action of SCF, the ligand for the c-kit-encoded receptor, when FL was used in combination with other growth factors it manifested potent co-stimulating activity. The action of FL with other growth factors was in many cases synergistic, and at the least, the effect of the combination of FL with another growth factor was additive. FL synergized with granulocyte/macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) to enhance the proliferation of granulocyte/macrophage progenitor cells (CFU-GM), with granulocyte (G)-CSF to enhance the proliferation of granulocyte progenitor cells (CFU-G), and with M-CSF to enhance the proliferation of macrophage progenitors (CFU-M). It also had additive to synergistic effects when added with GM-CSF, IL-3, G-CSF, or M-CSF in combination with SCF. This synergism was apparent in both the numbers of CFU-GM-, CFU-G-, and CFU-M-derived colonies formed *in vitro* and the number of cells per colony. The colonies formed from progenitors stimulated with the combination of FL and SCF plus other colony-stimulating factors or cytokines came from progenitor cells that were more immature than those colonies that formed from cells grown in the presence of only one or two cytokines. That FL acted on the more primitive subsets of progenitor cells was consistent with the effects of FL on progenitors known to occur earlier in maturation than CFU-GM, CFU-G, and CFU-M. FL enhanced the proliferation of high-proliferative-potential colony-forming cells (HPP-CFC) and long-term culture-initiating cells. FL also enhanced colony formation of early (BFU-MK) and more mature (CFU-MK)-subsets of megakaryocytic progenitors in the presence of thrombopoietin (TPO). FL had minimal effects on enhancing erythropoietin-responsive erythroid (BFU-E) and multipotential (CFU-GEMM) progenitors. Although there are reports that FL has an effect *in vitro* on BFU-E and CFU-GEMM, the effect was modest and abbreviated in that the erythroid cell-containing colonies that formed were small in size. FL also enhanced colony formation of CFU-GM, HPP-CFC, and other myeloid progenitor cells (MPCs) when used in combination with either P1XY321, a GM-CSF/IL-3 fusion protein, or the combination of IL-6 with the soluble IL-6 receptor. In fact, FL synergized with essentially all the colony-stimulating factors and those interleukins that had MPC growth-stimulating/enhancing activity. The effects of FL when used with other cytokines are enhanced even further when SCF is added to the mix.

The expansion of hematopoietic stem cells and MPCs *ex vivo* is a goal of clinical medicine, and FL has been found to be a critical component of the cytokine combinations used to expand MPCs and possibly stem cells *ex vivo*. The most potent combination of cytokines used for *ex vivo* expansion is currently believed to be FL, TPO, and SCF, although additional cytokines have been added to this three-factor combination, sometimes with enhanced effects. There is no question that these *ex vivo* conditions expand MPCs. However, whether or not these *ex vivo* expansion procedures actually result in expansion or even maintenance of human hematopoietic stem cells with long-term repopulating capability (e.g., the competitive long-term marrow repopulating stem cell) is still an open question. There is some evidence that the three-factor combination does *ex vivo* expand the human cell population capable of repopulating sublethally irradiated mice with severe combined immunodeficiency (SCID). At this time, the mouse SCID repopulating cell (SRC) assay is considered the best assay for detecting human hematopoietic stem cells, but it is not a perfect assay as it does not allow for rescuing a lethally irradiated animal, the hallmark for a stem cell assay and one that is used to define mouse stem cells. Also, there are large discrepancies in the literature as to the extent of *ex vivo* expansion of human SRC. In this context, the membrane-bound form of FL may be more potent than the soluble form of FL in enhancing the *ex vivo* expansion of human stem cells and more immature subsets of MPCs.

### B. FL Effects on Proliferation of Lymphoid Progenitor Cells

FL has been found to act with IL-7 to support expansion of murine thymic B-cell progenitors, murine B-cell commitment and development from uncommitted progenitors, promotion of stromal cell-independent expansion and differentiation of human fetal pro-B cells, and growth of $CD43^+$ $B220^{low}$ progenitor cells (very primitive B cells) but not of the more mature pro- or pre-B-cell compartments. In combination with transforming growth factor-β (TGF-β), FL potentiates the *in vitro* development of Langerhans-type dendritic cells.

### C. FL-Responsive Progenitors as Targets for Negatively Acting Cytokines

Progenitor cells responsive to stimulation by a combination of growth factors are the more immature subsets of progenitors, those with enhanced expansive capabilities. A number of cytokines have been shown to suppress the proliferation of myeloid progenitors, especially the more immature subsets of these cells. Members of the chemokine family of cytokines and also TGF-β and tumor necrosis factor α (TNFα) have demonstrated suppressive activities on FL-responsive MPCs. Chemokines fall into four subfamilies denoted by their cysteine–cysteine (CC), CXC (where X is another amino acid), C, and $CX_3C$ motifs. More than 20 members within the CC, CXC, and C subfamilies, of the more than 60 chemokines known to exist, can suppress the proliferation of immature subsets of human and murine MPCs, including those responsive to the co-stimulating effects of FL. TGF-β and TNFα have similar effects on the growth of murine $Sca1^+Lin^-$ cells. In the mouse system, the $Sca1^+Lin^-$ phenotype is usually a marker for murine hematopoietic stem cells. The effects of chemokines, TGF-β, and TNFα on suppression of the proliferation and growth of FL-responsive MPCs and murine $Sca1^+Lin^-$ cells has been confirmed at the level of single target cells. This strongly suggests that the suppressive effects on the FL-responsive target MPCs/stem cells are direct. The single-cell experiments are crucial to determining direct actions, since numerous cytokines have multiple actions, including the capacity to induce the production/release of cytokines from cell populations that can act in a paracrine fashion. Thus, unless studies such as this are performed at the level of the single isolated cell, it will not be possible to rule out an indirect effect mediated through the induced release of another cytokine or cytokines that are the final mediator of the biological effect noted.

### D. FL Effects on Survival of Progenitor Cells

All primary cells will undergo programmed cell death (apoptosis) when removed from the presence of cytokines. In addition to its capacity to augment/co-stimulate the proliferation of progenitor cells, FL acts as a survival/anti-apoptosis factor. Survival/apoptosis can be evaluated *in vitro* by removing cells from growth factor (even in the presence of serum) and then subjecting the cells to delayed addition of the growth factors. Thus, cells can be plated in the absence or in the presence of the putative survival/anti-apoptotic cytokine (which should not have the capacity to stimulate or enhance proliferation of the target population by itself, either because it is not active in this context or because it is used at a concentration lower than the concentration at which it is active). By delaying the addition of the proliferation-stimulating cytokines (e.g., colony-stimulating factors), it is possible to evaluate the survival-enhancing activity of the test material. That this effect is actually anti-apoptotic requires verification by the use of apoptosis detection assays (e.g., annexin binding, caspase 3 activation). In the context of these types of studies, FL was found to act as a survival/anti-apoptotic activity for CFU-GM, BFU-E, and CFU-GEMM in $CD34^{+++}$ bone marrow and cord blood cells and for CFU-M. FL inhibits apoptosis of acute myelogenous leukemia (AML) cells and this effect is associated with enhanced expression of the anti-apoptotic genes coding for Bcl-2 and Bax. These anti-apoptotic effects of FL on myeloid and B-lymphoid progenitor cells could be counterbalanced by chemokines, TGF-β, and TNFα. FL has also been shown to protect the murine M1 leukemic cell line from leukemia inhibitory factor (LIF)-induced differentiation and suppression of "self-renewal."

It is of interest that up-regulation of Flt3 receptor expression on murine bone marrow $Sca1^+Lin^-$ $c\text{-}kit^+$ cells was accompanied by a loss of self-renewal capacity but sustained lymphoid-restricted reconstitution potential. How this finding relates to the proliferation- and/or survival-enhancing effects of FL remains to be determined.

## IV. BIOLOGICAL EFFECTS OF FL *IN VIVO*

### A. Effects on Hematopoiesis and MPC Mobilization

Mice with a targeted disruption of the FL receptor, Flt3, demonstrated normal mature hematopoietic cell populations, but they had decreased numbers of primitive B-lymphoid progenitors. Also, phenotypically defined stem cell populations from these mice had decreased T-cell and myeloid repopulation capability in lethally irradiated secondary recipient mice in a competitive stem cell-repopulating assay, with the greatest decrease apparent in the lymphoid lineage.

Recombinant soluble FL has been administered to mice and primates to evaluate the effects on hematopoiesis in these animals. When administered to mice for 15 days at a dose of 10 μg/mouse, there was an increase in nucleated cellularity in the spleen and peripheral blood. This was coincident with increases in splenic B cells, myeloid cells, and nucleated erythrocytes. There were increased numbers of granulocytes, lymphocytes, and monocytes in the blood and increased numbers of CFU-GM, BFU-E, and CFU-GEMM in bone marrow, spleen, and blood. Increases were also noted in the numbers of CFU-spleen cells (a more mature member of the stem cell compartment that is assayed *in vivo*) and in phenotypically defined populations of stem cells. Administration of FL to mice and rabbits was radioprotective but was most effective in this activity if given prior to the irradiation.

The FL-induced mobilization of stem and progenitor cells to the blood is of clinical relevance as growth factor-mobilized stem and progenitor cells can and are being used for clinical transplantation. When administered to primates at 200 μg/kg/day, there was a slow increase over time in the numbers of peripheral blood MPCs that peaked 2 weeks after start of the injections. In addition, there were enhanced numbers of circulating lymphoid and osteoclast progenitors. FL synergized with G-CSF in primates to enhance mobilization to the blood of the different types of progenitors.

FL and G-CSF synergy in the mobilization of stem and progenitor cells was also seen in mice. Administration of 100 μg/kg/day FL in combination with G-CSF synergized to mobilize hematopoietic stem cells with a $\text{Sca1}^{+\text{Lin}\,-\,\text{c}}\text{–kit}^{+}$ phenotype and with both short- and long-term engrafting capability. These cells induced by FL alone or in combination with G-CSF could protect lethally irradiated mice and resulted in long-term multilineage engraftment. The synergy between FL and G-CSF for mobilization was apparent not only in the numbers of stem/progenitor cells mobilized but also in the speed with which the cells were mobilized.

### B. Effects on Dendritic Cells and Natural Killer Cells

An interesting outcome of the studies evaluating the *in vivo* effects of FL in mice was the observation that FL enhanced the numbers of dendritic cells, potent antigen-presenting cells, and potential effector cells for immune therapy of tumor development.

FL administration resulted in enhanced numbers of functionally mature dendritic cells, with the markers MHC class II, CD11c, DEC205, and CD86, which were capable of presenting alloantigen or soluble antigen to T lymphocytes or in priming an antigen-specific T-cell response *in vivo*. Increased numbers of these dendritic cells were seen in bone marrow, gastrointestinal lymphoid tissue, liver, lymph node, lung, peripheral blood, peritoneal cavity, spleen, and thymus. Enhanced numbers of cells were also seen in mouse liver that had an immature dendritic cell phenotype with poor T-cell allostimulatory activity. However, propagation of these immature dendritic cells *in vitro* with GM-CSF and IL-4 induced a more mature dendritic cell phenotype and enhanced functional activity. When administered to mice with G-CSF, FL induced an increase in the numbers of plasmacytoid dendritic cells in bone marrow and spleen.

*In vivo* administration of FL to mice also resulted in increased numbers of functional natural killer (NK) cells in bone marrow, thymus, blood, spleen, and liver; this effect possibly occurs through the induced expansion of NK-cell progenitors (Pro-NK cells).

## V. FL AND MALIGNANCY/THERAPY

It is not entirely clear what role the FL/Flt3 axis plays in malignancy or indeed whether it plays any role at all. However, in addition to stimulatory/co-stimulatory effects of FL on normal cells, FL stimulates the proliferation of blast cells from some patients with acute myelogenous leukemia *in vitro*, and this effect is synergized when FL is added with G-CSF, GM-CSF, IL-3, or SCF.

In humans, FL is predominately synthesized as a transmembrane protein that must undergo proteolytic cleavage to generate a soluble form of the molecule. When murine stem/progenitor cells were transduced with retroviral vectors encoding murine transmembrane FL, the increased expression of this gene resulted in predisposition of Flt3 receptor-positive progenitor cells to leukemic transformation. Also, retroviral-mediated gene transfer of human FL enhanced the proliferation and intracellular activity of mitogen-activated protein kinase (MAPK) in the Flt3-expressing human leukemia cell line AML-5. FL was detected on the surface of the transduced AML-5 cells and although there was some increase in the numbers of AML-5 colony-forming cells, the main effect was to enhance the proliferation of individual

AML-5 colony-forming cells to form much larger colonies. Information on intracellular signaling induced by FL as well as information on Flt3 mutations is presented in Section VI.

### A. FL Therapy in Animal Models

FL has shown efficacy in modifying disease progression in a number of animal models of solid tumors. In a murine model of metastatic lung cancer (Lewis lung carcinoma), administration of FL delayed tumor growth, but this was a temporary effect. In a murine model of metastasis, FL reduced the number of hepatic metastases, and this was coincident with increased numbers of dendritic cells, $CD4^+$ T cells, and $CD8^+$ T cells. Depletion of NK cells in these mice reduced the anti-tumor effects of FL, indicating that NK cells are also mediators of the FL effects. Increased anti-tumor effects in this model were apparent when FL was used in combination with IL-12.

Mice challenged with syngeneic breast cancer cells were protected by administration of soluble FL, but this protective effect was transient. However, transducing the murine breast cancer cells with murine or human FL genes by retroviral-mediated gene transduction decreased tumor development and protected mice from a subsequent challenge with untransduced tumor cells. Genes for either the membrane-bound form or the soluble form of FL were protective in this context. It was believed that the protective effects might be exerted through an effect on antigen-presenting cells but might also be mediated in part by NK cells since immunodepletion of activated NK cells with anti-asiolo-$GM_1$ blocked the anti-tumor activity.

It remains to be determined how effective FL therapy might be for certain tumors and exactly how these anti-tumor effects are being mediated. It is not likely that FL treatment of tumors that express Flt3 would be helpful, and in fact this might be counterproductive since FL acts as a proliferation-/co-proliferation-inducing and/or survival-enhancing cytokine for normal and leukemic Flt3-expressing cells.

## VI. Flt3 RECEPTOR

### A. Structure of Flt3 Receptor

The human Flt3 has 993 amino acids and the murine Flt3 has 1000 amino acids. They are 86% identical at the amino acid level. The Flt3 receptor is a single transmembrane protein and has an extracellular region composed of five immunoglobulin-like domains, a single transmembrane region, a juxtamembrane (JM) domain, a conserved tyrosine kinase domain spaced by a kinase insert region, and a C-terminal region. The Flt3 receptor thus belongs to the type III receptor tyrosine kinase family, which also includes the receptors for M-CSF, SCF, and platelet-derived growth factor (PDGF).

The murine and human Flt3 receptors have 9 and 10 potential sites for N-linked glycosylation, respectively, in their extracellular domains. The protein is glycosylated at one or more of these sites and undergoes carbohydrate remodeling as it migrates from the endoplasmic reticulum to the cell surface. The Flt3 receptor protein immunoprecipitated from cells treated with tunicamycin (an inhibitor of N-linked glycosylation) has a molecular mass of 110 kDa, which is the predicted size of the protein backbone alone. Immunoprecipitation of cells transfected with the Flt3 receptor reveals two proteins of 130–143 and 155–160 kDa. Pulse–chase experiments showed that the larger protein arose from the smaller protein, presumably as a result of glycosylation processing. Consistent with this interpretation is the finding that only the 158 kDa species is found on the cell surface. There do not appear to be any O-linked sugars on the protein.

### B. Expression of Flt3 Receptor in Normal Tissues

Murine Flt3 is expressed mainly in hematopoietic tissues, brain, gonads, and placenta. The significance of Flt3 expression in nervous tissues is not known, but the major site of expression and function appears to be hematopoietic tissues. In mouse bone marrow (BM), Flt3 expression is restricted to primitive stem/progenitor cells. In humans, Flt3 mRNA is expressed in most lymphohematopoietic organs including bone marrow, thymus, spleen, liver, and lymph nodes and weakly in placenta. No expression is seen in either B or T cells, but monocytes or granulocytes are weakly positive. In normal human bone marrow, expression of Flt3 is limited to $CD34^+$ cells, a population enriched for hematopoietic stem/progenitor cells. However, recent studies show that $CD34^-$ lineage negative BM cells also express Flt3. More than 60% of $Flt3^+$ human BM cells co-express CD33, a myeloid cell-surface antigen, suggesting that Flt3 may be expressed on subsets of myeloid progenitor and/or mature cells. Most human $CD34^+$ BM and cord blood cells express Flt3, and most GM progenitors express Flt3, whereas $CD34^+Flt3^+$ cells are depleted in erythroid progenitors. Flt3 appears to be shut off before erythroid differentiation and gradually down-regulated during GM differentiation. Myeloid-derived and lymphoid-derived dendritic cell (DC) progenitors appear to express Flt3, because they respond to FL in combination with other cytokines. However, mature DCs do not seem to express Flt3.

## C. Expression of Flt3 Receptor on Hematopoietic Cell Lines and Leukemia

Expression of Flt3 receptor has been extensively studied in mouse and human hematopoietic cell lines. No Flt3 expression is seen in any of the mouse T-cell, macrophage, erythroid, megakaryocyte, or mast-cell lines examined or most early mouse B-cell lines, but it has been reported in several mature B-cell lines. In contrast, human Flt3 is expressed in a high percentage of human myeloid and monocytic cell lines. All myeloma, erythroid, and erythroblastic cell lines are Flt3 negative, and only a few megakaryocytic cell lines are positive. Among lymphoid cell lines, pro-B- and pre-B-cell lines are Flt3 receptor positive, whereas all T-cell lines, NK-cell lines, and Hodgkin's cell lines are negative.

Interestingly, Flt3 receptors are frequently seen on most AML blasts and pre-B acute lymphoblastic leukemia (ALL) cells. The majority of adult AML samples from all French American British (FAB) classes are positive for Flt3 receptor expression, as are all B-lineage ALL samples. In contrast, both T-cell and B-cell lymphomas are negative for Flt3 receptor expression. Almost all chronic-phase or accelerated-phase CML samples are negative for Flt3 receptor expression. However, approximately two-thirds of the samples from chronic myelogenous leukemia (CML) patients in blast crisis are Flt3 receptor positive.

## D. Mutation of Flt3 Gene

Mutations of receptor tyrosine kinases, including c-kit, PDGF receptor (PDGFR), and Flt3, have been found in human leukemia. In acute myeloid leukemia, an internal tandem duplication (ITD) mutation of the juxtamembrane domain of the Flt3 gene has been found in 20–30% of patients. In Flt3/ITD, a fragment of the juxtamembrane domain-coding sequence was arranged in direct head-to-tail succession. Its location and length vary from sample to sample, but the duplicated sequence is always in-frame. Thus, mutant Flt3 contained a long JM domain although other domains were not affected. All duplications involve sequences that contain one to four tyrosine residues. These additional tyrosines could potentially play a role in the autophosphorylation of the receptor. Flt3/ITD mutations result in constitutive activation of Flt3 and subsequent activation of downstream MAPK and Stat5 even without ligand stimulation. Flt3/ITD-transfected murine IL-3-dependent cell lines, such as Ba/F3, 32D, and FDC-P1, are able to proliferate without IL-3 and cause leukemia-like disease when injected into syngeneic mice. Clinically, in AML patients, these mutations appear to be associated with higher white blood cell counts and poorer survival. These results suggest that abnormal regulation of Flt3 may contribute to leukemic transformation and that the Flt3 receptor may represent a therapeutic target in AML.

Recently, a point mutation of D835 in the kinase domain of human Flt3 was found in some AML patients. The D835 residue is located in a region called the activation loop of receptor tyrosine kinases (RTKs), and mutations in D835 spontaneously activate tyrosine kinase activity and induce factor-independent proliferation of hematopoietic cell lines. A similar point mutation was found in the c-kit gene (D816). In contrast to Flt3/ITD mutations, D835 mutations were not associated with poor prognosis.

## E. Signal Transduction through the Flt3 Receptor

Similar to other type III RTKs, Flt3 is activated by ligand-induced dimerization and transphosphorylation of tyrosine residues in the cytoplasmic domain. Subsequent signal transduction is mediated through the association and/or phosphorylation of cytoplasmic substrates that propagate the signal. Several groups have reported the intracellular signaling pathways activated by either wild-type Flt3 or constitutively active mutant Flt3 in different cell systems. Since the Flt3 receptor was cloned before the ligand was found, the early studies examined the putative signal components of wild-type Flt3 using chimeric receptors containing the extracellular domains of either c-kit or M-CSF receptor (c-fms) fused to the transmembrane and cytoplasmic domains of murine Flt3. The chimeric receptor transduces activation signals through association with and/or phosphorylation of a number of cytoplasmic proteins, including the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), RAS GTPase-activating protein, phospholipase C-γ, Vav, growth factor receptor-binding protein 2 (Grb2), and Src homology and collagen protein (Shc). Recent studies using full-length wild-type Flt3 have confirmed most of the above findings and identified several new signaling components.

### F. PI3-Kinase/Akt Pathway

PI3-kinase is a heterodimeric protein containing an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110). It was one of the first major signal molecules identified in Flt3 signaling. Activation of murine Flt3 induces tyrosine phosphorylation of p85, the association of p85 with Flt3 through the SH2 domain, and subsequent activation of PI3-kinase. Murine Flt3 has two potential p85-binding sites, $Y^{922}$FVM and $Y^{958}$QNM. It has been shown by phosphopeptide competition and mutagenesis studies that, in contrast to c-kit and CSF-1R, p85 binds to Y958 in the carboxy tail of the receptor. A mutant receptor in which Y958 had been changed to F958 could not bind p85 and did not activate PI3-kinase upon activation. Yet the mutant receptors, when expressed in fibroblasts or in the interleukin 3-dependent cell line Ba/F3, still provided a mitogenic signal comparable to that of wild-type receptors. The Y958F mutation had no effect on the internalization of the receptor.

Compared with murine Flt3, human Flt3 does not have a p85-binding motif at the carboxyl-terminus but does have one at the second kinase domain, $Y^{919}$IIM. PDGF receptor, CSF-1 receptor, and c-kit all have the p85-binding site in the kinase insert domain. Co-immunoprecipitation experiments failed to detect the association between human Flt3 and p85, suggesting that, in contrast to murine Flt3, p85 does not bind directly to human Flt3. Instead, p85 associated with Shp-2,SH2-domain-containing phosphatase (SHIP), Cbl (casitas B-lineage lymphoma), and Gab2 (Grb2-associated binder 2). Immunoblotting with anti-phosphotyrosine antibody failed to detect the phosphorylation of p85 in these studies. These results suggest that human Flt3 may use different mechanisms to activate PI3-kinase. In addition to PI3-kinase, activation of Flt3 induced the transient activation of Akt, a serine/threonine protein kinase downstream of PI3-kinase.

### G. MAPK Pathway

Several studies have found that the stimulation of Flt3 ligand activates extracellular signal-related kinase (ERK). The activation of ERK is required for the mitogenic signaling of Flt3 ligand as PD98059, a specific MAPK kinase inhibitor, inhibited cell proliferation induced by FL in a dose-dependent manner. The activation of ERK is likely mediated by the adapter protein Grb2, which has been shown to bind directly to both murine and human Flt3. There are two putative Grb2-binding sites in the C-terminus of Flt3. It remains to be determined whether Grb2 binds to both sites or to either of them.

In addition to Grb2, Shc, SHIP, Shp-2, Cbl, and Cbl-b become prominent tyrosine-phosphorylated proteins after activation by Flt3. Interestingly, Shc does not bind directly with Flt3, and it associates with tyrosine-phosphorylated SHIP. In contrast to other members of the type III RTKs, Shp-2 does not bind directly to Flt3. Rather it associates with Grb2. In human myeloid cell lines and pro-B-cell lines, Cbl and Cbl-b are tyrosine phosphorylated and associate with p85 on ligand stimulation. Gab1 (Grb2-associated binder 1) and Gab2, two scaffolding adapter proteins, have recently been identified as new components of Flt3 signaling. Both Gab1 and Gab2 are transiently tyrosine phosphorylated and interact with Shp-2, Grb2, and p85.

### H. Jak/Stat Pathway

The Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway represents an extremely rapid membrane-to-nucleus signaling system and is widely used by members of the cytokine receptor superfamily. Jaks are cytoplasmic tyrosine kinases with four mammalian members: Jak1, Jak2, Jak3, and Tyk2 (protein tyrosine kinase-2). Stats are latent cytoplasmic transcription factors that become activated after recruitment to an activated receptor complex. Subsequently, these active Stats translocate to the nucleus to affect gene expression. Seven Stat proteins have been identified in mammalian cells: Stats1 to 6, including Stat5a and Stat5b, which are encoded by distinct genes. In addition to the cytokine receptor superfamily, a number of RTKs, including c-kit, PDGFR, and M-CSF receptor, have been shown to activate the Jak/Stat pathway. Among Stat proteins, only Stat5 has been found to be involved in Flt3 signaling. It has been shown that Stat5 may mediate the proliferative effect of FL on hematopoietic progenitor cells. Thus far, Jak kinases have not been implicated in Flt3 signaling.

## VII. FUTURE DIRECTIONS

Compared with other type III RTKs, little is known about the intracellular signaling pathways of Flt3. Considering the important role of FL/Flt3 in normal hematopoiesis, studies of Flt3 signaling mechanisms should shed light on how FL functions in normal hematopoiesis. More importantly, this knowledge may provide insight into the pathogenesis of leukemia and thus help in designing strategies to slow the progression of and possibly cure certain forms of leukemia.

## Glossary

**Flt3** The fms-like tyrosine kinase 3 receptor that acts as the cell surface mediator of biological effects induced by its ligand upon specific receptor binding.

**Flt3 ligand (FL)** A type 1 transmembrane protein that in soluble or transmembrane form binds to Flt3 and elicits the intracellular signaling cascade that guides hematopoietic stem and progenitor cells to proliferate, survive, and develop. For most activities, FL has modest effects by itself, but it is a potent co-stimulating molecule and actively synergizes with other cytokines for optimal biological effects.

**hematopoiesis** The production of blood and immune cells. The mature end-stage blood and immune cells are generated from a rare population of hematopoietic stem cells that have the capacity to self-renew and/or differentiate into multilineage blood and immune cells. Stem cells give rise to rare populations of progenitor cells, which have little or no self-renewal capacity and are more restricted/committed to specific cell lineages. Progenitor cells give rise to precursor cells, which are the first morphologically recognized dividing cells within a specific blood/immune cell lineage. Stem/progenitor cell production is regulated by cytokines and cell–cell interactions that stimulate, enhance, and/or suppress the proliferation, self-renewal, differentiation, survival/apoptosis, and/or migration (homing/mobilization) of these cells.

## See Also the Following Articles

**Angiogenesis • Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Erythropoietin, Biochemistry of • Granulocyte Colony-Stimulating Factor (G-CSF) • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Leukemia Inhibitory Factor (LIF) • Protein Kinases • Tumor Necrosis Factor (TNF)**

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# Follicle-Stimulating Hormone (FSH)

William L. Miller
*North Carolina State University*

I. INTRODUCTION
II. STRUCTURE OF FSH
III. FSH RECEPTOR
IV. FSH ACTION IN THE TESTIS
V. FOLLICULAR GROWTH AND REGULATION
VI. INDUCTION OF OVULATION WITH FSH
VII. REGULATION OF FSH
VIII. SUMMARY

**Follicle-stimulating hormone (FSH) is an α/β heterodimer produced only in pituitary gonadotropes, which constitute 3–8% of all pituitary cells. FSH acts on granulosa cells and Sertoli cells to ensure maturation of eggs and sperm in females and males, respectively.**

## I. INTRODUCTION

Follicle-stimulating hormone (FSH) is named for its ability to stimulate follicle growth in females, as depicted in Fig. 1. Follicle-stimulating hormone stimulates the division and function of granulosa cells that surround and nurture the developing oocyte (egg) in the follicle.

Spermatogenesis also relies heavily on FSH, which induces Sertoli cell division in early life. In later life, both FSH and testosterone stimulate Sertoli cells to nurture B spermatogonia as they develop into sperm. A number of hormones from the hypothalamus, the gonads, and the pituitary itself help regulate FSH. This article first deals with the effect(s) of FSH on sperm and egg maturation (including the use of FSH to solve infertility problems in the female) and then it outlines the complex nature of FSH regulation in mammals.

## II. STRUCTURE OF FSH

A ribbon diagram of human FSH is shown in Fig. 2. Two different subunits (α/β) are held together tightly by noncovalent bonds. The human α-subunit, encoded by a single gene on chromosome 6q12.2, is 92 amino acids long, contains five cysteines that stabilize it by intrasubunit disulfide bonds, and is N-glycosylated on asparagines 52 and 78. Up to 30% of its final weight comes from carbohydrate. The α-subunit is found in other hormones, such as luteinizing hormone (also made in gonadotropes), thyroid-stimulating hormone (made in pituitary thyrotropes), and human chorionic gonadotropin (made in placental tissue).

The β-subunit of human FSH is encoded by a single gene on chromosome 11p13. It is 111 amino acids long, contains six cysteines that stabilize its conformation with disulfide bonds, and is N-glycosylated on asparagines 7 and 24. As with the α-subunit, approximately 30% of the β-subunit of FSH is carbohydrate by weight. Both FSHβ and luteinizing hormone β (LHβ) gonadotropin subunits are produced in pituitary gonadotropes and bind to the common α-subunit described above, which is produced in excess.

The carbohydrate content of FSH varies significantly throughout the estrous/menstrual cycle and has been thought to be important in FSH regulation. At the beginning of the cycle, the carbohydrate contains more sialic acid and is, therefore, more acidic, but more basic forms predominate at the end of the cycle. The more acidic forms have longer half-lives because they are protected longer from degradation, but they have less biological activity. Because of the trade-off in longevity versus potency, it has never been established clearly whether the changes in glycosylation are biologically significant.

Inactivating mutations for FSH are very rare so infertility is not likely to come from faulty FSH. Both the α- and β-subunits of FSH are encoded by single-copy genes and are vulnerable to a standard rate of mutation. However, there are no mutations of the α-subunit known to inactivate FSH, and there are very few individuals known, worldwide, to harbor inactivating mutations of the FSH-specific β-subunit. Women with homozygous inactivating mutations for FSH suffer from amenorrhea and are infertile. Men lacking functional FSH have normal or slightly delayed puberty, but have small testes and have low sperm count. Heterozygous mutations of FSH do not alter reproductive function.

## III. FSH RECEPTOR

Follicle-stimulating hormone acts only by binding its receptor, shown in Fig. 3. This is a classical seven-transmembrane G-protein-coupled receptor

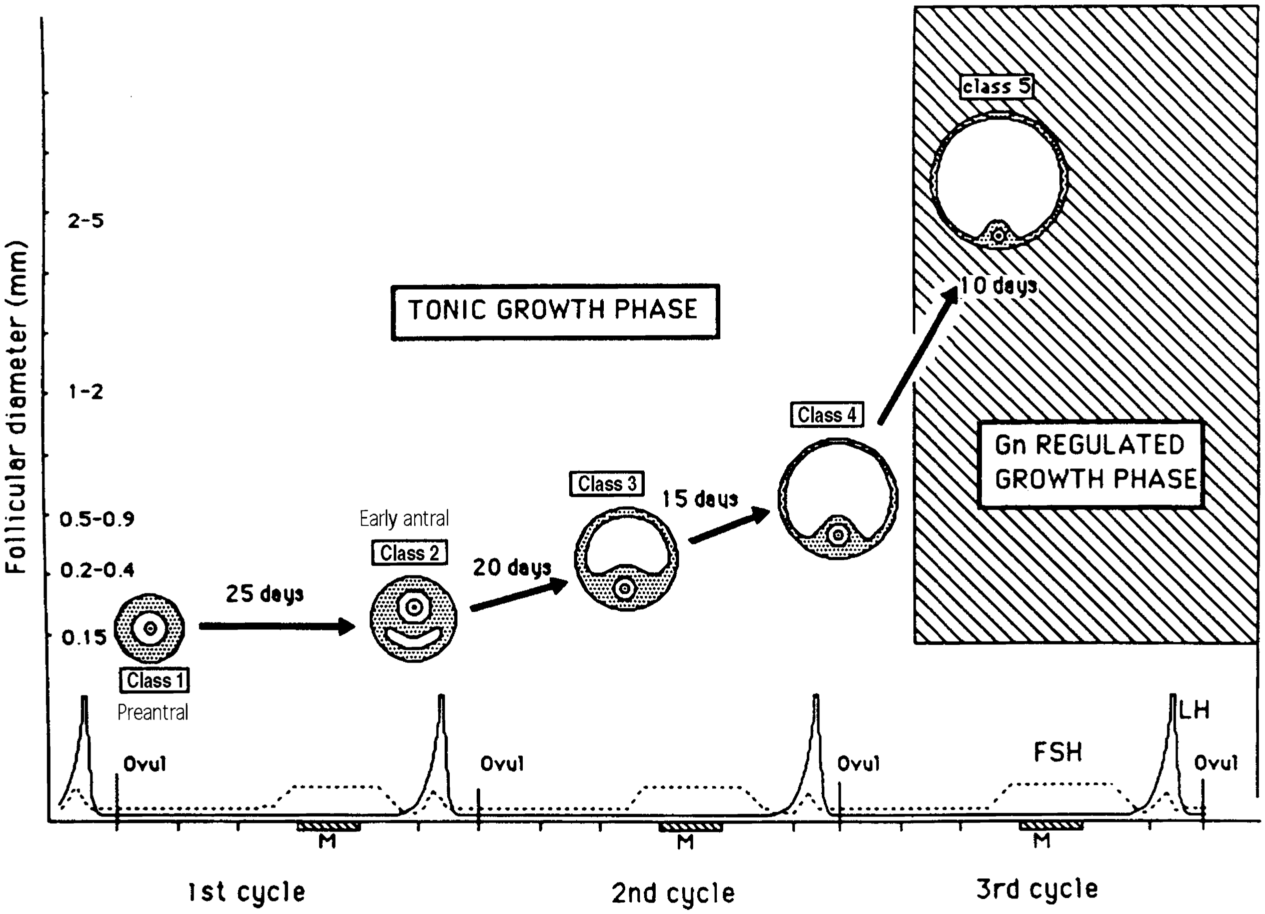

FIGURE 1 Schematic representation of human ovarian follicle development. Primordial follicles entering the growth phase form primary follicles (class 1). This is followed by gonadotropin-independent (tonic) growth (classes 1 to 4) and eventually gonadotropin (Gn)-dependent growth. Note that the overall development from a class 1 to a class 5 follicle takes three cycles. Modified from Gougeon, A. (1986) *Hum. Reprod.* **1**, 81–87, with permission.

that is found only on granulosa or Sertoli cells. The FSH receptor activates heterotrimeric G-proteins having $G\alpha_s$-subunits that stimulate production of the intracellular "second messenger" cyclic AMP, which, in turn, activates protein kinase A to phosphorylate a number of proteins such as enzymes and transcription factors that induce or inhibit the expression of many genes, some of which have been identified. Evidence also indicates that heterotrimeric G-proteins containing $G\alpha_i$- and G11/q-subunits are mobilized to activate protein kinase C and several map kinases and to increase intracellular calcium levels, but the physiological importance of these mechanisms is not clear. The complete array of genes and proteins affected by the FSH receptor in granulosa or Sertoli cells has not yet been determined, but the effects of FSH are widespread within granulosa and Sertoli cells.

Very few individuals have defective FSH receptors. The few known mutations of FSH receptors are shown in Fig. 3. Seven cases involve inactivating point mutations (open boxes) and one mutation causes the receptor to be hyperactive (filled circles). Defective FSH receptors give rise to the same infertility and developmental symptoms observed with defective FSH (see above).

## IV. FSH ACTION IN THE TESTIS

Sertoli cells are the only cells with FSH receptors in males. As noted in Fig. 4, FSH stimulates Sertoli cells to nurture spermatogonia as they mature into spermatocytes. It is noteworthy that failure of FSH to stimulate Sertoli cells usually leads to reduced male fertility, but this is "clinically hidden" and not obvious by merely looking at a man since it causes

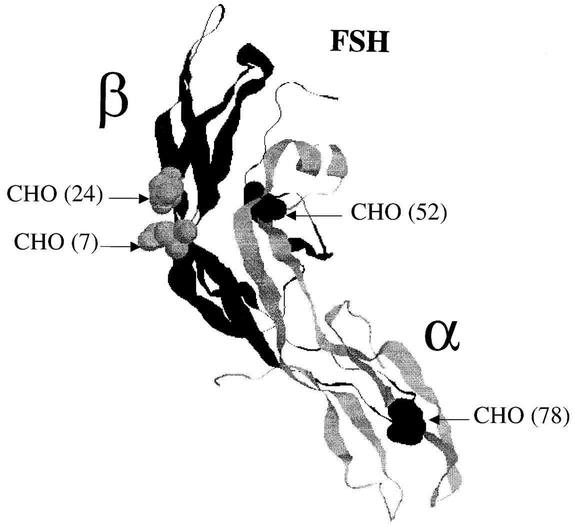

**FIGURE 2** Ribbon diagram of FSH showing the noncovalently associated α-subunit (light gray) and β-subunit (black). Glycosylation sites are shown at specific amino acids on both subunits [indicated by CHO (carbohydrate) with the amino acid position number given in parentheses]. Reprinted from Fox, K. M., *et al.* (2001) *Mol. Endocrinol.* **15**, 378–389, with permission from The Endocrine Society.

no change in testosterone and associated secondary male characteristics. Thus, males may appear virile but have very few or no sperm.

Male contraception has long been an interest of reproductive biologists, and significant effort has been devoted toward disrupting FSH action to prevent spermatogenesis (a male contraceptive method). Indeed, interrupting FSH action by immunization against FSH has been shown to block spermatogenesis in adult monkeys. Recent data gathered from both mice and men have caused controversy about this approach to contraception, however, because males that have never been able to respond to FSH from birth are still fertile. They have only 25% of the Sertoli cells of normal males, have smaller testes, and have sperm counts that are 75% lower than those of normal males, but they still produce enough sperm to be fertile. The current hypothesis reconciling the effects of FSH loss during adulthood with those of FSH loss from birth suggests that alternate hormone pathways can develop *in utero* to permit sperm production in males lacking FSH or FSH receptors from birth, whereas removal of FSH in adults occurs after alternate pathways are able to develop. This hypothesis argues that specifically targeting FSH production in adult males may still be an effective means of blocking spermatogenesis.

Another approach to male contraception involves nonselectively depressing all pituitary gonadotrope function to decrease both FSH and LH. This approach, however, requires steroid hormone replacement (testosterone) to maintain masculine body characteristics and libido since LH maintains male sex drive and body characteristics by inducing Leydig cells to produce the male sex steroid, testosterone.

## V. FOLLICULAR GROWTH AND REGULATION

The female granulosa cell is analogous to the male Sertoli cell. It is the only cell type in females that contains FSH receptors and its function is to nurture the developing gamete (oocyte) within the ovarian follicle (Fig. 1). Since granulosa cells also produce estrogens, which induce secondary female sex characteristics and maintain the menstrual cycle, a failure of FSH not only causes infertility, but also manifests itself "clinically" in women.

In Fig. 1, granulosa cells are shown directly contacting the oocyte. They comprise the gray area that surrounds the oocyte and provide it with growth factors such as epidermal growth factor and transforming growth factor-β (TGF-β) family members. There is a critical flow of hormone information between the oocyte and granulosa cells that is not well defined but is initiated and maintained by FSH. Since granulosa cells also produce estrogens, they are responsible for the increase in estrogens during the follicular phase that triggers the gonadotropin surge leading to the climactic act of ovulation (see Figs. 1 and 5).

Fig. 1 focuses on the maturation of a single "dominant" follicle as it develops over the course of several human menstrual cycles. It also highlights the very important period of FSH rise that influences follicular development (the dotted line in Fig. 1 represents increased FSH secretion during the "follicular phase"). This rise in FSH is thought to be the only physiologically important rise in FSH during the reproductive cycle although FSH also rises during the LH surge on day 0.

Women have approximately 400,000 primordial follicles at puberty, each containing a single egg that can develop into a mature oocyte, as shown in Fig. 1. Between puberty and age 27, women lose a majority of these eggs. Each month, more than 1000 follicles enter their final developmental stage (class 5) but only one becomes dominant and has the chance to release its mature egg following the LH surge. After age 27, the loss of eggs per month is significantly decreased, but the number of developing oocytes is decreased as well.

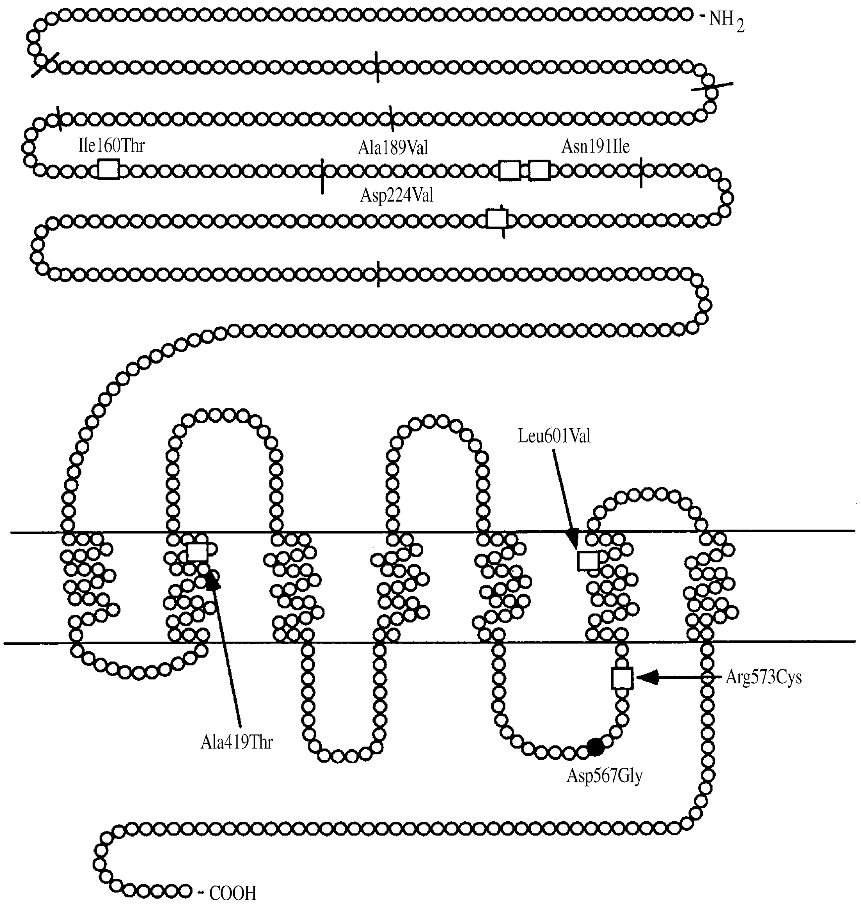

**FIGURE 3** The amino acid sequence for the human FSH receptor. This schematic shows the locations of seven inactivating mutations (open squares) and one activating mutation (filled circle) known to occur in the human FSH receptor. The short lines across the amino acid chain show the combination sites for the 10 exons of the gene encoding the human FSH receptor. Reprinted from Themmen and Huhtaiemi (2000), with permission from The Endocrine Society.

The decline in FSH during the follicular phase of the cycle (see Fig. 5) plays a critical role in selecting the single dominant follicle that eventually releases its egg to the uterus. The dominant follicle becomes relatively independent of gonadotropin support after it reaches 10 mm in diameter and continues to grow and flourish as FSH declines during the latter part of the follicular phase (day −7 to day 0). This single dominant follicle synthesizes the estrogen needed to trigger the gonadotropin surge that causes the follicle to burst and release its fully mature oocyte on day 0 (Figs. 1 and 5). Only the most mature follicle eventually becomes independent of gonadotropin production; the others (>99.9% of the follicles!) stop developing as FSH falls below a "threshold level" that is required for their maintenance and they undergo atresia (apoptosis and self-absorption).

Fig. 5 shows that FSH reaches its highest level on day 0, but as noted above, this transient increase seems to have little impact on overall follicle development. The primary effect of FSH comes from its early and continuous rise during the follicular phase and menses in humans that constantly stimulates granulosa cells once it reaches a high enough threshold level to influence them. Finally, Fig. 5 shows an increase in progesterone produced by the corpus luteum, which forms from the spent dominant follicle. Progesterone dominates the reproductive cycle for 14–16 days during the "luteal phase." Declining levels of estrogens and progesterone in the absence of pregnancy elicit menstruation in the primate. This is followed by the follicular phase that matures and selects another dominant follicle for ovulation.

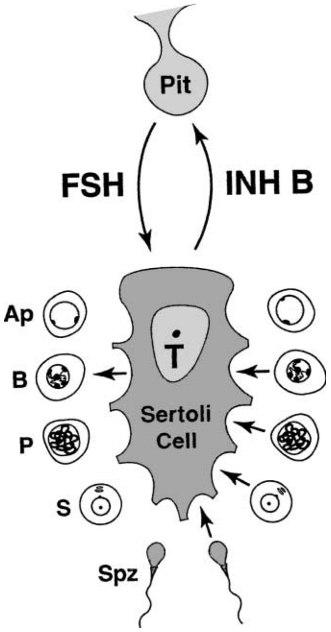

FIGURE 4 A model for the regulation of sperm production in primates. According to this model, FSH amplifies a basal level of spermatogenesis that is dependent on intratesticular testosterone (T). The degree of amplification is directly related to the circulating concentration of FSH, and FSH drive is relayed to the germinal epithelium via the production of a paracrine factor by the Sertoli cell. This paracrine factor favors the survival of differentiated B spermatogonia (B), which leads to an increase in the number of subsequent generations of germ cells. The FSH concentration is regulated by the rate of secretion of inhibin B (INH B) by the Sertoli cell. Inhibin B exerts a brake on FSH secretion by suppressing FSHβ gene expression. The mechanism that controls the rate of inhibin B secretion by the testis is controversial but in the present model a signal(s) from the differentiated germ cells is proposed to positively regulate inhibin B production by the Sertoli cell. The intensity of the putative germ cell signal is posited to be related to the number of differentiated germ cells. P, Primary spermatocyte; S, round spermatid; Spz; elongating spermatid and testicular spermatozoa; Pit, pituitary gland. Reprinted from Plant and Marshall (2001), with permission from The Endocrine Society.

## VI. INDUCTION OF OVULATION WITH FSH

Follicle-stimulating hormone is used to induce ovarian follicle development in women with hypothalamic amenorrhea (low gonadotropins), "unexplained infertility," or endometriosis. These women are treated with FSH to cause ovulation induction that is termed "controlled ovarian hyperstimulation." In these women, enough FSH is given to produce four to six ovulations since the success rate with only one ovulation is low (20%). A "step-down" procedure is usually used, starting with high doses to recruit a large number of follicles and using lower doses during the final maturation stage of the follicles to mimic the decrease in FSH and selection of one (or two) dominant follicles that occurs during the normal reproductive cycle. Development of dominant follicles is monitored with transvaginal ultrasound and the goal is to produce one or two fetuses, which is usually met.

Some women are afflicted with polycystic ovary syndrome. Many of these women are relatively insensitive to insulin and may ovulate on their own when given insulin sensitizers or they may need both insulin sensitizers and FSH. These women are at the highest risk for multiple births from FSH treatment.

Reaching the correct level of stimulation is difficult because women exhibit significant variability in their individual requirements for FSH during their cycles. Also, FSH preparations are variable (postmenopausal urine gonadotropins, purified recombinant FSH). These different preparations seem equally efficacious when used properly. As noted above, it has become the general practice to adjust FSH dose as deemed necessary while monitoring follicle growth by ultrasound to obtain the formation of several dominant follicles.

When there is a need for *in vitro* fertilization, FSH preparations are administered to women at very high levels so that FSH receptors on the granulosa cells are saturated. This permits many of the developing follicles to attain ovulatory size. The oocytes from these multiple ovulatory-size follicles are retrieved prior to the normal time of ovulation and fertilized *in vitro*. In this case, the number of embryos transferred back into the womb is highly controlled and the remaining embryos are cryopreserved for later transfer.

## VII. REGULATION OF FSH

The production of FSH is regulated primarily at the transcriptional level of its β-subunit, which is the limiting subunit for FSH production in pituitary gonadotropes. Transcription of the FSHβ subunit is controlled by a number of hormones including hypothalamic gonadotropin-releasing hormone (GnRH), TGF-β family members, and follistatin, which is produced in the pituitary and gonads. Gonadal steroids are also important but often work indirectly through regulation of GnRH and/or TGF-β family members.

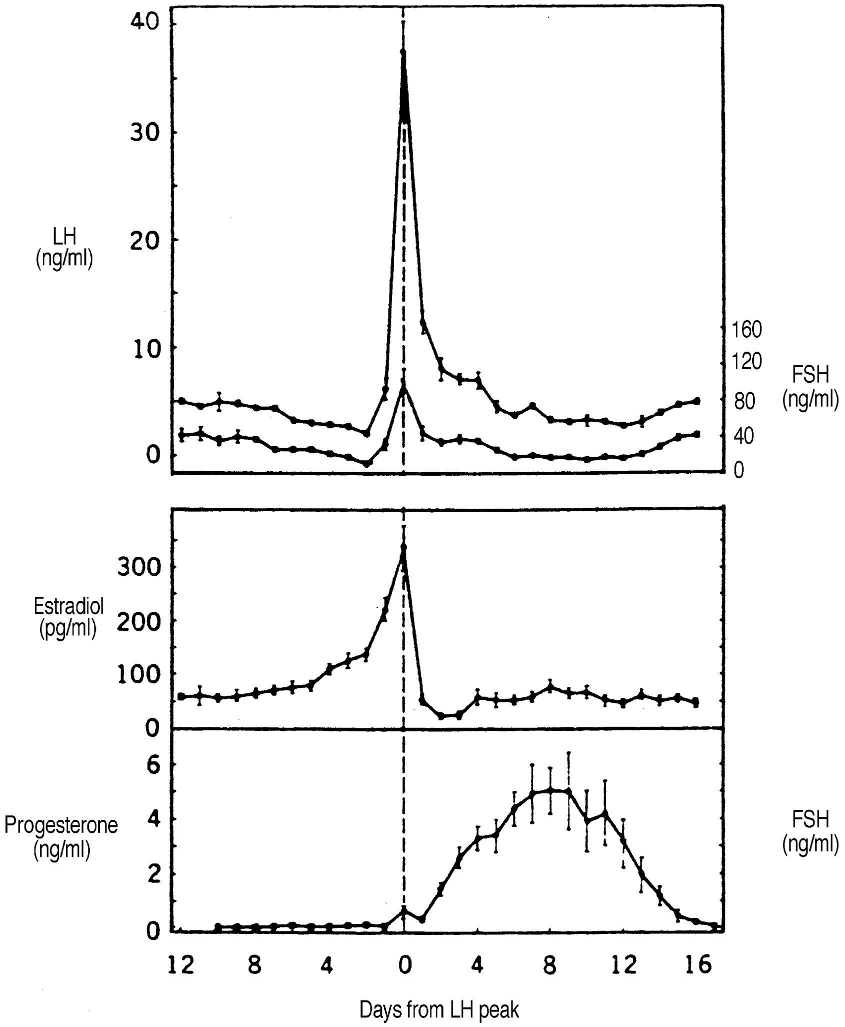

FIGURE 5 Profile of the concentrations of follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone throughout the rhesus monkey menstrual cycle. Reprinted from Knobil, E. (1974) *Rec. Prog. Horm. Res.* 30, 1–36, with permission.

## A. Gonadotropin-Releasing Hormone

GnRH is a major inducer of FSHβ expression. When GnRH action is blocked in rats by GnRH antagonists or antibodies that bioneutralize GnRH, overall FSH levels fall 50–60%. When GnRH is absent from birth, as in hypogonadal mice (hgp mice), the females produce 60% less FSH than normal mice and hgp males produce only 17% of the normal FSH level. These hypogonadal mice have even less LH than FSH and are infertile. Correction of the defective GnRH gene in mice corrects all gonadal failure. A similar defect in GnRH secretion is found in humans with Kallmann's syndrome (olfactory–genital hypoplasia). Normal FSH production and normal reproductive function can be established in these individuals by providing GnRH pulses by pump at hourly intervals although the procedure is costly and cumbersome for the patient.

There is evidence (*in vitro* and *in vivo*) that GnRH can directly induce FSHβ transcription. Direct induction of FSHβ transcription is likely to be a small part of this GnRH effect, however, because GnRH also stimulates general protein translation and gene transcription in gonadotropes in a pleiotropic manner.

## B. TGF-β Members: Induction of FSH

Although GnRH is a major inducer of FSH, TGF-β family members seem to have even more influence over FSH expression. As shown in Fig. 6, activins are

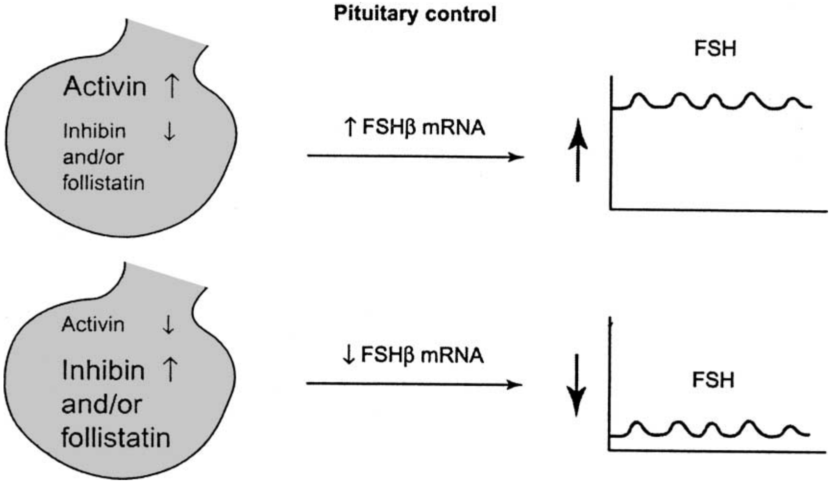

**FIGURE 6** Schematic representation of the local pituitary loop involving activin, inhibin, and follistatin and their impact on FSH secretion. This model predicts that increases in pituitary activin or decreases in inhibin and follistatin will increase FSH secretion. In contrast, a decrease in activin or increases in follistatin–inhibin will lower FSH secretion. Reprinted from Padmanabhan, V., and McNeilly, A. S. (2001) *Reproduction* **121**, 21–30, with permission.

strong inducers of FSH and inhibins and follistatin (which blocks activin action) are powerful inhibitors of FSH.

It has been known for 15 years that activins A and B can induce FSHβ transcription, and it has recently been reported that three other TGF-β family members also induce FSHβ. These are bone morphogenetic proteins 6, 7, and 15 (BMP6, BMP7, and BMP15), which are less potent than the activins but may be more abundant. The mRNAs for all of these TGF-β family members are present in the pituitary either in gonadotropes or in other pituitary cells so they may all stimulate FSHβ transcription by autocrine and/or paracrine methods.

Although activin B can be induced by GnRH under certain defined conditions, it is not known whether this induction is central to FSH regulation *in vivo*. It is possible that these TGF-β inducers of FSHβ transcription are expressed in a constitutive manner to provide constant stimulation for FSH production or, perhaps, certain family members are regulated specifically to control FSH expression at discrete periods of time. This is an area of research that is ripe for exploration.

## C. Inhibition of FSH

Inhibition of FSHβ transcription is accomplished by follistatin and inhibins. Follistatin is a molecule that binds and bioneutralizes activins and BMPs. Although it is produced at many locations in the body, it is secreted locally in the pituitary and is thought to work primarily in this local environment to control expression of FSH. Follistatin can be induced by GnRH under certain defined conditions and in certain species, but it is not certain how crucial this regulation is to overall control of FSH *in vivo*.

Inhibins A and B are α/β heterodimers of the TGF-β family that are strong inhibitors of FSHβ transcription (most TGF-β family members are like activins or BMPs, which are β/β homodimers or β/β heterodimers). The inhibins appear to bind their own receptors, which block FSH production by interfering with activin receptors. Inhibins are produced primarily in the ovaries and testes and exert considerable negative control over FSH production in the pituitary. Castration or ovariectomy leads to a large increase in FSH, which is generally attributed to the cessation of gonadal inhibin secretion. It is not clear, however, how much influence gonadal inhibins have over day-to-day regulation of FSH since there is little convincing evidence to show that blood levels fluctuate enough to cause the changes observed in Fig. 6.

## D. Gonadal Steroids

Finally, gonadal steroids such as estradiol and progesterone alter production of FSH, but they generally act indirectly by changing GnRH secretion during the estrous period of the female cycle. It is clear that estrogens and progestins can act directly at the promoter of FSHβ to inhibit its transcription, but the importance of this inhibition has not been established *in vivo* in sheep and this type of inhibition does not occur in rodents such as mice. Sometimes GnRH secretion is stimulated and sometimes it is inhibited by steroids, but this is somewhat variable among species except for the stimulation given by estrogens during the preovulatory surge of LH.

## VIII. SUMMARY

Follicle-stimulating hormone is produced in pituitary gonadotropes and acts on Sertoli and granulosa cells to ensure maturation of sperm and eggs in males and females, respectively. It can be used to hyperstimulate ovarian function to produce follicles in women who have trouble ovulating naturally for a number of reasons. Whereas FSH is absolutely required for egg production, sperm production occurs without FSH, but is stimulated significantly by it. Regulation of FSH is controlled by hypothalamic GnRH, TGF-β family members made in the pituitary and gonads, and gonadal steroids such as estrogens and progestins.

## Glossary

**corpus luteum** The progesterone-producing tissue in the ovary that is formed from dominant follicles under the influence of luteinizing hormone.

**follistatin** A molecule that binds and inactivates β/β transforming growth factor-β family members, thereby indirectly inhibiting follicle-stimulating hormone transcription.

**gonadotropes** Pituitary cells that make and secrete gonadotropins (follicle-stimulating hormone and luteinizing hormone).

**gonadotropin-releasing hormone** A hypothalamic decapeptide released in a pulsatile way that stimulates gonadotropes to produce and/or secrete follicle-stimulating hormone and luteinizing hormone.

**gonadotropins** Follicle-stimulating hormone and luteinizing hormone, which are made in the pituitary; they regulate the gonads (ovary and testis) in mammals.

**granulosa cells** Located in the ovarian follicle, these are the only female cells that respond to follicle-stimulating hormone. They nurture the developing oocyte and produce estrogens in the ovarian follicle.

**Sertoli cells** The only male cells that respond to follicle-stimulating hormone (FSH). They respond to FSH by dividing (early in life) and by supporting spermatogenesis in the adult.

**transforming growth factor-*β* (TGF-*β*) family members** β/β-type members are released from the pituitary and induce FSH; inhibitory TGF-β members (α/β type) are released by the gonads.

## See Also the Following Articles

**Corpus Luteum in Primates • Extracellular Matrix and Follicle Development • Fecundity Genes • Folliculogenesis • Gonadotropin-Releasing Hormone (GnRH) • Inhibins, Activins, and Follistatins • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Ovulation • Spermatogenesis, Hormonal Control of**

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# Folliculogenesis

Jock K. Findlay
*Prince Henry's Institute of Medical Research, Australia*

I. FOLLICULOGENESIS
II. HORMONAL CONTROL OF FOLLICULOGENESIS

**The functions of the ovary are the production of fertilizable oocytes and hormones in a timely manner. To achieve this requires a complex interplay between the growth and development of the ovarian follicles that house the oocytes and the extra- and intraovarian hormones and growth factors that regulate these processes. This article gives a description of ovarian folliculogenesis and provides an overview of the hormonal regulation of folliculogenesis in the ovarian cycle.**

## I. FOLLICULOGENESIS

The adult ovary is a multifunctional, dynamic organ with compartments that vary according to its physiological status. Folliculogenesis is the process describing the growth and development or atresia of follicles, through a series of morphological and functional stages from primordial to ovulatory. Follicles in this growth trajectory can be divided into two morphological categories based on the presence or absence of a fluid-filled cavity, or antrum, in the granulosa compartment. Preantral follicles are primordial, primary, or secondary, whereas antral follicles are either tertiary or ovulatory (also called Graafian follicles). Primordial follicles can be defined as nongrowing, with all other types being committed to growth, differentiation, and ovulation or to atresia.

Primordial follicles consist of a primary oocyte arrested in meiotic prophase surrounded by a single layer of squamous pregranulosa cells and with no basal lamina or zona pellucida. These follicles can remain in this state for long periods, up to 40 years in women, and are found principally in the stromal cortex. Although said to be "quiescent", the primordial follicles are metabolically active. Some begin a very slow transformation into an intermediate stage before becoming primary follicles. These intermediate follicles contain a mixture of squamous and cuboidal granulosa cells but exhibit no change in oocyte or follicle size. In small primary follicles, the oocytes are surrounded by a single layer of cuboidal granulosa cells confined by an outer basal lamina and separated from the oocyte by the zona pellucida. The zona layer is traversed by cytoplasmic processes of the granulosa cells that form gap junctions with the oocyte. Growth in these primary follicles is recognized by an increase in oocyte diameter and an increase in granulosa cell number. Oocyte growth is not accompanied by the resumption of meiosis, which occurs after the preovulatory surge of gonadotropins.

Secondary follicles are larger than primary follicles and consist of the primary oocyte with its zona pellucida surrounded by several layers of granulosa cells, a basal lamina, and a theca layer that forms when there are two or three granulosa cell layers. The thecal cells are recruited from interstitial stromal cells. There is a marked rise in the mitotic rate of the somatic cells and formation of the blood and lymph networks in the theca layer. The granulosa/oocyte compartment of the follicle within the basal laminar remains avascular up to the time of ovulation and relies on gap junctions between these cells to form a syncytium to transport nutrients, intercellular regulators, and metabolic waste.

The appearance of the antrum heralds the formation of the tertiary follicle. The oocyte is surrounded by cumulus cells derived from the granulosa cells. Although the mitotic rate of the granulosa and theca cells begins to decline in tertiary follicles, follicles continue to increase in size because of an increase in antral fluid volume. Functional changes in the granulosa layer finally transform the follicle to be capable of releasing an oocyte at ovulation and luteinization of the somatic cells in response to the preovulatory surge of gonadotropin.

## II. HORMONAL CONTROL OF FOLLICULOGENESIS

Primordial follicles leave the pool committed irreversibly to growth or atresia. The number making this commitment each day depends on the species and age and is small (up to 10 or 20) compared with the total number available. The mechanism by which a particular follicle is recruited for growth is not known, but no two recruited follicles will be functionally identical once they are in the growth trajectory. The commitment to leave the primordial pool is independent of the cyclic changes in the pituitary hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin. This leads to the conclusion that local mechanisms may be involved. The location of the primordial follicle within the ovary, or intrafollicular factors, perhaps originating from the oocyte, may be determining factors. Alternatively, the trigger may involve release from inhibition by some mechanism that is not yet understood. FSH may have an indirect influence through its action on larger follicles to produce paracrine factors that can modify the recruitment of primordial follicles.

Committed preantral primary and secondary follicles enter a slow tonic growth phase that is independent of gonadotropins but facilitated by them. In primary follicles, granulosa cell proliferation and atresia rates are low and are independent of the cyclic changes in pituitary hormones. Atresia is characterized by degeneration of the oocyte. The granulosa cells express FSH receptors, which facilitate proliferation through the action of FSH on cyclin D2, an essential component of the cell cycle machinery. They do not express the LH receptor (LHR), do express low levels of aromatase, and make very little estrogen. This phenotype of the granulosa cell is most likely regulated by the inhibitory actions of growth and differentiation factor 9 (GDF9) and/or bone morphogenic factor-15 (BMP-15) from the oocyte. In turn, GDF9 and/or BMP-15 production by the oocyte may be maintained in a positive feedback loop by kit ligand from the undifferentiated granulosa cells. The granulosa cells also express high levels of androgen receptor (AR) and increasing levels of estrogen receptor (ER), particularly ER-β. Theca cells form at this time and become LH responsive through the acquisition of LHR, a process that is influenced by the oocyte, most likely via GDF9. The thecal production of androgen is driven by LH, facilitated by insulin-like growth factor-I (IGF-I) and inhibin, and inhibited by activin, all granulosa cell paracrine regulators. Thecal androgen production, coupled with the low levels of aromatase in the granulosa cells, results in a high androgen to estrogen ratio in these follicles. Androgen action via the AR on granulosa cells facilitates their slow proliferation and eventually FSH-induced differentiation in late preantral follicles.

As the preantral follicles expand through the proliferation of granulosa cells, it is hypothesized that there is a gradient of influence of the oocyte on the granulosa cells by paracrine factors such as GDF9 and BMP-15. As a result, the more distal, mural granulosa cells of secondary follicles are under less influence from the oocyte and become more differentiated by acquiring more ER and IGF-I receptors. They also respond to FSH by increasing aromatase activity, thus increasing their capacity to produce 17β-estradiol (E2) from thecal androgen. This marks the shift from FSH independence to FSH dependence and coincides with the appearance of the antrum and the transformation to tertiary follicles. These follicles now require FSH to maintain their growth and viability or they succumb to atresia. The balance between growth and atresia is at the mercy of cyclical fluctuations in FSH and LH in the blood. There is also differentiation of the granulosa population into three layers related to the proximity to the oocyte. The layer closest to the oocyte comprises the cumulus cells, which remain under the influence of the oocyte until the time of ovulation, and has a phenotype distinct from the antral and mural layers. The mural layer closest to the basement membrane has the highest levels of expression of steroidogenic enzymes and gonadotropin receptors.

The follicle(s) selected for ovulation comes from these early antral, FSH-dependent tertiary follicles. Selection or cyclic recruitment is accomplished through a timely rise in the circulating levels of FSH such as occurs at menstruation in women or after exogenous gonadotropin treatment. This results in an increase in the mitotic index of the granulosa cells and increased capacity of the follicle to synthesize E2 due to an increase in the FSH-dependent aromatase activity. There is also an enhanced supply of androgen substrate as theca cells respond with increased sensitivity to LH. The androgen to estrogen ratio is now in favor of E2. Inhibin B production by the granulosa cells also increases in response to the rise in FSH. Inhibin B and E2 suppress the circulating levels of FSH below the threshold necessary to maintain viability of the remaining antral follicles, thereby establishing dominance. The dominance established over the other nonselected follicles persists until after ovulation when the corpus luteum is formed. Dominance is maintained by the selected follicle in the face of falling levels of FSH, through three mechanisms. The first is due to the actions of paracrine and autocrine regulators in the dominant follicle such as IGF-I, activin, transforming growth factor-β, and E2, which sensitize the follicular cells to the gonadotropins. The second mechanism is the acquisition of LHR on the granulosa cells so that they are driven by the increasing frequency of LH pulses as ovulation approaches, rather than depending on the falling levels of FSH. The third is the suppression of FSH levels by E2 and inhibin B, and subsequently by inhibin A, produced by the dominant follicle.

A short-loop positive feedback system operating within the dominant or Graafian follicle drives E2 production to a level sufficient to trigger the preovulatory gonadotropin surge by the pituitary

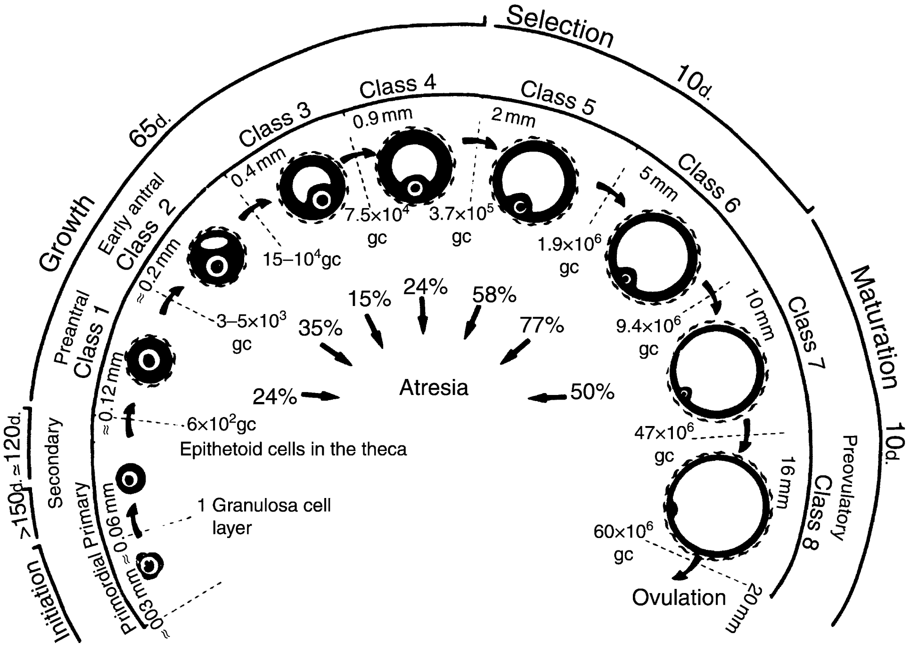
Selection
10d.
Growth
65d.
Maturation
10d.
Initiation
>150d. ≈120d.
Secondary
Primordial Primary
Preantral Class 1
Early antral Class 2
Class 3
Class 4
Class 5
Class 6
Class 7
Preovulatory Class 8
≈0.03 mm
≈0.06 mm
≈0.12 mm
≈0.2 mm
0.4 mm
0.9 mm
2 mm
5 mm
10 mm
16 mm
20 mm
1 Granulosa cell layer
$6\times10^2$gc
Epithetoid cells in the theca
3–$5\times10^3$ gc
15–$10^4$gc
$7.5\times10^4$ gc
$3.7\times10^5$ gc
$1.9\times10^6$ gc
$9.4\times10^6$ gc
$47\times10^6$ gc
$60\times10^6$ gc
Ovulation
24%
35%
15%
24%
58%
77%
50%
Atresia

FIGURE 1 Classification of follicles in the human ovary. Reprinted from Gougeon, A. (1986). Dynamics of follicular growth in the human: A model from preliminary results. *Hum. Reprod.* 1, 1–87, by permission of Oxford University Press.

gland and ovulation ensues. The LH surge triggers the resumption of meiosis of the oocyte, the expansion of the cumulus cells, and the cascade of inflammatory-like processes in the follicle wall to allow release of the oocyte–cumulus complex. It also triggers the onset of luteinization of the granulosa and theca cells and the formation of the corpus luteum.

Less than 1% of follicles that leave the primordial pool ever ovulate and it takes them 320 days to reach that point in the human ovary (Fig. 1). It is only during the last 15 days that the dominant follicle is gonadotropin-dependent. Intrafollicular regulators from the oocyte and somatic cells play a very important local role throughout folliculogenesis.

## Acknowledgments

The author acknowledges the assistance of Sue Panckridge with the figures and the National Health and Medical Research Council of Australia (RegKeys Nos. 983212, 241000, and 198705) for financial support.

## Glossary

**antral follicles** Are either tertiary or ovulatory, also called Graafian follicles, and are characterized by the presence of a fluid-filled antrum in the granulosa layer.

**atresia** The process whereby oocytes are lost from the ovary other than by ovulation.

**cumulus cells** The layer of granulosa cells surrounding the oocyte that make up the cumulus oophorus.

**dominance** The status of the follicle destined to ovulate, which has a regulatory influence over other antral follicles.

**follicle** Consists of a germ cell or oocyte surrounded by somatic cells and is the fundamental reproductive element of the ovary.

**folliculogenesis** The growth and development or atresia of follicles, through a series of morphological and functional stages from primordial to ovulatory.

**gonadotropins** The glycoprotein hormones follicle-stimulating hormone and luteinizing hormone, which are secreted by the pituitary gland and which stimulate ovarian cells.

**granulosa cells** Hormone-producing somatic cells that surround the oocyte and become differentiated into cumulus cells, which are closest to the oocyte, and antral and mural cells.

**local regulation** Autocrine, paracrine, or juxtacrine regulation of cells by growth factors or cytokines made locally within a tissue.

**preantral follicles** Are primordial, primary, or secondary, depending on the size of the oocyte, the number and type of granulosa cells, and the presence of theca cells.

**primary oocyte** An oocyte in the process of undergoing the first meiotic division, arrested in meiotic prophase.

**recruitment** The process whereby a primordial follicle leaves the resting pool and becomes committed to growth, development, and ovulation or instead undergoes atresia.

**selection** The process whereby an antral follicle(s) is saved from atresia by a rise in follicle-stimulating hormone and develops as the dominant follicle.

**steroidogenic enzymes** A family of enzymes, many of which belong to the P450 gene family, that catalyze the synthesis and metabolism of the sex steroid hormones, estrogens, androgens, and progestogens.

**theca cells** Hormone-producing somatic cells differentiated into a vascularized interna and externa layer, recruited from the stroma to lie around the basal lamina outside the granulosa layer.

**zona pellucida** A layer of highly glycosylated proteins regulated by the oocyte Fig α gene; it forms the unique surface coat of the oocyte and is traversed by extensions of cumulus cells that form gap junctions with the oocyte.

## See Also the Following Articles

**Extracellular Matrix and Follicle Development • Fecundity Genes • Follicle-Stimulating Hormone (FSH) • Folliculogenesis, Early • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Prolactin (PRL)**

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# Folliculogenesis, Early

Ursula A. Vitt and Aaron J. W. Hsueh
*Stanford University School of Medicine*

I. INTRODUCTION
II. PRIMORDIAL FOLLICLE DEVELOPMENT
III. PRIMARY FOLLICLE GROWTH
IV. PREANTRAL FOLLICLE GROWTH
V. SUMMARY

**Early folliculogenesis is the process by which ovarian follicles grow and develop from the primordial stage to the point at which the antral cavity is formed. This development is controlled by a number of paracrine factors that are derived from all three compartments of the follicle—the oocyte, granulosa cells, and theca cells.**

## I. INTRODUCTION

The development of the mammalian ovary is characterized by the division and differentiation of primordial germ cells into oogonia and the subsequent endowment of a fixed number of primordial follicles. This pool of primordial follicles is gradually depleted during reproductive life. The follicles develop through primordial, primary, and preantral stages before acquiring an antral cavity (see Fig. 1). Some primordial follicles begin to grow as soon as they are formed but most enter a state of suspended animation. Primordial follicles are characterized by the centrally located oocyte surrounded only by a thin layer of flattened granulosa cells. The primary follicle has cuboidal-shaped granulosa cells that proliferate during the formation of preantral follicles concomitant with an increase in oocyte size. Once follicles reach the small antral stage, most of them undergo atresia unless rescued by follicle-stimulating hormone (FSH). Under the influence of gonadotropins, the antrum is formed and the selected antral follicles further increase in size until they reach the preovulatory stage. Early folliculogenesis denotes the development of follicles up to the time of antral formation and has to date been believed to be largely independent of the influence of endocrine hormones and regulated mainly by paracrine factors. All three compartments of ovarian follicles (oocyte, granulosa cells, and theca cells) have been shown to produce paracrine hormones determining the development of the other cellular compartments. This article examines the role of paracrine factors expressed during early follicle stages whose function is at least partially understood. A summary of these factors and the follicle stages affected is given in Table 1.

## II. PRIMORDIAL FOLLICLE DEVELOPMENT

### A. Formation and Maintenance of the Primordial Follicle Pool

The processes that lead to the initial endowment of a fixed number of primordial follicles at the onset of growth, development, and ovulation or instead undergoes atresia.

**selection** The process whereby an antral follicle(s) is saved from atresia by a rise in follicle-stimulating hormone and develops as the dominant follicle.

**steroidogenic enzymes** A family of enzymes, many of which belong to the P450 gene family, that catalyze the synthesis and metabolism of the sex steroid hormones, estrogens, androgens, and progestogens.

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## II. PRIMORDIAL FOLLICLE DEVELOPMENT

### A. Formation and Maintenance of the Primordial Follicle Pool

The processes that lead to the initial endowment of a fixed number of primordial follicles at the onset of

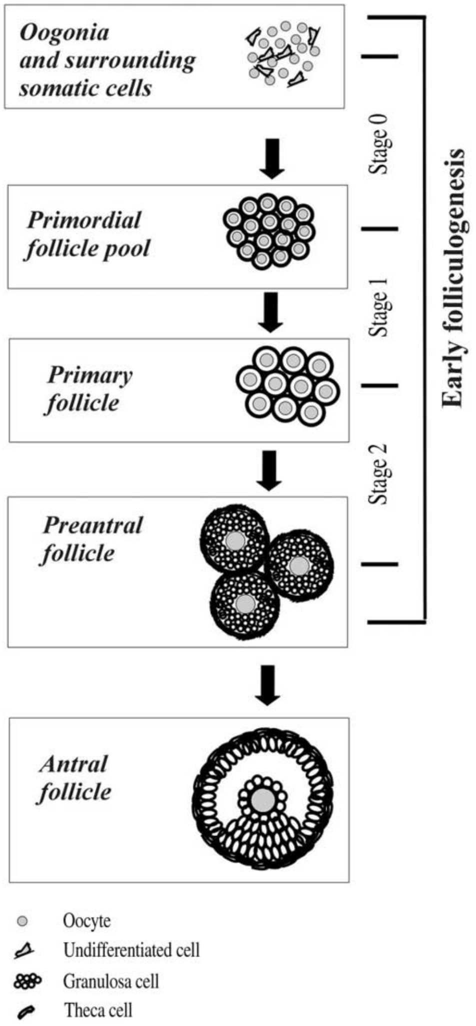

**FIGURE 1** Folliculogenesis starts with the endowment of primordial follicles by the association of oocytes with surrounding somatic cells. The formed primordial follicles grow and develop through primary and preantral stages characterized by granulosa cell and theca cell proliferation concomitant with oocyte growth.

folliculogenesis are poorly understood. Of several paracrine factors that have been found to be expressed during follicular endowment, only nerve growth factor (NGF) has been shown to be essential for the incorporation of oocytes into follicular structures. This is revealed in the phenotypes of NGF null mice, which have a small number of follicles due to a reduction of somatic cell proliferation associated with follicle formation. NGF is secreted by the oocyte and thus represents a control of somatic cell proliferation by the germ cell before the formation of the primordial follicle.

In addition to NGF, other paracrine factors that have been found to be expressed during follicle endowment include Kit ligand (KL) and transforming growth factor-β (TGF-β). KL (also known as stem cell factor) is expressed during follicle endowment and is believed to play a key role in the maintenance of the primordial follicle pool. KL is expressed in a soluble form and a membrane-bound form by somatic cells and signals through the c-kit receptor, a proto-oncogene expressed primarily by the oocyte. Even though KL has been shown to be essential for primordial germ cell migration in some mice with KL mutations, it is not an essential factor for primordial follicle endowment as follicle development can progress up to primary stages in mice lacking KL. The role of TGF-β in primordial follicle formation is unknown as TGF-β1 null mice are embryonic lethal.

**TABLE 1** Factors Involved in Early Folliculogenesis

| | | Stage[b] | | |
|---|---|---|---|---|
| **Ligand** | **Expression cell type**[a] | **0** | **1** | **2** |
| NGF | Stroma | XS[c] | | |
| Angiogenin | GC | XS | XS | XS |
| TGF-β1 | GC | XS | XS | XS |
| Kit ligand | GC | XU[d] | XS | XS |
| AMH | GC | XU | XS | XS |
| IGFI | GC/TC | XU | XU | XS |
| PTHRP | Oo, GC, TC | XU | XU | XU |
| GDF9 | Oo | | XS | XS |
| GDF9B | Oc | | XS | XS |
| Activin | GC | | XS | XS |
| TGF-β2 | GC/TC | | XU | XS |
| EGF | GC | | XU | XS |
| Estrogen | GC | | XU | XS |
| Progesterone | GC | | | XS |
| IGFII | GC/TC | | | XS |
| HGF | TC | | | XS |
| KGF | TC | | | |
| FGF8 | Oo | | | XS |
| FGF2 | GC/TC | | | XS |
| Inhibin | GC | | | XS |
| FSH | Endocrine | | | XS |
| LH | Endocrine | | | XS |
| GH | Endocrine | | | XS |
| NT3 | ? | | | XU |
| GMCSF | Oo, GC, TC | | | XU |
| POMC | GC | | | XU |
| Thymosin | GC | | | XU |

[a] Oo, oocytes; GC, granulosa cells; TC, theca cells.

[b] Stage 0, primordial follicle; Stage 1, primary follicle; Stage 2, preantral follicle.

[c] Expressed and function studied.

[d] Expressed but function unclear.

Its function in later follicular stages is the enhancement of mitogenic effects induced by other factors such as FSH, which stands in contrast to the antimitogenic effects of TGF-β in other cell types.

Once the primordial follicle pool is established, a gradual depletion of this pool occurs throughout life. Several factors have been described to control, directly or indirectly, this depletion. In addition to the protective action of KL, both leukemia inhibitory factor (LIF) and insulin-like growth factor-I (IGF-I) have additive effects on the preservation of primordial follicles via inhibition of oocyte apoptosis. LIF is a pleiotropic cytokine of the interleukin-6 family and was named for its ability to inhibit the proliferation of a myeloid leukemic cell line. To date, its function in the primordial follicle other than inhibition of apoptosis is unknown but it represents, like KL, a germ cell protection signal derived from somatic cells. Similarly, the protective effect of granulosa cell-derived IGF-I is transmitted to IGF-I receptors on the oocyte.

Another factor involved in the maintenance of primordial follicles is anti-Müllerian hormone (AMH). The specific cellular function of AMH in primordial follicles is unknown but the phenotype of AMH null mice shows an increase in primordial follicle recruitment leading to early depletion of the primordial follicle pool. As both the AMH ligand and its receptor are expressed in somatic cells, it represents an autocrine as well as a paracrine control mechanism of primordial follicle preservation. Furthermore, factors such as parathyroid hormone-related hormone (PTHRP) are present in both oocytes and granulosa cells of primordial follicles and are known to have an anti-proliferative effect but neither their specific function in primordial folliculogenesis nor their mutant phenotypes are known.

## B. Initiation of Primordial Follicle Growth

To date, the mechanisms leading to the initiation of primordial follicle growth are unknown. Some authors believe that some primordial follicles start to grow as soon as they are formed and that a constant feedback loop between growing follicles and nongrowing follicles determines the number of follicles starting to grow at any given point in time. One of the main changes occurring during the transition from the primordial to the primary follicle stage is the formation of an outer layer of theca cells. Thus, the association of the germ cell with epithelial granulosa cells in the primordial follicle is followed by the interaction of the follicular structure with the surrounding mesenchyme. Paracrine factors such as activin A, which interacts with extracellular matrix proteins, could play a key role in this interaction and have a stimulatory effect on the initiation of follicle growth. In addition, theca cell differentiation has been shown to be controlled by KL, which promotes primordial follicular growth.

Furthermore, other factors such as growth differentiation factor 9 (GDF9) have been shown to lead to a decrease in the number of primordial follicles and a subsequent increase in the number of growing follicles, suggesting that they might play a role in the initiation of primordial follicle growth. In all studies completed thus far, however, the increase in follicle progression to preantral and larger follicles might lead to an enhancement of primordial follicle growth, and thus, these results may be secondary and not represent a direct action of a given factor on primordial follicle growth. This is especially true for GDF9 as its expression in primordial follicles is unknown. In contrast to stimulatory factors, changes in the secretion of growth-inhibiting factors could also affect the initiation of primordial follicle growth.

## III. PRIMARY FOLLICLE GROWTH

After initiation of growth, primordial follicles progress to primary follicles with distinct layers of granulosa and theca cells closely associated with the centrally located oocyte. The growth of the primary follicle is characterized by the proliferation of both granulosa and theca cells, leading to the formation of the preantral follicle with several layers of both cell types. Several of the paracrine factors mentioned above that affect primordial follicle growth are also expressed during this stage and could control primary follicle growth.

GDF9 plays a key role in the control of primary follicle growth as reflected by the infertile phenotype of GDF9 null mice. Ovaries lacking GDF9 exhibit a complete block in follicle development at the primary stage. Thus, the oocyte largely controls primary follicle growth via secretion of GDF9, which has been shown to be a potent mitogenic agent of granulosa cells. In addition to GDF9, GDF9B, its closest paralogue, is co-expressed with GDF9 by the oocyte. The phenotype of homozygous GDF9B mutant Inverdale sheep revealed striking similarities with GDF9 mutant mice. This observation and the fact that GDF9B stimulates granulosa cell proliferation *in vitro* indicate that GDF9B shares functional similarities with GDF9 even though primary follicle growth is largely unaffected in GDF9B mutant mice. The species differences in GDF9B function are of interest as GDF9B could represent a potential factor leading to the vast differences in ovarian follicle development between different species.

At the primary follicle stage, KL also has essential effects on follicle growth. The naturally occurring Steel Panda mutant mice as well as KL null mutant mice display ovarian phenotypes similar to that of GDF9 null mice. At this stage of folliculogenesis, receptors for KL are present on the oocyte as well as on theca cells. Thus, primary follicle growth is controlled by oocyte-derived GDF9 as well as by granulosa cell-derived KL, which controls both oocyte and theca cell development.

Furthermore, TGF-β and IGF-I as well as their receptors are expressed in primary follicles. Their influence on primary follicle development is, however, not well studied. The same applies to angiogenin, a potent mediator of blood vessel formation, which is expressed in granulosa cells of primary follicles. Even though blood vessel formation in the ovary is an essential requirement for folliculogenesis, the specific roles of angiogenin in follicle growth are unclear.

## IV. PREANTRAL FOLLICLE GROWTH

As more layers of somatic cells surround the oocyte, mainly as the result of granulosa cell proliferation, the preantral follicle is formed. Most of the paracrine factors involved in primordial and primary growth continue to be secreted and could influence preantral follicle development. In addition, a large number of other paracrine factors were found to be expressed during preantral follicle development. This increased number of secreted factors could be due to the fact that preantral follicle stages are easier to retrieve and a much larger number of studies were performed using preantral and larger follicles than were performed using follicles from earlier stages.

The first step of preantral follicle development includes mainly granulosa cell proliferation promoted by mitogenic agents. Again, GDF9 and KL have been shown to promote follicle growth at this stage. Another mitogenic agent, epidermal growth factor (EGF), which is also expressed in primary follicles, stimulates preantral follicle growth. GDF9 enhances the number of growing preantral follicles, probably due to its proliferative action. In contrast, KL and EGF contribute to the growth of the preantral follicle via suppression of apoptosis. Furthermore, KL induces theca cell proliferation and secretion of hepatocyte growth factor (HGF). The latter, via stimulation of KL expression, leads to a positive feedback loop that promotes the growth of both granulosa cells and theca cells. Thus, in addition to the ongoing paracrine communication between granulosa cells and the oocyte, another level of communication is introduced by further growth and development of theca cells. *In vitro* studies suggest that the two somatic cell types modulate each other by affecting proliferation, differentiation, and responsiveness to gonadotropins in a reciprocal manner. The example of KL and HGF mentioned above is only one of these paracrine interactions. Another very well-studied interaction is the theca cell-derived secretion of androgens, which promote granulosa cell responsiveness to FSH and serve as substrates for the estrogen-producing granulosa cells.

In addition to several paracrine factors present in the preantral follicle, inhibins, activins, estradiol, and progesterone represent the most important hormones secreted by the ovary and are responsible for both endocrine feedback regulation of the anterior pituitary and paracrine regulation of local ovarian functions. FSH stimulates inhibin secretion by granulosa cells, and inhibins, in turn, suppress FSH secretion by the pituitary, thus forming a negative feedback loop. Furthermore, inhibin null mice display sex cord stromal tumors, showing that inhibins function as tumor suppressors. In contrast, activins stimulate the release of FSH from the pituitary. In addition, activins are the first paracrine factors secreted by preantral follicles that have been reported to suppress primary follicle growth.

Ovarian estrogens suppress pituitary gonadotropin secretion. In addition to endocrine effects, estrogens are autocrine factors because estrogen receptors are localized in granulosa cells and treatment with estrogens has been shown to promote follicle growth and granulosa cell differentiation.

In addition to ovarian secretion of endocrine hormones, pituitary control of preantral follicle development is established by the synergistic effects of FSH and luteinizing hormone (LH) on preantral follicle growth. Although the best known effect of FSH is its stimulation of estradiol production by granulosa cells, the main function of FSH in the preantral follicle is to drive the formation of the antrum. The growth-promoting effect of FSH is probably due to its differentiative action and subsequent follicle antrum formation. Concomitant with the formation of the antral cavity and thus termination of preantral follicle growth is the differentiation of two subsets of granulosa cells. The mural granulosa cells have a higher level of LH receptor expression and a greater steroidogenic capacity than the cumulus granulosa cells located adjacent to the oocyte. GDF9, secreted by the oocyte, is known to inhibit FSH-induced differentiation. A gradient of oocyte-derived GDF9 in combination with FSH has been reported to support the formation of these two subsets of granulosa cells.

Several paracrine factors secreted by granulosa cells in the follicle modulate the effects of FSH. Both IGFs and fibroblast growth factors are believed to modulate FSH-induced steroidogenesis. Many more factors have been described to be present in the preantral follicle, such as propioomelanocortin and granulocyte/macrophage colony-stimulating factor. However, their specific functions in preantral follicle development are not well studied.

## V. SUMMARY

The early development of the ovarian follicle is controlled by a variety of paracrine factors that are derived from all three compartments of the follicle. The secretion of growth-promoting and unidentified inhibiting factors by the oocyte is essential for the onset of follicle growth and different stages of follicle development. However, the oocyte itself is again influenced by paracrine factors secreted by surrounding granulosa cells. The granulosa cells communicate with feedback loops with the outer theca cells and both cell types are controlled by pituitary-derived hormones. During the early stages of ovarian development, folliculogenesis can commence without pituitary control. At later stages, granulosa cells and the anterior pituitary communicate via different hormonal feedback loops to ensure successful follicle development.

## Glossary

**antrum** Extracellular matrix-containing cavity that divides the oocyte from granulosa cells in the antral follicle.

**apoptosis** Programmed cell death.

**atresia** Follicle degeneration.

**endowment** The first step of follicle formation in which the oocyte is associated with surrounding somatic cells to form a primordial follicle.

**folliculogenesis** The process by which follicles grow and mature from primordial to preovulatory stages.

**preantral follicle** Oocyte surrounded by several layers of granulosa and theca cells. Includes all growing follicle stages after the primary follicle stage up to the formation of the antrum.

**primary follicle** Oocyte surrounded by one clearly visible cuboidal-shaped layer of granulosa cells and a thin layer of theca cells.

**primordial follicle** First stage of follicle development, consisting of the oocyte surrounded by a thin layer of granulosa cells.

**steroidogenesis** The production and secretion of steroids (in the ovary, mainly progesterone, androstenedione, and estradiol) by differentiated somatic cells.

## See Also the Following Articles

**Anti-Müllerian Hormone • Apoptosis • Extracellular Matrix and Follicle Development • Fecundity Genes • Follicle-Stimulating Hormone (FSH) • Folliculogenesis • Luteinizing Hormone (LH) • Nerve Growth Factor (NGF) • Oocyte Development and Maturation • Ovulation**

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# Follistatins

See *Inhibins, Activins, and Follistatins*

# Follitropin (Follicle-Stimulating Hormone) Receptor Signaling

James A. Dias
*New York State Department of Health and State University of New York*

I. INTRODUCTION
II. FOLLITROPIN
III. FOLLITROPIN RECEPTOR
IV. FOLLITROPIN RECEPTOR SIGNALING
V. FOLLITROPIN SIGNAL ATTENUATION
VI. GENETIC ABNORMALITIES

**Gonadotrope cells of the anterior pituitary produce follicle-stimulating hormone (follitropin) in response to tropic signals from the hypothalamus and the gonads. Follitropin is a glycoprotein hormone. All of the glycoprotein hormones have an α-subunit that is identical in primary sequence but that may vary in the extent of glycosylation. The specificity of binding is conveyed by the β-subunit, which is unique for each hormone.**

## I. INTRODUCTION

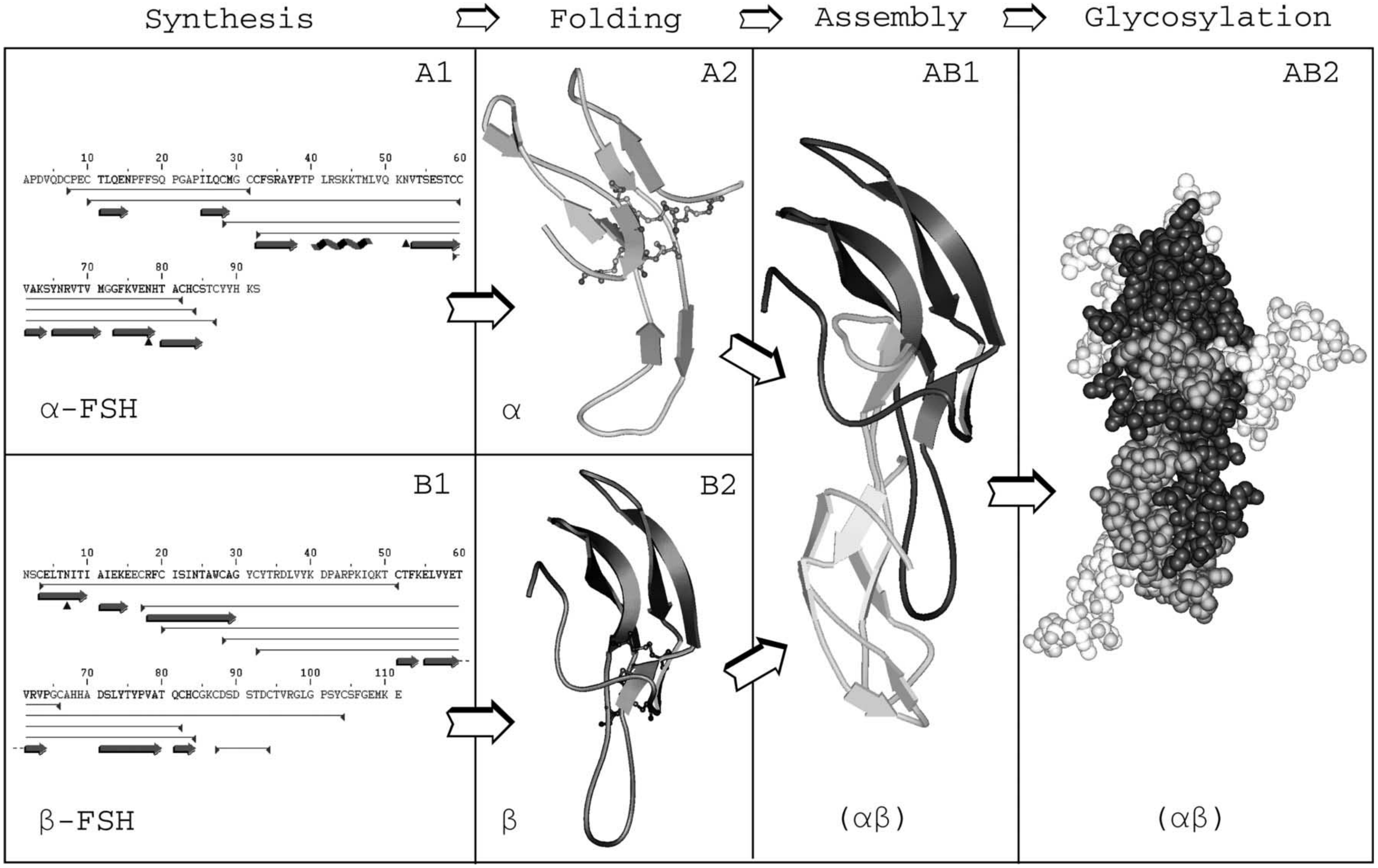
Secretion of follitropin into the blood is under control of the hypothalamus. Hypothalamic neurons release gonadotropin-releasing hormone into the portal system, and—despite its small size (decapeptide) and rapid clearance kinetics—the releasing hormone reaches high concentrations in the pituitary portal system due to a retrograde blood flow. Gonadal (testicular or ovarian) steroids also regulate the expression of gonadotropin genes in gonadotropes. Estrogens can up-regulate secretion and testosterone and progesterone can decrease expression. Inhibins and activins, nonsteroidal hormones produced by the gonads, also control follitropin secretion. Inhibin and activin can suppress or increase, respectively, gonadotropin secretion.

When follitropin is secreted into the blood from anterior pituitary gonadotropes, it binds to and activates follitropin receptors in the Sertoli cells of the testis or the granulosa cells of the ovary. In females, development of ovarian follicles and production of mature viable oocytes are thus under control of follitropin. In the absence of follitropin signaling, ovarian follicular development is arrested at a stage prior to antrum formation. In males, follitropin is essential for maintaining the production of many high-quality gametes. The following discussions describe the molecular mechanisms underlying formation of the follitropin–follitropin receptor complex, as well as how binding leads to the activation process and how the activation process is regulated.

## II. FOLLITROPIN

The pituitary glycoprotein hormone follitropin consists of two subunits, an α-subunit of 92 amino acids and a β-subunit of 114 amino acids (see Fig. 1). Each subunit is a product of a different gene from different chromosomes. There are four glycoprotein hormones, and each of the four β-subunits of these glycoprotein hormones is from a unique gene. Thus, there are four β-subunit genes, but there is only one α-subunit gene. Following synthesis on the ribosome and translocation into the endoplasmic reticulum, each subunit folds into domains (see schematic in Fig. 1). The α-subunit folds much more rapidly than the β-subunit and may serve as a scaffold on which the β-subunit is assembled. Each subunit has the same tertiary conformation, although the subunits differ in primary sequence. This conformation, or protein fold, is characteristic of a family of proteins called cystine knot proteins. Cystine knot proteins have many different functions despite a similar pattern of folding. One additional difference between cystine knot proteins is that some are heterodimeric, like follitropin, and others are homodimeric. In order for follitropin to have activity, it must form the heterodimer. In the endoplasmic reticulum, disulfide bonds of the cystine knot are formed within the α- and β-subunits and then follitropin is assembled by a combination of the two subunits (Fig. 1).

Depending on the species, each follitropin subunit has one or two sites that allow a process of carbohydrate attachment (glycosylation) that is linked to asparagine residues (Fig. 1, panel AB2). The signal for asparagine-linked glycosylation in proteins can be predicted if the primary sequence contains the motif Asn-X-Ser/Thr, where X can be any amino acid. Folding, assembly, secretion, and circulatory half-life of follitropin are affected by glycosylation of the subunits. In the blood, many glycosylation variants of follitropin exist, all having the same primary sequence but with different degrees of carbohydrate complexity. The physiological and analytical significance of this heterogeneity


Synthesis
Folding
Assembly
Glycosylation
A1
10 20 30 40 50 60
APDVQDCPEC TLQENPFFSQ PGAPILQCMG CCFSRAYPTP LRSKKTMLVQ KNVTSESTCC
70 80 90
VAKSYNRVTV MGGFKVENHT ACHCSTCYYH KS
α-FSH
B1
10 20 30 40 50 60
NSCELTNITI AIEKEECRFC ISINTAWCAG YCYTRDLVYK DPARPKIQKT CTFKELVYET
70 80 90 100 110
VRVPGCAHHA DSLYTYPVAT QCHCGKCDSD STDCTVRGLG PSYCSFGEMK E
β-FSH
A2
α
B2
β
AB1
(αβ)
AB2
(αβ)

is considerable. Assembly of the subunits into a heterodimeric protein in the endoplasmic reticulumx is necessary for proper glycosylation and full activity.

The biological activity of any hormone is usually measured in a bioassay. Historically, bioassays were carried out by measuring effects of hormone treatments in animals (*in vivo*). Now, bioassays are commonly carried out in the laboratory using isolated cells or cellular components (*in vitro*). However, because of considerations of metabolic clearance, final activity of any hormone must still be measured *in vivo*. The activity of follitropin is measured by its ability to bind to the follitropin receptor and to induce signal transduction *in vitro*, and by its ability to stimulate ovarian weight gain *in vivo*. Follitropin levels are measured in blood using sensitive immunoassays. Generally, the quality of an immunoassay is measured by the ratio of bioassay activity and immunoactivity (B/I ratio; optimum ratio = 1.0). Because follitropin is glycosylated and has many glycosylation variants (glycoforms) in blood, the B/I ratios of follitropin change with various physiological states, thus making its measurement problematic.

Incomplete glycosylation of follitropin can lead to altered clearance kinetics. It has been noted that desialylated follitropin is cleared more rapidly from the blood than is native follitropin. Because the glycoform profile can change in blood depending on physiological state as well as during pharmacological intervention, it has been of interest to determine what role carbohydrate plays in follitropin function, beyond prolonging clearance. In this regard, it has been noted that removal of carbohydrate from follitropin α-subunit site N76 has dramatic effects on the thermal stability of the hormone, which could affect its storage properties. Importantly, removal of carbohydrate from site N52 results in increased affinity of binding to receptor with a concomitant decrease in signal transduction. The etiology of these effects likely rests in the stabilizing effect that carbohydrate has on protein.

Because only heterodimeric follitropin is active in the body, it has been recognized for some time that both subunits must participate in formation of the active complex with the follitropin receptor. Notably, two residues, which are conserved in follitropin from humans to fish, have been found to be essential for follitropin binding to its receptor. These are Asp-93 on the β-subunit and Lys-52 on the α-subunit. The crystal structure of human follitropin shows that a distance of about 6 Å separates the side chains of these two residues. In addition, several residues in the carboxyl-terminal tail of the α-subunit, juxtaposed with these two essential residues, are also critical for high-affinity binding. Thus, the most critical residues, which are essential for follitropin binding to its receptor, are brought together not only by protein folding but also by the assembly of the two subunits. However, until the crystal structure of the follitropin–follitropin receptor complex is determined, it cannot be predicted how many other residues contribute in important, yet not critical, ways to formation of the stable complex.

In summary, follitropin is modular, with each subunit folding into higher order domains to form a tertiary structure, and finally associating, one α-subunit and one β-subunit, to form an active principal. The most critical residues for binding to receptor are brought together in three-dimensional space as a result of the association of the two subunits. The formation of this glycosylated, active complex, which contains 206 amino acids and four glycosylation sites and has a molecular mass in excess of 35,000 Da, and its secretion into the blood are the first steps in follitropin action. Contact with the follitropin receptor is the next step.

## III. FOLLITROPIN RECEPTOR

Follitropin receptor is a single-subunit protein of 678 amino acids. Unlike follitropin, which is composed of two subunits, the follitropin receptor is a single polypeptide chain. However, the receptor can be

**FIGURE 1** Biosynthesis of follitropin. The primary structure of human follitropin (follicle-stimulating hormone) subunits, α-FSH (A1) and β-FSH (B1), predisposes a set of secondary structure elements, β-sheets (indicated by arrows). Following synthesis and transfer across the endoplasmic reticulum, each subunit folds into a tertiary structure and disulfide bonds form (indicated by ball-and-stick models in A2 and B2). Assembly is required to form the active heterodimeric protein (AB1; for clarity, the β-subunit is darker). During assembly, FSH is glycosylated [AB2; carbohydrate shown in the space-filling model in Corey–Pauling–Kultun (CPK) colors], thereby prolonging its half-life in blood (for an explanation of CPK colors, see, e.g., http://beta.rcsb.org/pdb/pe/shared/cpk-rgb.htm). All structural models prepared using Web Lab Viewer Pro, M.S.I., Inc.

thought of as a modular protein with several domains. For example, it contains a large extracellular domain that is necessary and sufficient to bind heterodimeric follitropin. The extracellular domain is 345 amino acids, roughly 1.5 times the size of follitropin heterodimer. The extracellular domain is also glycosylated. A second module of the receptor, the transmembrane domain, is composed of 273 amino acids. This part of the receptor is predicted to traverse the membrane seven times, giving rise to a heptahelical module that is the signal transducer of the receptor. An interesting aspect of this second module is that it forms three small extracellular loops that are 18, 20, and 11 amino acids long and three small intracellular loops that are 9, 20, and 24 amino acids long. These loops are likely to play important roles in the consummation of a signal transduction event following hormone binding. Finally, a third domain of the receptor is its cytoplasmic tail, which is composed of 65 amino acids. One can readily appreciate that each module of the receptor has complexity, and that the environment surrounding each module will play a key role in its functionality, especially with regard to signal transduction.

## IV. FOLLITROPIN RECEPTOR SIGNALING

It is generally accepted that follitropin signals through the follitropin receptor via a guanosine nucleotide binding protein (G-protein)-coupled pathway. The G-proteins are heterotrimers that bind to the cytoplasmic domains of G-protein-coupled receptors. On activation, the G-proteins bind guanosine triphosphate (GTP), and then they dissociate into an $\alpha$-subunit ($G_{s\alpha}$) and a stable heterodimeric $\beta\gamma$ subunit complex ($G_{\beta\gamma}$). The $G_{s\alpha}$ subunit binds to and activates adenylate cyclase. There are 12 different forms of $G_{\alpha}$. Some are stimulatory ($G_{s\alpha}$), some are inhibitory ($G_{i\alpha}$), and others have specific ion-channel or enzymatic activation properties. The $G_{\beta\gamma}$ subunit binds to and activates phospholipase C, which in turn hydrolyzes phospholipids to inositol triphosphates and prostaglandin.

Follitropin activates the adenylate cyclase pathway indirectly by acting as a guanine nucleotide exchange factor. When follitropin binds to its receptor, a conformation of the receptor is stabilized, which predisposes the stimulatory subunit of heterotrimeric G-protein to release guanosine diphosphate (GDP) and to bind GTP. The binding of GTP to the G-protein $\alpha$-subunit causes dissociation of the G-protein $\alpha$-subunit from the G-protein $\beta\gamma$ -subunit complex. The G-protein $\alpha$-subunit can now activate adenylate cyclase. Adenylate cyclase is thus activated by the G-protein $\alpha$-subunit, which has guanosine triphosphate bound. The activation of adenylate cyclase leads to catalysis of adenosine triphosphate (ATP) to form cyclic adenosine monophosphate (cAMP).

An elevation of intracellular cAMP leads to activation of protein kinase A. This kinase thereby regulates the activity of its substrate proteins. Protein kinase A is activated by the binding of cAMP to its regulatory subunit. The activation of this kinase leads to pleiotropic effects. Most notably, the phosphorylation of cAMP response element binding proteins and their subsequent effects as transcriptional regulators have been extensively studied. More recently, it has been appreciated that cross talk between signaling pathways can occur with follitropin treatment. Thus, the mitogen-activated protein kinase pathway can be activated by protein kinase A, an indirect effect of follitropin stimulation.

Follitropin has also been shown to stimulate phosphorylation and activation of protein kinase B in granulosa cells. This kinase is involved in cell survival. Because follitropin plays a role in selection of follicles and prevention of atresia, this particular relationship is an attractive mechanism by which to explain how follitropin prevents apoptosis.

## V. FOLLITROPIN SIGNAL ATTENUATION

Follitropin receptor signaling is attenuated in four ways. First, phosphorylation of the receptor appears to be associated with decreased sensitivity to agonist-mediated stimulation (desensitization). This mechanism is rapid (minutes) and does not require a change in concentration of receptor at the cell surface. Desensitization occurs by phosphorylation of the receptor on its intracellular loops.

On occupancy by follitropin, the follitropin receptor becomes susceptible to phosphorylation. Phosphorylation of the follitropin receptor occurs on the first and third intracellular loops of the receptor. A specific kinase that interacts with the follitropin receptor has not been identified. However, it is possible to phosphorylate the receptor by treatment of cells with nonspecific stimulator of protein kinase C. Protein kinase C can thereby regulate the activity of substrate proteins such as the follitropin receptor. These enzymes are activated and regulated by a variety of factors, including calcium, inositol triphosphates, ceramide, and phosphorylation and adapter proteins. Also, overexpression of G-protein receptor kinase in cells can enhance phosphorylation of the follitropin receptor. An important distinction between these two kinases is that the G-protein receptor kinase can phosphorylate the receptor efficiently only if follitropin is bound. Thus, ligand-induced phosphorylation by the G-protein receptor kinase family is one mechanism for attenuation of follitropin signaling.

The second mechanism of follitropin receptor signal attenuation is via removal of occupied receptor from the cell surface, a process called down-regulation. The process of movement of receptor from the cell surface into clathrin-coated pits is referred to as internalization. Although receptor turnover occurs to some extent without ligand occupancy, ligand-induced internalization is faster. When follitropin binds the follitropin receptor and phosphorylation of the receptor occurs, the phosphorylated receptor is bound by β-arrestin. Binding of the receptor by β-arrestin greatly enhances the rate of internalization of the receptor via clathrin-coated pits. Mutation of phosphorylation sites in the first intracellular loop of the follitropin receptor results in a decrease in internalization, demonstrating that this loop in particular is important for internalization through an arrestin-mediated pathway.

Additional mechanisms lead to attenuation of follitropin-mediated signaling. A family of enzymes called phosphodiesterases accomplishes the conversion of cAMP to AMP. The catalysis of this second messenger provides a third level of control of the cellular response to follitropin by decreasing levels of this activator of protein kinase A. The actions of phosphodiesterases are neither spatially nor temporally restricted. Their broad role in all signaling pathways cannot be understated. However, one specific role related to follitropin receptor signaling is that of attenuating the follitropin-mediated signal. Likewise, it is important to remember that although follitropin has a spatial constraint, the second messenger cAMP has few boundaries. Its inactivation in particular cellular compartments must be appreciated to understand fully how follitropin signaling is not necessarily a linear phenomenon. Moreover, follitropin can regulate the expression levels of phosphodiesterase. Thus, prolonged exposure of Sertoli cells to follicle-stimulating hormone can lead to multilog increases of phosphodiesterase isoform four mRNA and log increases in activity. Surprisingly, when phosphodiesterase 4 knockout mice were examined, they exhibited decreased ovulation, suggesting that this isoenzyme may also play a role in preventing desensitization of the ovary to gonadotropin signaling. In fact, treatment of ovarian follicles with an inhibitor of this isoform causes ovulation, mimicking a gonadotropin (LH) effect, suggesting that inhibitors of phosphodiesterase might be used to treat gonadotropin resistance.

A recently discovered protein family functions as regulators of G-protein signaling (RGS). These proteins contain an RGS domain, which is necessary for negatively regulating $G_\alpha$ protein subunits. This diverse protein family provides a fourth mechanism of desensitization; RGS proteins terminate signaling of G-proteins that have already been activated. In this sense, they are not like kinases that inactivate receptors. They act to dampen a G-protein activity at the level of the activated G-protein by accelerating the hydrolysis of GTP. Although these proteins have not been directly studied in the follitropin receptor system, their ubiquitous nature assures that they will be found to play a key role in regulation of follitropin signaling.

## VI. GENETIC ABNORMALITIES

It might be expected that mutations of the genes encoding either follitropin or the follitropin receptor will lead to disorders in reproduction. Indeed, several mutations identified in the follitropin β and follitropin receptor genes are associated with fertility disorders. Not surprisingly, mutations in follitropin genes that change codons for those cysteine residues necessary for disulfide bond formation (C51G, C82R), as well as premature stop codons, result in infertility. No mutations in the α-subunit have been reported in patients with infertility, probably because of the absolute requirement for the α-subunit for formation of the active thyrotropin heterodimer and for normal thyroid function.

Several mutations reported for the follitropin receptor do diminish follitropin-mediated signal transduction. When the codon for Ala-172 is mutated to the codon for valine, females present with ovarian dysgenesis and arrested follicular development. High follitropin levels in serum are indicative of a lack of ovarian feedback inhibition at the pituitary. Males are less affected, but they show decreased testicular volume. Arrested follicular development is noted when mutation occurs in the codons for Ile-143, Arg-556, Asp-207, and Leu-584.

Mutations in the extracellular domain of follitropin receptor (I143T, D207V, A172V, and N174I) cause a defect in signaling due to a dramatic decrease of receptors on the cell surface. The decrease in receptors at the cell surface leads to a decrease in follitropin-mediated signal transduction and to lowered maximal levels of cAMP, as compared to wild-type receptor. Mutations that affect binding only slightly but that do affect signal transduction have been reported. These are at codons in the intracellular loop 3 (R556C) or in the sixth (L584V) or second (A402T) transmembrane domains. Only one activating mutation of follitropin receptor has been found, in the third intracellular loop (D550G). This is in direct contrast to the lutropin receptor, in which many inactivating and activating mutations have been discovered. The lack of activating mutations in the follitropin receptor has suggested to some that the conformational stability of the follitropin receptor differs from that of the lutropin receptor.

## Glossary

**adenylate cyclase** Nucleotidase that converts adenosine triphosphate to cAMP.

**desensitization** Transient loss of cellular responsiveness to tropic hormone.

**down-regulation** Transient loss of cell surface receptors following exposure to tropic hormone.

**glycoprotein hormones** Family of heterodimeric (two dissimilar subunits) glycosylated proteins in vertebrates that includes thyrotropin, follitropin, lutropin, and choriogonadotropin.

**G-protein** Heterotrimeric (containing three dissimilar subunits) guanosine triphosphate hydrolase enzyme.

**MAP kinase** Family of mitogen-activated protein kinases that phosphorylate proteins on serine or threonine residues.

**phosphodiesterases** Cyclic nucleotidases that hydrolyze cAMP and cGMP.

**protein kinase C** Family of enzymes that phosphorylate serine or threonine residues in proteins.

**protein kinase A** Family of tetrameric ($R_2C_2$) enzymes that phosphorylate tyrosine residues in proteins.

**RGS proteins** Newly discovered family of proteins that are regulators of G-protein signaling.

## See Also the Following Articles

**Extracellular Matrix and Follicle Development • Follicle-Stimulating Hormone (FSH) • Folliculogenesis • Heterotrimeric G Proteins • Protein Kinases**

## Further Reading

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# FSH

See ***Follicle Stimulating Hormone***

# Gastrin

Jens F. Rehfeld
*University of Copenhagen*

I. HISTORICAL INTRODUCTION
II. STRUCTURE—FUNCTION RELATIONSHIPS
III. THE GASTRIN GENE AND mRNA
IV. PROGASTRIN MATURATION
V. CELLULAR GASTRIN RELEASE
VI. THE GASTRIN RECEPTOR
VII. SUMMARY AND PERSPECTIVE

**This article describes the biology of gastrin, an important gastrointestinal hormone that regulates gastric acid secretion and mucosal growth in the stomach. After a historical introduction, the article focuses on the structure of gastrin peptides, structure–function relationships, gastrin biogenesis and expression, and cellular release. The biology of the gastrin receptor is also described. In addition, the article alludes to the phylogenesis, ontogenesis, and oncogenesis of gastrin peptides and concludes by discussing the perspectives of recent gastrin research.**

## I. HISTORICAL INTRODUCTION

Gastrin is the master hormone of the gastrointestinal tract, just as insulin is the master hormone of the pancreas. Gastrin was discovered in 1905 in the wake of Bayliss' and Starling's breakthrough discovery of secretin in 1902. It was John Edkins from London who found that the antral mucosa of the stomach released a gastric acid-stimulating factor into the blood. He named the factor gastrin, an abbreviation for "gastric secretin." Hence, secretin and gastrin were the first peptide hormones to be discovered.

In 1964, gastrin was purified and identified as a peptide of 17 amino acids (Fig. 1). The C-terminal sequence of gastrin-17 and that of another gastrointestinal hormone, cholecystokinin (CCK), were subsequently shown to be identical and to constitute the active site of both hormones. Thus, biologically active gastrin and CCK peptides have the same C-terminal pentapeptide sequence, and this sequence has been exceedingly well conserved throughout evolution over the past 500 million years. The homology of the active site, as well as other structural similarities, defines gastrin and CCK as members of the same hormone family.

During the past three decades, the concept of gastrin as a simple peptide hormone from the upper

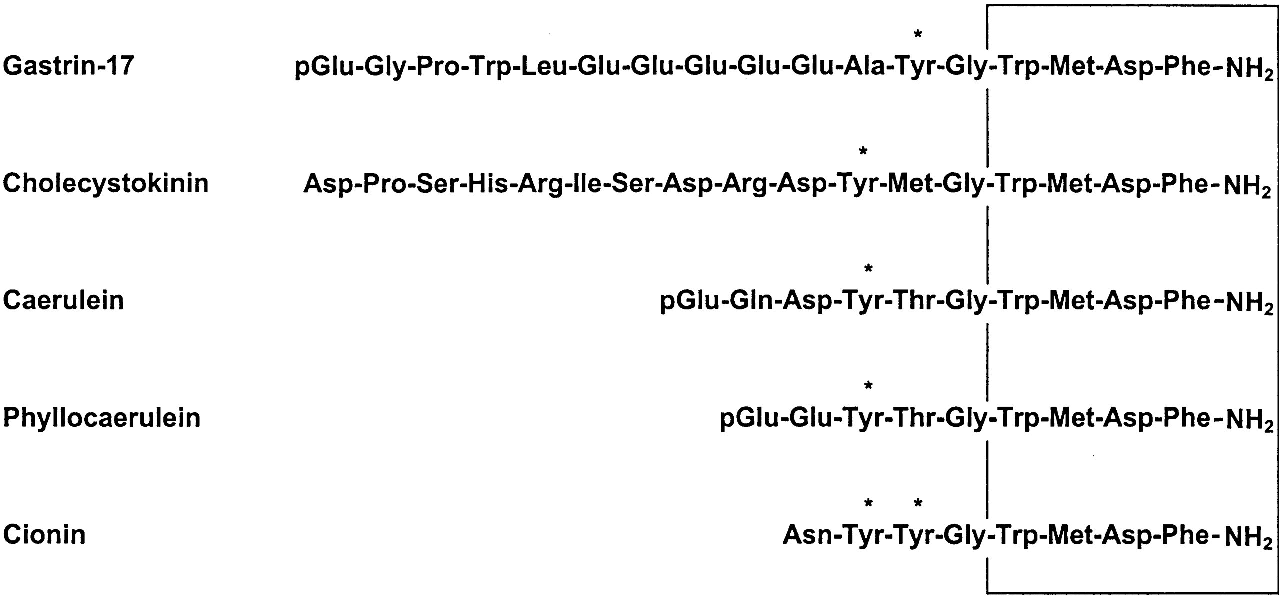
Gastrin-17
pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH₂
Cholecystokinin
Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂
Caerulein
pGlu-Gln-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH₂
Phyllocaerulein
pGlu-Glu-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH₂
Cionin
Asn-Tyr-Tyr-Gly-Trp-Met-Asp-Phe-NH₂
*

FIGURE 1 The C-terminal amino acid sequences of members of the gastrin–cholecystokinin family. The asterisks over the tyrosine residues indicate O-sulfation.

digestive tract has changed considerably. Now gastrin is known to occur in multiple molecular forms in plasma and tissue. Moreover, the gastrin gene is expressed in a cell-specific manner in a variety of neurons, endocrine cells, and other epithelial cells outside the gastrointestinal tract. Moreover, gastrin has been shown to stimulate growth in the stomach and perhaps elsewhere. Today, the view is favored that growth stimulation is a major function of gastrin.

Along with an increased focus on growth effects, clinical interest in gastrin has expanded from its role in duodenal ulcer disease to its significance in diabetes mellitus and in tumor development in the stomach, pancreas, and colorectal mucosa. However, gastrin may, in accordance with its widespread expression, also be involved in growth and neoplasias elsewhere.

## II. STRUCTURE—FUNCTION RELATIONSHIPS

Most biologically active peptides occur in families whose members display structural homology. The occurrence of peptide families is believed to reflect evolution from a single ancestral gene by duplication and subsequent mutations. Also, gastrin and CCK are, as mentioned, members of the same family, and it is possible that the putative common ancestor resembles a dityrosyl-sulfated peptide, cionin, which has been isolated from neurons in protochordates. The frog skin peptides caerulein and phyllocaerulein are also members of the gastrin–CCK family (Fig. 1). At our present state of knowledge, however, CCK and gastrin are the only members of the family in mammals.

All known biological effects of gastrin and CCK peptides reside in the conserved common C-terminal tetrapeptide amide sequence (Fig. 1). Modification of this sequence grossly reduces or abolishes its receptor binding and biological effects in terms of gastric acid secretion and gastric mucosal growth. The different N-terminal extensions of the common C-terminal pentapeptide amide sequence increase the biological potency and the specificity of gastrin/CCK-B receptor binding. Of particular importance for receptor specificity is the tyrosine residue at position 6 of mammalian gastrin and at position 7 of CCK peptides, as counted from the C-terminal phenylalanine (Fig. 1). The gastrins are consequently defined as peptides that stimulate gastric acid secretion and have the C-terminal sequence Tyr-X-Trp-Met-Asp-Phe-$NH_2$ (where X in most mammalian species is a glycine residue). The tyrosine residue is partly sulfated in gastrins, and the gastrin/CCK-B receptor binds sulfated and unsulfated ligands equally well.

The active-site homology of gastrin with CCK has posed large problems in the study of these peptides, because the active site is strongly immunogenic and because the gastrin/CCK-B receptor binds peptides having the shared C-terminal sequence with almost equal affinity. Human gastrin peptides display extensive homology with corresponding peptides from other species. The extent of the species differences has been elucidated by cDNA deduction of the preprogastrin structures for various mammals. Comparison of the sequences shows that the active site and structures around major processing sites are well preserved (Fig. 2).

## III. THE GASTRIN GENE AND mRNA

The single-copy gene for human gastrin is located on chromosome 17q. The cloning of mammalian gastrin and CCK genes shows that the genes are structurally similar, both in the overall exon–intron organization and in certain peptide-coding sequences. The gastrin gene spans 4.1 kb of chromosomal DNA and contains two introns of 3041 and 130 bp, respectively (Fig. 3). Antral G cells generate a single mRNA of 0.7 kb, which encodes the 101-amino-acid preprogastrin in human and mouse, whereas other mammalian preprogastrins have 104 amino acids due to a prolonged C-terminal flanking peptide (Fig. 2). The first exon encodes the 5′-untranslated region (5′-UTR) (Fig. 3). Gastrin cDNAs derived from tumor mRNA are identical to those isolated from mRNA in normal tissues.

Several studies have identified important regulatory domains in the human gastrin gene promoter using rodent cell systems. Hence, a cell-specific regulatory element has been located in the cap–exon 1 region of the human gastrin gene, and a pancreatic islet cell-specific regulatory domain in the gastrin promoter, containing adjacent positive and negative DNA elements, has also been identified. This regulatory domain may be a switch controlling the transient transcription of the gastrin gene in the pancreatic islet cells during fetal and neonatal development. The transcription of the gastrin gene is stimulated by epidermal growth factor (EGF) and inhibited by somatostatin. The EGF-responsive element is of interest for the elucidation of the growth-promoting and oncological significance of gastrin, since EGF/transforming growth factor-α (TGF-α) is expressed in a variety of carcinoma cell lines derived from the colorectal mucosa, ovary, pancreas, and stomach.

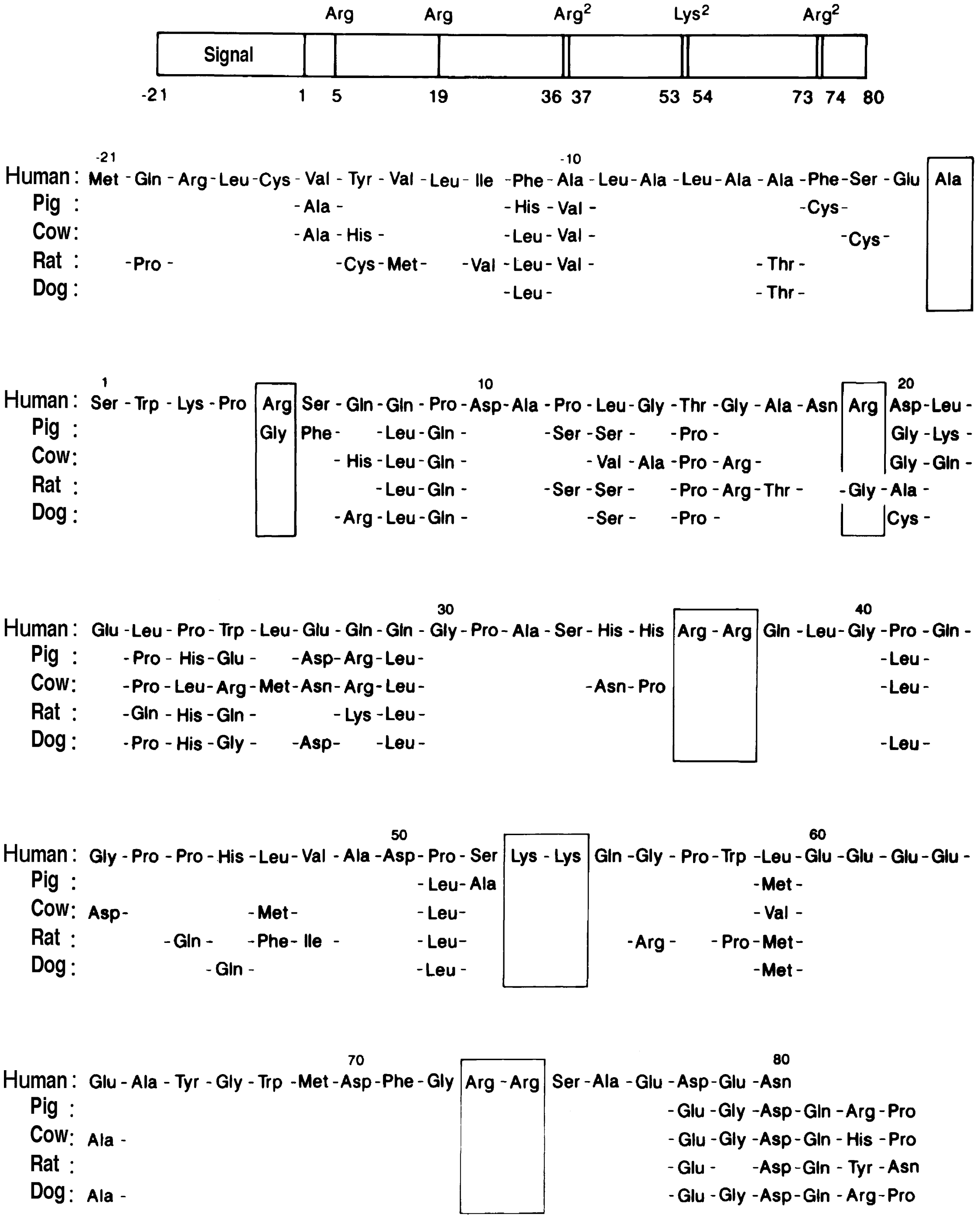
Arg Arg Arg2 Lys2 Arg2
Signal
-21 1 5 19 36 37 53 54 73 74 80
-21 -10
Human: Met - Gln - Arg - Leu - Cys - Val - Tyr - Val - Leu - Ile - Phe - Ala - Leu - Ala - Leu - Ala - Ala - Phe - Ser - Glu Ala
Pig : -Ala - -His - Val - -Cys-
Cow: -Ala - His - -Leu - Val - -Cys-
Rat : -Pro - -Cys - Met- -Val - Leu - Val - -Thr-
Dog: -Leu- -Thr-
1 10 20
Human: Ser - Trp - Lys - Pro Arg Ser - Gln - Gln - Pro - Asp - Ala - Pro - Leu - Gly - Thr - Gly - Ala - Asn Arg Asp - Leu -
Pig : Gly Phe- -Leu - Gln - -Ser - Ser - -Pro - Gly - Lys -
Cow: - His - Leu - Gln - - Val - Ala - Pro - Arg - Gly - Gln -
Rat : -Leu - Gln - -Ser - Ser - -Pro - Arg - Thr - -Gly - Ala -
Dog: -Arg - Leu - Gln - -Ser - -Pro - Cys -
30 40
Human: Glu - Leu - Pro - Trp - Leu - Glu - Gln - Gln - Gly - Pro - Ala - Ser - His - His Arg - Arg Gln - Leu - Gly - Pro - Gln -
Pig : -Pro - His - Glu - -Asp - Arg - Leu- -Leu-
Cow: -Pro - Leu - Arg - Met - Asn - Arg - Leu- -Asn - Pro -Leu-
Rat : -Gln - His - Gln - - Lys - Leu-
Dog: -Pro - His - Gly - -Asp- -Leu- -Leu-
50 60
Human: Gly - Pro - Pro - His - Leu - Val - Ala - Asp - Pro - Ser Lys - Lys Gln - Gly - Pro - Trp - Leu - Glu - Glu - Glu - Glu -
Pig : - Leu - Ala -Met-
Cow: Asp- -Met- -Leu- -Val-
Rat : -Gln - -Phe - Ile - -Leu- -Arg - -Pro - Met-
Dog: -Gln - -Leu- -Met-
70 80
Human: Glu - Ala - Tyr - Gly - Trp - Met - Asp - Phe - Gly Arg - Arg Ser - Ala - Glu - Asp - Glu - Asn
Pig : -Glu - Gly - Asp - Gln - Arg - Pro
Cow: Ala - -Glu - Gly - Asp - Gln - His - Pro
Rat : -Glu - -Asp - Gln - Tyr - Asn
Dog: Ala - -Glu - Gly - Asp - Gln - Arg - Pro

FIGURE 2 The cDNA-deduced amino acid sequences of preprogastrins from five mammalian species. Boxes indicate established proteolytic processing sites, mainly at di- and monobasic sites. Note the preserved sequences around the active site (residues 68–71) and around major processing sites.

Therefore, neoplastic expression of the gastrin gene may be due to transcriptional stimulation by EGF and/or TGF-α.

Thus far, no tissue-specific splicing or use of alternative promoters has been reported. Therefore, the tissue-specific molecular pattern of gastrin peptides is due to differences in posttranslational processing, rather than to alternative RNA splicing.

In the developing rat colon, gastrin mRNA concentrations increase from birth to adulthood,

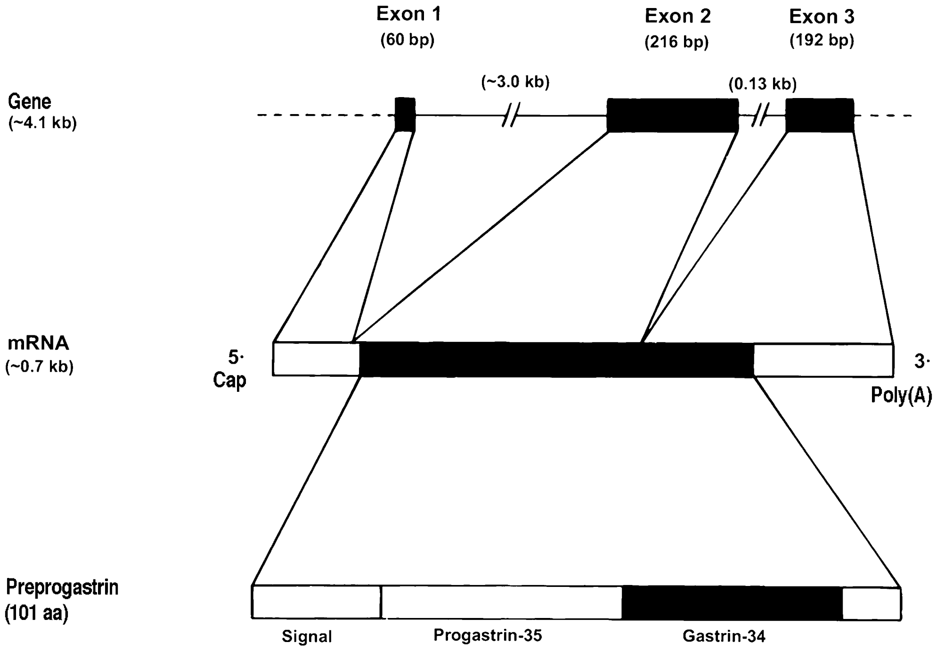

FIGURE 3 Structure of the human gastrin gene, mRNA, and prepropeptide. Exons are shown as boxes, and introns are shown as straight lines. The black area of the mRNA indicates the coding region. Numbers indicate the number of base pairs or kilobase pairs in each section of the gene. In preprogastrin, the position of gastrin-34 is shown.

apparently without a corresponding increase in peptide synthesis. Therefore, expression of the gastrin gene also appears to be regulated at the translational level. Comparison of rat, pig, and human cDNA sequences shows remarkably conserved nucleotide sequences in the 5′- and 3′-untranslated regions. It is therefore possible that such a sequence in the 5′-UTR may determine the translational efficiency of the expression cascade. The 3′-UTR may be involved in regulation of polyadenylation, termination of transcription, or message stability. Further studies on gastrin mRNA are necessary to explain these observations. The expression of the gastrin gene is ontogenetically regulated. Consequently, expression of the gastrin gene in tumors may involve factors that normally are expressed only in fetal life.

## IV. PROGASTRIN MATURATION

The antral G cells are the main site of gastrin synthesis. A small number of G cells are also present in the proximal duodenum. After antrectomy, however, the duodenal G cells "antralize" and increase their synthesis considerably. Gastrin biosynthesis studies have thus far focused on antral tissue. Combining the results of these studies with general knowledge about peptide hormone synthesis provides a clear picture of the processing pathway of antral progastrin (Fig. 4).

After translation of gastrin mRNA in the endoplasmic reticulum and co-translational removal of the N-terminal signal peptide from preprogastrin, intact progastrin is transported to the Golgi apparatus. In the *trans*-Golgi network, the first posttranslational modifications occur. These are O-sulfation of the Tyr-66 residue neighboring the active site, and the first endoproteolytic convertase cleavage at two monobasic processing sites. From the *trans*-Golgi network, vesicles carry the processing intermediates toward the basal part of the G cells, where the gastrin peptides are stored in characteristic secretory granules. The cleavage by prohormone convertases 1 and 2 (PC1/3 and PC2), the exoproteolytic carboxypeptidase E trimming, and the subsequent glutamine cyclization, corresponding to the N-termini of gastrin-34 and gastrin-17 (Figs. 2 and 4), continue during the transport from the Golgi to the immature secretory granules. The last, decisive processing step in the synthesis of gastrin then occurs during storage in the secretory granules. The secretory granules contain the amidation enzyme, which removes glyoxylate from the immediate precursors, the glycine-extended

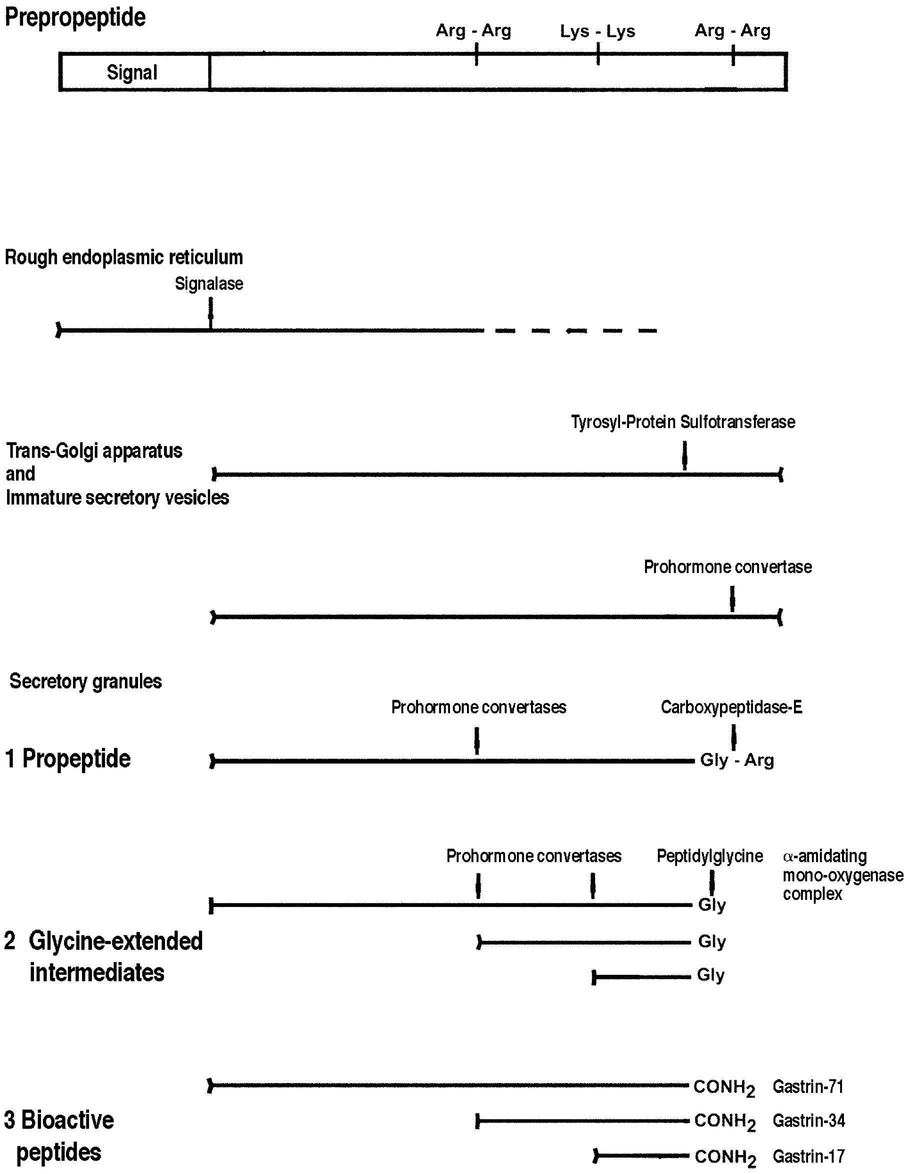
Prepropeptide
Signal
Arg - Arg
Lys - Lys
Arg - Arg
Rough endoplasmic reticulum
Signalase
Tyrosyl-Protein Sulfotransferase
Trans-Golgi apparatus and Immature secretory vesicles
Prohormone convertase
Secretory granules
Prohormone convertases
Carboxypeptidase-E
1 Propeptide
Gly - Arg
Prohormone convertases
Peptidylglycine
α-amidating mono-oxygenase complex
Gly
2 Glycine-extended intermediates
Gly
Gly
3 Bioactive peptides
CONH2 Gastrin-71
CONH2 Gastrin-34
CONH2 Gastrin-17

FIGURE 4 Schematic illustration of the posttranslational processing of preprogastrin in the antral G cell.

gastrins, to complete the synthesis of bioactive carboxyamidated peptides (Fig. 4). Amidation of gastrin is an all-or-none activation process that is carefully controlled. Activation of the enzymatic amidation process requires copper, oxygen, and ascorbic acid as co-factors and a pH of approximately 5. Carboxyamidiation of peptides is known to require two sequentially acting enzymes: a peptidylglycine α-hydroxylating monooxygenase, derived from the N-terminal part of the enzyme precursor, and a peptidyl-α-hydroxyglycine α-amidating lyase, derived from the remaining intragranular region of the precursor.

## V. CELLULAR GASTRIN RELEASE

As a result of the elaborate biosynthesis process, the antral G cells release a heterogeneous mixture of progastrin products from the mature secretory granules into the blood. In human, a small percentage of these products are nonamidated precursors, mainly glycine-extended gastrins, whereas more than 95% are α-amidated bioactive gastrins. Of these, 85% are gastrin-17, 5–10% are gastrin-34, and the rest is a mixture of gastrin-71, gastrin-14, and the short gastrin-6 (Fig. 4). Approximately half of the amidated gastrins are tyrosine-sulfated. Due to gross differences in metabolic clearance rates, the distribution pattern of gastrins in peripheral plasma changes, so that larger gastrins with their long half-lives predominate over gastrin-17 and shorter gastrins. Hence, in peripheral blood, gastrin-34 is the predominant form of gastrin.

Increased gastrin synthesis changes the molecular pattern in plasma further. Abnormally increased antral synthesis occurs in human by achlorhydria, as seen in pernicious anemia. In antrum-sparing pernicious anemia, the translational activity of gastrin mRNA in G cells seems to be so high that the enzymes responsible for the processing of progastrin cannot keep up with the maturation of the peptide. Consequently, G cells release more unprocessed and incompletely processed nonamidated progastrin products when the rate of synthesis is increased. Also, the carboxyamidated gastrins are less sulfated and the N-terminus of progastrin is cleaved to a lesser degree. Precursors, processing intermediates, and long-chained carboxyamidated gastrins, such as gastrin-71 and gastrin-34, are, as mentioned, cleared at a relatively slow rate from the circulation and therefore accumulate in plasma when the rates of synthesis and release are increased.

Gastrin peptides are also released from cell types other than the antroduodenal G cells. Quantitatively, these other cells contribute only a little to circulating gastrin, partly because the secretion seems to serve local purposes and partly because the biosynthetic processing is cell-specific. Thus far, expression of progastrin and its products has been encountered outside the antroduodenal mucosa in endocrine cells] in the ileum and the colon, in endocrine cells in the fetal and neonatal pancreas, in pituitary corticotrophs and melanotrophs, in oxytocinergic hypothalamo-pituitary neurons, in a few cerebellar and vagal neurons, in the adrenal medulla of some species, in the bronchial mucosa, in postmenopausal ovaries, and in human spermatogenic cells. As shown in Table 1, the concentrations and presumably also the synthesis in the extra-antal tissues are far below those of the antral "main factory."

The function of gastrin synthesized outside the antroduodenal mucosa is not yet fully known, but an obvious possibility is paracrine or autocrine regulation of growth. Second, it is possible that the low concentration of peptides is without significant function in the adult, but is a relic of a more comprehensive fetal synthesis for local stimulation of growth. A third possibility is that the low cellular concentration and, hence, low tissue concentration are due to constitutive rather than regulated secretion. With constitutive secretion, there is no storage of peptides in the cells despite a considerable amount being released per unit of time.

TABLE 1 **Expression of the Gastrin Gene at the Peptide Level in Normal Adult Mammalian Tissue**

| Tissue | Total translation product (pmol/g tissue) | Precursor percentage |
|---|---|---|
| Gastrointestinal tract | | |
| Antral mucosa | 10,000 | 5 |
| Duodenal mucosa | 400 | 20 |
| Jejunal mucosa | 40 | 30 |
| Ileal mucosa | 20 | 85 |
| Colonic mucosa | 0.2 | 100 |
| Neuroendocrine tissue | | |
| Cerebellum | 5 | 20 |
| Vagal nerve | 8 | 10 |
| Adenohypophysis | 200 | 98 |
| Neurohypophysis | 30 | 5 |
| Adrenal medulla | 2 | 100 |
| Pancreas | 2 | 95 |
| Genital tract | | |
| Ovaries | 0.5 | 100 |
| Testicles | 6 | 100 |
| Spermatozoa | 2 | 55 |
| Respiratory tract | | |
| Bronchial mucosa | 0.3 | 100 |

*Note*. Orders of magnitude based on examination of different mammalian species.

Although it is possible that the extra-antral synthesis of gastrin is without function in the adult organism, recognition of the phenomenon has considerable biomedical interest. Hence, tumors originating from cells that express the gastrin gene at low levels in adult organisms may produce significant amounts of gastrin in tumors. Well-known examples are pancreatic, duodenal, and ovarian gastrinomas that give rise to the Zollinger–Ellison syndrome.

## VI. THE GASTRIN RECEPTOR

The gastrin/CCK-B receptor is the predominant receptor for gastrin and CCK peptides in the central nervous system. It is expressed with particularly high density in the cerebral cortex. The gastrin/CCK-B receptor is less selective than the CCK-A receptor, which binds only sulfated CCK peptides. In contrast, the gastrin/CCK-B receptor binds both sulfated and nonsulfated gastrin and CCK peptides as well as short C-terminal fragments of CCK and gastrin, all with similar high affinity. The gastrin/CCK-B receptor is abundantly expressed in the brain, in ECL (enterochromaffin-like) cells in the stomach, and also at a lower level in the exocrine and endocrine pancreas of human. The canine gastrin receptor is a 453-amino-acid protein that also contains seven transmembrane domains and belongs to the G-protein-coupled receptor superfamily (Fig. 5). Like the CCK-A receptor, it has a number of N-terminal glycosylation sites and several serine and threonine residues for phosphorylation but no tyrosine phosphorylation site. The gastrin receptor also activates phospholipase C, degrades inositol-phospholipids, mobilizes intracellular calcium, and activates protein kinase C. Valine-319 in the sixth transmembrane domain of the human gastrin/CCK-B receptor determines the binding specificity of the nonpeptide receptor antagonists. This receptor site for antagonist binding differs from that of agonist binding, as shown also for other peptide receptors. Hence, allosteric binding of nonpeptide antagonists is a common feature of the G-protein-coupled, seven-transmembrane-spanning family of peptide receptors. Alternative splicing of gastrin/CCK-B receptor mRNA has been observed. The significance of the splicing in terms of receptor specificity is still unknown.

## VII. SUMMARY AND PERSPECTIVE

Gastrin is an important regulatory peptide system. Although originally discovered and named as a simple gastrointestinal hormone, gastrin peptides are now recognized as widespread intercellular messengers in many compartments of the body. In addition to their classical blood-borne hormonal activity as regulators of gastric acid secretion via ECL-cell release of histamine, gastrin peptides are neurotransmitters and growth factors. In addition, the far-reaching phylogeny indicates a vital role for gastrin and gastrin-like peptides in the evolution of multicellular life for more than 500 million years.

The gastrin system of peptides continues to be a subject of extensive interest and research. In recent years, it has been suggested that the progastrin molecule itself as well as intermediate processing products such as the glycine-extended gastrins may have specific growth-promoting effects. Accordingly, it has been suggested that target cells in normal and neoplastic tissue express specific receptors for these precursors and processing intermediates. The suggestions are interesting, but they are still hypothetical ideas that require further and substantial evidence. A necessary milestone that remains to be reached

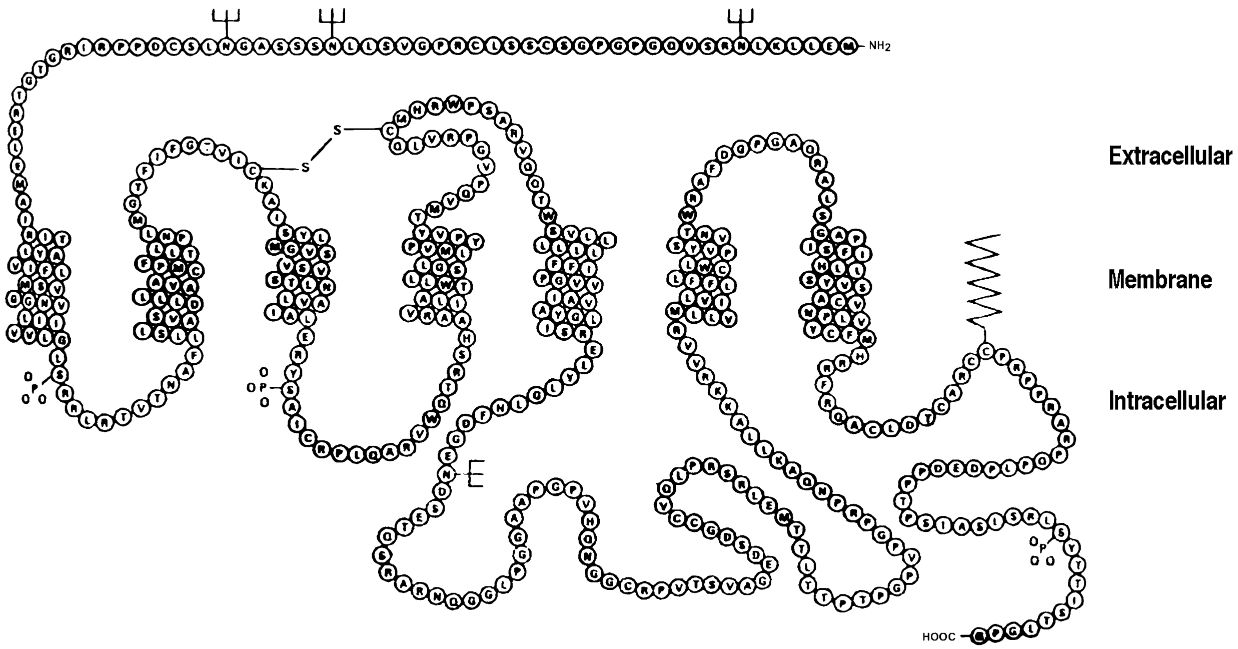

FIGURE 5 Schematic model of the rat gastrin/CCK-B receptor. The cDNA-deduced amino acid sequence (single-letter code) shows putative transmembrane helices, consensus sites for N-linked glycosylation (tridents), serine and threonine phosphorylation ($-PO_3$), disulfide bridge (–S–S), and cysteine palmitoylation (jagged line). Figure courtesy of Stephen A. Wank (National Institutes of Health, Bethesda, MD).

is covalent identification of appropriate high-affinity receptors. Until then, the gastrin concept is based on the activity of the well-known carboxyamidated gastrins.

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**gastrin cells (G cells)** Endocrine cells dispersed in the antral part of the gastric mucosa and in the proximal part of the duodenal mucosa. G cells produce by far most of the gastrin in the adult organism. The synthesis of gastrin is regulated by negative feedback from gastric acid via antral somatostatin cells.

**transforming growth factor-α (TGF-α)** A growth factor closely related to EGF (epidermal growth factor). TGF-α is often expressed in neoplastic tissue together with gastrin, and TGF-α (or EGF) stimulates gastrin gene transcription.

## See Also the Following Articles

**Cholecystokinin (CCK) • Gastrointestinal Hormone (GI) Regulated Signal Transduction • Gastrointestinal Hormone-Releasing Peptides • Insulin Secretion • Secretin • Vagal Regulation of Gastric Functions by Brain Neuropeptides**

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# Gastrointestinal Hormone (GI) Regulated Signal Transduction

MARK R. HELLMICH
*University of Texas Medical Branch, Galveston*

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II. GI HORMONE SIGNALING: AN OVERVIEW
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VIII. FUTURE DIRECTIONS

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# Gastrointestinal Hormone (GI) Regulated Signal Transduction

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**Gastrointestinal hormones, peptide messengers that are released from endocrine cells, are distributed throughout the gastric mucosa, small intestine, and colon in response to nervous activity, physical stress, and chemical stimulation coincident with the intake of food. Acting in an endocrine, paracrine, or neurocrine manner, gastrointestinal hormones regulate a variety of specific biological responses by the stomach, small intestine, pancreas, liver, and gallbladder to optimize the physiological conditions necessary to permit efficient digestion and absorption of protein, carbohydrates, and fat from the lumen of the intestine.**

## I. INTRODUCTION

Gastrointestinal (GI) hormones act in concert with the nervous system to regulate exocrine and endocrine GI secretion, cell proliferation, differentiation, and motility. The GI tract produces many different peptide hormones and GI-active neuropeptides, some of which are listed in Tables 1 and 2, respectively. There are myriad GI peptides that activate signal transduction pathways; the following discussion presents a summary of the current knowledge of the intracellular signal transduction pathways involved in normal physiological actions of a select few GI hormones (gastrin, cholecystokinin, and secretin) on specific GI tract target cells: gastric parietal cells, enterochromaffin-like cells, and pancreatic acinar cells.

## II. GI HORMONE SIGNALING: AN OVERVIEW

All GI hormones are polypeptides that bind to receptors belonging to the superfamily of guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs). GI hormone-stimulated signal transduction begins with the binding of the peptide hormone to its cognate cell surface receptor. Highly specific interactions between individual amino acid residues of the peptide hormone and amino acids of the receptor cause a conformational change in the receptor, which transduces the informational content of the peptide hormone across the plasma membrane to intracellular signal transduction networks. These networks consist of second-messenger-producing enzymes, protein kinases, phosphatases, scaffold and adapter proteins, transcription factors, and other regulatory proteins, which act in a synergistic and combinatorial manner to elicit specific cellular responses to the hormone stimulus.

As members of the GPCR superfamily, all GI hormone receptors share common structural motifs, including seven transmembrane-spanning α-helical domains, and all function as ligand-regulated guanine nucleotide exchange factors for intracellular heterotrimeric G-proteins. Heterotrimeric G-proteins are composed of the products of three gene families encoding α, β, and γ subunits. The agonist-activated GPCR catalyzes the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) bound to the $G_\alpha$ subunit, as well as the dissociation of GTP-$G_\alpha$ from its cognate $G_{\beta\gamma}$ dimer. The activated GTP-$G_\alpha$ and $G_{\beta\gamma}$ subunits, in turn, regulate the activity of various intracellular effector proteins, such as phospholipases, adenylyl cyclases, protein kinases, membrane ion channels, and members of the Ras family of GTP-binding proteins. Approximately 20 mammalian $G_\alpha$ subunits have been identified and, based on structural similarities, are divided into four distinct subfamilies: the cholera-toxin-sensitive αs subunits, which stimulate adenylyl cyclase and thus increase levels of intracellular cAMP; the pertussis-toxin-sensitive αi/o subunits, which inhibit adenylyl cyclase activity; the pertussis-toxin-insensitive αq/11/14 subunits, which stimulate membrane phospholipases; and the α12/13 subfamily, which appear to provide

TABLE 1 **Gastrointestinal Hormones**

| Hormone | Major actions |
|---|---|
| Gastrin | ↑ Acid and pepsin secretion from gastric mucosa; ↑ ECL proliferation |
| CCK | ↑ Pancreatic enzyme secretion and gallbladder contraction |
| Secretin | ↑ Pancreatic bicarbonate and $H_2O$ secretion |
| Motilin | ↑ Intestinal motility |
| Enteroglucagon | ↑ Enterocyte proliferation |
| Somatostatin | ↓ Gastric acid secretion and gastric motor activity |
| Gastric inhibitory peptide | ↑ Glucose-stimulated insulin secretion and ↓ gastric acid secretion |
| Pancreatic polypeptide | ↓ Pancreatic bicarbonate secretion |
| Peptide YY | ↓ Gastric motor activity and acid secretion |

TABLE 2 GI Active Neuropeptides

| Neuropeptide | Major actions |
|---|---|
| Bombesin (gastrin-releasing peptide) | ↑ Gastrin release |
| Substance P | ↑ Smooth muscle contraction mediates inflammatory response |
| Vasoactive intestinal peptide | ↑ Smooth muscle relaxation and pancreatic bicarbonate secretion |
| Calcitonin gene-related peptide | ↑ Somatostatin release and smooth muscle contraction |

a link between GPCRs and the activation of the Ras-related GTP-binding protein, Rho.

In addition to the $G_\alpha$ subunits, 12 $G_\gamma$ subunits and 6 $G_\beta$ subunits have been cloned. When released from the activated GTP-α subunit following receptor stimulation, $G_{\beta\gamma}$ dimers activate intracellular signaling molecules such as phosphatidylinositol 3-kinases (PI3-kinases), select isoforms of adenylyl cyclase, and receptor kinases, which play an important role in attenuating agonist-induced receptor activation. The mechanisms of GI hormone-stimulated intracellular signal transduction have been extensively characterized in a number of experimental models. Several excellent reviews have recently been published and are listed in the bibliography at the end of this article.

## III. MAJOR GI HORMONES AND RECEPTORS

Classical hormones are secreted from endocrine glands or cells into the bloodstream and are distributed to their targets through the general circulation. GI hormones are a subset of hormones produced by and released from endocrine cells of the gut in response to a meal that have other gut cells as their primary targets. Not all GI hormones function in a truly endocrine fashion—that is, many GI peptides are not discharged into blood vessels to act at a distant (target) site, but rather they act in either a paracrine or neurocrine manner. Of the many regulatory peptides found in the GI tract, five peptides can be considered “true” GI hormones. There is ample evidence showing they are released into the blood from one site of the GI tract in response to a meal and act at a distant target site to regulate the processes of nutrient digestion, absorption, and transit. The five major GI hormones that act predominantly in an endocrine manner are gastrin, cholecystokinin (CCK), secretin, gastric inhibitory peptide (GIP), and motilin. Currently, little is known about the intracellular signaling pathways mediating the actions of GIP and motilin, thus this review focuses on the signaling pathways regulated by gastrin, CCK, and secretin (Fig. 1).

### A. Gastrin

In the GI tract, gastrin regulates gastric acid secretion from parietal cells, histamine release from enterochromaffin-like (ECL) cells, and normal and neoplastic cell proliferation. Gastrin is released from G cells located in the gastric antrum and duodenum. The major circulating forms of gastrin are the amidated gastrin 1–17 (G-17) and big gastrin, gastrin 1–34 (G-34). During the interdigestive (basal) state, most of the gastrin detected in human serum is G-34. Unlike other species, in humans the duodenal mucosa produces significant amounts of G-34. After a meal,

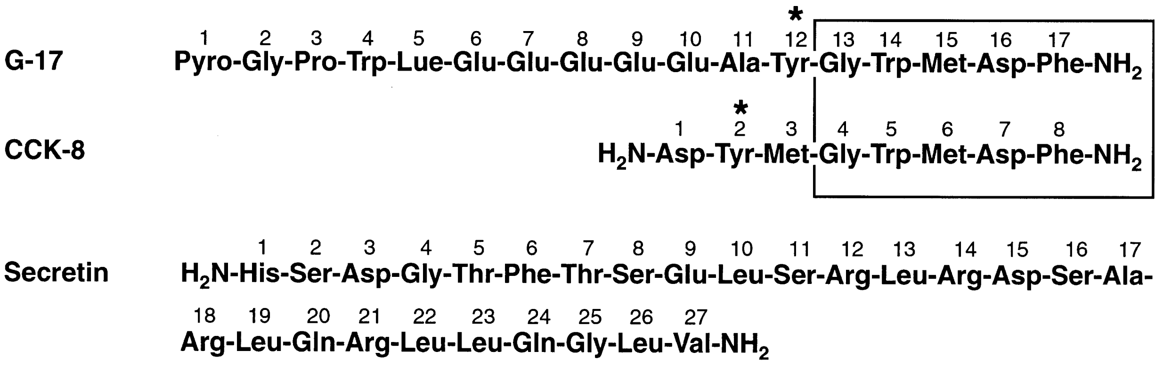
G-17
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
*
Pyro-Gly-Pro-Trp-Lue-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH2
CCK-8
1 2 3 4 5 6 7 8
*
H2N-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH2
Secretin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
H2N-His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-
18 19 20 21 22 23 24 25 26 27
Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH2

FIGURE 1 Amino acid sequences of gastrin 1–17 (G-17), cholecystokinin 1–8 (CCK-8), and secretin. The last five amino acid residues of G-17 and CCK are identical (box). The sites of sulfation are indicated ( ∗ ). Pyro, Pyroglutamyl.

however, large quantities of antral gastrin, primarily G-17, are released and provide most of the stimulus for gastric acid secretion. Gastrin regulates acid release directly by stimulating HCl-producing parietal cells and indirectly by stimulating ECL cells to increase production and release of the biogenic amine, histamine, which binds to $H_2$ histamine receptors on the parietal cell and enhances gastrin-stimulated acid secretion.

## B. Gastrin/CCK-B Receptor

The gastrin receptor was originally cloned from an expression cDNA library constructed with canine parietal cell messenger RNA (mRNA) and was found to be identical to the cholecystokinin-B (CCK-B) receptor (CCK-BR) cloned at about the same time by other laboratories from rat brain and the rat pancreatic acinar carcinoma cell line, AR42J. To date, cDNA for CCK-BR has been cloned from rat brain and stomach, rat pancreatic tumor cells, human brain, calf pancreas, guinea pig gallbladder and stomach, and from ECL and parietal cells from the rodent *Mastomys natalensis*. A comparison of cDNA sequences from these various species indicates that the structure of CCK-BR is highly conserved, with an overall predicted amino acid sequence identity of 72% and pairwise amino acid sequence identities of 84–93%. Within the same species, CCK-BR shares approximately 50% sequence identity with the CCK-A receptor (CCK-AR), which is the primary physiological receptor for the gastrin-related peptide, CCK (Fig. 1).

Translation of the rat and canine CCK-BR cDNAs predicts proteins composed of 452 and 453 amino acid residues, respectively. Hydropathy analysis indicates that CCK-BR has a structure common to GPCRs, i.e., the seven transmembrane-spanning segments. In addition, CCK-BR contains at least three consensus sites for N-linked glycosylation (Asn-X-Ser/Thr), which is consistent with the 74- and 78-kDa glycoproteins identified by affinity cross-linking studies using $^{125}$I-labeled [Leu or NorLeu$^{15}$]-gastrin-2–17 and gastric mucosal extracts enriched for parietal cells. Also, CCK-BR has in the first and second extracellular loops two conserved cysteine residues, which may form a disulfide bridge required for stabilization of its tertiary structure, and a cysteine in the COOH-terminal tail domain, which may serve as a membrane-anchoring palmitoylation site.

## C. CCK

Although CCK is structurally related to gastrin, its major targets are the pancreas and gallbladder. In the pancreas, CCK is an important regulator of digestive enzyme secretion and acinar cell growth. In the gallbladder, CCK is a potent stimulus of gallbladder wall contractions and plays a role in relaxation of the sphincter of Oddi, which guards the exit of the common bile duct into the duodenum. CCK also plays a role in potentiating the effects of secretin and the related peptide, vasoactive intestinal peptide (VIP), on pancreatic bicarbonate secretion and growth regulation of the exocrine pancreas.

CCK is expressed in intestinal I cells, located in the duodenum and jejunum, as well as in neurons of the enteric, peripheral, and central nervous systems. It is released from I cells into the bloodstream in response to the presence of fatty acids longer than eight carbon atoms and proteins in the form of short peptides and single aromatic amino acids in the lumen of the duodenum.

In humans, CCK occurs predominantly as COOH-terminal amidated 58- and 8-amino-acid peptides that arise from the posttranslational processing of a 115-amino-acid preprohormone. Additional forms of CCK (CCK-39 and CCK-33) have been described in various species, including humans. All of these forms of CCK have similar molar potencies for the stimulation of pancreatic enzyme secretion from *in vitro* preparations of rat pancreatic acini. CCK-8 is the shortest peptide that exhibits biological activity. However, to achieve full potency, the tyrosine seven residues from the COOH-terminus must be sulfated (Fig. 1).

## D. CCK-A Receptor

The first receptor for CCK (CCK-AR) was cloned from a rat pancreatic acinar cell cDNA library using degenerate oligonucleotide primers as probes. Hydropathy analysis of the CCK-AR cDNA indicated seven putative transmembrane-spanning domains. The deduced sequence of the rat CCK-AR predicts a 429-amino-acid protein with a calculated molecular mass of 48 kDa. Consistent with ligand affinity cross-linking experiments, which identified a CCK binding protein with a molecular mass of approximately 90 kDa in pancreatic acini, three potential sites for N-linked glycosylation have been identified on the CCK-AR extracellular domains. Other structural features of potential functional importance include three consensus sites for protein kinase C (PKC) phosphorylation in the third intracellular loop and a cysteine residue in the COOH-terminal tail domain that may serve as a membrane-anchoring palmitoylation site.

To date, CCK-AR cDNA clones have been characterized from guinea pig gallbladder, pancreas, and gastric chief cells; human gallbladder; and rabbit gastric mucosa. The CCK-AR is highly conserved among these different species, with an overall amino acid sequence homology of 80% and a pairwise amino acid sequence identity of 87–92%. Unlike the CCK-BR, which binds sulfated-CCK and gastrin with similar affinities, CCK-AR is highly selective for sulfated-CCK and binds gastrin poorly.

### E. Secretin

Secretin was the first peptide hormone discovered. The principal biological action of secretin is to stimulate the release of bicarbonate and water from pancreatic acinar cells. Secretin is secreted from S cells located in the duodenum and, to a lesser extent, from the jejunum, when the acidity of the duodenal contents fall below a pH of 4.5.

Unlike other peptide hormones, only a single form of secretin has been identified. Biologically active secretin is 27 amino acids long (Fig. 1) and is derived from the posttranslational processing of a 104-amino-acid residue peptide preprohormone. Secretin has structural homology with glucagon, gastric inhibitory peptide (GIP), and vasoactive inhibitory peptide.

### F. Secretin Receptor

The first secretin receptor cDNA was isolated using an expression cloning strategy and mRNA from the cell line NG108-15. The secretin receptor cDNA encodes a GPCR containing 449 amino acids with a mass of approximately 49 kDa. In addition, the secretin receptor contains five potential sites of N-glycosylation, 10 extracellular cysteine residues, and three intracellular consensus sites for protein kinase C-mediated phosphorylation. The predicted amino acid sequences of the rat and human secretin receptors are 81% identical.

Following cloning of the secretin, VIP, calcitonin, and parathyroid hormone receptors, it became apparent that, although these receptors shared common structural and functional characteristics with the superfamily of GPCRs, they were sufficiently different from the rhodopsin/β-adrenergic family of GPCRs to warrant assigning them to a new branch of the phylogenetic tree. Currently, five classes (A–E) of GPCRs are recognized. The secretin receptor defines class B GPCRs and, in addition to those listed above, includes receptors for glucagon, glucagon-like peptide-1 (GLP-1), GIP, and pituitary adenylate cyclase-activating polypeptide (PACAP). Gastrin/CCK-BR and CCK-AR are members of the class A rhodopsin-like receptors.

Secretin is the preferred ligand of the secretin receptor and binds with relatively high affinity. Competition binding studies found that 50% of bound [$^{125}$I]-labeled secretin was displaced by 1 n$M$ of unlabeled secretin. In addition to secretin, the secretin receptor will bind VIP and glucagon but with much lower affinity. A concentration of 1 μ$M$ of unlabeled VIP was required to displace 50% of the labeled secretin bound to the secretin receptor.

## IV. GASTRIN REGULATION OF GASTRIC ACID SECRETION

The stimulation of gastric acid secretion from the parietal cell in response to a meal is a complex process involving the integration of signals from the nervous systems, GI hormones, and the biogenic amine histamine. The parietal cells receive input from the neurotransmitter acetylcholine, released from neurons of the vagus nerve, from the GI hormone gastrin, released by G cells of the gastric antrum, and from ECL cells, which produce and release histamine (Fig. 2). Gastrin plays a central role in the regulation of acid secretion from parietal cells by both direct and indirect mechanisms.

### A. Parietal Cells

The parietal cells, located in the fundus of the stomach, secrete a solution of 0.15 $M$ HCl and 7 m$M$ KCl, with traces of other electrolytes. The concentration of hydrogen ion in parietal cell secretions is greater than 1 million times the concentration in the plasma. The low pH of gastric secretions initiates denaturation of ingested proteins and converts the inactive protease pepsinogen, released from gastric chief cells, into the active enzyme pepsin. The acid also kills many of the bacteria that enter the stomach, thereby reducing the number of potentially pathogenic organisms that reach the intestines.

The final step in acid secretion from parietal cells involves translocation of gastric $H^+$,$K^+$-ATPase from its intracellular vesicular compartment to the apical plasma membrane. $H^+$,$K^+$-ATPase is related to the family of P-type cation pumps, which includes

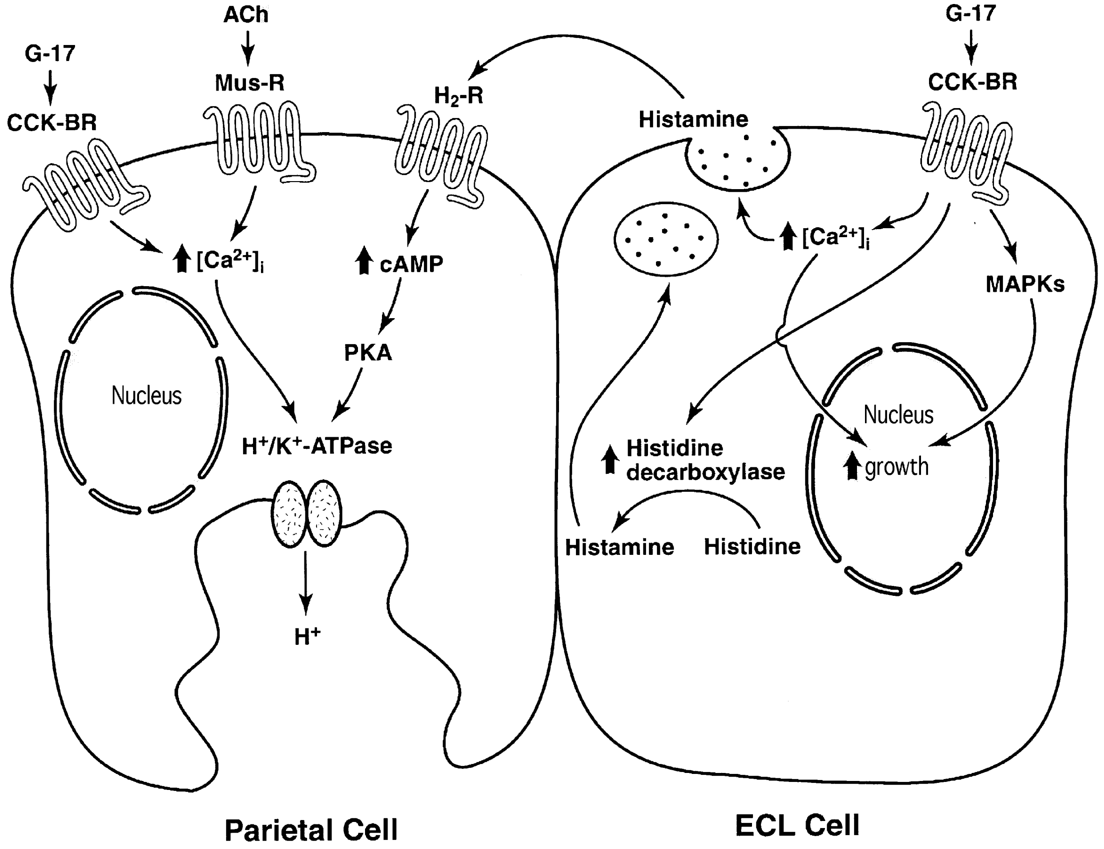

FIGURE 2 Summary of intercellular regulation of gastric acid secretion from a gastric parietal cell and an enterochromaffin-like (ECL) cell. CCK-BR, Cholecystokinin-B receptor; ACh, acetylcholine; $H_2$-R, histamine receptor; PKA, protein kinase A; MAPKs, mitogen-activated protein kinases.

$Na^+$,$K^+$-ATPase. $H^+$,$K^+$-ATPase consists of a catalytic α-subunit and a highly glycosylated β-subunit. It uses the energy from ATP hydrolysis for the electroneutral countertransport of $H^+$ for $K^+$. In unstimulated parietal cells, $H^+$,$K^+$-ATPase is sequestered within a population of cytoplasmic vesicles called tubulovesicles, which are spatially insulating from the gastric lumen. Also, the tubulovesicle membrane has a very low permeability to KCl, limiting the activity of the pump even though there is ample ATP around the enzyme. Activation of acid secretion is achieved when (1) the tubulovesicles fuse with the apical secretory membrane, thus recruiting functional $H^+$,$K^+$-ATPase to the expanding microvillar surface, and (2) the apical membrane becomes permeable to KCl.

## B. Gastrin Stimulates $H^+$,$K^+$-ATPase Translocation

Gastrin stimulates the translocation of tubulovesicles containing $H^+$,$K^+$-ATPase to the apical membrane in a process involving membrane docking and fusion. Studies of classical secretory systems, from those of budding yeast to those of the mammalian nervous system, have revealed an evolutionarily conserved multimeric complex of proteins that regulate secretory vesicle docking and fusion. Integral membrane proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Ras-related GTP-binding proteins of the Rab subfamily are essential components of the docking and fusion machinery. Vesicle docking and membrane fusion require the highly regulated interaction of the vesicle membrane proteins known as synaptobrevins [also called vesicle (v)-SNAREs or vesicle-associated membrane proteins (VAMPs)] and the plasma membrane proteins, i.e., syntaxins, and synaptosomal-associated proteins (SNAPs), collectively referred to as t-SNAREs. All SNAREs are associated with the membrane via a single transmembrane domain or acylation, and all possess one or more α-helices capable of forming coiled-coiled structures.

Vesicle docking and fusion to the plasma membrane involve, in part, the formation of a complex between v-SNAREs and t-SNAREs, as well as regulation by additional factors, including Rabs. Rabs are a large family of evolutionarily conserved small GTP-binding proteins; over 40 members have been identified in mammalian cells. Different Rabs are found on vesicular elements of secretory and endosomal pathways and are required for vesicle docking and fusion to target membranes. Several proteins and their isoforms that function in vesicle docking and fusion have been identified in parietal cells. These include syntaxin isoforms 1 through 4, VAMP-2 (synaptobrevin), SNAP-25, Rab2, Rab11, Rab25, and secretory carrier membrane proteins (SCAMPs).

## C. Gastrin Stimulates an Increase in $[Ca^{2+}]_i$

The precise signaling pathways linking CCK-BR activation with tubulovesicle docking and fusion have not been defined. However, it is known that gastrin stimulation of parietal cells activates phospholipase C (PLC), leading to the generation of the second messengers inositol 1,4,5-triphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) from the hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate ($PIP_2$). Soluble $InsP_3$ activates ligand-gated $Ca^{2+}$ channels that are located in the endoplasmic reticulum, resulting in a rapid and transient elevation in the concentration of free cytoplasmic $Ca^{2+}$ ($[Ca^{2+}]_i$). Elevated levels of $[Ca^{2+}]_i$ and DAG activate PKC in a number of cell types and, presumably, in parietal cells, although currently there is no direct evidence for a role of PKC in gastrin-stimulated acid secretion. Gastrin-stimulated increases in $[Ca^{2+}]_i$ could, however, (1) promote vesicular motor activity through the activation of downstream $Ca^{2+}$/calmodulin-dependent myosin light chain kinase, (2) regulate actin filament turnover, or (3) promote interactions between SNAREs and the $Ca^{2+}$-dependent phospholipid-binding protein annexin (Fig. 3). Further studies are necessary to examine the potential role of these pathways in gastrin-stimulated $H^+$,$K^+$-ATPase translocation and acid secretion.

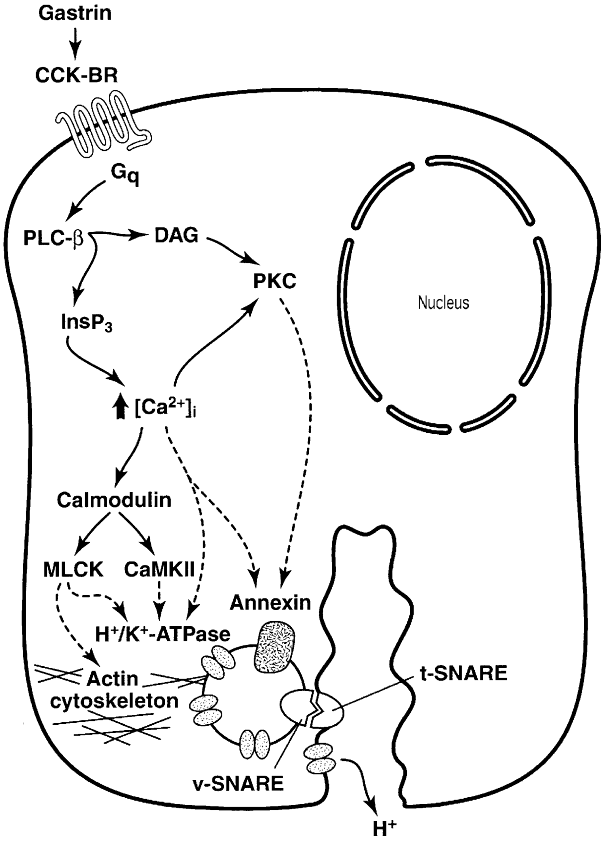

FIGURE 3 Parietal cell. Summary of pathways mediating gastrin-stimulated acid secretion from the gastric parietal cell (dashed lines indicate proposed pathways). CCK-BR, Cholecystokinin-B receptor; PLC-β, phospholipase C-β; DAG, diacylglycerol; PKC, protein kinase C; $InsP_3$, inositol 1,4,5-trisphosphate; MLCK, myosin light chain kinase; CaMKII, calmodulin kinase II; v-SNARE, vesicle soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, target membrane SNARE.

## D. Gastrin Potentiates Histamine-Stimulated Acid Secretion

Gastrin regulates acid secretion, in part, by binding to CCK-BR on parietal cells to stimulate acid release; however, in most species this direct effect of gastrin is relatively weak. More significant to the overall acid production by parietal cells are the indirect effects of gastrin on ECL cell histamine release. Gastrin binds to CCK-BR on the ECL cells, where it stimulates both production and release of histamine. Gastrin increases histamine production by stimulating histidine decarboxylase activity (Fig. 4). Treatment of ECL cells with pertussis toxin inhibits the effects of gastrin on histidine decarboxylase activity, suggesting the involvement of $G_{i/o}$ in this process. Once released from ECL cells, histamine, acting in a paracrine fashion, binds to $H_2$-type histamine receptors on the parietal cell to greatly enhance acid secretion through a cAMP- and protein kinase A (PKA)-dependent pathway (Fig. 2). Where gastrin- and histamine-stimulated signaling pathways converge to potentiate acid secretion is currently unknown.

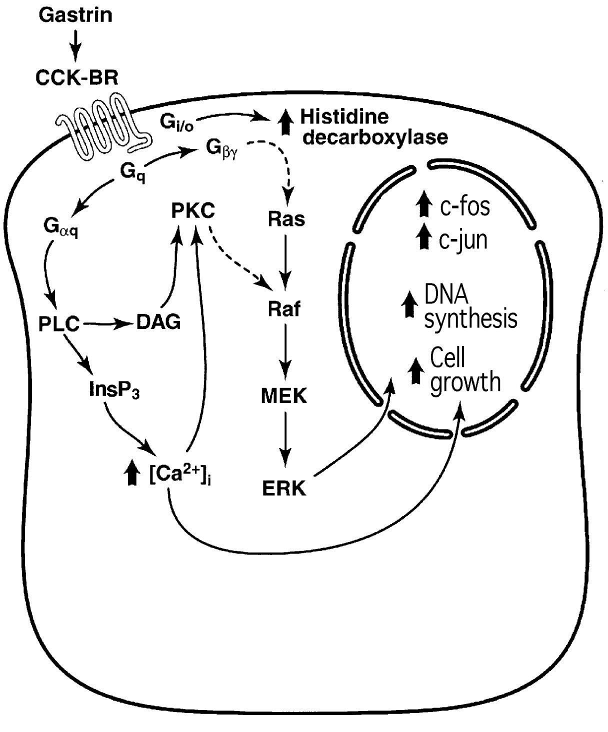

FIGURE 4 Enterochromaffin-like cell. Summary of pathways mediating gastrin-stimulated growth in the enterochromaffin-like cell (dashed lines indicate proposed pathways). CCK-BR, Cholecystokinin-B receptor; PKC, protein kinase C; PLC, phospholipase C; DAG, diacylglycerol; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.

## V. GASTRIN REGULATION OF CELL GROWTH

In addition to its role in gastric acid secretion, gastrin also acts as a potent growth factor and has been implicated in a variety of normal and abnormal biological processes, including maintenance of the gastric mucosa, proliferation of ECL cells, and neoplastic transformation. It is well established that in rats and humans, hypergastrinemia (higher than normal concentration of gastrin in the blood) is associated with ECL cell proliferation and occasionally with the development of gastric carcinoid tumors. Also, there is epidemiological evidence implicating hypergastrinemia as a risk factor for the development of colorectal cancer, and studies using genetically altered mice have demonstrated the proliferative effects of gastrin on the normal stomach and colonic mucosa. Many cell lines have been used to study the signal transduction pathways associated with the proliferative effects of gastrin. Some of these cell lines express an endogenous CCK-BR and others have been transfected with expression plasmids containing the CCK-BR cDNA. Accumulating evidence indicates that mitogen-activated protein kinases (MAPKs) play a central role in gastrin-stimulated cell proliferation.

### A. Mitogen-Activated Protein Kinases

MAPKs are serine/threonine-directed kinases that are activated by a variety of stimuli, including growth factors, cytokines, neurotransmitters, hormones, extracellular matrix proteins, and cell stress. MAPKs regulate a number of cellular processes, including gene transcription, protein translation, metabolism, and the function of the cytoskeleton. As a result, MAPKs are involved in the control of cell growth, differentiation, and apoptosis. These actions are carried out by phosphorylating downstream target proteins and by carrying information to the nucleus or cytoskeleton through translocation within the cell. Currently, five MAPK pathway subfamilies have been identified, including the extracellular signal-regulated kinases (ERK1 and ERK2), the c-Jun N-terminal kinases (JNKs), the 38-kDa MAPKs (p38 MAPKs), ERK5, and ERK3/ERK4.

MAPKs are activated by a kinase cascade composed of three families of protein kinases acting in series. Each MAPK is activated by an upstream MAPK kinase (MAPKK), which is, in turn, activated by a MAPK kinase kinase (MAPKKK). All MAPKKs are dual-specificity kinases that activate MAPKs by phosphorylating both the tyrosine and threonine residues present in the consensus sequence (T-X-Y). Six MAPKK family members have been identified and are designated MEK1–MEK5 and ERK3 kinase. Upstream of MAPKKs are the MAPKKKs, which include protein kinases such as Raf-1, Raf-A, and Raf-B, transforming growth factor-β (TGF-β)-activated kinase (TAK), and thousand-and-one-amino acid kinase (TAO). MAPKKKs receive information from cell surface receptors, usually through a small GTP-binding protein of the Ras superfamily. The Ras superfamily members include Ras, Rho, Rac, and Cdc42, all of which, like the Gα subunits of heterotrimeric G-protein, are activated when bound with GTP.

### B. MAPK Activation through PKC- and $Ca^{2+}$-Dependent Pathways

Gastrin-stimulated ERK activation has been reported in a variety of cells, including the human gastric cancer cell line AGS-B, Rat1 fibroblasts, Chinese hamster ovary (CHO) cells, NIH 3T3 cells, and in the rat pancreatic cell line AR42J. In AR42J cells, gastrin activates PKC, stimulates MAPK pathways, activates the Ras-related protein Rho, and induces c-*fos* gene expression. Inhibition of either PKC or the agonist-stimulated increase in $[Ca^{2+}]_i$ blocks gastrin-stimulated ERK activation in AR42J cells, indicating that MAPK activation requires PKC activity. Another indication of the critical role of PKC in gastrin-stimulated signal transduction is the observation that inhibitors of PKC block ERK-mediated phosphorylation and activation of the transcription factors Elk1 and Sap-1a, which regulate the activity of the serum response element (SRE) located in the c-*fos* promoter. The precise mechanism by which gastrin-stimulated PKC activates ERK is unknown. However, there is evidence in AR42J cells that gastrin stimulates rapid tyrosine phosphorylation of a complex of proteins composed of the adapter protein Shc (Src homology and collagen), Grb2 (growth factor receptor-bound protein 2), and the guanine nucleotide exchange factor Sos (son-of-sevenless), which, in turn, leads to the activation of Ras and Raf. However, in other cells, gastrin apparently regulates MAPK through a Ras-independent activation of Raf.

Gastrin stimulates both a rapid release of $Ca^{2+}$ from intracellular stores and an influx of $Ca^{2+}$ across the plasma membrane from the extracellular fluid. Depending on cell type and gastrin concentration, CCK-BR stimulation induces either a rapid and sustained increase in $[Ca^{2+}]_i$ or oscillatory increases in $[Ca^{2+}]_i$. $Ca^{2+}$ oscillations have been linked to CCK-BR regulation of cell growth because gastrin-stimulated increases in DNA synthesis are blocked when the C-terminal 44 amino acids of CCK-BR are removed. Although agonist stimulation of the truncation mutant of CCK-BR causes an elevation in $[Ca^{2+}]_i$, $Ca^{2+}$ oscillations are not induced. These data demonstrate the importance of the pattern of change in $[Ca^{2+}]_i$ in defining the specific cellular response to a particular signal. The idea that the pattern of change in $[Ca^{2+}]_i$ can encode specific cellular responses is supported by recent data showing that altering both the amplitude and the duration of intracellular $Ca^{2+}$ oscillation affects the patterns of expression of specific genes. Neither the mechanism by which gastrin induces $Ca^{2+}$ oscillations nor the molecular events linking $Ca^{2+}$ oscillation to gastrin-stimulated cell growth have been discovered.

### C. Gastrin Stimulates ECL Cell DNA Synthesis

Unlike parietal cells, ECL cells are capable of proliferation and are stimulated to do so by gastrin. Gastrin increases the number of isolated rat ECL cells in culture, suggesting a direct mitogenic effect, and stimulates replication of ECL cells *in vivo* as measured by $[^3H]$thymidine incorporation. Moreover, hypergastrinemia is associated with an increase in ECL cell numbers in humans and rats and with the occurrence of gastric ECL cell carcinoid tumors. Using ECL cells isolated from the small sub-Saharan rodent, *Mastomys natalensis*, it was found that gastrin stimulated a dose-dependent increase in DNA synthesis measured by an increase in the incorporation of $[^3H]$thymidine. The gastrin-induced increase in DNA synthesis was blocked by the selective MAPKK inhibitor, PD98059. These results contrast those seen with parietal cells from the same animal, which showed that gastrin had no effect on either MAPK activity or $[^3H]$thymidine incorporation.

## VI. CCK REGULATION OF PANCREATIC ENZYME RELEASE AND CELL GROWTH

In the pancreas, CCK is a major regulator of digestive enzyme secretion in response to a meal. The pancreatic acini are the functional units for pancreatic exocrine secretion. The primary function of the pancreatic acinar cell is to synthesize, package, and secrete a variety of digestive enzymes, including trypsinogen, chymotrypsinogen, lipases, and pancreatic amylase. In addition to stimulating secretion, it has recently become clear that CCK also is an important factor in the regulation of acinar cell growth, energy product, gene expression, and protein synthesis. All of these responses are initiated on binding of CCK to the CCK-AR located on the basolateral membrane surface of pancreatic acinar cells (Fig. 5).

### A. CCK Stimulates Phospholipase Activity and an Increase in $[Ca^{2+}]_i$

CCK-stimulated amylase release from pancreatic acinar cells requires an agonist-induced increase in $[Ca^{2+}]_i$. It appears that the G-proteins involved in CCK-mediated increases in $[Ca^{2+}]_i$ belong to the $G_q$ subfamily of heterotrimeric G-proteins, which, through the activation of PLC, stimulate an $InsP_3$-dependent increase in $[Ca^{2+}]_i$. Pancreatic acinar cells contain all three $G_q$ family members, which include $G_q$, $G_{11}$, and $G_{14}$. Studies using G-protein α-subunit-specific antibodies have demonstrated that CCK activation of PLC is inhibited by antibodies directed against the COOH-terminal domains of

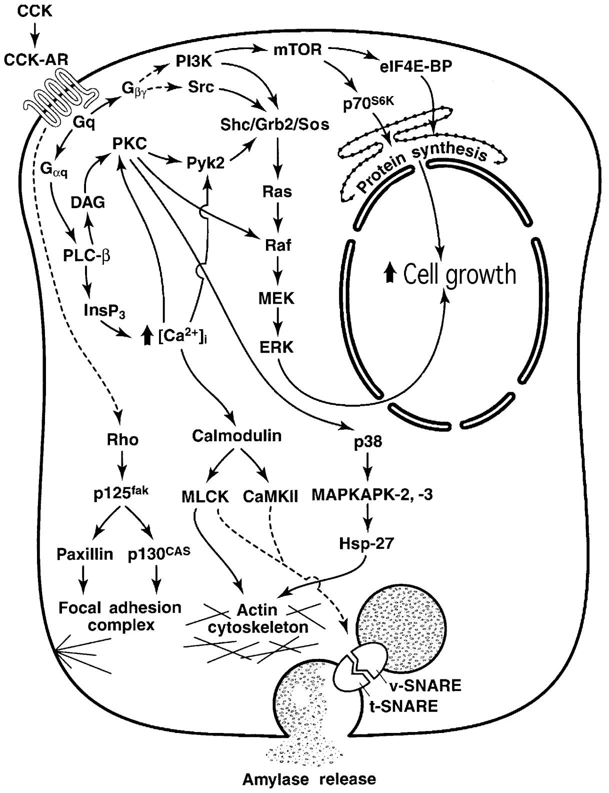

FIGURE 5 Pancreatic acinar cell. Summary of signal transduction pathways mediating cholecystokinin (CCK)-stimulated pancreatic acinar cell growth and amylase secretion (dashed lines indicate proposed pathways). PI3-K, Phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin (protein); eIF4E-BP, elongation initiation factor binding protein; MAPKAPK, mitogen-activated protein kinase-activated protein kinase (see Figs. 3 and 4 for other abbreviations).

$G_{\alpha q}$ and $G_{\alpha 11}$, indicating the involvement of these G proteins in CCK stimulation of PLC activity.

In addition to $G_{\alpha}$ subunits, the $G_{\beta\gamma}$ subunits of G-proteins also activate some isoforms of PLC. Rat pancreatic acinar cells express several PLC phosphatidylinositol-specific isoforms, including PLC-β1, -β3, -γ1, and -δ1. Treatment of acinar cells with a low concentration of CCK induces an oscillating $Ca^{2+}$-activated chloride current across the plasma membrane. The activation of this chloride channel requires an increase in $[Ca^{2+}]_i$ and, therefore, its activity is frequently used as an experimental indicator of elevated levels of $[Ca^{2+}]_i$. Addition of purified $G_{\beta\gamma}$ subunits, via a micropipette, induced intracellular $Ca^{2+}$ oscillations, mimicking the effect of CCK on the $Ca^{2+}$-activated chloride current, whereas addition of a $G_{\beta\gamma}$ scavenger blocked the $Ca^{2+}$-activated chloride current induced by CCK. These data suggest that $G_{\beta\gamma}$ plays a role in mediating the effects of CCK on the regulation of $[Ca^{2+}]_i$, perhaps through the regulation of specific $G_{\beta\gamma}$-sensitive PLC isoforms. However, studies using specific anti-PLC antibodies have found that only an anti-PLC-β1 antibody blocked CCK-stimulated PLC activity. PLC-β1 is generally believed to be regulated by $G_{\alpha q}$ and not $G_{\beta\gamma}$, suggesting that the effects of $G_{\beta\gamma}$ on $[Ca^{2+}]_i$ are not mediated by PLC.

In addition to $InsP_3$ and DAG, a third product of PLC activity, arachidonic acid, is involved in CCK-stimulated amylase secretion from rat pancreatic acinar cells. Several laboratories have found that CCK-induced intracellular $Ca^{2+}$ oscillation and amylase release are mediated by arachidonic acid. Increased arachidonic acid production in response to CCK can occur through two distinct pathways. One pathway involves the action of PLC and diglyceride lipase on phosphoinositides and another involves phospholipase $A_2$-mediated hydrolysis of phosphatidylcholine. Both pathways appear to be involved in CCK stimulation of amylase release from acinar cells.

## B. CCK Activates $Ca^{2+}$-Dependent Protein Kinases

The elevation in $[Ca^{2+}]_i$ induced by CCK initiates the activation of various $Ca^{2+}$-dependent protein kinase cascades, including $Ca^{2+}$-dependent isoforms of PKC and $Ca^{2+}$-activated calmodulin (CaM)-dependent kinases. Calmodulin is a ubiquitous 19-kDa $Ca^{2+}$-binding protein that is responsible for activating several acinar cell enzymes, including multiple protein kinases, in a $Ca^{2+}$-dependent manner. Seven distinct CaM kinases have been characterized. Four of these have been identified and/or purified from pancreatic tissue, including myosin light chain kinase (MLCK), CaM kinase I, CaM kinase II, and CaM kinase III. MLCK is believed to mediate the increased phosphorylation of myosin light chains observed in pancreatic acinar cells following stimulation with CCK. Furthermore, an inhibitor of MLCK, ML-9, blocks amylase release from acini, indicating an important role for this kinase in CCK-stimulated secretion.

Another important family of protein kinases activated, in part, by $Ca^{2+}$ is the PKC family. The PKC enzymes are widely distributed in many tissues and phosphorylate a wide variety of substrates on serine and threonine residues. Three groups or subfamilies of PKC isoforms have been characterized based on similarities of their biochemical properties and amino acid sequences. The three subfamilies are the conventional (sometimes referred to as classical), novel, and atypical protein kinases. The conventional PKC enzymes, which include PKC α-I, β-I, β-II, and γ, are activated by phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), DAG, and $Ca^{2+}$. The novel PKC isoforms (δ, ϵ, η, θ), on the other hand, are activated by phorbol esters and DAG but not by $Ca^{2+}$. Following treatment of cells with phorbol esters, there is a translocation (activation) of PKC from the cytosol to the membranes, as defined by cell fraction experiments. CCK stimulation of pancreatic acinar cells causes the translocation of PKC enzyme activity to the membrane fraction. Also, a considerable amount of evidence supports a role for PKC in the regulation of CCK-stimulated amylase secretion. Prolonged incubation of cells with either TPA or other phorbol esters will down-regulate PKC activity within the cells. Down-regulation of PKC in guinea pig acini decreases both the magnitude and the sensitivity of CCK-stimulated amylase release without affecting the response to $Ca^{2+}$ ionophore or VIP. Several recent studies have focused on identifying the isoforms of PKC present in acini. Western blotting using subtype-specific antibodies shows the presence of PKC α, β, δ, ϵ, and ξ. However, only the novel PKC δ and ϵ are translocated to a membrane fraction on CCK stimulation, suggesting that these $Ca^{2+}$-independent subtypes may mediate the stimulatory effects of CCK on amylase secretion.

### C. ERKs Mediate CCK-Stimulated Acinar Cell Growth

Of the six MAPK pathways identified, three are involved in CCK-stimulated signal transduction—the ERK, JNK, and p38 MAPK cascades. In isolated rat pancreatic acini and in the rat pancreas *in vivo*, CCK stimulates a rapid and transient increase in the level of phosphorylation and kinase activity of two isoforms of ERK, ERK1 and ERK2 (also known as p44 and p42 MAPK, respectively). The activating phosphorylation of ERK1 and ERK2 results from the activation of the upstream, dual-specificity MAPK kinases, MEK1 and MEK2. Several pathways leading to MEK activation have been identified. In pancreatic acinar cells, epidermal growth factor (EGF) receptor, a tyrosine kinase receptor, activates MEKs and hence ERKs via the Shc/Grb2/Sos protein complex, as well as Ras and Raf. In contrast to EGF, CCK only weakly affects Shc/Grb2/Sos complex formation and has no measurable effect on Ras activity. However, CCK stimulation of ERK activity is blocked by inhibitors of PKC, suggesting that the major mechanism for CCK activation involves PKC-mediated, Ras-independent activation of Raf. Further support for a Ras-independent activation of ERK by CCK in acinar cells is provided by experiments using an adenoviral vector expressing a dominant-negative N17Ras. Expression of N17Ras in rat acinar cells did not affect CCK-stimulated amylase release, intracellular $Ca^{2+}$ mobilization, or ERK activation, but completely blocked EGF-stimulated ERK activation. These data support the conclusion that, in contrast to EGF, CCK stimulates ERK activity through a Ras-independent mechanism.

### D. CCK Activation of JNK: A Possible Role in Pancreatitis

Although JNKs may play a role in cell proliferation, their major role appears to be in the regulation of apoptosis, or programmed cell death, in response to stress stimuli. CCK activates two isoforms of JNK (p46 and p55) in pancreatic acini both *in vitro* and *in vivo*; however, the activation is slower than that of ERKs and also requires higher concentrations of hormone. It is well established that high concentrations of CCK can induce pancreatitis in rats and mice and that activation of JNK is one of the earliest events associated with pancreatitis. Interestingly, inhibition of JNK with a selective inhibitor blocks the edema associated with pancreatitis, suggesting a possible beneficial role for JNK in mediating the stress response. However, a role for CCK in the regulation of pancreatic JNK activity under normal conditions has not been established.

### E. p38 MAPK Regulates Actin Cytoskeleton Organization

The p38 MAPKs are activated both by stresses (UV irradiation, heat, and osmotic stress) and by growth stimuli (hematopoietic growth factors). CCK rapidly activates p38 MAPK in rat pancreatic acinar cells in a time- and dose-dependent manner. In contrast to JNK activation, which requires higher (superphysiological) concentrations of CCK, maximal activation of p38 MAPK occurs between 0.3 and 1.0 n*M*. Interestingly, treatment of rat acini with higher concentrations of CCK (10 n*M*) leads to a decrease in p38 MAPK activity, suggesting that agonist concentration may be an important determinant in the selective activation of different MAPK pathways. CCK stimulation of rat acinar cells causes disruption of the actin cytoskeleton, which may be important for SNARE-mediated docking and fusion of zymogen granules with the plasma membrane. Inhibition of p38 MAPK with the selective inhibitor SB203580 blocks the effects of CCK on actin organization. Several downstream targets of CCK-activated p38 MAPK have been identified, including MAP kinase-activated protein (MAPKAP)-2 kinase and -3, and heat-shock protein (Hsp)-27; however, the functions of these proteins in acinar cell physiology remain largely unknown.

### F. CCK Regulates Protein Synthesis through the PI3-K–mTOR–p70$^{S6K}$ Pathway

CCK regulation of digestive enzyme secretion and acinar cell growth requires new protein synthesis. The PI3-K–mTOR–p70$^{S6K}$ pathway is the primary regulator of protein synthesis. The p70$^{S6K}$ protein is one of two kinases that phosphorylate the small ribosomal subunit protein S6, thereby regulating the synthesis of mRNA with 5′-polypyrimidine tracts. The mammalian target of rapamycin (mTOR) protein stimulates protein synthesis by phosphorylating the elongation initiation factor binding protein eIF4E-BP (also called PHAS-1), which is the binding protein for the translation initiation factor, eIF4E. In a nonphosphorylated state, eIF4E-BP is bound to eIF4E. After phosphorylation by mTOR, eIF4E-BP dissociates from eIF4E; eIF4E then forms a complex with the scaffolding protein eIF4A and the RNA helicase eIF4G, and initiates translation. CCK stimulates phosphorylation of p70$^{S6K}$ and eIF4E-BP in rat pancreatic acini. CCK-stimulated phosphorylation of p70$^{S6K}$ is blocked by rapamycin, which, on binding to its intracellular receptor FKBP-12, inhibits mTOR, and by wortmannin, an inhibitor of PI3-K. Phosphorylation of eIF4E-BP and release of eIF4E are inhibited by wortmannin. The net result of treating pancreatic acinar cells with either rapamycin or wortmannin is inhibition of CCK-stimulated protein synthesis. The mechanism by which CCK activates PI3-K in acinar cells is currently unknown. However, in other cell types, $G_{\beta\gamma}$ mediates GPCR activation of the PI3-K pathway. Further studies are required to determine if a similar pathway is utilized in acinar cells.

### G. CCK Regulation of Tyrosine Kinase Activity and Rho GTPase

CCK stimulation of rat pancreatic acini causes a rapid and dose-dependent phosphorylation of tyrosine residues on p125$^{Fak}$, p130 Crk-associated substrate (p130$^{CAS}$), and paxillin. The phosphorylation of these proteins does not require activation of PKC or an increase in $[Ca^{2+}]_i$ and is associated with profound alterations in the organization of the actin cytoskeleton and the assembly of focal adhesions. Treating the cells with *Clostridium botulinum* C3 toxin, an ADP-ribosyltransferase that inhibits Rho GTPase activity, blocks the CCK-stimulated tyrosine phosphorylation of p125$^{Fak}$ and paxillin, suggesting a role for Rho in the tyrosine phosphorylation of these proteins. Elucidation of the signaling mechanisms coupling CCK to Rho-mediated changes in cytoskeleton organization will, however, require additional investigations.

Recently, Pyk2, a Ca$^{2+}$-dependent kinase related to p125$^{Fak}$, was found to be activated on CCK stimulation of acinar cells. In other cell types, Pyk2 activates ERKs through an Shc/Grb2/Sos complex. Whether a similar mechanism regulates CCK activation of ERKs in pancreatic acinar cells is not known.

## VII. SECRETIN-STIMULATED SIGNALING TRANSDUCTION

Secretin is a member of a larger family of related peptides that includes GIP, VIP, parathyroid hormone (PTH), growth hormone-releasing hormone (GHRH), and exedens. All of these peptides share a high degree of amino acid sequence homology, are widely distributed in the body, and exert pleiotropic physiological effects, in many instances by acting in a paracrine manner. Secretin is the principal hormonal stimulant of pancreatic and biliary bicarbonate ($HCO_3^-$) and water secretion and it may contribute to pancreatic enzyme secretion. High concentrations of secretin may also contribute to gastric secretion of pepsinogen, inhibit gastric acid secretion and postprandial gastric emptying, and stimulate secretion of bicarbonate and epidermal growth factor from Bunner's glands.

The effects of secretin on target tissues result from activation of adenylyl cyclase and the subsequent generation of 3′, 5′-cyclic adenosine monophosphate (cAMP) and the activation of cAMP-dependent PKA. Studies assessing the presence of $G_{\alpha s}$ in secretin receptor-expressing cells and the ability of guanosine triphosphate (GTP) analogues to diminish radiolabeled secretin binding support the idea that secretin stimulates cAMP production through the $G_{\alpha s}$-mediated activation of adenylyl cyclase.

Additional molecular targets of secretin stimulation include cAMP-dependent activation of the cystic fibrosis transmembrane regulator Cl$^-$channels and translocation of the water channel, aquaporin-1, from subcellular organelles to the cell surface of rat cholangiocytes. The latter response to secretin stimulation is inhibited by treating the cholangiocytes with colchicine, an inhibitor of microtubule polymerization. Finally, secretin-stimulated increases in cAMP result in activation of the slowly activating $K^+$ current ($I_{Ks}$) in rat pancreatic acinar cells. The signaling pathways linking cAMP to activation of these various channels are not known. Presumably, the activation of PKA is required, but further investigations are needed to confirm this mechanism.

## VIII. FUTURE DIRECTIONS

Application of modern molecular biology to the study of GI hormone-regulated signal transduction has revealed complex webs of interrelated signaling pathways that regulate diverse and sometime overlapping and competing biological responses. The challenge for future investigation is to unravel the webs of signaling pathways, with the goal of understanding how specific cellular responses are generated. It is generally accepted that an important determinant of signaling specificity is the cellular context in which it occurs. One way to study signaling pathways in the appropriate physiology and cellular context is with genetically altered animal models. Transgenic and gene "knockout" animals provide a powerful tool for examining the effects of defined genetic changes in the context of the whole animal. One needs only to consider possible species differences when extrapolating the results from experimental animal models to humans. Other areas that warrant future effort are the development of human transgenic and gene-disrupted gastrointestinal cell lines, and improved methodologies for quantitative kinetic analyses of signaling pathways. After all, the real challenge to unraveling the complex signaling networks is to distinguish between what is functionally possible and what is biologically important.

## Glossary

**cholera toxin** An A-B-type bacterial toxin produced by the bacterium *Vibrio cholerae*; catalyzes transfer of the ADP-ribose moiety of nicotinamide dinucleotide to the α-subunit of G-proteins of the $G_s$ subfamily, resulting in their constitutive activation.

**enterochromaffin-like cells** Histamine-producing cells located in the gastric glands of the stomach.

**mitogen** Substance that induces an increase in cell proliferation, usually by stimulating DNA, RNA, and protein synthesis.

**pancreatitis** Acute or chronic inflammation of the pancreas.

**paracrine** Describes the relationship between a hormone-releasing cell and its target cell. Hormones that act in a paracrine manner are released from a cell into the extracellular space and act on adjacent target cells without reaching the general circulation.

**parietal cells** Acid-producing cells located in the gastric glands of the stomach.

**pertussis toxin** An A-B-type bacterial toxin produced by the bacterium Bordetella pertussis; catalyzes transfer of the ADP-ribose moiety of nicotinamide dinucleotide to the α-subunit of G-proteins of the $G_{i/o}$ subfamily, resulting in their inactivation.

**protein kinases** Class of enzymes that catalyze the transfer of the γ phosphate from ATP to the amino acid side chains of substrate proteins. Typically, the phosphate is transferred to serine, threonine, or tyrosine residues within a protein. Generally, the activity of a protein kinase is directed to a specific consensus site, consisting of several amino acids in a defined sequence.

## See Also the Following Articles

**Cholecystokinin (CCK) • Gastrin • Protein Kinases • Secretin**

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# Gastrointestinal Hormone-Releasing Peptides

Jan-Michel Otte*, Jaroslaw Walkowiak†, and Karl-Heinz Herzig*,‡

*Christian-Albrechts-Universität, Germany ● †Karol Marcinkowski University of Medical Sciences, Poland ● ‡University of Kuopio, Finland

I. CHOLECYSTOKININ/SECRETIN-RELEASING FACTORS
II. FUTURE PERSPECTIVES

**Since the discovery and isolation of the first gastrointestinal hormones, secretin, gastrin, and cholecystokinin, more than 100 different biologically active gut peptides have been discovered. These peptides regulate gut motility, secretion, digestion, and adsorption of nutrients. In the past decade, much interest has been generated regarding the hypothesis that the secretion of gastrointestinal hormones is regulated, in part, by releasing factors produced in the gut. The purpose of this article is to describe these releasing factors.**

## I. CHOLECYSTOKININ/SECRETIN-RELEASING FACTORS

### A. Lumenal Cholecystokinin-Releasing Factor

Lumenal cholecystokinin (CCK)-releasing factor (LCRF) was isolated originally from a jejunal perfusate of conscious rats. The peptide has a molecular mass of 8136 Da and is composed of 70–75 amino acids. LCRF exhibits a unique amino acid composition with no sequence homology. Microsequence analysis revealed only a partial N-terminal sequence of 41 residues; the remainder of the LCRF sequence structure has not been described. LCRF-like immunoreactivity has been found throughout the gut, including the pancreas, with the highest levels in the small intestine. Immunohistochemistry showed strong staining of the epithelium of intestinal villi, Brunner's glands, myenteric plexus, gastric pits, and pancreatic ductules and islets. The natural endogenous peptide and different synthetic fragments ($LCRF_{1-41}$, $LCRF_{1-35}$, and $LCRF_{11-25}$, but not $LCRF_{1-6}$) have significant CCK-releasing activity.

The N-terminal fragment of LCRF, $LCRF_{1-35}$, significantly stimulated CCK release in a dose-dependent manner (5–200 n*M*) from dispersed human intestinal mucosa cells. In the rat, diversion-stimulated pancreatic exocrine secretion is inhibited by somatostatin, peptide YY (PYY), and the bile acid taurocholate. One report shows that in conscious rats, somatostatin does not affect lumenal LCRF levels but inhibits diversion-stimulated CCK release and pancreatic enzyme secretion, suggesting that LCRF secretion is not regulated by somatostatin. The effect of PYY or bile acids on LCRF-stimulated CCK release has not been described. Another report shows that a diversion-stimulated increase of LCRF in the duodenal lumen was not inhibited by atropine and that intraduodenal and intravenous administration of the synthetic somatostatin analogue octreotide inhibited CCK release and lowered LCRF and pancreatic secretion stimulated by bile and pancreatic juice diversion in rats, indicating that somatostatin may have a role in the regulation of LCRF secretion. Further work needs to be done to examine the role of endogenous LCRF in nutrient-induced CCK secretion and its interaction with other CCK-releasing factors.

### B. Diazepam-Binding Inhibitor

In the search for a CCK-releasing factor, another peptide, diazepam-binding inhibitor (DBI), was isolated and sequenced from porcine intestinal mucosa; the peptide consists of 86 amino acids and has a molecular mass of 9810 Da. DBI was first isolated from rat brain and was named for its ability to displace diazepam from the γ-aminobutyric acid type A receptor. DBI has been detected in a number of organs outside the central nervous system, with the highest concentration in the liver, duodenum, testis, kidney, and adrenals. $DBI_{1-86}$ and $DBI_{32-86}$ have been found in the porcine duodenum. The primary structure of this protein is highly conserved. In agreement with its wide distribution, multiple biological functions have been described for this protein. In addition, DBI has been reported to function as a high-affinity acetyl coenzyme A-binding protein in the liver, to stimulate steroidogenesis in adrenal cells, to act as an autocrine/paracrine regulator of Leydig cell function, and to inhibit glucose-stimulated insulin release. DBI immunoreactivity is detected in the endocrine pancreas and in the secretory epithelial cells of the gut. Intraduodenal infusion of synthetic porcine DBI in rats significantly stimulates pancreatic amylase output and CCK release, which is completely blocked by the CCK antagonist MK326. In anesthetized rats with pancreaticobiliary and intestinal cannula, a 2-fold increase in pancreatic protein secretion accompanied by a 10-fold increase in CCK plasma levels has been observed. These increases were paralleled by a 4-fold increase in lumenal DBI levels. Furthermore, after 5 h of bile–pancreatic diversion and return of CCK levels to baseline, intraduodenal infusion of peptone significantly stimulated DBI release, which was blocked by a DBI antibody. Previously, it has been shown that peptone, but not casein, maltose, or L-amino acids, stimulated the secretion of CCK-releasing peptide into the lumen. 5-HT$_3$ and substance P antagonists blocked peptone-stimulated secretion. Based on these findings, Li and Owyang suggested that peptone stimulates serotonin release, which activates the sensory substance P neurons in the submucosal plexus. Signals are then transmitted to cholinergic interneurons and epithelial CCK-releasing peptide (CCK-RP)-containing cells via cholinergic secretomotor neurons (Fig. 1).

Regulation of DBI-induced CCK release has been shown *in vitro* in isolated intestinal mucosal cells. Somatostatin inhibited DBI-stimulated CCK release in a dose-dependent manner. This inhibition could be reversed by pertussis toxin, indicating that somatostatin acts via its receptor on CCK cells (I cells) coupled to an inhibitory guanine nucleotide-binding protein of adenylate cyclase. In contrast to somatostatin, neither PYY nor taurocholate affects DBI-stimulated CCK release, suggesting that these substances act through other mechanisms to inhibit feedback-stimulated exocrine pancreatic secretion.

## C. Secretin-Releasing Peptide

Intestinal secretin regulates pancreatic water and bicarbonate secretion. Secretin is released from S cells in the upper small intestine by various stimuli such as gastric acid, fatty acids, and bile salts. In addition, several neuropeptides such as pituitary adenylate cyclase-activating polypeptide (PACAP), gastrin-releasing peptide (GRP), and serotonin via 5-HT$_{2/3}$ receptors stimulate the release of secretin. Diversion of pancreatic juice from the duodenum increases plasma levels of secretin in addition to CCK,

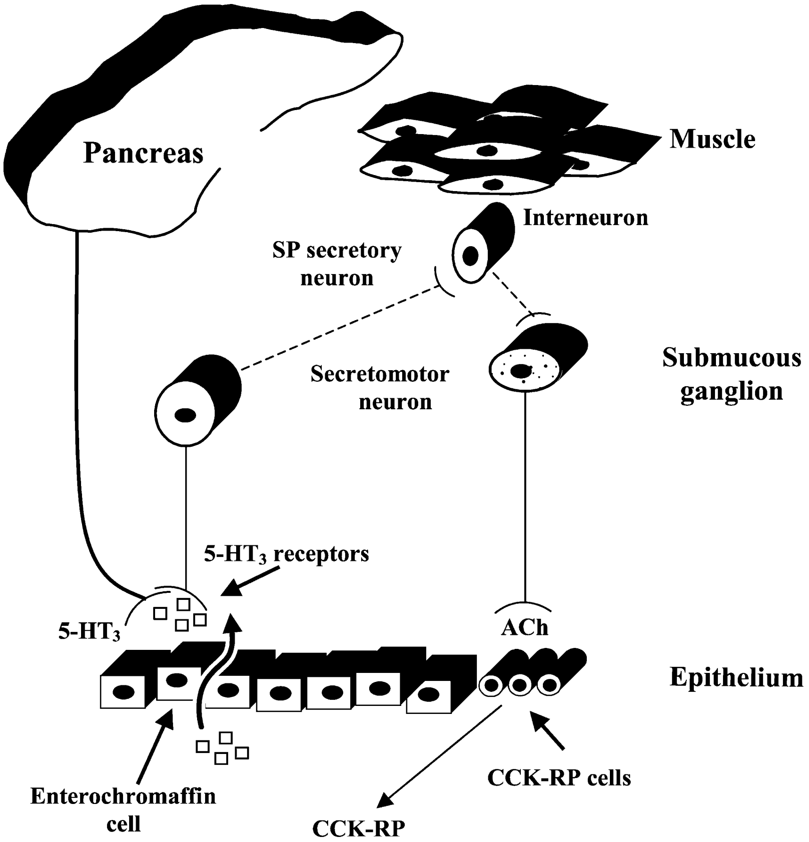

FIGURE 1 Model of the neuroendocrine control of the secretion of CCK-releasing peptide (CCK-RP). Peptone stimulates CCK-RP secretion by activating intestinal submucosal cholinergic neurons. Ach, acetylcholine. Adapted from Li, Y., and Owyang, C. (1996). Peptone stimulates CCK-releasing peptide secretion by activating intestinal submucosa cholinergic neurons. *J. Clin. Invest.* 97, 1463–1470.

indicating a regulatory feedback control mechanism for secretin release. To further investigate the mechanism of this feedback regulation, donor rats were intraduodenally perfused with 0.01% hydrochloric acid. Reinfusion of the adjusted pH and threefold concentrated perfusate into a recipient rat significantly stimulated volume flow, bicarbonate output, and plasma secretin levels. These effects were completely blocked by intravenous infusion of rabbit anti-secretin serum 15 min prior to the intraduodenal perfusion of the concentrated perfusate from the donor rats. Treatment of the perfusate with trypsin also abolished the effect, suggesting that acid-stimulated secretin release is mediated by a peptide produced in the upper small intestine. The release and action of this peptide have been shown to be neuromodulated. Recently, a secretin-releasing peptide has been demonstrated in canine pancreatic juice. Two peptides of 14,061 and 14,053 Da have been isolated and sequenced with identical or substantial sequence homology to the canine pancreatic phospholipase A2. Testing commercially available porcine pancreatic phospholipase A2 revealed a dose-dependent stimulation of secretin release from murine intestinal CCK cells (STC-1 cells) and from isolated rat duodenal mucosa. Preincubation of the concentrated intestinal perfusate from donor rats with polyclonal rabbit porcine phospholipase A2 antibodies significantly suppressed secretin-releasing activity in recipient rats. The physiological relevance of duodenal and pancreatic phospholipase A2 still needs to be investigated and defined.

## D. Monitor Peptide

Diversion of pancreatic juice or the intraduodenal instillation of protease inhibitors results in a reduced tryptic activity in the duodenal lumen and enhances CCK-stimulated pancreatic enzyme secretion. Intraduodenal trypsin infusion, on the other hand, inhibits pancreatic exocrine secretion. In addition to this negative feedback mechanism in the absence of pancreatic secretion entering the duodenum, a "positive feedback" mechanism has been demonstrated in which a secreted product in the pancreatic juice itself stimulates further secretion by CCK release. Funakoshi and co-workers isolated a novel peptide from pancreatic juice and named it "monitor peptide" for its putative function of monitoring the intraduodenal environment. Purification and sequencing of monitor peptide revealed 61 amino acids exhibiting substantial homology to the family of pancreatic secretory trypsin inhibitors. The human form of monitor peptide has not been characterized. Despite being a member of the family of protease inhibitors, monitor peptide directly stimulates CCK release during vascular perfusion of the intestine devoid of intralumenal proteases and from isolated, perfused intestinal mucosal cells. Monitor peptide directly increased intracellular calcium concentrations in an enriched population of intestinal CCK cells. In addition, a reversible, temperature- and pH-dependent binding to dispersed intestinal mucosal cells has been shown. Monitor peptide-binding sites exist throughout the small intestine, corresponding to the distribution of CCK cells. $^{125}$I-monitor peptide-binding sites have been found only on the villi and not in the crypts of the small intestine. CCK stimulated the transcription of monitor peptide in a $Ca^{2+}$-dependent and protein kinase C-independent manner. In chronically ethanol-fed rats, monitor peptide mRNA levels were elevated more than four times in the pancreas, indicating that monitor peptide may contribute to the observed hypersecretion during chronic alcohol consumption.

## II. FUTURE PERSPECTIVES

Another process, apart from pancreatic secretion, that is also regulated by a complex interaction of neural, paracrine, and endocrine signals from the brain and gastrointestinal tract involving different releasing peptides, is gastric acid secretion (Fig. 2). Gastric acid secretion reflects a balance between histamine-induced stimulation and somatostatin-induced

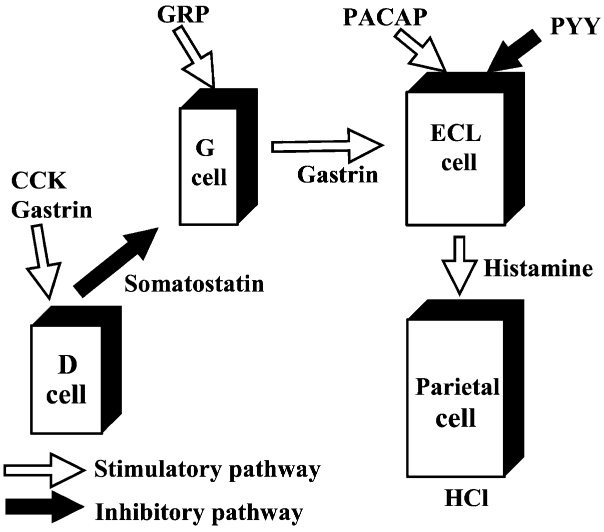

FIGURE 2 Gastric acid secretion regulated by releasing factors influencing gastrin and histamine release.

inhibition, with gastrin acting as a “histamine-releasing peptide” and CCK and CGRP acting as “somatostatin-releasing peptides.” In a recent animal study, endothelin, met-enkephalin, PYY, PACAP, and vasoactive intestinal peptide (VIP) were also shown to have strong stimulatory, concentration-dependent effects on ECL-cell histamine mobilization.

Gastrointestinal hormone-releasing peptides also have an important role in nutrient-mediated insulin secretion. These releasing peptides, the incretins, functionally link the intestine and pancreatic beta cells, thereby generating the “entero-insular axis” (Fig. 3). Incretins contributing to the entero-insular axis in a paracrine or neuronal fashion include gastric inhibitory polypeptide (GIP), glucagon-like polypeptide-1 [GLP-1 (7–36) amide], islet amyloid peptide (Amylin), CCK, GRP, and VIP. To date, the most physiologically relevant incretins are GIP and GLP-1 (7–36) amide.

In addition, peptides that play a role in the regulation of gastrointestinal function either by their own actions or by the release of other peptides have recently been identified. These include leptin, ghrelin, orexin, and apelin. Ghrelin can be regarded as an important regulatory peptide that potentially links enteric nutrition with growth hormone secretion, growth, and food intake. Orexin has been proposed to stimulate the secretion of insulin, leptin, and serotonin. Finally, apelin is the newest member of the growing family of CCK-releasing peptides. The physiological significance of apelin in protease-mediated feedback regulation of CCK and pancreatic exocrine secretion and its interaction with DBI and LCRF still needs to be investigated.

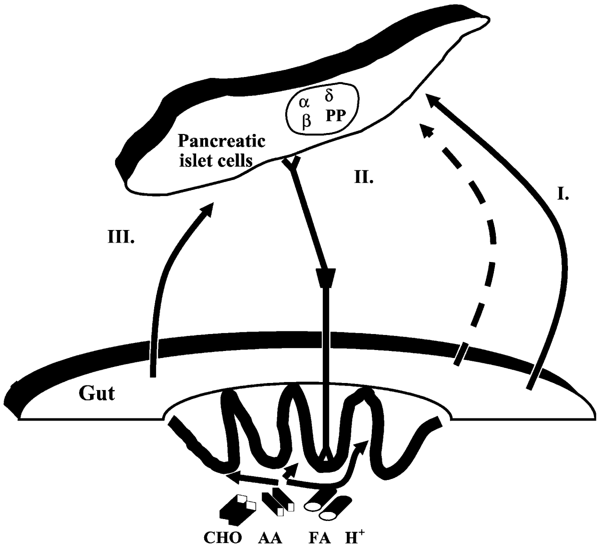

FIGURE 3 Entero-insular axis: I, endocrine transmission; II, neurocrine transmission; III, substrate stimulation. CHO, carbohydrates; AA, amino acids; FA, fatty acids; $H^+$, gastric acid. Adapted from Creutzfeldt, W. (1979) The incretin concept today. *Diabetologia* **16**, 75–85.

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## Glossary

**cholecystokinin** A peptide hormone that is secreted by mucosa endocrine cells (I cells) of the upper small intestine and that stimulates pancreatic exocrine secretion, stimulates gallbladder contraction, and inhibits gastric emptying.

**diazepam-binding inhibitor** One of the putative releasing factors for cholecystokinin.

**lumenal** Facing the interior of a cell or the interior cavity (lumen) of an organ; e.g., a releasing factor may be secreted directly into the intestinal lumen where it acts on the lumenal side of gut hormone cells localized in the intestinal epithelium.

**negative feedback** A type of feedback regulation in which the final product of a pathway acts to inhibit the first reactions in the pathway.

**releasing factor** A small peptide/protein produced in the gastrointestinal tract and/or pancreas that stimulates the secretion of gut hormones; levels of these releasing factors in the gut lumen may be regulated, in part, by degradation from pancreatic enzymes.

## See Also the Following Articles

**Cholecystokinin (CCK) • Gastrin • Protein Kinases • Secretin**

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# GH

See *Growth Hormone*

# Ghrelin

GUIYUN WANG, HEUNG-MAN LEE,
ELLA W. ENGLANDER, AND GEORGE H. GREELEY JR.
*Shriners Hospitals for Children and University of Texas Medical Branch, Galveston*

I. INTRODUCTION
II. STRUCTURE
III. DISTRIBUTION
IV. ACTIONS
V. REGULATION OF GHRELIN EXPRESSION AND SECRETION

**Ghrelin is a recently discovered ligand for the growth hormone secretagogue receptor. Ghrelin is expressed primarily in stomach endocrine cells; administration of ghrelin to animals stimulates growth hormone secretion, food intake, and weight gain. Overall, present findings suggest that ghrelin has a role in regulation of energy balance and expenditure and serves as an endocrine link between the stomach, pituitary, and hypothalamus.**

## I. INTRODUCTION

Growth hormone (GH) secretion from the pituitary gland is under the regulation of two hypothalamic hormones, GH-releasing hormone (GHRH) and somatotropin release-inhibiting factor (SRIF; somatostatin). GHRH stimulates GH release whereas SRIF inhibits GH secretion. Development of synthetic GH-releasing peptides (GHRPs) (i.e., GHRP-6, hexarelin) that stimulate GH secretion through a non-GHRH receptor has led to the hypothesis that there are unique, endogenous GH-releasing peptides distinct from GHRH. This non-GHRH receptor has been determined to be a growth hormone secretagogue receptor. Cloning of a novel G-protein-coupled receptor for the GH-releasing peptides bolstered the hypothesis of unique GH-releasing peptides, and, more recently, the endogenous ligand for the growth hormone secretagogue receptor was cloned. Two separate groups concurrently characterized and named the native ligand, one group calling the peptide ghrelin and the other group naming it motilin-related peptide. Ghrelin is the name used in this article.

## II. STRUCTURE

Ghrelin is a 28-amino-acid peptide (Fig. 1) with an n-octanoylated Ser-3 residue. The n-octanoylation is essential for biological activity. Although present data show that only the first five amino acid residues are necessary to maintain the biological activity of ghrelin, additional work must be done to show that the pentamer retains all of the biological activities of ghrelin (1–28). The cDNAs for rat, mouse, dog, and human ghrelin have been cloned. Human ghrelin is homologous to rat ghrelin with the exception of two residues. A second endogenous ghrelin variant from a missing rat stomach extract has been described; this

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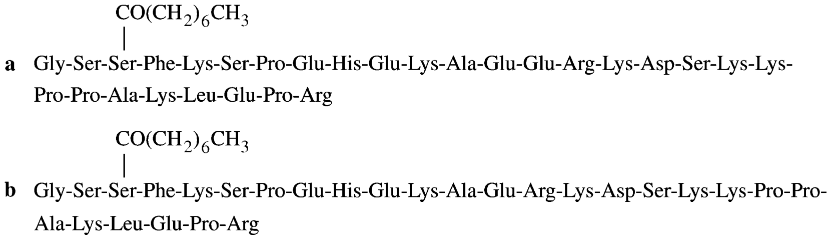

**a**

$$\text{Gly-Ser-}\overset{\overset{\text{CO(CH}_2\text{)}_6\text{CH}_3}{|}}{\text{Ser}}\text{-Phe-Lys-Ser-Pro-Glu-His-Glu-Lys-Ala-Glu-Glu-Arg-Lys-Asp-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Glu-Pro-Arg}$$

**b**

$$\text{Gly-Ser-}\overset{\overset{\text{CO(CH}_2\text{)}_6\text{CH}_3}{|}}{\text{Ser}}\text{-Phe-Lys-Ser-Pro-Glu-His-Glu-Lys-Ala-Glu-Arg-Lys-Asp-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Glu-Pro-Arg}$$

**FIGURE 1** Amino acid sequences of (a) rat ghrelin-28 and (b) *des*-$Gln^{14}$-ghrelin.

ligand, called *des*-$Gln^{14}$-ghrelin, is a 27-residue peptide and its sequence is identical to that of ghrelin except for a glutamine. *Des*-$Gln^{14}$-ghrelin has an n-octanoyl modification at Ser-3, like ghrelin, which is essential for biological activity. Genomic sequencing and cDNA analysis indicate that *des*-$Gln^{14}$-ghrelin is not encoded by a gene distinct from the ghrelin gene but is encoded by an mRNA created by alternative splicing of the ghrelin gene, making this the first described example of a novel mechanism that produces peptide multiplicity.

## III. DISTRIBUTION

Reports on the endocrine cell types expressing ghrelin in the stomach are conflicting. A mouse study demonstrates that ghrelin is expressed in stomach enteroendocrine cells that also express either chromogranin A (CGA) (a marker of endocrine cells), serotonin, or somatostatin. The same report shows that ghrelin is also expressed in stomach cells that do not express CGA or serotonin. Additionally, the mouse study reports that ghrelin expression is detected in the testis but not in various brain regions (including the olfactory bulb, hippocampus, cerebellum, cortex, and striatum) nor in mammary glands, uterus, ovary, kidney, thymus, liver, and spleen. Immunohistochemical localization of ghrelin shows a few ghrelin-containing cells in mouse crypts and villi of the duodenum and among the mucous cells of Brunner's glands. A separate rat/human study shows that stomach ghrelin is not co-expressed with somatostatin, serotonin, or histamine and is strictly localized in the X/A-like endocrine cell of the oxyntic mucosa, which accounts for approximately 20% of the endocrine cells in the oxyntic gland. The rat study shows that ghrelin-immunoreactive cells are abundant from the neck to the base of the rat oxyntic gland and rare in the pyloric gland. Ghrelin-immunoreactive cells are also found in the upper small intestine and are marginally dispersed in the distal small intestine and colon.

Radioimmunoassay (RIA) studies show a gradient of ghrelin expression in the rat gastrointestinal tract, with the mucosal layer of the stomach fundus expressing the highest concentration and lower levels in the antrum of the stomach, duodenum, jejunum, ileum, and colon. Ghrelin is not expressed in the fetal rat stomach, although its expression increases dramatically during the second and third postnatal weeks.

There are conflicting reports on the expression of ghrelin in the rat pancreas, kidney, and liver. Ghrelin and its receptor are reported to exist in the mouse kidney. Ghrelin is also expressed in other, nonenteric sites, including the placenta and hypothalamus. The ghrelin receptor shows a widespread distribution, suggesting multiple paracrine, autocrine, and endocrine roles for ghrelin. The ghrelin receptor is found in the pituitary gland, hypothalamus, stomach, heart, lung, pancreas, intestine, and adipose tissue.

## IV. ACTIONS

The biological activities of ghrelin indicate that ghrelin has a global role in the regulation of body metabolism and energy expenditure. Ghrelin administered systemically and intracerebroventricularly (ICV) stimulates GH secretion in rats, and systemically administered ghrelin stimulates GH secretion in humans (Table 1). In humans, ghrelin is more potent than GHRH and hexarelin and causes an approximately 15-fold elevation in plasma GH levels. The rise in plasma GH levels persists for longer than 60 min. In rats, the stimulatory effect of ghrelin on GH secretion is both rapid and transient because plasma GH values peak within 10 min after its administration and return to basal values within 40–60 min. The plasma GH responses to ghrelin, when expressed as a percentage of basal values, are similar during troughs and peaks of the spontaneous

TABLE 1 **Profile of Stimulatory and Inhibitory Effects of Ghrelin on Hormone Secretion in Rats and Humans**[a]

| Hormone | Rat | | Human (systemic administration) |
|---|---|---|---|
| | Systemic administration | Intracerebroventricular administration | |
| ACTH | nt | + | + |
| TSH | nt | − | nt |
| GH | + | + | + |
| PRL | nt | nt | + |
| Gastrin | + | nt | nt |
| Insulin | + | nt | nt |
| Cortisol | nt | nt | + |
| Aldosterone | nt | nt | + |
| Epinephrine | nt | nt | + |

[a] ACTH, Adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; GH, growth hormone; PRL, prolactin; nt, not tested; +, stimulatory; −, inhibitory.

pulsatile GH secretion in rats. Ghrelin will also stimulate GH secretion when given to rat pituitary cells in culture. In rodents, ghrelin given systemically stimulates release of gastrin and insulin. In rats, ICV-administered ghrelin stimulates ACTH and prolactin secretion and inhibits TSH secretion (Table 1). Leptin and prolactin are unchanged by ghrelin administration in rats. In humans, systemic ghrelin stimulates ACTH, prolactin, cortisol, aldosterone, epinephrine, and GH secretion. Changes in luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) secretion are not observed in response to ghrelin administration in humans. The prolactin, ACTH, and cortisol-releasing actions of ghrelin are consistent with the stimulatory effects of hexarelin, a synthetic GH-releasing peptide. The releasing action of ghrelin on GH, ACTH, and prolactin are probably mediated at the pituitary and hypothalamic levels. Because the cortisol-releasing effect of hexarelin is absent when the pituitary is separated from the hypothalamus, the stimulatory action of ghrelin on cortisol secretion is most likely mediated by ACTH. The epinephrine-releasing action of ghrelin is probably a direct action on the adrenals because binding sites for ghrelin are present on the adrenal glands.

Another intriguing biological activity of ghrelin is its ability to stimulate food intake and weight gain when given to rodents either ICV or systemically. This is not surprising, however, because synthetic GHRPs stimulate food intake and growth in rats. Ghrelin also reduces fat utilization in rats. Ghrelin was found to be more potent than any other orexigenic peptide except neuropeptide Y (NPY). In mice, the rank order of potency for increasing ingestive behavior was NPY > ghrelin > agouti-related protein (AGRP) > orexin A > orexin B > melanin-concentrating hormone (MCH). The orexigenic activity of systemically administered ghrelin is dependent on vagal afferent pathways and is consistent with earlier reports that central administration of GHRP-6 and KP-102, two synthetic GH secretagogues, stimulates food intake and weight gain in rats. Mechanistically, the orexigenic actions of ICV-administered ghrelin are apparently mediated by brain circuits for NPY and AGRP, two peptides with potent orexigenic actions in the rodent brain. The orexigenic action of ghrelin is blocked by ICV injection of an NPY/Y1 receptor antagonist and ghrelin antagonizes the leptin-induced reduction in food intake through the activation of hypothalamic NPY/Y1 receptor pathway.

In terms of stomach effects, ghrelin, given systemically or ICV, stimulates gastric acid secretion in rats. Systemic ghrelin also stimulates gastric motility in rats and either centrally or systemically administered ghrelin stimulates gastric emptying in mice. The stimulatory action of ghrelin on acid secretion is mediated via a vagus-dependent cholinergic pathway based on the fact that its effects are blocked by atropine or bilateral cervical vagotomy but not by a histamine $H_2$ receptor antagonist. Because intravenous ghrelin stimulates gastrin secretion in rats, the stimulatory action of ghrelin on acid secretion may be mediated partly by gastrin. Ghrelin administered ICV induces expression of c-*fos* in neurons of the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve. These are regulatory sites for gastric secretion in the brain. Together, these findings suggest that ghrelin has a role in the vagal-mediated control of gastric function.

## V. REGULATION OF GHRELIN EXPRESSION AND SECRETION

Because ghrelin is postulated to be involved in regulation of metabolism and energy expenditure, we would anticipate that ghrelin expression and secretion are influenced by metabolic hormones. Stomach ghrelin expression is up-regulated under conditions of negative energy balance and down-regulated with a positive energy balance. In fasted rats, stomach ghrelin expression and secretion increase significantly, and with refeeding ghrelin expression and secretion decrease. Insulin and leptin, given acutely, up-regulate ghrelin expression in rodents. Interestingly, plasma ghrelin levels are reduced in obese humans, which is thought to reflect excess caloric intake. This human study suggests that the decrease in plasma ghrelin levels is due to elevated insulin or leptin levels. The human study also concluded that the decreased plasma ghrelin levels are a physiological adaptation to the positive energy balance associated with obesity.

## Glossary

**antrum of the stomach** Gastrin-producing portion of stomach.

**ghrelin** A stomach peptide hormone that stimulates growth hormone secretion and food intake.

**growth hormone-releasing hormone** Hypothalamic hormone that stimulates growth hormone release.

**growth hormone-releasing peptide** Peptide that stimulates growth hormone secretion through a receptor other than a growth hormone-releasing hormone receptor.

**growth hormone secretagogue receptor** The unique receptor for a growth hormone-releasing peptide.

**oxyntic mucosa** Acid-secreting portion of stomach.

**somatostatin** Hypothalamic somatotropin release-inhibiting factor that inhibits growth hormone secretion.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Eating Disorders • Gastrin • Gastrointestinal Hormone-Releasing Peptides • Growth Hormone-Releasing Hormone (GHRH) and the GHRH Receptor • Growth Regulation: Clinical Aspects of GHRH • Somatostatin**

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# GI

See *Gastrointestinal Hormone*

# Gibberellins

Valerie M. Sponsel
*University of Texas, San Antonio*

I. INTRODUCTION
II. HISTORY
III. STRUCTURE
IV. BIOSYNTHESIS AND METABOLISM
V. USE OF MUTANTS
VI. MODE OF ACTION IN CEREAL GRAIN
VII. REGULATION OF STEM GROWTH BY GAs
VIII. SUMMARY

**Gibberellins are tetracyclic diterpenes that are found in plants and fungi. A few of the 126 gibberellins identified to date are known to be active hormones that are involved in seed germination, seedling emergence, stem elongation, and flower and fruit development. The gibberellin receptor has not yet been conclusively identified.**

## I. INTRODUCTION

To date, 126 gibberellin A (GA) compounds from plants and fungi have been characterized. They have been named in chronological order of their discovery ($GA_1$ to $GA_n$). All GAs are diterpenes with the same tetracyclic skeleton, but they differ in the types and positions of functional groups. Although individual plant species may contain >20 different GAs, probably only one GA in each species has intrinsic biological activity and is the active hormone. The others are either precursors or deactivation products of the active hormone. GAs are present in vegetative tissues at concentrations ranging from 0.1 to 100.0 ng $g^{-1}$ fresh weight. The concentrations may be several orders of magnitude higher in developing seeds, which historically have been an excellent source of GAs for characterization. Developing seeds have also been useful sources of biosynthetic enzymes for determining metabolic pathways. Moreover, germinating cereal grains have been a model system for studying GA action because of the role GAs play in inducing synthesis and secretion of α-amylase. Another notable action of GAs is on stem growth. Some plants treated with biologically active GAs in excess of their natural levels can be made to grow exceedingly tall. In contrast, plants treated with growth retardants that inhibit GA biosynthesis and plants in which GA biosynthesis is genetically blocked are dwarfs. GAs act in stem growth via an enhancement of both cell division and cell elongation. GAs also promote flowering, male fertility, and fruit development.

## II. HISTORY

Gibberellins get their unusual name from the fungus *Gibberella fujikuroi*, from which they were first isolated. *Gibberella fujikuroi*, a pathogen of rice, causes severe stem overgrowth, commonly known as "foolish seedling disease." Early work on the characterization of growth-promoting factors produced by the fungus led to the first isolation of GAs. It became apparent in the 1950s that plants contain similar GAs to those in *Gibberella*, although at much lower levels than in the fungus. Application of GAs to a variety of dwarf or nonflorally induced plants led to striking effects on growth and flowering, paving the way for the designation of GAs as a new class of plant hormone.

## III. STRUCTURE

Gibberellins are tetracyclic diterpenes with an *ent*-gibberellane ring structure (Fig. 1). They contain either 20 or 19 carbon atoms. The $C_{20}$ GAs, e.g., $GA_{12}$ (Fig. 1), which have the full complement of 20 carbons, are precursors of the $C_{19}$ GAs. The $C_{20}$ GAs do not have bioactivity per se. Although there are large numbers of $C_{19}$ GAs, many of these do not have all the structural requirements for bioactivity, which include the presence of a 3β-hydroxyl group and absence of a 2β-substituent. Of the $C_{19}$ GAs that fulfill these criteria, $GA_1$ (Fig. 1), $GA_3$, $GA_4$, and $GA_7$ are the most active. Their high biological activity

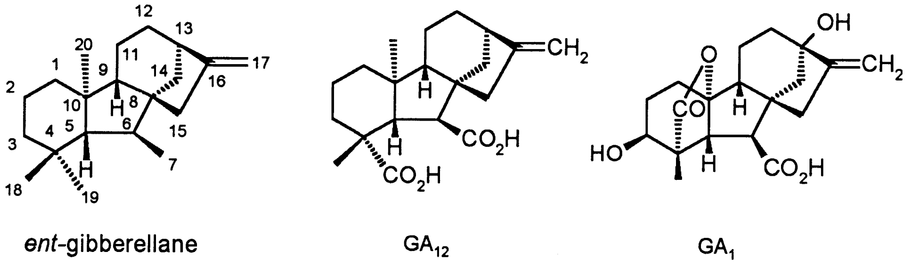

**FIGURE 1** The *ent*-gibberellane skeleton, which is common to all gibberellins (GAs); GA$_{12}$ is a C$_{20}$ GA with the full diterpenoid complement of 20 carbons, and bioactive GA$_1$ is a C$_{19}$ GA that has lost carbon 20.

undoubtedly explains why these GAs were among the very first to be isolated. In most species, GA$_1$ is considered to be the active hormone. However, in *Arabidopsis thaliana*, GA$_4$ (13-deoxyGA$_1$) appears to be more bioactive than GA$_1$, and is suggested to be the active hormone. There is also evidence that additional GAs may have activity in the control of flowering. GA$_3$, also known as gibberellic acid, is the major GA in *G. fujikuroi* and is produced commercially from fungal fermentations. GA$_3$ has many commercial uses in horticulture, agronomy, and brewing.

## IV. BIOSYNTHESIS AND METABOLISM

Biosynthesis of GAs in plants can be divided into three parts. The first part occurs in plastids and involves biosynthesis of a tetracyclic hydrocarbon, *ent*-kaurene (Fig. 2). In the second part of the pathway, which occurs in the endoplasmic reticulum, *ent*-kaurene is oxidized sequentially and is converted to the first-formed GA, GA$_{12}$ (Fig. 3). The third part of the pathway occurs in the cytosol and it can produce the multiplicity of C$_{20}$ and C$_{19}$ GAs known to be present in most plants.

The diterpenoid nature of GAs indicates that they are synthesized from isopentenyl diphosphate (IPP), which is the universal 5-carbon building block for all terpenoids. The classical route to IPP is the acetate/mevalonate (MVA) pathway. This pathway is located in the cytosol and leads to sesquiterpenoids (containing 15 carbon atoms) and triterpenoids (30 carbons). In the past 10 years, a second biosynthetic route to IPP has been discovered in bacteria, algae, some protozoa, and plants. This is the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway (Fig. 2), and in eukaryotes this new pathway occurs only in plastids. IPP formed in chloroplasts and other types of plastids is further converted to monoterpenes (10 carbons), diterpenes (20 carbons), and tetraterpenes (40 carbons). Therefore, DOXP-derived IPP might be expected to be the precursor of GAs, because they are diterpenes. Although the synthesis of GAs by this route has not been demonstrated unequivocally, it is generally assumed that the DOXP pathway will be the major biosynthetic route to GA precursors.

Briefly, in the DOXP pathway, a two-carbon unit (derived by decarboxylation of pyruvate) is added to glyceraldehyde 3-phosphate to give DOXP. The reaction is catalyzed by DOXP synthase and seems to be a point of regulation in the pathway. The gene encoding DOXP synthase in several plants is expressed in a developmental and tissue-specific manner. The next reaction, catalyzed by DOXP reductoisomerase, involves the reduction and intramolecular rearrangement of the linear DOXP to give a branched structure, 2-*C*-methyl-D-erythritol 4-phosphate (MEP). The route from MEP to IPP is only partially defined (Fig. 2), but all reactions so far elucidated take place in plastid stroma.

Subsequent condensation of IPP and its isomer, dimethylallyl diphosphate, gives the monoterpene, geranyl diphosphate (GPP). Two molecules of GPP combine to give the linear diterpene, geranylgeranyl diphosphate (GGPP). At GGPP the pathway branches in several directions, with separate branch pathways leading to the carotenoids, to the phytoene side chain of chlorophyll, to the nonaprenyl side chain of plastoquinone, and to the *ent*-kaurenoids and GAs. Only the branch to the *ent*-kaurenoids and GAs is considered here.

En route to GAs, cyclization of the linear GGPP to the tetracyclic *ent*-kaurene occurs in two stages (Fig. 2). GGPP is converted first to a bicyclic compound, *ent*-copalyl diphosphate, by *ent*-copalyl diphosphate synthase (CPS). *ent*-Copalyl diphosphate is converted to the tetracyclic compound *ent*-kaurene in a reaction catalyzed by *ent*-kaurene synthase (KS). CPS is present at much lower levels than KS, and so CPS is thought to have an important "gatekeeper"

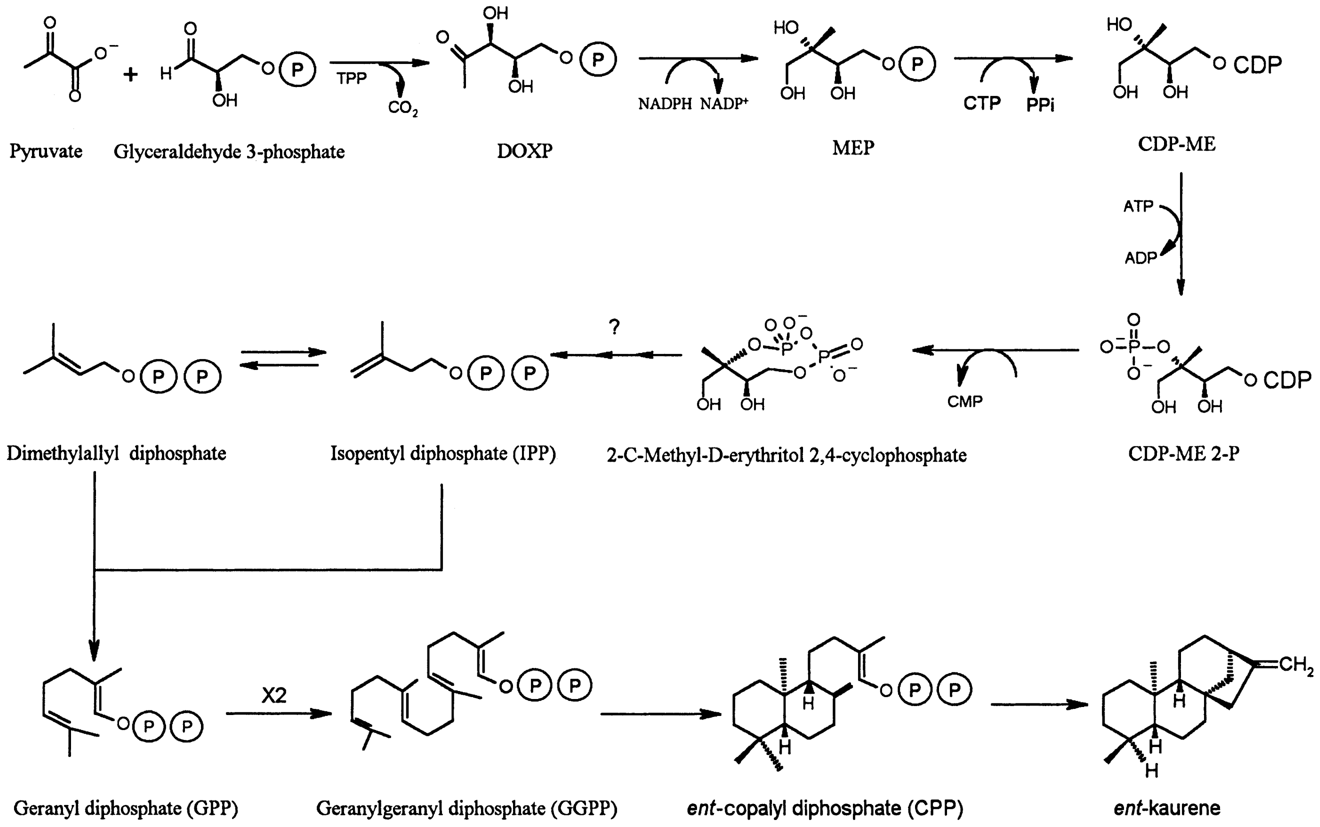
Pyruvate
Glyceraldehyde 3-phosphate
TPP
CO2
DOXP
NADPH NADP+
MEP
CTP PPi
CDP-ME
ATP
ADP
CDP-ME 2-P
CMP
?
2-C-Methyl-D-erythritol 2,4-cyclophosphate
Isopentyl diphosphate (IPP)
Dimethylallyl diphosphate
Geranyl diphosphate (GPP)
X2
Geranylgeranyl diphosphate (GGPP)
ent-copalyl diphosphate (CPP)
ent-kaurene

FIGURE 2 The 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway to isopentenyl diphosphate, and subsequent formation of the diterpene, *ent*-kaurene. CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-ME 2-P, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate.

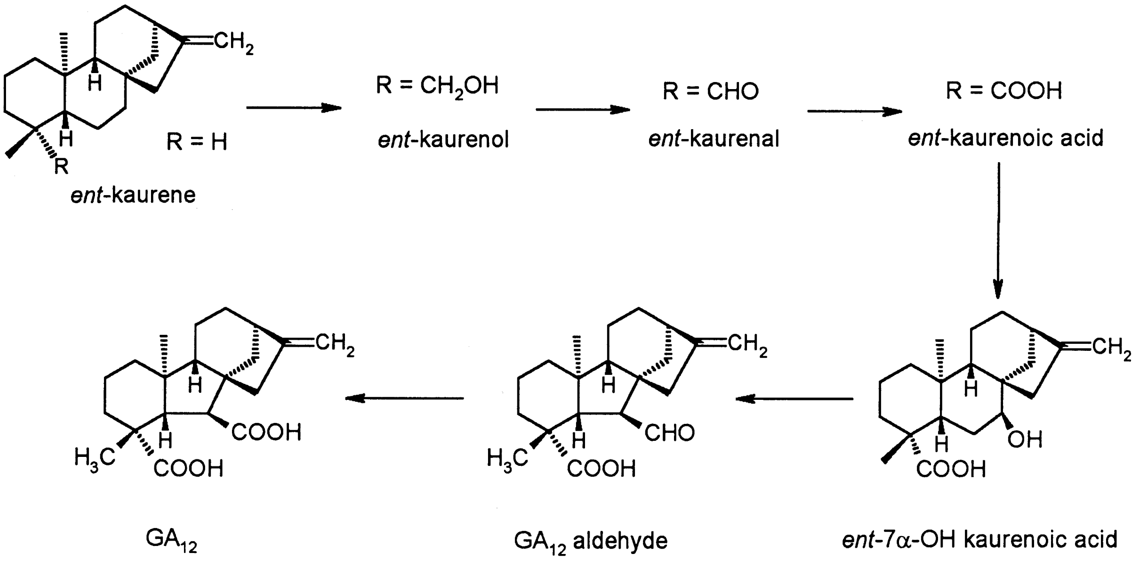

FIGURE 3 The multistep conversion of *ent*-kaurene to $GA_{12}$.

function on the branch of the terpenoid pathway that is committed to *ent*-kaurenoids and GAs.

The next part of the pathway (Fig. 3) involves the sequential oxidation of *ent*-kaurene at carbon 19 and is catalyzed by a membrane-bound, cytochrome P450 enzyme, *ent*-kaurene oxidase. This catalytic activity has been thought for some time to be associated with the endoplasmic reticulum, although recently *ent*-kaurene oxidase from *Arabidopsis* was reported to be localized to the chloroplast outer envelope. *ent*-Kaurene oxidase is multifunctional and can catalyze three oxidations: *ent*-kaurene → *ent*-kaurenal → *ent*-kaurenol → *ent*-kaurenoic acid (Fig. 3). The next enzyme in the pathway, *ent*-kaurenoic acid oxidase, is also a multifunctional P450 monooxygenase and is associated with the endoplasmic reticulum in *Arabidopsis* and many other species. It can catalyze the sequence: *ent*-kaurenoic acid → 7β-hydroxykaurenoic acid → $GA_{12}$-aldehyde → $GA_{12}$ (Fig. 3). These reactions involve the conversion of a 6-membered B ring to a 5-membered B-ring and the formation of a carboxylic acid at carbon 7. The biosynthetic pathway leading to $GA_{12}$ appears to be the same in all plants examined to date, but it can diverge from there on. The next reactions, comprising the last part of the pathway, occur in the cytosol.

There are multiple positions in the GA molecule for subsequent oxidation. The enzymes that catalyze these reactions are soluble 2-oxoglutarate-dependent dioxygenases. The sequential oxidation of carbon 20, until it is eventually lost as $CO_2$, yields a GA containing 19, instead of 20, carbon atoms (Fig. 4). The carboxyl at C-4 forms a lactone at C-10, and this γ-lactone is essential for bioactivity. Also necessary for bioactivity is the presence of a 3β-hydroxyl group or some other functionalization of carbon 3, including a 2,3 double bond. The introduction of hydroxyl groups can occur "early" or "late" in the pathway, and because the dioxygenases do not have absolute substrate specificity, parallel and interconnecting pathways may occur.

Because several of the enzymes in the pathway are multifunctional, as few as four enzymes can catalyze the 10 reactions needed to convert *ent*-kaurene to a bioactive GA. This is a much smaller number than expected. However, despite the small number of different enzymes that are needed, it is now known that some of the enzymes, particularly the dioxygenases, are encoded by small gene families. This means that there are multiple isoenzymes of some of the enzymes. In *Arabidopsis*, for instance, there are as many as five different 20-oxidases. The expression of individual members of a gene family can be tissue specific and developmentally regulated. Moreover, regulation by intrinsic and/or extrinsic factors may operate on selective isoenzymes only.

With regard to sites of synthesis, there is incontrovertible evidence that GA biosynthesis occurs in the developing seeds of many species. The stages of GA biosynthesis from GGPP have been demonstrated repeatedly in cell-free systems from endosperm preparations and other seed parts. Other sites of GA biosynthesis include actively growing tissues such as shoot tips, expanding leaves, and floral organs.

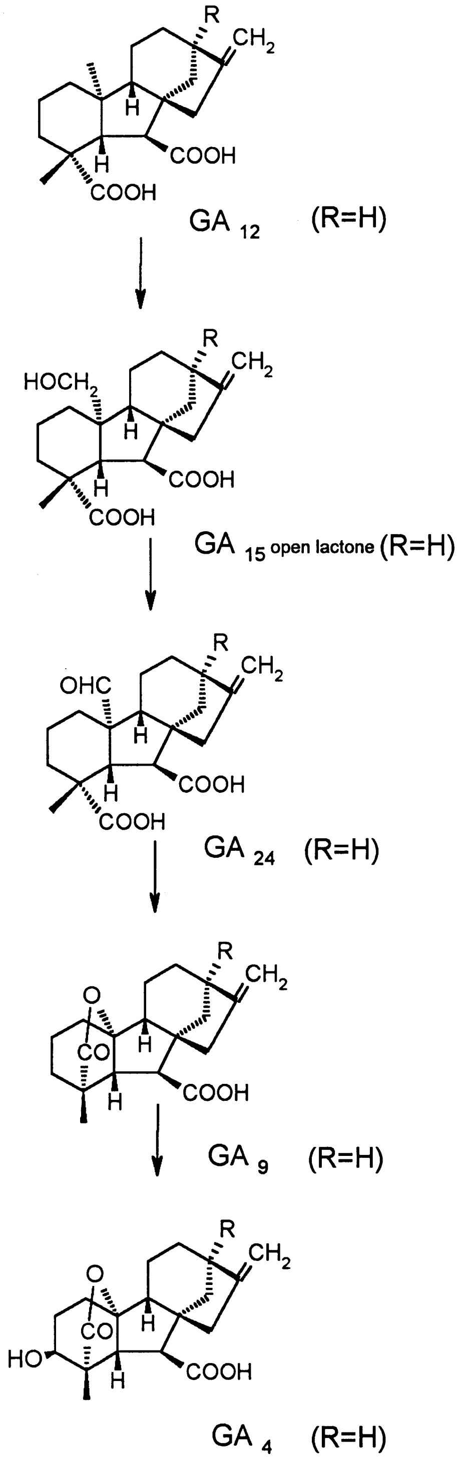

**FIGURE 4** The three-step conversion of $GA_{12}$ to $GA_9$ is catalyzed by a multifunctional GA 20-oxidase and results in the formation of the $C_{19}$ GA, $GA_9$. Hydroxylation at carbon 3 is required to form a bioactive GA, $GA_4$. Any of these GAs can be a substrate for a 13-oxidase, potentially giving the following sequence: $GA_{53}$, $GA_{44}$-open lactone, $GA_{36}$, $GA_{20}$, and $GA_1$, in all of which R = OH.

The evidence for GA biosynthesis in these tissues comes from studying the expression of genes that encode key enzymes in the GA biosynthetic pathway. The use of reporter genes for these studies has been particularly instructive. In many cases, the site of GA synthesis correlates with the site of GA action. Although localized movement of GAs may occur—for instance, within regions of the shoot apex or between different seed parts—there is little evidence that long-distance transport is required.

Alteration in GA biosynthesis in response to environmental factors is one important way that light, for example, can have an impact on plant growth and development. GA biosynthesis is affected by both light quantity and quality. In *Arabidopsis* and spinach a stem-expressed 20-oxidase is up-regulated in shoots after exposure to long days, coincident with stem elongation and prior to flowering. 3-Oxidation in lettuce and *Arabidopsis* seeds is enhanced in red illumination and is important for germination. In both of these examples, up-regulation of an enzyme catalyzing a rate-limiting step in the biosynthetic pathway leads to an increase in the level of bioactive GA.

## V. USE OF MUTANTS

In the past, there have been concerted efforts to identify all GAs in a given plant species, to determine the GA metabolic pathways within the plant, and to characterize a comprehensive series of mutants. The GA-responsive stem length mutants of corn, pea, and *Arabidopsis* have been particularly useful. For each of these species a series of mutants that showed a graded severity of phenotype, from extreme dwarf to semidwarf, were either generated or obtained from natural sources (e.g., *Arabidopsis*; Fig. 5). Each of these mutants was shown to be blocked at a specific stage in GA biosynthesis between *ent*-kaurene and the active hormone. Treatment of a particular mutant with an *ent*-kaurenoid or GA beyond the metabolic block can restore normal growth. If a mutant is blocked for 3β-hydroxylation (*ga4* in *Arabidopsis*, *d1* in corn, *le* in pea,), 3-deoxy GAs cannot restore growth, whereas $GA_4$ or $GA_1$ can, providing evidence that the active GA must be 3β-hydroxylated.

In addition to having an extreme dwarf phenotype, the *ga1*, *ga2*, and *ga3* mutants of *Arabidopsis* are also male-sterile. Flower buds fail to open and no seeds are set (Fig. 5). Treating these mutant plants with GA not only restores normal stem elongation but also restores fertility. Moreover, seeds of these

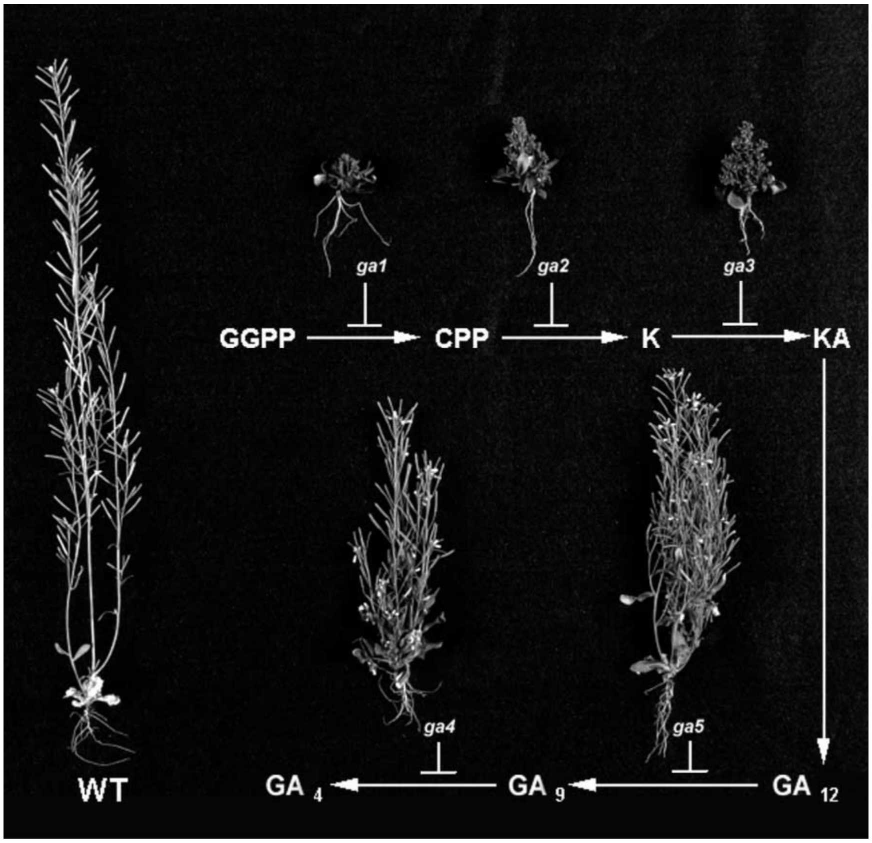

**FIGURE 5** *Arabidopsis thaliana*. The Landsberg *erecta* ecotype (wild type) and five mutants, *ga1–ga5*. The position of the metabolic block in the GA biosynthetic pathway is shown for each mutant. All plants are photographed at 7 weeks. Only wild-type, *ga4*, and *ga5* plants have flowered and set seed. The *ga1*, *ga2*, and *ga3* mutants are male-sterile dwarf plants.

three GA-deficient mutants of *Arabidopsis* cannot germinate unless they are treated with GA, underscoring the requirement for GAs in several different phases of a plant's life cycle.

Using GA biosynthesis mutants, it has been shown that stem length is proportional to the content of bioactive GA. This has been clearly demonstrated for pea stem length and $GA_1$ content. Thus, factors that control the pool size of $GA_1$ or other active GA in the target tissue will be important for determining growth. Control points include the regulation both of biosynthesis of the active hormone and of its subsequent deactivation. In *Arabidopsis*, the 20-oxidases and 3-oxidases are regulated by feedback. The action of GA in promoting growth down-regulates transcription of genes that encode enzymes occurring earlier in the GA pathway. The precise mechanism whereby this regulation occurs is not known. Deactivation involves 2β-hydroxylation and may also involve conjugation to glucose. The 2β-hydroxylase is regulated by a feedforward mechanism.

Other mutants that have been characterized have altered responses to GAs. These mutants, which can be identified by their short or very tall phenotypes, are unlike the previously described mutants because their stem height often bears no direct correlation to their level of endogenous $GA_1$ (or $GA_4$). This is because the feedback control of the biosynthetic pathway requires GA-induced growth. In the absence of growth and the consequent absence of feedback regulation, the transcription of genes encoding 3-oxidase and 20-oxidase enzymes is up-regulated, which results in very high GA levels in severely dwarfed individuals. In contrast, other mutants can be unusually tall, even if the levels of endogenous GAs are reduced to almost zero by chemical or genetic means. Both of these types of mutants are useful tools with which to dissect the signal transduction pathway, and are discussed later.

## VI. MODE OF ACTION IN CEREAL GRAIN

The cereal aleurone system has been the most well-investigated site of GA action. During germination, $GA_1$ from the embryo moves to the aleurone, which is a layer or layers of cells that envelope the starchy endosperm (Fig. 6). Within the aleurone layer, GA from the embryo induces the synthesis and secretion of hydrolytic enzymes, including several isoforms of α-amylase. α-Amylase catalyzes the breakdown of starch in the nonliving starchy endosperm, yielding usable forms of fixed carbon to nourish the growing seedling (Fig. 6). Cereal grains would therefore appear to be excellent material from which to isolate a GA receptor, and attempts to do so are ongoing.

The site of GA perception appears to be the plasma membrane of aleurone cells. The evidence for this is twofold. First, $GA_4$ that has been covalently linked to agarose beads to prevent its uptake into the cell can still induce α-amylase production. Second, GA injected directly into aleurone cells has no bioactivity. Two GA-binding proteins that specifically bind bioactive GAs have been isolated from aleurone cells, but unequivocal evidence that either or perhaps both (as a dimer) are the GA receptor is not yet available. Despite this serious gap in our understanding of GA perception, there is now a considerable amount of evidence for downstream signaling events that transduce the GA signal into the well-defined responses of α-amylase production and secretion. There are some small differences in GA signaling in grains of different genera (barley, rice, wheat, and wild oats are all used extensively), and the following description draws together experimental data from several different systems.

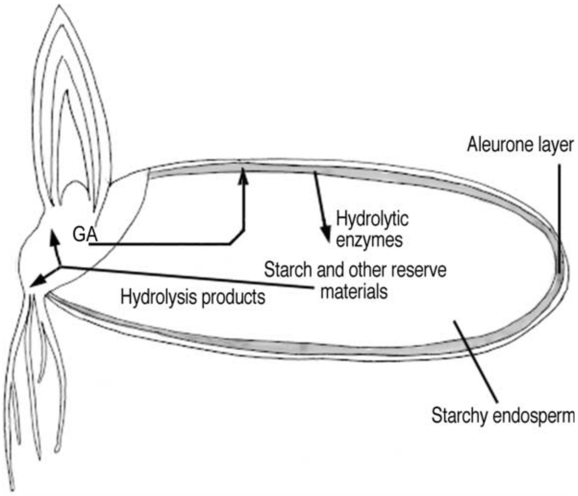

**FIGURE 6** Cereal grain showing that GA from the embryo moves to the aleurone. *De novo* synthesis of amylase and other hydrolytic enzymes occurs in the aleurone. These enzymes break down reserve materials in the starchy endosperm in order to nourish the growing seedling.

Components of the GA signal transduction chain have been identified in the plasma membrane, cytosol, and nucleus (Fig. 7). Several lines of evidence indicate that bioactive GA, perceived at the plasma membrane and bound to a hypothetical receptor protein, interacts with a membrane-localized heterotrimeric G-protein. G-protein agonists can induce α-amylase gene expression in wild oat protoplasts in the absence of GA. Moreover, guanosine triphosphate (GTP)-γ-S, which binds $G_\alpha$ subunits and holds them in an activated form, stimulates α-amylase expression, whereas guanosine diphosphate (GDP)-β-S, which holds the subunits in an inactivated form, prevents GA induction of α-amylase gene expression. Additional evidence linking a putative heterotrimeric G-protein with GA signal transduction comes from the observation that the *dwarf1* (*d1*) mutant of rice, which has impaired sensitivity to GA, has a defective $G_\alpha$ subunit.

Based on identification of both $Ca^{2+}$-dependent and -independent events, there appears to be a dichotomy in the GA signal transduction pathway within the cytoplasm of barley aleurone cells (Fig. 7). One branch of the signaling pathway leads to rapid increases in intracellular calcium concentration, particularly in the peripheral cytoplasm, whereas the other branch leads to induction of α-amylase gene expression. Sustained lowering of $Ca^{2+}$ levels for several hours does not affect α-amylase gene expression but inhibits α-amylase secretion. On the other hand, syntide 2 (an inhibitor of phosphorylation) inhibits GA induction of α-amylase but does not inhibit the rapid increase in $Ca^{2+}$ concentration that is observed with GA treatment. These results show that effects of GA on α-amylase gene expression are independent of the $Ca^{2+}$ effects, although intracellular $Ca^{2+}$ concentration is important in α-amylase secretion. In the *d1* mutant of rice, previously mentioned because it defines a mutation in a $G_\alpha$ subunit of a heterotrimeric G-protein, both $Ca^{2+}$-dependent and -independent responses to $GA_3$ are perturbed at all but the highest dose of $GA_3$. This global disruption of GA responses would reinforce the position of a $G_\alpha$-protein early in the GA signal transduction pathway (Fig. 7).

The mechanism for elevating intracellular $Ca^{2+}$ concentration is presently unknown, but it has been suggested to involve increased flux of $Ca^{2+}$

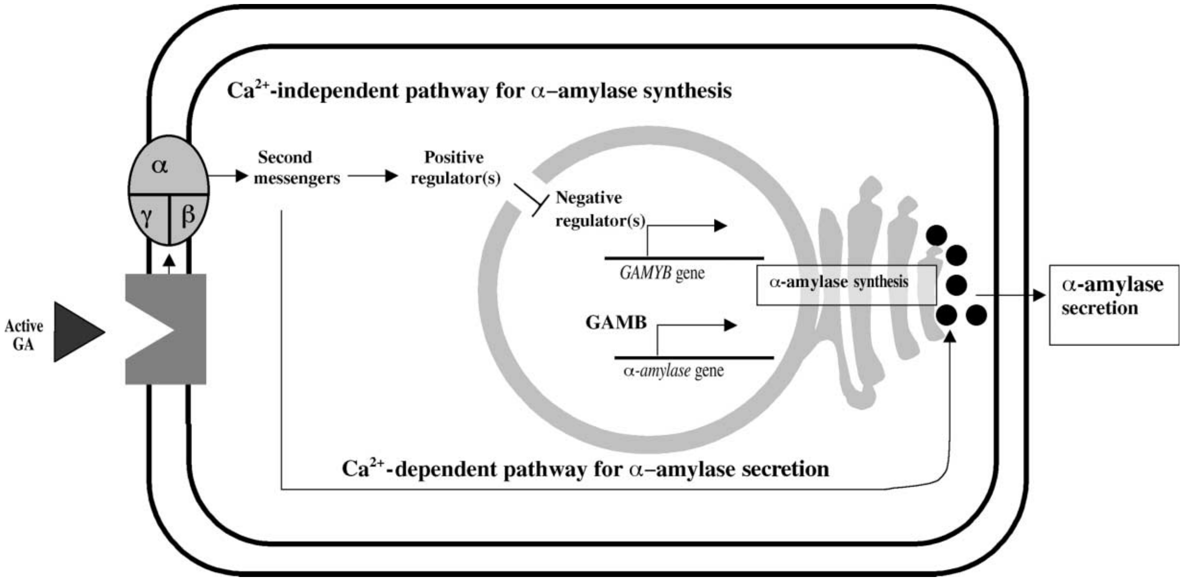

FIGURE 7 Simplified scheme to show some putative players in GA signal transduction in a generic cereal aleurone cell. The GA receptor is thought to be membrane localized, and the signal may be transduced through a heterotrimeric G-protein, the α-subunit of which is encoded by the *D1* locus in rice. The transduction pathway bifurcates into $Ca^{2+}$-dependent and $Ca^{2+}$-independent branches. The latter branch is under the control of both positive (GID1 in rice, GSE in barley) and negative (SLR in rice, SPY and SLN in barley, Rht1 in wheat) regulators. In the presence of a GA signal the negative regulation is removed, allowing GAMYB transcription factors to be formed, α-amylase genes to be transcribed, and α-amylase protein to be synthesized and secreted.

from the apoplast and/or a release of $Ca^{2+}$ from an intracellular storage pool. GA also causes an elevation of calmodulin (CaM) levels in the aleurone. Targets for the elevated levels of $Ca^{2+}$ and CaM in the cytoplasm may be transporters, channels, or other membrane proteins at several cellular locations, some of which may be directly involved in the secretory process.

Use of guanylyl cyclase inhibitors prevents a transient increase in cyclic GMP (cGMP) normally seen in barley aleurone layers 2 h after GA treatment. These inhibitors also reduce the accumulation of α-amylase mRNA and that of the gibberellin-associated MYB transcription factor, GAMYB. For this and other reasons, it has been suggested that cGMP may be a component of the transduction chain, provisionally placed downstream of the G-proteins and upstream of MYB. Interestingly, there is evidence in tomato for phytochrome A activation of a heterotrimeric G-protein, which in turn uses cGMP and $Ca^{2+}$ in separate transduction chains to bring about anthocyanin production and chloroplast development.

Reversible phosphorylation is also involved in the GA signal transduction chain. The aforementioned syntide 2, which essentially blocks phosphorylation by competing with natural substrates, inhibits GA induction of gene expression. In contrast, okadaic acid, which is a phosphatase inhibitor, negates the effects of GA on $Ca^{2+}$ accumulation, on gene expression, and on α-amylase secretion. This inhibitor therefore must act early (i.e. before the transduction chain branches) or, if it acts later it, it must act at two sites to inhibit both branches of the signal transduction chain.

The induction of gene expression by GA has been studied in detail. Within their promoter regions, amylase genes contain a GA response element (GARE) to which GA-inducible nuclear proteins bind. The GAMYB proteins have been identified as GA-inducible transcription factors that bind to the conserved sequence TAACA/GA of GAREs and transactivate amylase genes. The same transcription factors may also transactivate other GA-inducible genes. Because the expression of *GAMYB* precedes the expression of amylase genes and does not require protein synthesis, it is suggested that *GAMYB* represent primary response genes. There is a possibility that GA acts to repress a repressor of GAMYB (this is discussed again briefly in the next section). Additional nuclear proteins can bind other sequences adjacent to the GARE that comprise a so-called GARC (GA response complex). The TATCCAC box in the promoter of amylase 1 is a suspected site of interaction. The identity of these cis-acting elements is as yet unknown, but they are envisaged to act as enhancers.

In summary, GA signaling in aleurone cells (represented schematically in Fig. 7) involves a putative membrane-bound receptor that may interact with a heterotrimeric G-protein to bring about a cascade of events within the cytoplasm. These may include, but are not limited to, an enhancement of guanylyl cyclase and an increase in cGMP, an elevation of intracellular $Ca^{2+}$, and the induction of transcription factors that facilitate α-amylase gene expression. α-Amylase is one of several hydrolytic enzymes that move to the starchy endosperm and catalyze the mobilization of reserve materials. The resulting breakdown products are essential for nourishment of the emerging seedling. Cereal grains in which amylase production is perturbed have impaired seedling emergence. Because cereals provide the world's population with a majority of its caloric needs and over half its protein, the importance of GA-induced amylase production in cereal aleurone cannot be overstated.

## VII. REGULATION OF STEM GROWTH BY GAs

One of the most easily recognized effects of GA is on stem elongation, and a number of stem length mutants with altered sensitivity to GA have been characterized. The mutants fall into two broad phenotypic categories: those that are dwarf and have reduced responsiveness to applied GAs and those that are as tall or taller than wild-type plants, irrespective of how much GA they contain. Biochemically, the mutants also fall into two main categories: those that have a mutation in a negative regulator of GA signaling and those that have a mutation in a positive regulator of GA signaling. The exact nature of a mutation (i.e. whether it is a loss- or gain-of-function mutation) will have an impact on the phenotype. The mutant loci discussed here are listed in Table 1.

It is now apparent that many plants contain a mutated form of a gene initially defined by the *GAI* (*gibberellin insensitive*) locus in *Arabidopsis*. *GAI* encodes a putative transcription factor that is a negative regulator of GA signaling. In wild-type plants, GA can derepress this regulator, allowing GA responsiveness. If a deletion occurs in a particular region of the *GAI* gene, identified by a DELLA motif in the amino acid sequence, then the alteration causes a semidominant gain-of-function mutation. This type of mutation (designated as the *gai1-1* allele) prevents GA from being able to derepress the negative regulator, or, to put it in other words, a mutation in the DELLA region makes *GAI* a constitutive repressor of the GA response. The resulting phenotype is a very dwarf plant that is GA insensitive.

*GAI* orthologues present in maize (*dwarf8*, *D8*), wheat (*reduced height1*, *RHT1*), barley (*slender*, *SLN*), and rice (*slender rice*, *SLR*) all encode a similar protein. So too does the *RGA* (*repressor of ga1-3*) gene in *Arabidopsis*, which was isolated as a suppressor of the GA-deficient phenotype of *ga1-3* mutants. The *d8* and *Rht1* semidominant mutations, like *gai1-1*, have a deletion in the DELLA region and are dwarf nonresponders to GA. Historically, this mutation has had a profound effect on the well-being of millions of people, because it is the mutation responsible for the development of semidwarf cereals adapted to the semitropics by Dr. Norman Borlaug in the Green Revolution of the 1960s. Interestingly, it has been shown that mutations in *GAI* and its orthologues in barley (*SLN*) and rice (*SLR1*) can give either slender or dwarf phenotypes, depending on the locations of the mutations. For example, in rice the slender phenotype is exhibited by plants in which a mutation occurs in the nuclear localization sequence of *SLR1*. In these recessive loss-of-function mutants, the GA response is constitutively derepressed, giving a very tall phenotype. Alternatively, a truncation in the DELLA region of *SLR1* (produced by transforming wild-type plants with *pSLRtr*) results in a semidominant gain-of-function mutation, which gives a GA-insensitive dwarf phenotype. This unusual situation in which the two mutations in the same gene can lead to opposite phenotypes has been demonstrated for *sln* and *gai* mutants too. An intragenic suppressor of *GAI* gives a recessive mutation, and these plants (*gai*) have a nondwarf phenotype that is in direct contrast to the severely dwarf phenotype of the *gai1-1* mutants (Table 1). It is assumed that this *gai* loss-of-function mutant does not have a slender phenotype because of the presence of a functional repressor of gibberellin (RGA) protein.

Recessive mutations at another locus, involving *Spindly* (*SPY)*, which was first defined in *Arabidopsis*, partly suppress the *gai* phenotype. This indicates that *SPY* may also be a negative regulator of GA signal transduction. It has been proposed that in the absence of GA, SPY activates GAI and RGA. In the presence of GA, SPY would be inactivated, or GAI and RGA might be modified to prevent interaction with SPY. The outcome would be the removal of negative

TABLE 1 Genetic Loci Known, from Mutant Analysis, to Affect GA Response

| Species | Locus[a] | Mutant | Type | Phenotype | Proposed action of wild-type gene product |
|---|---|---|---|---|---|
| *Arabidopsis* | *Spindly* | *spy* | Recessive | Tall | Negative regulator of GA signaling |
| Barley | *Spindly* | *Hvspy* | Recessive | Tall | Negative regulator of GA signaling |
| *Arabidopsis* | *RGA* | *rga* | Recessive | Semidwarf | Negative regulator of GA signaling |
| *Arabidopsis* | *GAI* | *gai1-1* | Semidominant | Dwarf | Negative regulator of GA signaling |
| *Arabidopsis* | *GAI* | *gai* | Recessive | Wild type | Negative regulator of GA signaling |
| Maize | *Dwarf8* | *d8* | Semidominant | Dwarf | GAI orthologue |
| Wheat | *Reduced ht1* | *Rht1* | Semidominant | Dwarf | GAI orthologue |
| Barley | *Slender* | *sln1* | Recessive | Tall | GAI orthologue |
| Rice | *Slender rice* | *slr1-1* | Recessive | Tall | GAI orthologue |
| Rice | *Slender rice* | *pSLRtr* | Semidominant | Dwarf | GAI orthologue |
| Rice | *Dwarf1* | *d1* | Recessive | Dwarf | Positive regulator of GA signaling |
| Rice | *GID* | *gid* | Recessive | Dwarf | Positive regulator of GA signaling |
| Barley | *GSE* | *gse* | Recessive | Dwarf | Positive regulator |

[a]Abbreviations: *RGA*, *repressor of ga1-3*; *GAI*, *gibberellin insensitive*; *GID*, *gibberellin-insensitive dwarf*; *GSE*, *gibberellin sensitive*.

regulation of the signaling pathway, allowing a GA response. It has recently been shown that SPY also affects plant development in addition to, but separate from, being involved in GA signaling. These additional roles for SPY have yet to be defined.

Studies utilizing green fluorescent protein (GFP) have shown that RGA is localized in the nucleus. However, given that neither RGA nor GAI possesses DNA-binding domains, it is likely that they interact with an additional factor (possibly a GAMYB transcription factor) that can bind to DNA. In barley, the gene product of *SLN*, which is the barley orthologue of *GAI* and thus a negative regulator of GA signaling, appears to be degraded in the presence of GA. This degradation of SLN is required before the expression of GAMYB in aleurone cells can be enhanced by GA. This result ties in a negative regulator of GA signaling (SLN), which was first defined through a mutant phenotype in stems with a known transcription factor (GAMYB) in the signaling pathway in aleurone. The GA-induced disappearance of RGA from *Arabidopsis* nuclei has also been reported, and again is consistent with the direct or indirect removal of a negative regulator by GA.

Compared to negative regulation, less is known about positive regulation of GA response pathways. Two loss-of-function (recessive) mutations, *d1* and *gid1* (*gibberellin-insensitive dwarf1*) in rice have been described. The mutant phenotypes are dwarf or semidwarf. As mentioned earlier, *D1* encodes a $G_\alpha$-subunit, implicating it early in signal transduction in aleurone cells. The function of *GID* has been examined in double mutants. Plants with an *slr1 gid1* genotype are phenotypically slender, indicating that *slr1* is epistatic to *gid1*. This is consistent with a role for SLR1 downstream of GID1. In preliminary results it has been shown that in wild-type plants SLR1, visualized by fusion of the gene to one encoding GFP, is localized to nuclei and disappears with GA treatment. In *gid1 SLR1-GFP* plants, the GFP signal is retained in the nucleus, implying that a functional GID1 protein is required for the GA-dependent removal of the negative regulation imposed by SLR1.

A putative positive regulator, encoded by the *GSE* (*gibberellin sensitivity*) locus, has been described in barley. A recessive loss-of-function mutation gives a dwarf phenotype with some reduction in sensitivity to GAs for both leaf elongation and amylase synthesis. In double mutants, *sln1* is epistatic to *gse*, implying that SLN1 is located downstream of GSE in the signal transduction chain.

Overall, the picture that is emerging for GA signaling in both stems and aleurone is of a series of negative regulators (e.g., SPY, and GAI and its orthologues, namely, RGA, SLR, SLN, Rht1, D8,) that keep the GA response pathway repressed in the absence of GA. GA removes the repression, either by acting directly or though one or more positive regulators (e.g., GID1) to inactivate or degrade the negative regulator(s) (Fig. 7).

A GA-binding protein has not been unequivocally identified in vegetative tissue, nor have many ultimate target genes of the GA signal been identified. Compared to the downstream events in the aleurone system, stem elongation and flowering responses are more complex to analyze. Nevertheless, progress has been made with several systems, notably one employing deep-water rice and its response to flooding. Deep-water rice is grown in Southeast Asia, where severe flooding can occur during the monsoon season. Rapid stem extension of up to 25 cm $day^{-1}$ after submergence is a consequence of an increased ratio of GA to abscisic acid. Applied GA, in the absence of flooding, can mimic the increases in cell division and the enhanced cell extension normally seen after submergence. Rice genes that are regulated by GA include those encoding proteins that function in the cell cycle and those involved in "loosening" the cell wall to allow elongation to occur. Expansins are a class of proteins that are closely correlated with the walls of elongating cells, but as yet have an unknown function. Other genes that might encode components in the transduction pathway have been sought in rice using a variety of approaches. The expression of a gene that codes for a leucine-rich repeat receptor-like protein kinase is up-regulated by GA in actively dividing and elongating cells. Also rapidly up-regulated in meristematic cells by GA treatment is the gene *Growth Regulating Factor1*, which encodes a putative transcription factor or activator. Further progress in elucidating the function of this and other GA-regulated genes in deep-water rice can be expected in the near future, and it will enhance greatly our understanding of the mode of action of GAs in stem growth.

## VIII. SUMMARY

The GA group is a daunting array of 126 individual compounds known to be present in plants and fungi. Much is known about their biosynthesis and metabolism, and structure/activity considerations lead us to believe that only a handful of GAs are active hormones in their own right. Their repertoire of activities includes promotion of signal events in the life cycle of a plant such as seed germination, seedling emergence, stem growth, flowering, and fruit development. Despite our inability to identify conclusively a GA receptor, much is now known about how the GA signal is transduced into the well-defined responses of amylase production and secretion in germinating cereal grains, and into less well-defined responses such as cell elongation.

## Glossary

**aleurone** Layers of cells surrounding the starchy endosperm in cereal grains; on stimulation by gibberellins during germination, the cells synthesize hydrolytic enzymes to break down starch and other storage materials in the starchy endosperm.

**$C_{20}$ gibberellins** Diterpenes with the full diterpenoid complement of 20 carbon atoms; the first of the products formed in the GA biosynthetic pathway.

**$C_{19}$ gibberellins** Diterpenes that, through metabolism, have lost carbon 20 and so contain only 19 carbon atoms.

**GA-insensitive dwarf mutants** Plants in which there is a defect in the ability to detect or respond to bioactive GA; cannot be normalized by application of GA.

**GA-sensitive dwarf mutants (GA biosynthesis mutants)** Plants in which one of the enzymatic steps in the GA biosynthetic pathway is blocked, resulting in reduced levels of GA; can be normalized by application of a GA normally produced in the pathway after the metabolic block.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Cytokinins • Ethylene • Jasmonates • Salicylic Acid**

## Further Reading

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# Glucagon Action

Mark L. Evans and Robert S. Sherwin
*Yale University School of Medicine*

I. INTRODUCTION
II. GLUCAGON RECEPTOR
III. SIGNAL TRANSDUCTION
IV. HEPATIC ACTIONS
V. NONHEPATIC SITES
VI. MINIGLUCAGON
VII. ABNORMAL GLUCAGON ACTION
VIII. DIAGNOSTIC USE/THERAPEUTIC USE

**Glucagon is a 29-amino-acid peptide hormone secreted from the alpha cells of the pancreas. The main target organ for glucagon is the liver, where it increases hepatic glucose production and release. Glucagon achieves this by having coordinated actions to increase gluconeogenesis and glycogenolysis and to inhibit glycolysis and glycogen synthesis, with the net effect of directing hepatic flux toward synthesis and release of glucose. These actions are opposed by insulin. In health, glucagon and insulin secretion and action are carefully coordinated to provide an integrated control of hepatic glucose metabolism.**

## I. INTRODUCTION

The mechanisms of glucagon action are outlined in this article; the focus is on the established hepatic effects of glucagon, but there is also discussion of some of the putative actions of glucagon on nonhepatic organs. Some of the conditions in which the physiology of glucagon action is disturbed are described. Several biological actions of glucagon have been exploited therapeutically and therefore some possible diagnostic and therapeutic uses for exogenous glucagon in clinical practice are detailed.

## II. GLUCAGON RECEPTOR

Like other peptide hormones, the intracellular actions of glucagon are initiated by binding to a cell surface receptor, which triggers a series of intracellular events mediated by second-messenger pathways. The glucagon receptor (GR) was first cloned in 1993 and is encoded on chromosome 17q25. The GR is a member of a large family of guanosine triphosphate (GTP)-binding protein-coupled receptors, all with seven transmembrane domains. Within this larger family of receptors, the glucagon receptor belongs to a subset of highly homologous receptors that includes receptors for peptides closely related to glucagon, i.e., glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide (GIP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), secretin, and growth hormone-releasing hormone (GRH). The subset also includes receptors for peptides unrelated to glucagon, such as corticotropin-releasing hormone (CRH), parathyroid hormone (PTH), and calcitonin.

## III. SIGNAL TRANSDUCTION

Glucagon binding to the cell surface GR triggers two complementary second-messenger pathways that act intracellularly to mediate the downstream actions of glucagon, the cyclic AMP (cAMP) pathway and the calcium-mediated pathway.

### A. cAMP Pathway

The best characterized mechanism for the transduction of glucagon binding to the GR into intracellular actions of glucagon involves formation of the second messenger, cAMP. Activation of the cAMP pathway is mediated by a GTP-dependent regulatory protein (G-protein) mechanism. Binding of glucagon to the GR results in an interaction with the stimulatory G-protein complex (GS). GS is a complex composed of α-, β-, and γ-subunits. In its trimeric state, the GS complex has the nucleotide guanosine diphosphate (GDP) bound to its α-subunit and is inactive. Glucagon binding to the GR acts to release the α-subunit of the GS by a process involving exchange of GDP for guanosine triphosphate. The liberated α-subunit is then active and able to stimulate the enzyme adenylyl cyclase, which converts ATP into the second messenger, cAMP. This process is limited by the endogenous GTPase activity of the α-protein, which will break down GTP to GDP, allowing reassociation of the inactive trimer.

Degradation of cAMP is catalyzed by the enzyme phosphodiesterase. Glucagon also acts to inhibit phosphodiesterase, probably by an inhibitory (GI) G-protein-mediated mechanism, to further increase cAMP signaling. The cascade of glucagon action continues with cAMP activation of cAMP-dependent protein kinase A (PKA). PKA is a heterotetrameric protein complex and, analogous to the activation of the GS, the process involves dissociation of the tetramer to free the catalytic subunits. The liberated catalytic (C) subunits of PKA catalyze the phosphorylation of serine or threonine residues in regulatory sites of key metabolic enzymes (to be described later). In addition to these rapid actions, PKA also alters gene expression through Ser-133 phosphorylation of the cAMP response element binding protein (CREB). CREB is a nuclear transcription factor that binds directly to DNA that contains a specific 8-base-pair sequence—the cAMP response element (CRE)—to alter gene expression.

In recent years, the importance of co-activators that enhance the actions of transcription factors has been recognized. For actions mediated by the cAMP/CREB pathway, two co-activators have been characterized—CREB-binding protein (CBP-1) and peroxisome proliferator-activated receptor-γ co-activator (PGC-1). CBP-1 is a co-activator for a number of transcriptional factors, including, but not limited to, CREB. Co-activators do not directly alter gene expression but do so by facilitating the actions of transcription factors. In the case of CBP, this probably involves a number of different mechanisms, including the acetylation of nuclear histones and the recruitment of other co-activators. PGC-1 is a recently described nuclear co-activator that facilitates the hepatic cAMP/CREB-mediated actions of glucagon to increase expression of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK). It seems likely that over the coming years, other co-activators and co-repressors acting on CREB-mediated gene expression will be described.

The cAMP pathway is a common effector mechanism for a number of hormones acting at cell surface receptors. For example, many of the hepatic effects of glucagon (see later) are also stimulated by β-adrenergic receptor-mediated activation of the cAMP pathway.

## B. Calcium Pathway

Although the cAMP pathway is the better characterized second-messenger pathway, glucagon also stimulates the complementary calcium/phospholipid pathway. Glucagon binding to GR results in the breakdown of cell membrane phospholipid, phosphatidylinositol 4,5-bisphosphate ($PIP_2$), by phospholipase C to two active compounds, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG). A rise in intracellular $InsP_3$ results in calcium release, initially from the endoplasmic reticulum but also from an influx of extracellular calcium into cells. Both calcium and the elevated levels of DAG activate protein kinase C (PKC), which is then able to alter activity by phosphorylating other intracellular enzymes (described later).

Although the cAMP and calcium pathways are described separately here, there are areas of overlap between the downstream effects (see later). There is also a synergism, so activation of one pathway will increase signaling through the other. For example, probably acting at multiple sites, cAMP amplifies signaling through the calcium pathway, from increased receptor affinity to amplification of the InsP3-mediated rise in intracellular calcium. Calcium is also able to stimulate CREB binding and thus alter gene expression, probably acting both by direct calmodulin-mediated and by indirect cAMP-mediated mechanisms.

# IV. HEPATIC ACTIONS

## A. Liver

Although glucagon actions in other tissues have been reported (and will be discussed later), the main target organ of glucagon action is undoubtedly the liver, where glucagon acts to increase glucose production and output. The portal anatomy is ideally designed so that glucagon (and insulin) secreted from the pancreas can bathe the liver in relatively high concentrations, compared with systemic levels, allowing changes in glucagon (and insulin) secretion to exert relatively rapid effects on hepatic metabolism.

In general, hepatic metabolism is characterized by the existence of opposing metabolic pathways—for example, glycogen synthesis and breakdown—with maintenance of homeostasis depending on an integrated balance between antagonistic pathways. Glucagon exerts its actions by having a coordinated action on these pathways, tending, for example, to increase glycogenolysis and simultaneously decrease glycogen synthesis. This fine control of hepatic metabolism is further increased by the opposing actions of insulin. Secretions of insulin and glucagon from the pancreas are, in general, inversely correlated in health, thus creating an exquisitely sensitive mechanism for controlling hepatic glucose homeostasis.

## B. Glycogenolysis/Glycogen Synthesis

The rapid effects of a rise in glucagon, i.e., increased hepatic glucose output, are mediated predominantly by increased glycogen breakdown. Glucagon enhances glycogen breakdown and inhibits glycogen synthesis by a series of coordinated changes in the activities of key enzymes controlling glycogen flux. The actions of glucagon on these regulatory enzymes are mediated by phosphorylation of key enzymes controlling glycogen synthesis/breakdown.

Glucagon increases glycogenolysis by activating the key enzyme, glycogen phosphorylase (Fig. 1). Glycogen phosphorylase is a key rate-limiting enzyme that is necessary for the breakdown of glycogen; it exists as a homodimer of subunits of about 100 kDa. Phosphorylation of glycogen phosphorylase on Ser-14 converts the enzyme from inactive phosphorylase b to active phosphorylase a. This phosphorylation is mediated by a regulatory enzyme, phosphorylase kinase, which in turn is also activated by phosphorylation. Phosphorylase kinase is a 1300-kDa protein complex made up of four different subunit types ($\alpha_4$, $\beta_4$, $\gamma_4$, and $\delta_4$) arranged as a hexadecamer. Phosphorylation of glycogen phosphorylase is catalyzed by the γ-subunit. Glucagon activates phosphorylase kinase by calcium-mediated activation (mediated by the δ-subunit) or by cAMP-dependent protein kinase phosphorylation of sites on the α- and β-subunits. This action of glucagon will be limited by the actions of glycogen-associated protein phosphatases (PP-1Gs), which will tend to convert active glycogen phosphorylase a back into inactive glycogen phosphorylase b.

This enhancement of glycogenolysis by glucagon is complemented by actions to inhibit glycogen synthesis. Again, this is mediated by phosphorylation of a key enzyme, glycogen synthase. Glycogen synthase is an 85-kDa protein with a number of potential phosphorylation sites at both N and C termini. Phosphorylation of serine residues of glycogen synthase converts it from the active "a" form into the inactive "b" form. Glucagon-induced phosphorylation (and thus inactivation) of glycogen synthase is probably mediated by a number of different kinases, including cAMP-dependent protein kinase. Glycogen synthase is reactivated by a specific glycogen-associated protein phosphatase (PP-1$G_L$). PP-1$G_L$ is also a site of glucagon action in that it is inhibited by the glucagon-driven rise in intracellular calcium and by inhibition by phosphorylase a.

In summary, glucagon will thus tend to promote a net glycogenolysis within liver parenchymal cells, resulting in increased flux of glucosyl units into glucose 6-phosphate. Glucose 6-phosphate may then be hydrolyzed by glucose 6-phosphatase, releasing glucose for hepatic export into the hepatic vein.

## C. Glycolysis/Gluconeogenesis

Glucagon also exerts coordinated actions on the opposing pathways of glycolysis and gluconeogenesis, inhibiting the former and increasing the latter

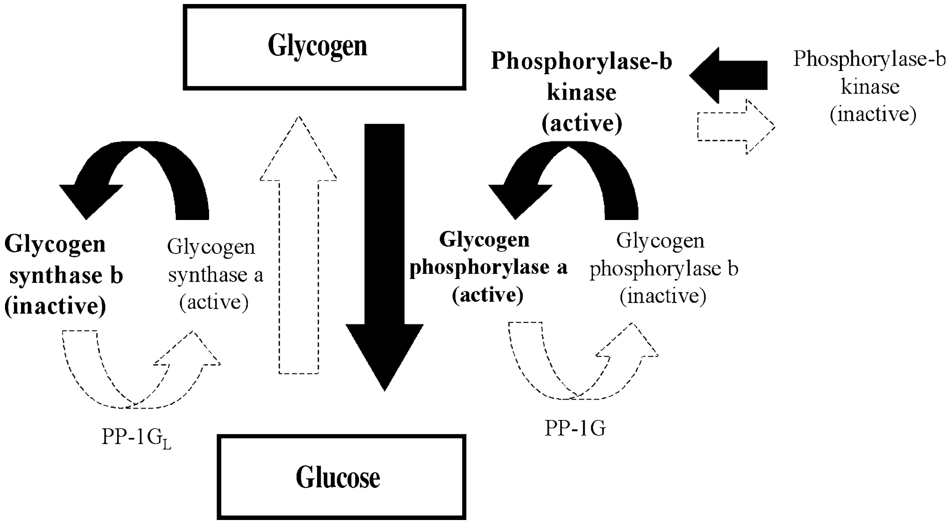
Glycogen
Phosphorylase-b kinase (active)
Phosphorylase-b kinase (inactive)
Glycogen synthase b (inactive)
Glycogen synthase a (active)
Glycogen phosphorylase a (active)
Glycogen phosphorylase b (inactive)
PP-1G$_L$
PP-1G
Glucose

FIGURE 1 Glucagon acts to promote glycogenolysis (actions shown by filled arrows). PP-1G, Glycogen-associated protein phosphatase.

in order to stimulate hepatic glucose production. The key regulatory step in glycolysis is the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate by the enzyme phosphofructo-1-kinase (PFK-1) (Fig. 2). In contrast to many other enzymes in glycolysis, the actions of PFK-1 are unidirectional, i.e., irreversible by PFK-1. During gluconeogenesis, fructose 1,6-bisphosphate is converted back into fructose 6-phosphate by the action of fructose 1,6-bisphosphatase. Thus the metabolic balance between glucose breakdown and formation depends on the balance between the activity of PFK-1 and fructose 1,6-bisphosphatase. This balance is controlled with the aid of the enzyme phosphofructo-2-kinase/fructose 2,6-bisphosphate (PFK-2), which is the site of action of glucagon on glycolysis/gluconeogenesis. PFK-2 acts to convert fructose 6-phosphate into fructose 2,6-bisphosphate, but, in contrast to the PFK-1 and fructose 1,6-bisphosphatase, the action of PFK-2 is reversible. The direction of action of PFK-2 is determined by its phosphorylation state: when phosphorylated it acts as a phosphatase and when dephosphorylated it acts as a kinase. Fructose 2,6-bisphosphate, the product of PFK-2 when dephosphorylated, is an allosteric activator of PFK-1.

Like the effects on glycogen metabolism, the short-term effects of glucagon on glycolysis/gluconeogenesis are exerted by phosphorylation of a key enzyme. Glucagon acts via protein kinase A to increase phosphorylation of PFK-2, thus converting it into a phosphatase and decreasing levels of fructose 2,6-bisphosphate. This will tend to decrease activity of PFK-1 and thus reduce flux through glycolytic pathways.

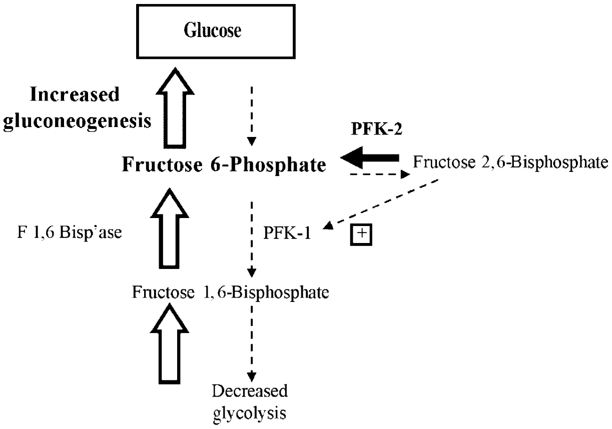
Glucose
Increased gluconeogenesis
PFK-2
Fructose 6-Phosphate
Fructose 2,6-Bisphosphate
F 1,6 Bisp'ase
PFK-1
+
Fructose 1,6-Bisphosphate
Decreased glycolysis

FIGURE 2 Glucagon acts to increase gluconeogenesis by inhibiting PFK1 (actions shown by large arrows). F1,6 bisp'ase, Fructose 1,6-bisphospatase; PFK-1, phosphofructokinase-1; PFK-2, phosphofructokinase-2/fructose 2,6-bisphospatase.

Glucagon also acts via CREB to alter gene expression, increasing levels of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, and glucose 6-phosphatase. Glucagon also decreases levels of the glucose transporter GLUT 2 and the hexokinase glucokinase, thus reducing liver glucose uptake and flux into glycolytic pathways. One important difference between the actions of glucagon on glycogenolysis/glycogen synthesis and actions on glycolysis/gluconeogenesis is that the latter is slower to reverse. This is probably of functional importance, because resynthesis of glycogen during refeeding after a fast, for example, will occur via gluconeogenesis and glycogen synthesis.

## D. Ketogenesis/Lipogenesis

Glucagon induces ketone formation and inhibits lipid synthesis in the liver. Lipid synthesis is inhibited by glucagon by phosphorylation of acetyl coenzyme A (coA) carboxylase, the first step in lipid synthesis. This effect is further enhanced by glucagon's actions to decrease glycolysis, which results in a decrease in acetyl coA, the substrate for acetyl coA carboxylase.

The net result is a decrease in levels of malonyl coA, the product of acetyl coA carboxylase. As well as being the first intermediate in the fatty acid synthesis pathway, malonyl coA has a key controlling influence on lipid metabolism. Increased levels of malonyl coA inhibit carnitine palmitoyltransferase I (CPT I), which transfers fatty acids across the mitochondrial membrane to undergo β-oxidation, resulting in formation of the ketones β-hydroxybutyrate and acetoacetate. A reduction in malonyl coA levels as a consequence of glucagon action thus results in increased β-oxidation of lipids and enhanced hepatic ketone formation. Insulin has opposite actions by decreasing levels of cAMP and thus decreasing protein kinase A phosphorylation of acetyl coA carboxylase.

## E. Physiological Role of Glucagon

In health, blood glucose levels are usually maintained within a remarkably tight range. In particular, glucose from the bloodstream is the main, if not the exclusive, fuel for the brain. If blood glucose falls, cognitive performance and brain functioning decrease. If blood glucose rises, as in diabetes, body tissues may be damaged. Glucose homeostasis is achieved largely by interplay between glucagon and insulin actions, although other hormones, the autonomic nervous system, and the hormone-independent effects of glucose per se also contribute. Mice in which the GR has been knocked out have a lower set point for glucose maintenance, with fed and fasted blood glucose levels about 30 mg/dl lower than in controls, suggesting that glucagon action plays an important, but not critical, role in maintaining euglycemia under normal conditions.

During exercise or stress, such as sepsis, hepatic glucose output rises. Human studies with somatostatin infusion to suppress glucagon secretion (and with replacement of insulin) suggest that the rise in glucagon seen during such stresses plays an important role.

## V. NONHEPATIC SITES

Although the classical target organ for glucagon action is the liver, as described above, glucagon receptors are known to exist in tissues in a number of other organs, suggesting putative actions of glucagon in some of these organs. Nevertheless, the main target of glucagon action is the liver.

### A. Adipose Tissue

Glucagon receptor mRNA is present in adipose tissue. Glucagon has been shown to have lipolytic actions *in vitro*. A number of *in vivo* studies have also demonstrated an augmentation of lipolysis by glucagon. However, the doses of glucagon used were supraphysiological and studies using more physiological doses, and more sensitive measurements of local tissue metabolism such as microdialysis, have failed to show any effect.

### B. Pancreas

Glucagon receptor mRNA has been detected in islets. *In vitro/ex vivo* studies suggest that glucagon potentiates insulin secretion from isolated islets, although the potency is considerably less than that of the insulin secretagogue GLP-1. Although the full significance of this remains to be determined, it is possible that local glucagon release from pancreatic alpha cells may exert a paracrine regulation on insulin secretion from beta cells.

It is also conceivable that GR on alpha cells may contribute to the feedback control of insulin secretion. In keeping with this, the GR knockout mice have markedly elevated circulating glucagon levels—two orders of magnitude higher than in wild-type animals and considerably higher than would be expected from their modestly lowered blood glucose. This suggests that there may be a blood glucose-independent feedback loop controlling glucagon secretion, with alpha cells monitoring bioactive glucagon by a GR-mediated process.

### C. Other Organs

Glucagon receptor mRNA has been identified by Northern blotting in rat heart, gastrointestinal tract, kidney, brain, spleen, ovary, and thymus, and in lesser amounts in adrenal gland, thyroid, and skeletal muscle. Glucagon has been demonstrated to have both inotropic and chronotropic effects on myocardium, and to be a vasodilator. In the kidney, glucagon has been shown to increase renal blood flow, glomerular filtration rate, and electrolyte excretion, although pharmacological doses are required. The kidney is also a gluconeogenic organ but, in contrast to its effects on the liver, glucagon has no actions on renal glucose production. In summary, the action of glucagon on these nonclassical target tissues is uncertain, with many of the effects being demonstrable only when pharmacological doses of glucagon are used. On the other hand, the presence of glucagon receptors suggests that there may be other, as yet unidentified, actions of glucagon in these organs.

## VI. MINIGLUCAGON

Miniglucagon was first discovered in 1990 as the product of the proteolytic cleavage of glucagon by a protease named the miniglucagon-generating endopeptidase (MGE). Miniglucagon is the COOH-terminal (19–29) fragment of glucagon cleaved by MGE at the Arg$^{17}$-Arg$^{18}$ doublet. Although first described in hepatic cells, MGE has been identified in pancreatic islets and other putative target tissues of glucagon, including heart, gastric mucosa, kidney, and spleen.

Miniglucagon has been demonstrated to be biologically active. Indeed, it is two orders of magnitude more potent than the intact glucagon molecule, which may more than compensate for the lower concentrations compared to full-length glucagon. In pancreatic islets, for example, miniglucagon colocalizes with glucagon in alpha cell secretory granules, with levels at about 3–4% of intact glucagon levels.

A number of putative actions have been suggested for miniglucagon, most notably in nonhepatic tissues. *In vitro* studies on insulin secretion from islets and/or isolated pancreata have shown that miniglucagon inhibits, and miniglucagon antiserum increases, insulin secretion. Similarly, in heart muscle there is some *in vitro* evidence that cleavage of glucagon into miniglucagon may be necessary, at least in part, for the inotropic effects of glucagon. However, the exact physiological role of miniglucagon and its interplay with glucagon action remain to be determined.

## VII. ABNORMAL GLUCAGON ACTION

### A. Glucagon in Diabetes

Abnormal glucagon secretion has long been recognized in type 1 diabetes. Circulating glucagon levels are elevated in absolute or relative terms and may not suppress normally with eating. This has lead to the suggestion that diabetes is a bihormonal disease, with hyperglucagonemia contributing to the development of hyperglycemia and possibly even the catabolic state that may be present at diagnosis of type 1 diabetes. As previously discussed, the actions of insulin and glucagon are closely coordinated so that the insulin deficiency that coexists in undertreated diabetes makes it difficult to disentangle the effects of elevated glucagon from those of hypoinsulinemia. It seems likely that the dominant influence is the lack of insulin. The exact contribution of hyperglucagonemia to remains to be determined.

At the other extreme, glucagon secretion may fail to rise during hypoglycemia in type 1 diabetes This reduction in defensive glucagon responses, particularly if then combined with secondary deficiencies in catecholamine and symptomatic responses to hypoglycemia, will significantly increase the risk of suffering from severe hypoglycemic episodes. The cause for this is uncertain.

### B. Glucagon Receptor Mutations

In epidemiological studies, a polymorphism of the glucagon receptor (glycine at codon 40 is substituted by serine, indicated by the notation G40S) has been associated with an increased risk of type 2 diabetes mellitus, central obesity, and hypertension. Other population studies have failed to show an association. The mechanism by which G40S may result in diabetes or obesity is uncertain given that *in vitro* mutagenesis studies suggest that the G40S polymorphism results in a decreased affinity of the GR for glucagon, which might be expected, at least at face value, to protect against hyperglycemia. For the association with hypertension, a decreased natriuretic effect of glucagon in the G40S kidney has been suggested as a mechanism, although, as previously discussed, the physiological effects of glucagon in the kidney are not clearly established.

### C. Glucagonomas

Glucagonomas are glucagon-secreting tumors of the pancreas (or rarely extrapancreatic) that may occur either in isolation or as part of a multiple endocrine neoplasia syndrome. Excessive secretion of glucagon leads to a glucagonoma syndrome characterized by the pathognomonic rash of necrolytic migratory erythema together with weight loss, glossitis, anemia, thromboembolism, gastrointestinal disturbances, and diabetes or impaired glucose intolerance.

## VIII. DIAGNOSTIC USE/THERAPEUTIC USE

### A. Diagnostic Use of Glucagon

Glucagon given as a 1-mg bolus by intravenous, intramuscular, or subcutaneous routes has been sparingly used as a diagnostic test in three areas of clinical practice. Although the tests are largely safe, about 20% of recipients suffer side effects, mostly nausea, dizziness, flushing, and transient syncope.

Glucagon has been used to measure the C-peptide response and thus, by inference, the insulin secretory reserve of diabetic patients. Although often assumed to distinguish between type 1 and type 2 diabetes without beta cell failure, in practice this is often complicated by type 1 patients with a persisting beta cell secretory reserve. For example, some patients may develop a "honeymoon" period, with minimal requirement for insulin lasting for several months after the onset of type 1 diabetes and a persistent C-peptide response to glucagon. Some type 1 patients may have an insidious onset of diabetes, i.e., the latent autoimmune diabetes of adult onset (LADA) with persisting C-peptide response during progression. Even in type 2 diabetes, C-peptide response has been in general an unreliable predictor of the need for insulin treatment.

In endocrinology clinics, glucagon has been used as a dynamic pituitary function test to measure growth hormone (GH) and adrenocorticotropic hormone (ACTH) secretory capacity, particularly in situations when insulin stress testing is desired but is contraindicated for medical reasons such as ischemic heart disease or epilepsy. The mechanism by which glucagon stimulates GH and ACTH release is poorly understood, but it may be a secondary effect mediated by catecholamine release. The latter effect has been employed by using a bolus of glucagon with measurement of plasma catecholamines in the diagnosis of phaeochromocytomas, although the test is not without risk and may not increase diagnostic sensitivity above that of simple nondynamic measurement of plasma and urine catecholamines.

Finally, the 1-mg bolus glucagon test has also been used in oncology clinics to distinguish different types of tumor hypoglycemia, with a blood glucose rise of at least 30 mg/dl suggesting that hypoglycemia is a result of hypersecretion of insulin or insulin-like growth factor-II (IGF-II), rather than other neoplastic causes such as hepatic failure. A glycemic response to glucagon may also indicate that glucagon infusion is a useful palliative therapeutic option for such patients if other treatments are ineffective.

## B. Therapeutic Use of Glucagon

Glucagon administered by subcutaneous, intramuscular, or intravenous routes is a rapid and effective treatment for insulin-induced hypoglycemia. Many patients with type 1 diabetes or their caregivers thus choose to have prefilled glucagon syringes at home for emergency rescue from severe hypoglycemia. An important caveat is that liver glycogen stores must be adequate for glucagon to be effective. The effects will also wear off relatively quickly, so that a successful rescue must be followed up by ingestion of carbohydrate to prevent a subsequent relapse (and, importantly, a critical review of the precipitating causes of the episode).

Glucagon has also been useful in rare cases of noniatrogenic hypoglycemia, either in cases of insulinoma or IGF-II-induced tumor hypoglycemia or in familial hyperinsulinemia. In these situations, glucagon must be given continuously, usually as a subcutaneous infusion.

The positive inotropic and chronotropic actions of pharmacological doses of glucagon have been utilized to treat drug-induced bradycardia, which can occur, for example, after overdoses of beta blockers, digoxin, or calcium blockers such as nifedipine or diltiazem. Although a role for glucagon in the treatment of bronchospasm, cardiogenic shock, and systolic/electromechanical cardiac arrest has even been suggested, the relative benefits of glucagon when compared with more conventional therapies remain to be determined and it seems unlikely that glucagon will replace more conventional therapies.

## Glossary

| | |
|---|---|
| **cAMP response element binding protein** | Nuclear transcription factor that mediates effects of cAMP to alter gene expression. |
| **cyclic adenosine monophosphate** | Second messenger formed inside cells in response to glucagon binding to its cell surface receptor, triggering a cascade of intracellular actions. |
| **glycogenolysis** | Glucagon-stimulated breakdown of glycogen stores in the liver. |
| **glycogen phosphorylase** | The main rate-limiting enzyme in glucagon-stimulated breakdown of glycogen. |
| **glycogen synthase** | The main rate-limiting enzyme in glycogen synthesis; inhibited by glucagon. |
| **miniglucagon** | Product of proteolytic cleavage of glucagon by endopeptidase; of uncertain physiological significance, despite its greater biological potency. |
| **phosphorylase kinase** | Regulatory enzyme that modifies the action of glycogen phosphorylase by phosphorylation. |

## See Also the Following Articles

**Diabetes Type 1 • Glucagon Gene Expression • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagonoma Syndrome • Glucagon Processing • Glucagon Secretion, Regulation of • Hypoglycemia in Diabetes**

## Further Reading

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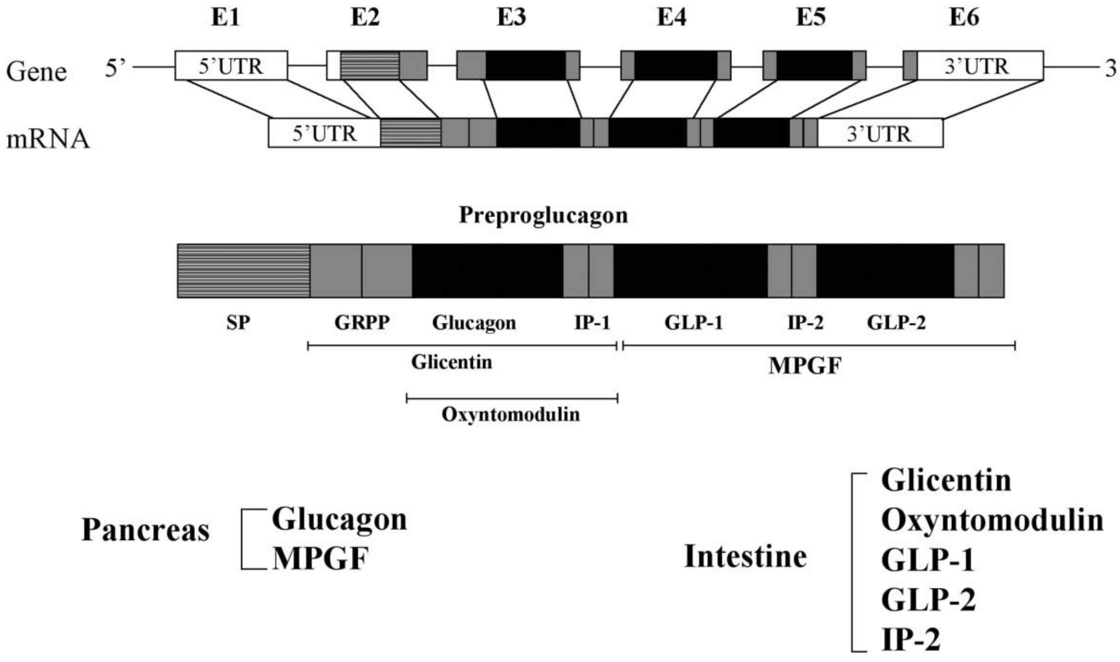

FIGURE 1 Structure of the proglucagon gene, depicting the genomic organization of the mammalian gene, the RNA, and the prohormone. Posttranslational processing of proglucagon-derived peptides occurs in the pancreas and intestine (bottom). E1–E6, Exons 1–6; UTR, untranslated region; SP, signal peptide; GRPP, glicentin-related polypeptide; IP-1, intervening peptide-1; GLP-1, glucagon-like peptide-1; MPGF, major proglucagon-derived fragment.

mice develop mild hypoglycemia due to glucagon deficiency. In contrast, the liberation of an intestinal profile of PGDPs, including glicentin, oxyntomodulin, and glucagon-like peptides (GLP-1 and GLP-2), derives from expression of the enzyme PC1/2 in enteroendocrine cells of the small and large intestines.

## II. PROGLUCAGON GENE EXPRESSION IN THE PANCREAS

The level of blood glucose is a major determinant of secretory activity from the islet A cell, with hyperglycemia inhibiting, and hypoglycemia stimulating, the release of glucagon into the circulation. Consistent with these findings, rats subjected to chronic hyperglycemic infusion for 5 days exhibit a significant reduction in islet proglucagon mRNA transcripts as assessed by *in situ* hybridization. Because hyperglycemia is frequently associated with enhanced insulin secretion, it is difficult to isolate the effects of glucose versus insulin on the islet A cell. Insulin administration inhibits proglucagon gene expression in normal and diabetic rodents; insulin-induced hypoglycemia, however, is associated with increased pancreatic proglucagon gene expression in the rat. Similarly, food deprivation leading to mild hypoglycemia is associated with induction of rat proglucagon gene expression. Studies of streptozotocin-induced diabetes in rats demonstrate increased levels of pancreatic proglucagon mRNA transcripts in

| | |
|---|---|
| RSLQDTEEKSRSFSASQADPLSDPDQMNEDKRHSQGTFTSDYSKYLDSRRQDFVQWLMNTKRNRNNIA | **Glicentin** |
| HSQGTFTSDYSKYLDSRRQDFVQWLMNTKRNRNNIA | **Oxyntomodulin** |
| HSQGTFTSDYSKYLDSRRAQDFVQWLMNT | **Glucagon** |
| HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG | **GLP-1(7-37)** |
| HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR | **GLP-1(7-36)**$^{\text{amid}}$ |
| HADGSFSDEMNTILDNLAARDFINWLIQTKITD | **GLP-2** |
| RNRNNIA | **IP-1** |
| DFPEEVAIVEELG | **IP-2** |

FIGURE 2 Amino acid sequence of the proglucagon-derived peptides liberated from proglucagon.

insulin-deficient diabetic rats, whereas the levels of proglucagon mRNA transcripts are reduced following correction of hyperglycemia following insulin administration. Whether changes in glucose alone, independent of insulin, regulate pancreatic proglucagon gene expression remains unclear, because correction of the hyperglycemia of experimental rodent diabetes with phlorizin is not associated with significant changes in the levels of pancreatic proglucagon mRNA. Despite the dominant inhibitory effect of insulin in the regulation of islet proglucagon gene expression, mice with targeted disruption of the insulin receptor gene exhibit no abnormalities in the levels of pancreatic proglucagon mRNA transcripts.

## III. PROGLUCAGON GENE TRANSCRIPTION IN ISLET CELL LINES

Proglucagon gene expression has been studied predominantly in immortalized rodent islet A cell lines, including rat RIN1056A, hamster InR1-G9 or HIT T15-G, and mouse αTC-1 cells. Incubation of RIN1056A cells with sodium butyrate induces cell cycle arrest, cell differentiation, and increased levels of proglucagon mRNA transcripts due to induction of proglucagon gene transcription. Similarly, phorbol esters activate proglucagon gene transcription in RIN1056A cells whereas the arginine-induction of rat islet proglucagon gene expression is abrogated by treatment of islets with the protein kinase C inhibitor H-7. Although cyclic adenosine monophosphate (cAMP), acting through protein kinase A (PKA), up-regulates levels of proglucagon mRNA transcripts in primary cultures of rat islets, glucagon-producing rodent islet cell lines frequently exhibit defects in cAMP-dependent signaling and fail to increase levels of proglucagon mRNA transcripts following treatment with dibutyryl cAMP or forskolin. In contrast, transfection of islet cell lines with the catalytic subunit of PKA bypasses the cAMP signaling defect and activates proglucagon gene transcription. Consistent with data from studies of mice and rats *in vivo*, insulin reduces the levels of proglucagon gene expression in islet cell lines via inhibition of gene transcription. Paradoxically, despite the lack of evidence implicating a direct role for glucose in rodent proglucagon gene expression *in vivo* or in cultures of rat islets *in vitro*, chronic incubation (1–5 weeks) of islet InR1-G9 cells with glucose-supplemented medium (11 m*M* glucose) resulted in up-regulation of proglucagon mRNA transcripts, compared to cells incubated in 5 m*M* glucose for comparable time periods. The glucose-stimulated induction of proglucagon mRNA transcripts was due to enhanced gene transcription, and was inhibited by cotreatment with insulin. Analysis of proglucagon mRNA decay following incubation with actinomycin reveals an estimated $t_{1/2}$ of ~12 h in RIN1056A rat islet cells; however, factors that regulate islet proglucagon mRNA stability *in vivo* remain poorly understood.

## IV. DELINEATION OF FUNCTIONAL DOMAINS WITHIN THE RAT PROGLUCAGON GENE PROMOTER

A majority of studies of proglucagon gene transcription have predominantly utilized the rat proglucagon gene promoter fused to heterologous reporter genes (e.g., genes encoding chloramphenicol acetyltransferase, luciferase, or growth hormone), followed by analysis of transcriptional activity in transfected islet cell lines. A majority of experiments have focused on the proximal sequences within the first 300 bp of the rat proglucagon promoter, because experiments in islet cell lines demonstrated that proglucagon promoter fusion genes containing additional 5′ flanking sequences extending to −2.3 kb exhibit levels of transcriptional activity comparable to that seen with sequences extending to −300 bp. Structural and functional characterizations of the rat promoter through deletional and mutagenesis studies have identified distinct cis-acting domains within the proximal 300 bp of the rat proglucagon promoter (Fig. 3). These domains, designated G1 through G5, bind transcription factors present in islet cell nuclear extracts and exhibit enhancer-like properties (G2–G5) or function as an islet A cell-specific promoter (G1) *in vitro*. The first three domains to be identified extend from −292 to −256 (G3), −192 to −179 (G2), and −114 to −74 (G1). Subsequent experiments subdivided the G3 sequence into an upstream domain A (5′-CGCCTGA) and a downstream domain B (5′-GATTGAAGGGTGTA-3), and an element partially overlapping original G1 sequences, designated G4, was identified spanning −140 to −100. More recently, DNA sequences located between G4 and G2, from −169 to −140, have been designated as the G5 element. A cAMP response element (CRE), 5′-TGACGTCA-3′, is located upstream of G3 from −300 to −292, and a phorbol ester response element has been mapped to sequences within G2. The finding that potassium-induced membrane depolarization activates glucagon gene

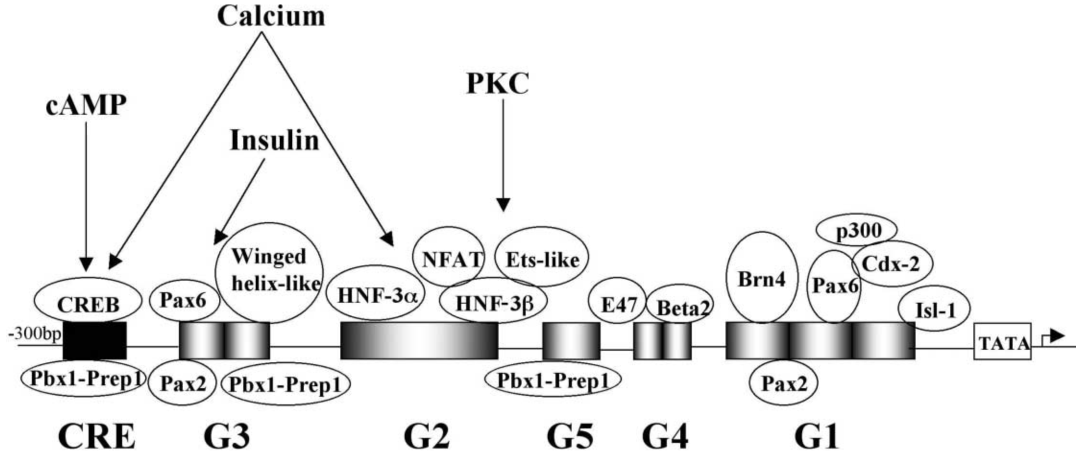

FIGURE 3 The proximal rat proglucagon gene promoter and its interaction with specific transcription factors. Specific functional subdomains, G1–G5, are located within the first 300 bp of the proximal rat proglucagon gene 5′ flanking region. cAMP, Cyclic adenosine monophosphate; PKC, protein kinase C; CREB, cAMP response element binding protein; HNF-3, hepatocyte nuclear factor-3 (see text for discussion of other factors).

transcription in a calcium-dependent manner has resulted in the mapping of the "depolarization response element" to sequences within the rat glucagon gene CRE. Mutational studies of the proximal rat proglucagon promoter have identified a second calcium response element within the region spanned by G2. Similarly, the inhibitory effect of insulin on rat proglucagon gene transcription has been mapped to the more distal domain A of the G3 element, and the available data suggest that insulin also exerts its effects through mechanisms converging on more proximal sequences located within the G1 element.

## V. MECHANISMS MEDIATING ISLET A CELL-SPECIFIC PROGLUCAGON GENE TRANSCRIPTION

The observation that pancreatic proglucagon gene transcription is restricted to islet A cells has focused attention on identification of DNA sequences and transcription factors that both activate transcription in the A cell and restrict proglucagon gene transcription to the A cell. The difficulty in obtaining purified populations of nonimmortalized A cells for gene transfer studies has necessitated use of immortalized islet cell lines derived from insulinomas (beta cells) or glucagonomas (A cells) for comparative studies of islet cell-specific proglucagon gene transcription. Indeed, some degree of transcriptional activation is observed following transfection of rat proglucagon promoter sequences into islet beta cell lines. The G2 and G3 elements display weak enhancer-like activity in islet, but not nonislet, cell lines, whereas the more proximal G1 element is required for restriction of transcriptional activation to the islet alpha cell lineage. Nevertheless, the results of electrophoretic mobility-shift assays or footprinting experiments using these DNA elements in conjunction with nuclear extracts from islet cell lines exhibiting different hormonal phenotypes produce comparable patterns of DNA–protein interaction, providing evidence for considerable complexity in the transcriptional specification of A cell-specific gene transcription. Furthermore, insulin and somatostatin gene enhancer sequences placed upstream of the proximal rat proglucagon promoter can activate transcription in islet cells when linked to the G1 element. Moreover DNA sequences within the proglucagon gene G3 element bind nuclear factors that also recognize related sequences within the somatostatin upstream enhancer element and the insulin gene E-1 promoter region, leading to the designation of the G3 domain A region as the pancreatic islet cell-specific enhancer sequence (PISCES) element. Given the common embryological origin of hormonally distinct yet related islet cell lineages, it is not surprising that islet hormone genes have evolved both common and unique mechanisms for specifying islet subtype-specific gene transcription.

Within the G1 proximal promoter element, at least four distinct patterns of DNA–protein complex formation, with varying binding specificity, can be identified using nucleotide sequences from $-100$ to $-52$ as probes interacting with islet A cell nuclear extracts. The formation of several G1 complexes appears to be relatively islet cell specific, and mutations within G1 that disrupt these DNA–protein interactions also result in diminished transcriptional activity in transfected islet A cells. Within the core G1 domain, the sequence CAGATG from −83 to −78 corresponds to E-1, an "E box-related" subdomain known to be important for expression of the somatostatin, insulin, and gastrin genes in islet cells. Two additional E box motifs are located within the adjacent G4 element, E-2, from −108 to −103, and E3, from −135 to −130. Functional studies using dominant negative helix–loop–helix (HLH) proteins suggest that the E-3 box likely represents the more functionally important sequence for transcriptional regulation. Although the insulin gene E boxes function as positive regulatory elements, the HLH protein E47 represses glucagon gene transcription mediated by E box binding sites, whereas the combination of E47 and Beta2 transcription factors activated glucagon promoter activity in InR1-G9 islet cells. Although Beta2-deficient mice develop diabetes and exhibit defects in formation of enteroendocrine cell lineages, whether Beta2 plays an essential role in control of glucagon gene expression independent of A cell lineage formation remains uncertain. Although the first 75 bp of the rat proglucagon gene promoter, comprising a portion of the proximal G1 element, imparts a degree of A cell specificity to transfected reporter genes, inclusion of the entire G1 domain extending to ~ −100 is required for optimal specificity in alpha versus beta cell lines. Sequences within the G5 element may also function to restrict proglucagon gene transcription to A cells, because sequential deletion of the G5 element modestly increases transcriptional activity of proglucagon promoter reporter genes in nonislet cell lines.

## VI. ISLET PROGLUCAGON GENE TRANSCRIPTION FACTORS

The observation that the proximal rat proglucagon promoter G1 element contains several TAAT nucleotide motifs corresponding to recognition sites for homeodomain transcription factors has provided impetus for assessment of the potential role of islet homeodomain proteins in the control of proglucagon gene transcription. The first islet transcription factor cloned using TAAT element DNA probes was Isl-1 (islet 1), a LIM domain homeobox protein that binds DNA sequences in the rat insulin gene E2 region. (The acronym LIM derives from the names of proteins Lin-11, Isl-1, and Mec-3.) Isl-1 also binds to TAAT motifs within the rat proglucagon gene G1 region and is a weak transactivator of proglucagon promoter activity in cell transfection studies. Nevertheless, mice with targeted disruption of the Isl-1 gene contain insulin mRNA transcripts yet exhibit defective islet A cell formation, implicating a role for Isl-1 in islet A cell development. Furthermore, inhibition of Isl-1 expression using antisense expression vectors in hamster islet InR1-G9 cells results in reduced levels of endogenous proglucagon mRNA transcripts. Whether Isl-1 is important for proglucagon gene expression in normal islet A cells awaits loss-of-function studies in human mutations or experiments designed to inactivate Isl-1 specifically in normal alpha cells *in vivo*. The beta cell transcription factor Pdx-1 also binds to the G1 element and is a potent repressor of proglucagon gene transcriptional activity and proglucagon mRNA transcripts, likely via DNA binding-independent mechanisms, in islet cell lines.

The *caudal* family homeobox protein Cdx-2/3, also cloned as an insulin gene enhancer binding protein, binds with high affinity to AT-rich motifs within the proglucagon gene G1 element and activates the proglucagon promoter *in vitro*. Increased expression of Cdx-2/3 also activates expression of the endogenous rodent proglucagon gene in transfected InR1-G9 cells. Two Cdx-2/3 isoforms are detected in rodent islet cells, including full-length bioactive Cdx-2/3, and an amino-terminally truncated protein that binds to the G1 element but does not exhibit transactivation potential. Determination of whether Cdx-2/3 is essential for islet proglucagon gene transcription in normal A cells *in vivo* is hampered by the early embryonic lethality of mice harboring inactivating mutations in the Cdx-2/3 gene.

Both Pax6 and Pax4 are expressed in the endocrine pancreas and play divergent roles in the control of cell lineage formation and proglucagon gene transcription. The paired homeodomain protein Pax6 binds to AT-rich motifs within both the G1 and G3 proximal promoter elements. The Pax6 protein activates G1- and G3-dependent transcription and is capable of synergistic transcriptional activation through heterodimer formation with Cdx-2/3, primarily through binding to the G1 element. The Pax-6:Cdx-2/3 transcriptional activation via G1 is further enhanced through interaction with the transcriptional co-activator p300. Mice with a targeted inactivation of the Pax6 gene, or mice expressing the dominant negative Pax6 SEY$^{NEU}$ allele exhibit marked abnormalities in the formation of islet cell lineages, reduced numbers of islet A cells, and decreased levels of proglucagon mRNA transcripts. Whether Pax6 is essential for proglucagon gene transcription in the adult A cell independent of its role in islet A cell development has not yet been determined.

The Pax4 gene is also essential for islet lineage development in that inactivation of the mouse Pax4 gene results in failure to form beta and delta cells, yet increased numbers of A cells. Also, Pax4 does not appear to be expressed in mature adult islet A cells, yet it is capable of acting as a transcriptional repressor of proglucagon gene expression when ectopically expressed in islet A cell lines. Intriguingly, the repression of transcription exerted by Pax4 may be independent of DNA binding, because Pax4 isoforms function as transcriptional repressors even in the absence of a DNA-binding domain. The integrated roles of Pax6 and Pax4 in islet cell development have led to a model whereby islet lineage allocation and phenotype may be dictated by both positive (Pax6) and negative (Pax4) control of cell development and hormone gene expression. The related homeobox transcription factor Pax2 is also expressed in islet alpha cells, binds to both the G3 and G1 promoter elements, and functions as a positive regulator of proglucagon gene transcription.

Brain 4 (Brn4), a POU domain transcription factor originally isolated from a rat hypothalamic cDNA library, binds to and transactivates promoters containing a DNA octamer motif recognized by related POU domain proteins. (POU is an acronym derived from the names of three transcription factors, Pit-1, Oct-1, and Unc-86, which are all characterized by a specific DNA-binding domain.) Brain 4 is also expressed in islet A cells, binds to AT-rich sequences within the 5′ end of the G1 element, and activates rat proglucagon gene transcription. Induction of Brn4 expression in beta cell lines is associated with activation of endogenous glucagon gene expression *in vitro*. Whether Brn4 is essential for proglucagon gene transcription in normal islets *in vivo* remains unclear; mice with targeted disruption of the Brn4 gene do not exhibit defects in islet glucagon gene expression. Members of the Maf transcription factor family also bind to G1 and enhance proglucagon gene transcription. Maf factors are capable of enhancing Pax6 transcriptional activity via increased Pax6 binding to G1 or though protein–protein interaction with Pax6 independent of DNA binding. Although c-Maf −/− mice have been generated via homologous recombination, a majority of c-Maf knockout embryos die on days E17.5–18.5, and the surviving mice exhibit microphthalmia; however, the status of endocrine pancreas and islet A cells in Maf −/− mice has not yet been reported.

Members of the hepatocyte nuclear factor-3 gene family (HNF-3), currently known as the Fox (fork-head box) transcription factors, bind to the rat proglucagon gene G2 element. HNF-3β (FoxA2) is expressed in islet A cell lines and overexpression of HNF-3β results in repression of proglucagon promoter activity in islet cell lines *in vitro*. Multiple HNF-3β isoforms can be detected in islet A cell lines that bind G2 with similar affinity, but only the full-length HNF-3β1 isoform represses transcriptional activity. HNF-3β also binds to the G3 element and transfection of a dominant negative mutant HNF-3β represses G3-dependent transcriptional activity in islet A cells. Targeted disruption of HNF-3β results in early embryonic lethality, precluding assessment of A cell formation and glucagon gene expression in the adult mouse, whereas beta-cell-specific inactivation of HNF-3β results in mice with hyperinsulinemic hypoglycemia, likely due to defective control of $K_{ATP}$ channel function.

The HNF-3 (FoxA) proteins exhibit similar patterns of DNA-binding specificity, and both HNF-3α (FoxA1) and HNF-3γ (FoxA3) also bind to the glucagon gene G2 promoter element. Although a functional role for HNF-3γ in control of proglucagon gene transcription remains to be established, HNF-3α activates G2-dependent proglucagon gene transcription in heterologous fibroblasts and in islet cell lines. Furthermore, HNF-3α −/− mice exhibit reduced levels of pancreatic proglucagon gene expression and a defective glucagon secretory response to hypoglycemia, implicating an essential role for HNF-3α in islet proglucagon gene transcription.

## VII. INTESTINAL PROGLUCAGON GENE EXPRESSION

Nutrient ingestion represents a primary determinant of proglucagon gene expression in enteroendocrine cells of the small and large intestines. Fasting reduces and feeding stimulates proglucagon gene expression in the rodent small bowel. High-fiber diets lead to up-regulation of proglucagon mRNA transcripts in the distal small bowel and colon, possibly due in part to a direct effect of fatty acids on induction of proglucagon gene expression. Indeed, lumenal infusion of long-chain triglycerides into the rat jejunum or intravenous infusion of fatty acids in parenterally fed rats stimulates intestinal proglucagon gene expression. Intestinal proglucagon gene expression is also up-regulated following major small bowel resection or induction of experimental diabetes in rodents. The mechanisms regulating intestinal proglucagon gene expression are poorly understood, although incubation of intestinal cell lines with specific nutrients such as peptones or fatty acids upregulates proglucagon gene expression, likely through effects on proglucagon gene transcription. Peptide hormones such as gastrin-releasing peptide also stimulate intestinal proglucagon expression via activation of proglucagon gene transcription.

## VIII. PROGLUCAGON GENE ENTEROENDOCRINE TRANSCRIPTION FACTORS

Compared to our understanding of islet proglucagon gene transcription, much less is known about control of proglucagon gene promoter activity in gut endocrine cells. Although the functional properties of the G1–G5 elements have been carefully defined in studies of islet-cell-specific proglucagon gene transcription, the potential importance of these proximal promoter elements in specifying intestine-specific gene transcription is less well understood. Evidence derived from analysis of proglucagon promoter activity following transfection of islet versus intestinal cell lines suggests important differences in the functional organization of proglucagon promoter elements in islet versus enteroendocrine cells. Transfection studies have identified a series of distal enhancer-like elements located between −2253 and −1292 bp upstream of the rat proglucagon gene transcription start site. This broad region of proglucagon gene 5′ flanking sequences, designated the glucagon gene upstream enhancer (GUE), appears to be functionally important for intestine-specific proglucagon gene transcription. Specific subdomains within the GUE exhibit positive and negative effects on reporter gene transcription, and GUE-derived probes bind proteins present specifically in GLUTag cell nuclear extracts. Nevertheless, proglucagon gene transcription factors that activate proglucagon gene expression specifically in gut endocrine cells have not yet been identified.

Both Cdx2/3 and Pax6 are expressed in enteroendocrine L cells, bind the G1 promoter element in electrophoretic mobility-shift assay (EMSA) experiments, and activate proglucagon gene transcription in transfected GLUTag mouse enteroendocrine cells. Mice expressing the dominant negative Pax6 SEY$^{NEU}$ allele exhibit markedly reduced levels of intestinal proglucagon mRNA transcripts in the small and large intestines. Whether this phenotype is principally due to the importance of Pax6 for enteroendocrine cell lineage formation or reflects an essential role for Pax6 in control of intestinal proglucagon gene expression remains unclear. In contrast to the central importance of HNF-3α for islet proglucagon gene expression, intestinal proglucagon gene expression appears normal in HNF-3α −/− mice.

## IX. ANALYSIS OF ISLET AND INTESTINAL PROGLUCAGON GENE TRANSCRIPTION IN TRANSGENIC MICE

The proglucagon promoter sequences required for expression of a reporter gene in murine islets and enteroendocrine cells have been broadly identified in a limited number of transgenic mouse studies. A 1252-bp fragment of the rat proglucagon gene promoter and 5′ flanking region extending from the Kpn I site and including 57 bp of exon 1 sequences directed expression of a cDNA encoding SV40 T antigen to islet A cells, resulting in the formation of glucagonomas in mice that were several months old. Transgene expression was also detected in the brain, but not in enteroendocrine cells of the gut. These findings imply that DNA sequences sufficient for islet-specific expression are distinct from those required to target correct enteroendocrine-specific expression. Furthermore, transgene expression under the control of the 1252-bp rat promoter was highly restricted to islet alpha cells, indicating that sequences within this promoter region were sufficient for specification of cell-specific transgene expression. Similarly, a comparable fragment of the rat proglucagon gene promoter extending from the Xho I site to exon 1 was sufficient for directing expression of a modified transforming growth factor-$\beta_1$ (TGF-$\beta_1$) cDNA to islet A cells *in vivo*. A larger rat proglucagon promoter fragment extending to −2252 bp containing the first 58 bp of exon was also expressed in islet A cells and in brain cells. Furthermore, transgene expression was detected in enteroendocrine cells of the small and large intestines, and these mice developed glucagon-producing endocrine tumors of the colon after weeks of transgene expression. Islet hyperplasia and endocrine tumor formation were also observed in the pancreas after several weeks, suggesting that the −2252 bp of rat proglucagon gene 5′ flanking sequences contained additional information, compared to the smaller 1252-bp promoter fragment, resulting in a greater degree of transgene expression compared to that observed with the −1252-bp promoter sequence. Transgene expression was also detected in the brain stem and hypothalamus of −2252-bp rat proglucagon promoter transgenic mice.

## X. HUMAN PROGLUCAGON GENE TRANSCRIPTION AND PROMOTER STUDIES IN MICE

Although a majority of studies of proglucagon gene transcription have utilized the rat promoter in transfection and transgenic experiments, there is little information available about the functional organization and activity of the human proglucagon gene promoter. Comparison of the human and rat proximal promoter sequences reveals a reasonable degree of sequence conservation within the G1–G5 promoter subdomains. Nevertheless, human proglucagon promoter and 5′ flanking sequences extending to −600 bp are transcriptionally inactive following transfection of rodent islet or intestinal cell lines. Furthermore, in contrast to the data obtained with reporter genes under the control of rat proglucagon promoter sequences, several kilobases of human proglucagon gene 5′ flanking sequences are not sufficient for activation of reporter gene expression in rodent islet cell lines, and human glucagon-producing islet or enteroendocrine cells have not yet been characterized and transfected with these reporter genes. The current findings indicate considerable species-specific differences in the structural and functional organization of the rat versus the human proglucagon gene promoter. Additional experimental data in support of the unique functional organization of the human proglucagon gene promoter derive from transgenic mice studies. A transgene containing the human growth hormone gene under the transcriptional control of ~1.6 kb of the human proglucagon gene promoter was expressed in the brain stem and in enteroendocrine cells, but not in mouse pancreatic islets. These results, interpreted within the experimental limitations of using murine cells for analysis of human promoter expression, provide further evidence suggesting that the human proglucagon gene promoter has evolved distinct functional mechanisms for regulation of islet-specific proglucagon gene transcription.

## XI. SUMMARY OF CURRENT KNOWLEDGE

Analyses of islet proglucagon gene expression, based on transfection experiments of rodent cell lines, transgenic mice, and physiological studies of glucoregulation in mice and rats, have delineated specific cis-acting domains and transcription factors that coordinately regulate rat proglucagon gene transcription specifically in islet A cells. Our knowledge of the DNA sequences and transcription factors important for control of proglucagon gene expression in enteroendocrine cells or neurons is considerably more limited. Furthermore, the available data from analysis of the human proglucagon promoter imply important functional species-specific differences, suggesting that the data obtained from analysis of the rat promoter may not necessarily be applicable to our understanding of human proglucagon gene expression. It seems likely that the development of additional experimental models using newly derived human endocrine cell lines, taken together with more detailed analysis of the factors regulating human proglucagon gene transcription, will be required to reconcile the differences observed in results from studies of rat and human proglucagon promoter control.

## Glossary

**diabetes** Metabolic syndrome characterized by an elevated blood glucose that results from a combination of insufficient insulin production and/or deficient insulin action.

**enteroendocrine cells** Specialized, phenotypically distinct endocrine cells; found within the mucosa of the gastrointestinal tract, they produce one or more peptide hormones.

**glucagon-like peptides** Two small peptides, glucagon-like peptide-1 and glucagon-like peptide-2, derived from posttranslational processing of proglucagon; exhibit sequence similarity to glucagon.

**intestine** The gastrointestinal tract, including the stomach and the small and large bowel tracts.

**islets** The islets of Langerhans; include four distinct highly specialized endocrine cell types that produce insulin beta cells), glucagon (alpha cells), somatostatin delta cells), or pancreatic polypeptide (PP cells).

## See Also the Following Articles

**Glucagon Action • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagonoma Syndrome • Glucagon Processing • Glucagon Secretion, Regulation of**

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# Glucagon-like Peptides: GLP-1 and GLP-2

Younes Anini and Patricia L. Brubaker
*University of Toronto*

I. INTRODUCTION
II. REGULATION OF GLP SECRETION
III. GLP METABOLISM
IV. GLP RECEPTORS
V. PHYSIOLOGICAL ACTIVITIES OF GLPs
VI. SUMMARY

**Glucagon-like peptides, produced in the intestine, are hormones that participate in the regulatory processes of insulin secretion and intestinal growth. Mechanisms controlling their synthesis and secretion, the nature of their receptors, and their biological and potential therapeutic effects are of great interest because of their potential in treating diabetes and intestinal insufficiency.**

## I. INTRODUCTION

Glucagon-like peptide-1 [GLP-1(7–36 $NH_2$)] and glucagon-like peptide-2 [GLP-2(1–33)] are intestinal peptide hormones that play important roles in the regulation of insulin secretion and intestinal growth, respectively. The physiology of these peptides and their potential roles in the treatment of diabetes and intestinal insufficiency, respectively, have been the subject of a number of recent studies. The gene encoding proglucagon, the precursor of GLP-1 and GLP-2, is expressed in pancreatic alpha cells and in endocrine L cells that are localized in the distal intestine and colonic mucosa. Mammalian proglucagon protein contains three homologous hormonal sequences: glucagon, GLP-1, and GLP-2. These sequences appear in this order in proglucagon, but are separated by two intervening peptides (IPs), IP-1 and IP-2, and are preceded by an N-terminal extension called glicentin-related pancreatic polypeptide (GRPP) (Fig. 1). Present at either end of each peptide are pairs of basic amino acids (Lys-Arg or Arg-Arg), representing sites of cleavage by the prohormone convertases (PCs), PC2 and PC1/PC3, in islet A cells and intestinal L cells, respectively.

In pancreatic alpha cells, proglucagon is processed to glucagon, GRPP, IP-1, and the major proglucagon-derived fragment (MPGF), which contains the unprocessed GLP-1, IP-2, and GLP-2 sequences. Although very low levels of GLP-1 are generated in alpha cells, GLP-1 and GLP-2 are major products of proglucagon processing in L cells, along with glicentin and oxyntomodulin, which contain unprocessed glucagon. The enteroendocrine cells producing the proglucagon-derived peptides (PGDPs), classified as L cells, are one of the most abundant endocrine cell types in the mucosa of the mammalian ileum, colon, and rectum. They are one of the so-called open-type endocrine cells (e.g., an elongated cell with apical microvilli reaching into the intestinal lumen and a base rich in endocrine granules near the basal lamina), which suggests that these cells can directly sense the lumenal contents. Most of the L cells reside

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Human       HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
Guinea pig  --------------------Q-LK--L-V
Chicken     ----------------------------N
Duck        ---------------T------------N
Anglerfish  --E---SN------ED-K--E--R----N
```

FIGURE 1 Primary structures of several vertebrate glucagons.

exerted mainly in the liver, where glucagon receptors are abundant. Activation of these G-protein-coupled receptors by the hormone activates signal transduction pathways, leading to glycogenolysis and stimulation of gluconeogenesis. These actions elevate the blood sugar level, opposing the hypoglycemic action of insulin and thus helping maintain normal blood glucose levels during fasting and in the postprandial period. The main stimulus to glucagon secretion is hypoglycemia, but amino acids, other hormones such as insulin, and autonomic activity also appear to regulate its release.

The pancreatic alpha cells are one of four distinct endocrine cell types that occur in the pancreatic islets. They contribute 15–20% of the islet cell mass, being second in abundance to the beta cells, which make up 65–70% of the islet cell population. Endocrine cell secretory products (e.g., insulin) are carried first to the liver via the portal vein and then into the periphery, a factor that enhances the direct action of glucagon on the liver to regulate blood glucose levels.

## II. BIOSYNTHESIS OF GLUCAGON

Early studies indicated that, like insulin, glucagon might be derived from a larger precursor. However, the prohormone was not identified until 1979 when Patzelt *et al.*, in pulse-chase labeling studies with isolated islets of Langerhans, demonstrated the existence of an 18-kDa protein that contained glucagon (Fig. 2). The 18-kDa proglucagon molecule was labeled after a very short pulse of radioactive amino acids and within 10 min underwent a slight shift toward a higher molecular weight, apparently due to O-glycosylation, thought to occur downstream of the glucagon moiety in the sequence. During the ensuing 2-h period, most of the proglucagon was converted via larger intermediate forms into the major products, consisting of glucagon and a ~10-kDa peptide termed the major proglucagon-derived fragment (MPGF).

The subsequent molecular cloning of glucagon cDNAs in several species confirmed these findings and provided the predicted amino acid sequence of preproglucagon, the encoded glucagon gene product (Fig. 3). Preproglucagon contains an N-terminal signal peptide in addition to the 18-kDa proglucagon sequence. However, besides glucagon, two additional glucagon-related sequences were found in proglucagon, within the C-terminal MPGF. These, like glucagon, were demarcated on either side by pairs of basic amino acids, suggesting that they could be released by proteolytic processing. Subsequent studies confirmed that these peptides, designated glucagon-like peptides (GLP-1 and GLP-2), are normally secreted from the proglucagon-expressing endocrine L cells in the intestinal mucosa. The L cells process proglucagon differently so as to liberate these two peptides, whereas the glucagon-containing N-terminal half of the proglucagon molecule is mainly released as a single intact entity known as glicentin

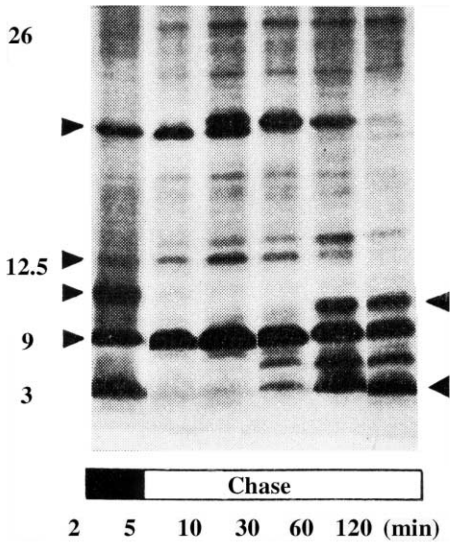
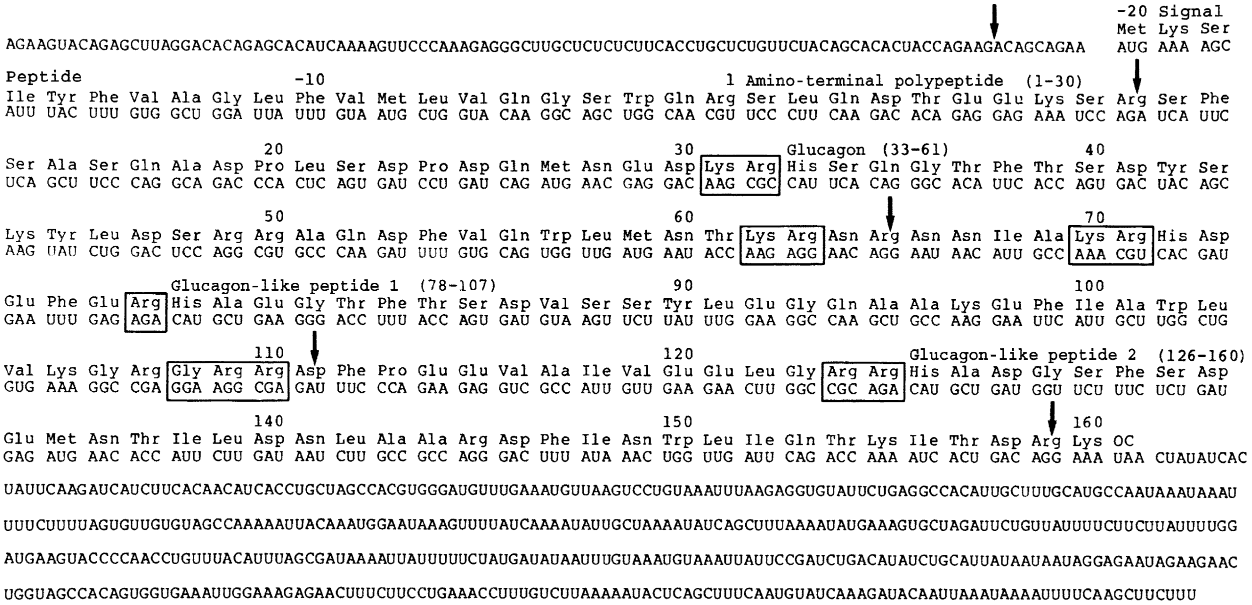


FIGURE 2 Biosynthesis of glucagon in rat islets of Langerhans. Pulse-chase labeling of rat islet proteins using [$^{35}$S]methionine as the tracer amino acid. After a 2-min pulse-labeling period, radioactivity was chased by dilution with a large volume of buffer containing an excess of unlabeled methionine. The glucose concentration was kept low (2.5 m*M*) to suppress insulin biosynthesis as much as possible. Proteins were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and bands were identified by autoradiography. Numbers on the left represent molecular weights of marker proteins. Arrowheads on the left (from top down) indicate proglucagon (18 kDa), prosomatostatin (12.5 kDa), preproinsulin (11.5 kDa), and proinsulin (9 kDa). Arrowheads on the right indicate the major proglucagon fragment (10 kDa) and glucagon and the insulin B chain (3 kDa). Modified from Patzelt *et al.* (1979).



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                                                                                                              -20 Signal
                                                                                                              Met Lys Ser
AGAAGUACAGAGCUUAGGACACAGAGCACAUCAAAAGUUCCCAAAGAGGGCUUGCUCUCUCUUCACCUGCUCUGUUCUACAGCACACUACCAGAAGACAGCAGAA   AUG AAA AGC

Peptide                    -10                                         1 Amino-terminal polypeptide  (1-30)
Ile Tyr Phe Val Ala Gly Leu Phe Val Met Leu Val Gln Gly Ser Trp Gln Arg Ser Leu Gln Asp Thr Glu Glu Lys Ser Arg Ser Phe
AUU UAC UUU GUG GCU GGA UUA UUU GUA AUG CUG GUA CAA GGC AGC UGG CAA CGU UCC CUU CAA GAC ACA GAG GAG AAA UCC AGA UCA UUC

                    20                                      30        Glucagon  (33-61)         40
Ser Ala Ser Gln Ala Asp Pro Leu Ser Asp Pro Asp Gln Met Asn Glu Asp|Lys Arg|His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser
UCA GCU UCC CAG GCA GAC CCA CUC AGU GAU CCU GAU CAG AUG AAC GAG GAC|AAG CGC|CAU UCA CAG GGC ACA UUC ACC AGU GAC UAC AGC

                    50                                      60                                  70
Lys Tyr Leu Asp Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr|Lys Arg|Asn Arg Asn Asn Ile Ala|Lys Arg|His Asp
AAG UAU CUG GAC UCC AGG CGU GCC CAA GAU UUU GUG CAG UGG UUG AUG AAU ACC|AAG AGG|AAC AGG AAU AAC AUU GCC|AAA CGU|CAC GAU

                Glucagon-like peptide 1  (78-107)           90                                 100
Glu Phe Glu|Arg|His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu
GAA UUU GAG|AGA|CAU GCU GAA GGG ACC UUU ACC AGU GAU GUA AGU UCU UAU UUG GAA GGC CAA GCU GCC AAG GAA UUC AUU GCU UGG CUG

                    110                                     120                 Glucagon-like peptide 2  (126-160)
Val Lys Gly Arg|Gly Arg Arg|Asp Phe Pro Glu Glu Val Ala Ile Val Glu Glu Leu Gly|Arg Arg|His Ala Asp Gly Ser Phe Ser Asp
GUG AAA GGC CGA|GGA AGG CGA|GAU UUC CCA GAA GAG GUC GCC AUU GUU GAA GAA CUU GGC|CGC AGA|CAU GCU GAU GGU UCU UUC UCU GAU

                    140                                     150                                 160
Glu Met Asn Thr Ile Leu Asp Asn Leu Ala Ala Arg Asp Phe Ile Asn Trp Leu Ile Gln Thr Lys Ile Thr Asp Arg Lys OC
GAG AUG AAC ACC AUU CUU GAU AAU CUU GCC GCC AGG GAC UUU AUA AAC UGG UUG AUU CAG ACC AAA AUC ACU GAC AGG AAA UAA CUAUAUCAC

UAUUCAAGAUCAUCUUCACAACAUCACCUGCUAGCCACGUGGGAUGUUUGAAAUGUUAAGUCCUGUAAAUUUAAGAGGUGUAUUCUGAGGCCACAUUGCUUUGCAUGCCAAUAAAUAAAU

UUUCUUUUAGUGUUGUGUAGCCAAAAAUUACAAAUGGAAUAAAGUUUUAUCAAAAUAUUGCUAAAAUAUCAGCUUUAAAAUAUGAAAGUGCUAGAUUCUGUUAUUUUCUUCUUAUUUGG

AUGAAGUACCCCAACCUGUUUACAUUUAGCGAUAAAAUUAUUUUUCUAUGAUAUAAUUUGUAAAUGUAAAUUAUUCCGAUCUGACAUAUCUGCAUUAUAAUAAUAGGAGAAUAGAAGAAC

UGGUAGCCACAGUGGUGAAAUUGGAAAGAGAACUUUCUUCCUGAAACCUUUGUCUUAAAAAUACUCAGCUUUCAAUGUAUCAAAGAUACAAUUAAAUAAAAUUUUCAAGCUUCUUU
```

**FIGURE 3** Structure of human preproglucagon mRNA and its encoded amino acid sequence. The mRNA encodes a 20-residue signal peptide (−20 to −1) followed by the 160-amino acid proglucagon sequence. The cleavage sites are boxed and vertical arrows indicate the positions of introns in the corresponding genomic sequence on chromosome 2 in humans. From Bell (1989) in *Endocrinology*, 2nd ed. (L. DeGroot, ed.) pp. 1263–1289, with permission.

(Fig. 4). The 69-residue glicentin peptide had been described originally as "gut glucagon" and had been isolated and characterized from intestinal mucosa.

GLP-1(1–37) is further processed at a single arginine residue in the L cells, giving rise to GLP-1(7–37), the major secreted GLP-1 bioactive form. It functions as a powerful "incretin," increasing insulin secretion in response to orally ingested food. The L cell secretory activity is regulated by other gut endocrine factors [such as glucose-dependent insulinotropic polypeptide (GIP)] that are released in response to feeding. GLP-1(7–37) acts on specific G-protein-coupled receptors in the beta cell that are distinct from the glucagon receptors, augmenting insulin secretion in response to the elevated blood glucose levels of the postprandial state.

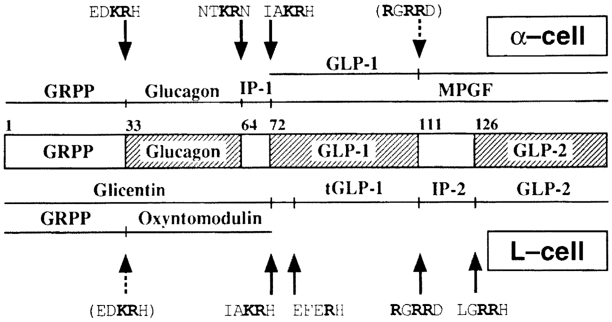
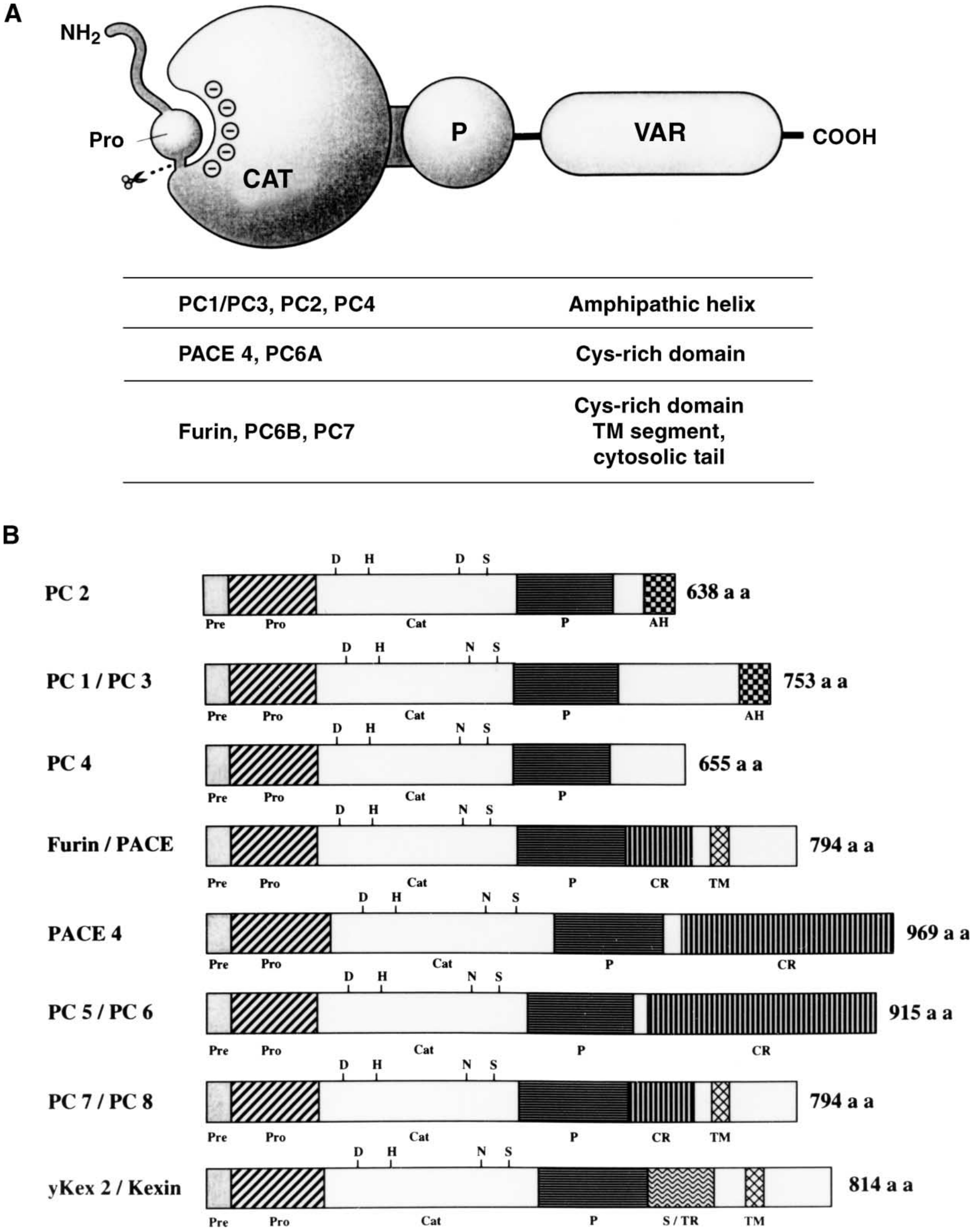


**FIGURE 4** Proglucagon processing. Schematic representation of the differences in cleavages of proglucagon in alpha cells (top) versus intestinal neuroendocrine L cells (bottom). Sequences near each cleavage site are shown above or below the sequence. GLP, Glucagon-like peptide; GRPP, glicentin-related polypeptide; IP, intervening peptide.

## III. THE PROHORMONE CONVERTASES

Since 1990, with the identification of furin and PC2, a seven-member family of proprotein-processing endoproteases that act within the secretory pathway has gradually emerged (Fig. 5). These proteases are all homologues of the yeast-processing protease (kexin) but are more diverse in their functional adaptations and localization within the secretory pathway. The catalytic domain of the convertases is a serine protease that is related to the subtilisin family of proteases, known as subtilases, and this subfamily is therefore known as the subtilisin-like proprotein convertases (SPCs). Most of the subtilases are nonspecific degradative endoproteases. However, special adaptations in the SPCs, including an additional ~150-residue well-conserved downstream domain, the P domain, have increased their specificity and introduced a special regulatory feature-a


A
NH2
Pro
CAT
P
VAR
COOH
B
PC 2
D H D S
Pre Pro Cat P AH
638 a a
PC 1 / PC 3
D H N S
Pre Pro Cat P AH
753 a a
PC 4
D H N S
Pre Pro Cat P
655 a a
Furin / PACE
D H N S
Pre Pro Cat P CR TM
794 a a
PACE 4
D H N S
Pre Pro Cat P CR
969 a a
PC 5 / PC 6
D H N S
Pre Pro Cat P CR
915 a a
PC 7 / PC 8
D H N S
Pre Pro Cat P CR TM
794 a a
yKex 2 / Kexin
D H N S
Pre Pro Cat P S / TR TM
814 a a

| | |
|---|---|
| PC1/PC3, PC2, PC4 | Amphipathic helix |
| PACE 4, PC6A | Cys-rich domain |
| Furin, PC6B, PC7 | Cys-rich domain<br>TM segment,<br>cytosolic tail |

FIGURE 5 Schematic representation of the structural features (A) and sequence organization (B) of the mammalian subtilisin-like proprotein convertases (SPCs). All seven family members have well-conserved signal peptides, proregions (Pro), catalytic domains (CAT), and P domains (P), but differ in their carboxyl-terminal domains (VAR), as indicated. Autocatalytic cleavage and release of the prodomain results in activation. Modeling studies predict that the P domain

requirement for micro- to millimolar calcium for activity.

All of the convertases have an inhibitory prodomain that immediately follows the signal peptide at the N-terminus and that must be removed to activate the proenzyme. Evidence has indicated that this cleavage is autocatalytic and in most cases occurs during the transport of the proprotease from the endoplasmic reticulum (ER) to the Golgi region where the prodomain dissociates and is degraded or inactivated. The cleavage sites for activation reflect the cleavage specificity and consist of polybasic sequences having the following motif: Arg·X·Lys/Arg·Arg. PC2 differs from the other convertases in that its activation occurs in the distal secretory pathway, most likely in the trans Golgi network (TGN) or early secretory granules (progranules). PC2 also requires the neuroendocrine protein 7B2 for activation. After proPC2 folds in the ER, it binds to 7B2, which facilitates its transport to the secretory granules and subsequent autocatalytic cleavage to yield the active form. The molecular basis for this effect of 7B2 on PC2 activation is not completely understood, but it may act through stabilization or alteration of proPC2 structure in a manner that facilitates the achievement of an active conformation following cleavage of the prosegment.

PC2 and PC1/PC3 are two members of the convertase family that are widely distributed within the brain and neuroendocrine system and thus are the major players in the processing of both neuropeptide and hormone precursors. PC4 is also similar to PC2 and PC3 in size and overall structure, but appears to be confined in its expression to the gonads, where it plays a role in fertility. In the intestine and perhaps also in brain, PC5A/PC6A may also play a role in maturing some hormone precursors. These convertase family members all have the appropriate characteristics and structural features that allow them to be sorted into regulated secretory vesicles/granules and to function in their acidic interiors. In contrast, furin, PC7/PC8, and PC5/PC6B all have transmembrane domains with cytosolic extensions that interact with factors that target them to the TGN and constitutive cargo vesicles, where they process proreceptors and the precursors of growth factors and/or plasma membrane proteins during their unregulated transfer to the cell surface or exterior. The pH optima of this latter group are mildly acidic (pH ~ 6.0–6.5), whereas the pH optima of the secretory granule convertases range from ~5.0 for PC2 to ~5.5 for PC3. Because of its earlier activation during intracellular transit, PC3 is believed to begin to act as early as the stage of secretory granule assembly in the TGN, whereas PC2 begins to act only in the secretory granules. Most prohormone processing, however, is believed to occur within early secretory granules after their formation. In the maturing secretory granules, PC3 undergoes further cleavages in its C-terminal region, truncating it from the full-length 753-residue active form to a ~616-residue form that appears to have increased activity, thus contributing to enhanced prohormone processing activity within the acidic maturing secretory granules.

Proinsulin was the touchstone for much of our knowledge on prohormone biosynthesis and processing. Early research on proinsulin conversion showed that a combination of trypsin-like (trypsin is a basic residue-specific serine endoprotease) and carboxypeptidase B (CPB)-like (CPB is a basic residue-specific exopeptidase that removes C-terminal basic residues) activities rapidly and quantitatively generated the major natural products of proinsulin conversion in the islet beta cell, i.e., native insulin and C-peptide, the entire proinsulin connecting segment without N- or C-terminal basic residues. This model system using pancreatic trypsin and CPB is currently being used commercially with great success to convert biosynthetic human proinsulin to insulin in the manufacture of insulin for treatment of diabetes. However, the natural process of conversion is catalyzed by the SPCs, PC2, and PC3, working together along with carboxypeptidase E (CPE), a homologue of the exocrine pancreatic CPB in the beta cell secretory granules, as illustrated in Fig. 6. PC3 acts first, selectively cleaving the B chain/C-peptide junction in proinsulin and thereby generating an intermediate (split 32,33 and/or *des*-31,32 proinsulin) that is a better substrate for PC2. PC2 in turn cleaves preferentially at the C-peptide/A chain junction to complete the liberation of insulin. CPE normally removes the newly exposed basic residues from the B chain of insulin and the C-peptide so rapidly that such

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folds to form an eight-stranded β-barrel that interacts with the catalytic domain through a hydrophobic patch. A note on alternative terminology of SPC, prohormone convertase (PC), and paired basic amino-acid-cleaving enzyme (PACE): furin = SPC1/PACE; PC2 = SPC2; PC1/PC3 = SPC3; PACE4 = SPC4; PC4 = SPC5; PC6 = SPC6; PC7 = SPC7/PC8/LPC.

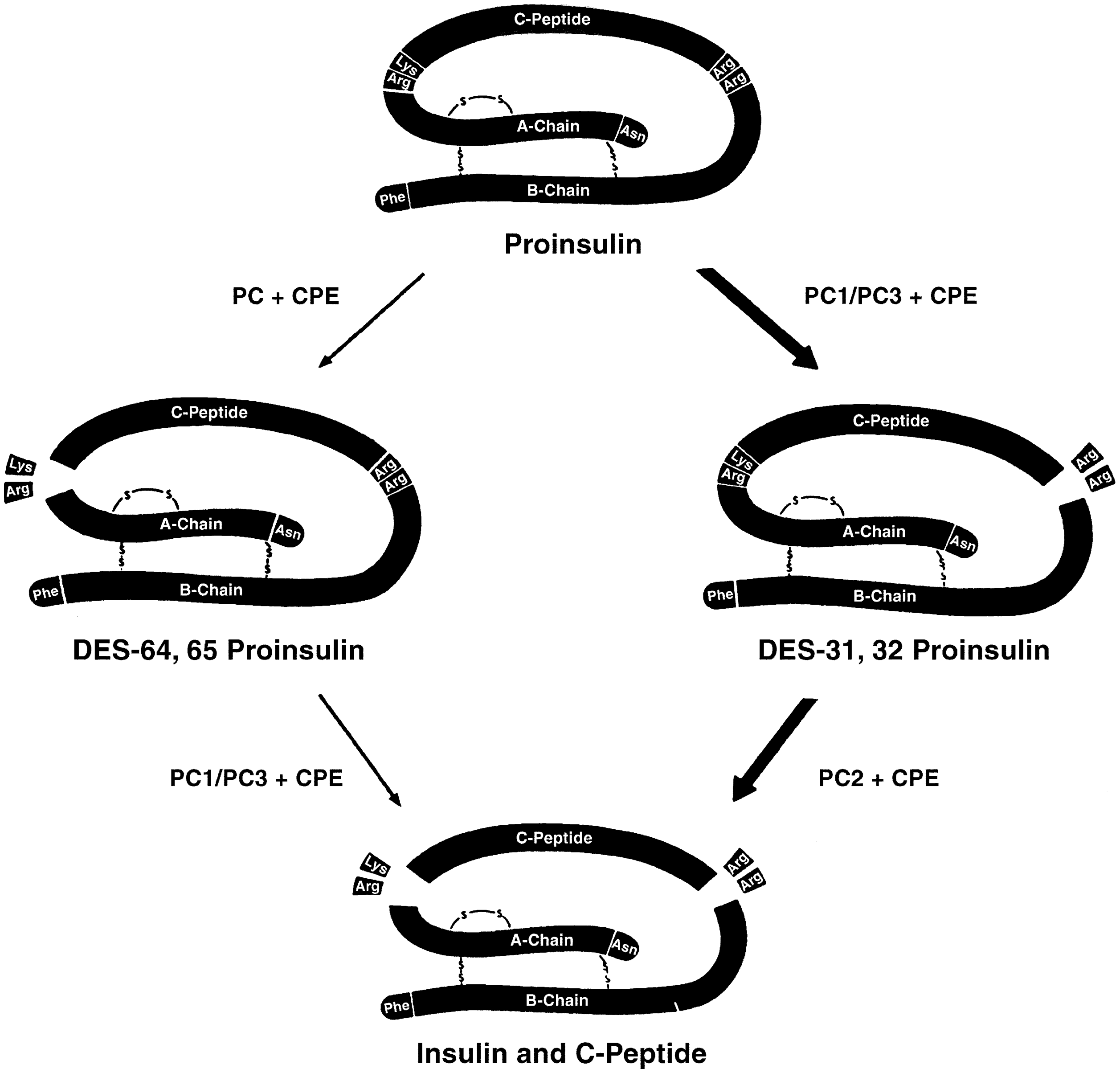
C-Peptide
Lys
Arg
Arg
Arg
A-Chain
Asn
Phe
B-Chain
Proinsulin
PC + CPE
PC1/PC3 + CPE
DES-64, 65 Proinsulin
DES-31, 32 Proinsulin
PC1/PC3 + CPE
PC2 + CPE
Insulin and C-Peptide

FIGURE 6 Processing of proinsulin to insulin and C-peptide by the prohormone convertases PC1/PC3 and PC2. Scheme depicts the preferential pathway on the right.

C-terminally extended intermediates are difficult to detect. Recent studies with PC2 null mice have demonstrated that proinsulin levels are elevated significantly (~30% vs <5%) and that *des*-31,32 proinsulin intermediates are also elevated, as expected. PC1/PC3 null mice, however, have a much more severe block in proinsulin processing, with levels as high as 85–90% and with significant accumulation of only *des*-64,65 proinsulin intermediates. These findings fully support the scheme shown in Fig. 6.

## IV. DIFFERENTIAL PROCESSING OF PROGLUCAGON BY PC2 AND PC1/3

In studies on glucagon biosynthesis in a T antigen-transformed alpha cell line, TCI-6, Rouillé and co-workers have demonstrated that PC2 acting

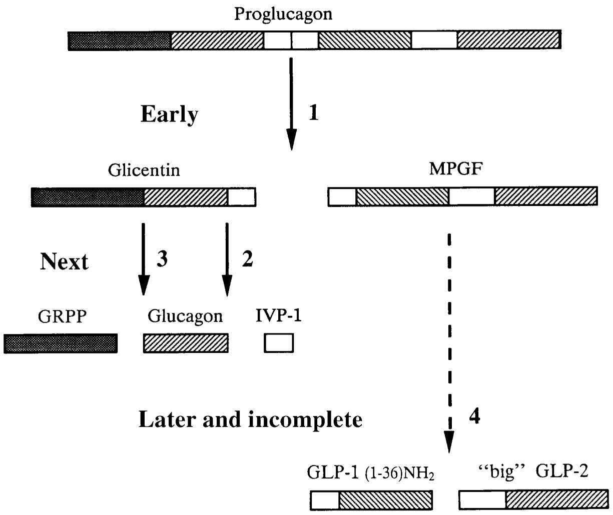

FIGURE 7 Proglucagon processing steps by prohormone convertase 2 in a pancreatic alpha cell-derived cell line, TCI-6. The cleavage at the interdomain site clearly precedes the further cleavage of glicentin to release glucagon. MPGF, Major proglucagon-derived fragment; GRPP, glicentin-related polypeptide; IVP-1, intervening peptide-1; GLP, glucagon-like peptide. Reproduced from Rouillé *et al.* (1994), with permission.

alone in these cells is able to reproduce the normal pattern of glucagon processing as seen in islets of Langerhans. Proglucagon is first cleaved at the interdomain site to release glicentin and MPGF. The glicentin is then further cleaved to glucagon, the C-terminal MPGF remains intact, and these products are secreted (Fig. 7). Further studies have fully elucidated the basis for the differential processing of proglucagon in the alpha cells versus the intestinal L cells, as shown in Fig. 8. The alpha cell pattern of processing of proglucagon is due to the presence of high levels of PC2 and the absence of significant levels of PC1/PC3 or other convertases. The importance of PC2 for the entire series of cleavages that release glucagon from proglucagon has now been confirmed by studies with isolated islets from PC2 and 7B2 null mice, which show a marked inhibition in proglucagon processing. These mice exhibit chronic hypoglycemia and flattened glucose tolerance curves, as expected if glucagon is lacking. Indeed, no active glucagon can be detected, despite the presence of marked alpha cell hyperplasia and large amounts of circulating higher molecular weight glucagon-like immunoreactive material. However, chronic treatment of these mice with glucagon via intraperitoneal osmotic minipumps for several weeks reverses the hypoglycemia and the alpha cell hyperplasia. A similar phenotype is seen in 7B2 null mice, which also lack active PC2.

By contrast, endocrine cells that express high levels of PC1/PC3 can be shown to process proglucagon efficiently to release glicentin and GLP-1, and probably GLP-2 as well. Efforts to identify conclusively the major intestinal L cell convertase as PC1/PC3 in canine intestine have not fully supported this notion, though, because immunoreactivity of both PC2 and PC1/PC3 appears to be present in the L cells. The reason for this discrepancy is not clear, but may be due to species differences between dogs and other mammals. Experiments using various endocrine cell lines that express both PC2 and PC1/PC3 indicate that these cells can process transfected proglucagon completely to all products-glucagon, GLP-1, and GLP-2. PC1/PC3 is also capable of cleaving at the single basic residue site in GLP-1 (proglucagon 72–107) to release active GLP-1(7–37). The proposed role of PC1/PC3 in the *in vivo* generation of GLP-1 in intestine has recently been confirmed by studies with PC1/PC3 null mice. These mice have markedly reduced levels of intestinal GLP-1 (J. Holst, personal communication).

Both PC2 and PC1/PC3 are able to rapidly perform the initial cleavage of proglucagon at the interdomain site between glicentin and the major proglucagon fragment (Fig. 8). These findings suggest that initial cleavage at this site may be required for the further, more specialized processing steps that follow. Recent protein modeling studies of have suggested a plausible explanation for the enhanced cleavage susceptibility of this site, as illustrated in Fig. 9. Further studies support the notion that proproteins such as proglucagon, proinsulin, proopiomelanocortin (POMC), and many others must have defined three-dimensional structures that serve to facilitate their relatively rapid intracellular transport, as well as their specific processing. Cell-specific proglucagon processing thus provides an excellent example of the regulation of specific hormone production from multifunctional precursors via differential tissue expression of prohormone convertases PC1/PC3 and PC2. The molecular basis for the sharply defined specificity of the various convertase cleavage sites within proglucagon, however, remains a mystery at this time.

## Acknowledgments

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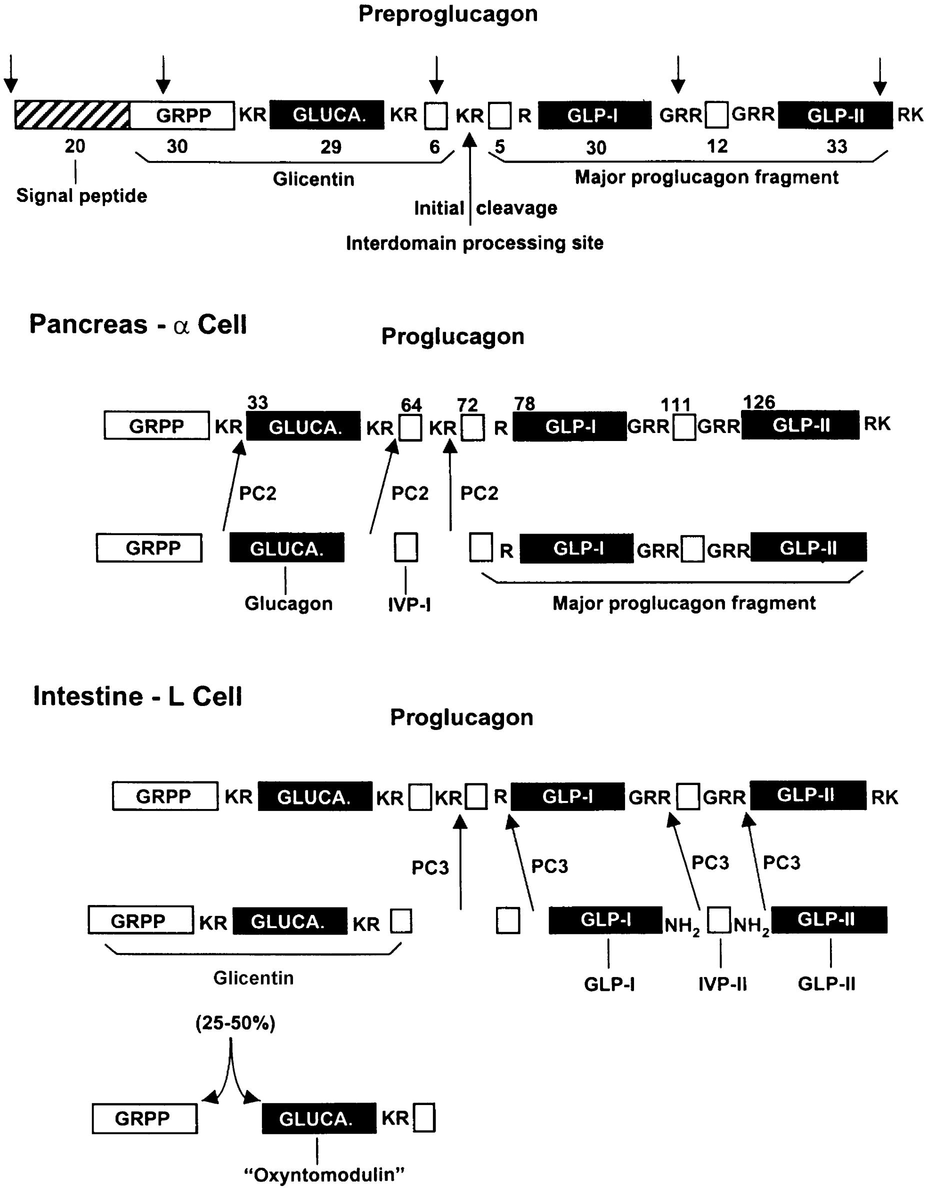
Preproglucagon
GRPP
KR
GLUCA.
KR
KR
R
GLP-I
GRR
GRR
GLP-II
RK
20
30
29
6
5
30
12
33
Signal peptide
Glicentin
Major proglucagon fragment
Initial cleavage
Interdomain processing site
Pancreas - α Cell
Proglucagon
33
64
72
78
111
126
PC2
PC2
PC2
Glucagon
IVP-I
Major proglucagon fragment
Intestine - L Cell
Proglucagon
PC3
PC3
PC3
PC3
NH2
NH2
GLP-I
IVP-II
GLP-II
Glicentin
(25-50%)
"Oxyntomodulin"

FIGURE 8 Differential processing of proglucagon by prohormone convertases (PC2 and PC1/PC3) in islet alpha vs intestinal L cells. Initial cleavage at the interdomain site by either convertase precedes the further specific processing of the right or left halves of the peptide. Abbreviations as in Fig. 7.

## Glossary

**glicentin** The N-terminal glucagon-containing half of the proglucagon molecule.

**gluconeogenesis** Production in the liver of glucose from noncarbohydrate precursors to support the blood sugar during fasting.

**hyperglycemia** Circulating blood glucose levels above normal values.

**hypoglycemia** Circulating blood glucose levels significantly below normal values.

**islets of Langerhans** Small clusters of neuroendocrine cells scattered throughout the pancreas, consisting of alpha, beta, delta, and gamma cells that produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively.

**major proglucagon-derived fragment** The C-terminal half of the proglucagon molecule containing the glucagon-like hormones glucagon-like polypeptide-1 and glucagon-like polypeptide-2.

**precursor processing** The specific proteolytic cleavage of larger precursor proteins to release biologically

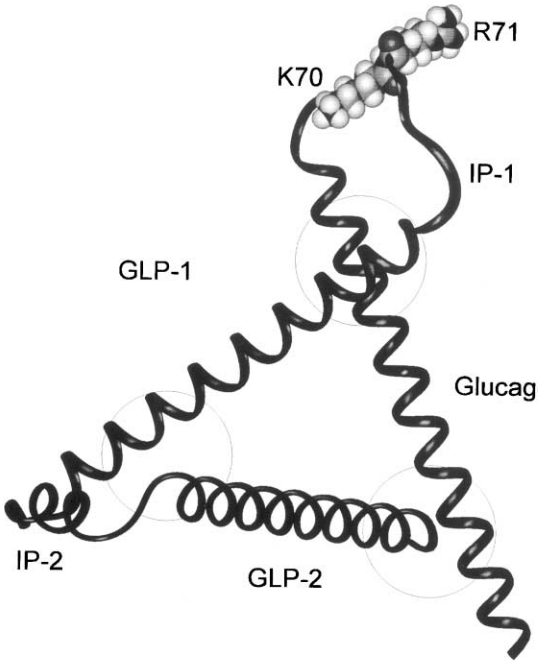

**FIGURE 9** Computer-generated model of the structure of proglucagon based on the structure of glucagon in crystalline glucagon. In this model, the three glucagon-related sequences in proglucagon form a triangular structure similar to the stacking of glucagon observed in crystals, in which central alpha-helical regions interact at either end through conserved hydrophobic patches (depicted by the circles). The interdomain cleavage site is in the loop at the top, where it is readily accessible to PC1/PC3 or PC2 in the first step of processing. Courtesy of G. Lipkind.

active peptides or proteins, e.g., insulin from proinsulin.

**preprohormone** The initial protein precursor, e.g., preproglucagon, of a peptide hormone, as encoded in its chromosomal gene; it contains additional information (signal peptide) specifying its intracellular routing via the secretory pathway.

**prohormone** The precursor, e.g., proglucagon, of an active peptide hormone or set of functionally and/or structurally related hormones; it is processed into its various active products within secretory granules in its cells of origin.

**signal peptide** An additional peptide sequence of 12–30 amino acids, having a hydrophobic central stretch of 8–12 strongly hydrophobic amino acids, usually located at or near the N-terminus of a nascent secreted protein, that serves as a signal for its segregative transfer into the secretory pathway. The signal peptide is then removed by the signal peptidase when this function has been completed.

**subtilisin-like proprotein convertases** The seven-member family (in mammals) of precursor-processing endoproteolytic enzymes that are all derived in evolution from the bacterial serine protease subtilisin; function in the secretory pathway of most of the body's cells to process a wide variety of protein precursors.

## See Also the Following Articles

**Glucagon Action • Glucagon Gene Expression • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagonoma Syndrome • Glucagon Secretion, Regulation of • Hypoglycemia in Diabetes**

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# Glucagon Secretion, Regulation of

Tamar Smith and John E. Gerich
*University of Rochester School of Medicine*

I. INTRODUCTION
II. EFFECTS OF SUBSTRATES ON GLUCAGON SECRETION
III. NEURAL EFFECTS ON GLUCAGON SECRETION
IV. HORMONAL EFFECTS ON GLUCAGON SECRETION
V. OTHER REGULATORY FACTORS
VI. DEVELOPMENTAL AND NUTRITIONAL ASPECTS OF GLUCAGON SECRETION
VII. GLUCAGON SECRETION IN DIABETES MELLITUS
VIII. SUMMARY AND CONCLUSIONS

**Glucagon is a single-chain 29-amino-acid 3.5 kDa peptide hormone encoded on chromosome 2. It is synthesized and secreted by alpha cells of the pancreatic islets of Langerhans following proteolytic cleavage of a prohormone precursor (proglucagon).**

## I. INTRODUCTION

Proglucagon is encoded by a single gene that contains six exons corresponding to six functional peptide domains. Alpha cell-specific transcription, translation, and modification lead to a 29-amino-acid final product that is formed into granules within the alpha-cell cytoplasm. The L-cells of the small intestine produce a similar precursor, which is processed differently than that of the pancreatic alpha cells so that different peptides are released. Once secreted from the pancreas, glucagon has a half-life of 3–6 min in plasma and is cleared from the circulation mainly by the liver and kidneys. Since pancreatic venous effluent directly enters the portal vein, portal venous glucagon concentration may be many times higher than that in the peripheral circulation.

Glucagon promotes the availability of endogenous fuels (primarily glucose) to maintain normoglycemia and protect against hypoglycemia. Glucagon's primary target organ is the liver but it may also act on adipocytes. Binding of glucagon to receptors in these tissues activates adenylate cyclase. The resulting increase in intracellular cyclic adenosine monophosphate (cAMP) levels alters the phosphorylation/dephosphorylation of enzymes that regulate glycogenolysis, gluconeogenesis, lipolysis, and ketogenesis (Table 1).

Glucagon synthesis involves a typical series of intracellular events including synthesis in the endoplasmic reticulum, transport of the prohormone to the Golgi apparatus for modification and packaging into granules, and the subsequent secretion of glucagon following migration of granules to the plasma membrane.

Recent advances in methodology (cytoplasmic $Ca^{2+}$ monitoring, patch clamp technique, availability of pure islet preparations) have led to a better understanding of the mechanisms involved in glucagon secretion. Changes in hormonally regulated cyclic nucleotides and calcium, in tandem with a nutrient-induced signal, have been implicated in the macromolecular events associated with glucagon secretion. It appears that nutrient-induced alterations in glucagon release are not mediated through changes in cellular cAMP levels since increased cAMP formation in alpha cells per se is insufficient to activate the glucagon secretory process by itself. Nutrient-induced glucagon release can be abolished by somatostatin, which inhibits cAMP formation and blocks cytosolic calcium increases. Somatostatin also inhibits phosphoinositide turnover and stimulates

TABLE 1 Key Enzymes Regulated by Glucagon

| | |
|---|---|
| *p*-Enolpyruvate carboxykinase | Up-regulated by glucagon; stimulation of gluconeogenesis (slow action) |
| Cyclic AMP-dependent protein kinase | Up-regulated by glucagon; phosphorylates several enzymes that regulate fuel metabolism |
| Phosphorylase b kinase | Activated by cyclic AMP-dependent protein kinase; stimulation of glycogenolysis |
| Glycogen synthase | Inactivated by cyclic AMP-dependent protein kinase; inhibition of glycogen synthesis |
| L-type pyruvate kinase | Inactivated by cyclic AMP-dependent protein kinase; stimulation of gluconeogenesis |
| Acetyl CoA carboxylase | Inactivated by cyclic AMP-dependent protein kinase; inhibition of lipogenesis and stimulation of ketogenesis |
| Hormone-sensitive lipase | Activated by glucagon in adipocytes via increase in cAMP |

phosphodiesterase, thereby augmenting the inhibition of cyclase production. Glucagon release can be amplified by exogenous cAMP (dibutyryl cAMP) and by agents such as epinephrine, which stimulate endogenous cAMP production in alpha cells. It has therefore been suggested that physiologic regulation of glucagon secretion entails the combined action of a nutrient-dependent signal and a cAMP-dependent signal. These most likely involve changes in intraislet calcium levels since glucagon release depends on the presence of extracellular $Ca^{2+}$, removal of which abolishes the secretory response of alpha cells to nutrients. Various factors including metabolic intermediates, nutrients, hormones, neuropeptides, and the autonomic nervous system can affect glucagon secretion (Table 2).

## II. EFFECTS OF SUBSTRATES ON GLUCAGON SECRETION

### A. Glucose

The primary regulator of insulin secretion *in vivo* is the plasma glucose concentration: increases suppress secretion and decreases stimulate it. Increases in the extracellular glucose concentration inhibit glucagon secretion. The threshold for glucose-induced inhibition of glucagon *in vitro* is 2–3 m*M*. Half-maximum inhibition and maximum inhibition of glucagon secretion *in vitro* occur at approximately 5.0 and 10.0 m*M* glucose, respectively (Fig. 1). On the other hand, a decrease in extracellular glucose concentration (≤3 m*M*) stimulates glucagon release (Fig. 2). In humans, a decrease in plasma glucose from ~90 to 70 mg/dl is sufficient to stimulate glucagon secretion. The ability of hexoses other than glucose to inhibit glucagon secretion is proportional to the alpha cell's capacity to metabolize the sugar, indicating that the provision of energy/generation of ATP is an important factor. Changes in glucose concentration also modulate the response of the alpha cell to various stimuli such as amino acids; e.g., infusion of glucose diminishes glucagon responses to arginine. This is a paradigm for the general process of integration of nutrient signals and other signals at the level of the alpha cells to modulate glucagon secretion.

The mechanism by which glucose affects glucagon secretion may result in part from the direct effects of glucose, the interrelationship between the alpha and beta cells (insulin), and other factors influenced by changes in glucose concentrations. Glucose probably has a direct effect since alpha-cell suppression can

TABLE 2 Classification of Major Factors Influencing Glucagon Secretion

| Substrates | Neural | Local | Hormonal | Ionic |
|---|---|---|---|---|
| Glucose | Sympathetic | Somatostatin | Insulin | Calcium |
| Amino acids | Parasympathetic | Serotonin | Growth hormone | Potassium |
| Free fatty acids | | Dopamine | Cortisol | |
| Ketone bodies | | Prostaglandins | Thyroid hormones | |
| β-Hydroxybutyrate | | γ-Amino butyric acid | Estrogens | |
| Acetoacetate | | | Androgens | |
| | | | Gastrointestinal hormones | |
| | | | Gastrin | |
| | | | Secretin | |
| | | | Pancreozymin (GIP,VIP) | |

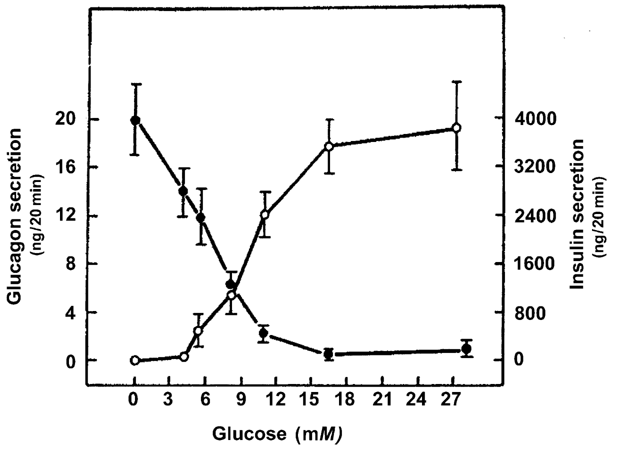

FIGURE 1 Dose–response relationships of the effect of glucose on the suppression of glucagon release and stimulation of insulin release in the perfused rat pancreas. Reprinted from Gerich *et al.* (1974) *J. Clin. Invest.* 54, 833–841, with permission.

occur at glucose levels too low to stimulate insulin release. Glucose-mediated inhibition of glucagon secretion is thought to be effected via the lowering of intracellular calcium, a positive mediator of glucagon secretion. Exposure of islets to glucose decreases cytoplasmic calcium. There is considerable evidence that an indirect effect of glucose, the stimulation of insulin secretion, also contributes to the rapidity and magnitude of inhibition in alpha-cell secretory activity.

Glucagon release is enhanced by inhibitors of ATP synthesis and is inhibited by fatty acids and ketones, which are presumed to be acting as metabolic fuels. Because most secretory systems require energy, the energy within the alpha cell must be compartmentalized. It should be noted, however, that glucose may act through mechanisms independent of energy production, since the α-glucose anomer is more effective than the β-glucose anomer at suppressing glucagon release. Another possible mediator for glucose-induced suppression is the stimulation of γ-aminobutyric acid (GABA) release from pancreatic beta cells. GABA is co-localized with insulin within beta cells at concentrations comparable to those in the central nervous system. Enzymes responsible for the synthesis and degradation of GABA are present in the beta cell. GABA is released from the beta cell in concert with insulin. There is evidence that GABA subsequently interacts with the $GABA_A$ receptor to open membrane chloride channels, resulting in alpha-cell hyperpolarization and a net decrease in the frequency of action potentials in alpha cells. Thus, GABA may exert a suppressive effect on the alpha cell that is independent of glucose.

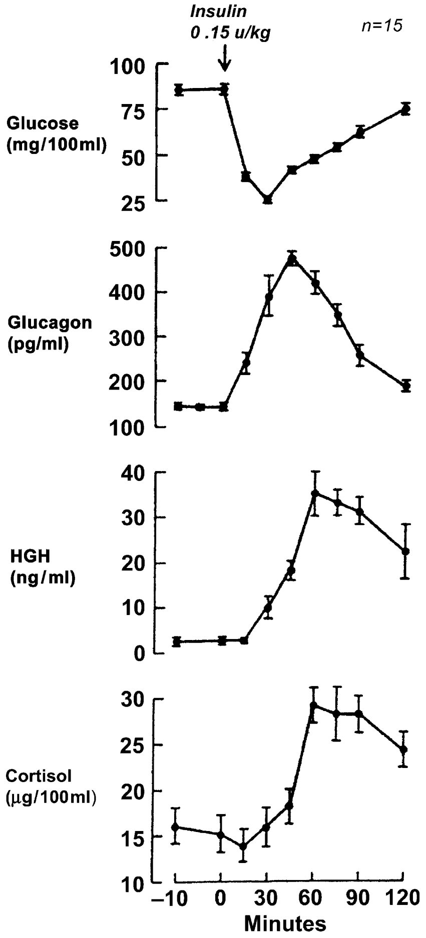

FIGURE 2 Effect of insulin-induced hypoglycemia on plasma glucagon levels in normal human volunteers. Reprinted from Gerich, J. E., Schneider, V., Dippe, S. E., Langlois, M., Noacco, C., Karam, J. H., and Forsham, P. H. Characterization of the glucagon response to hypoglycemia in man. *J. Clin. Endocrinol. Metab.* 38, 77–82 (1974), with permission. Copyright The Endocrine Society.

## B. Amino Acids

Amino acids induce biphasic glucagon release in human and other species (Fig. 3) and are probably as important in stimulating glucagon secretion as hypoglycemia. This effect is glucose dependent and is best observed at low glucose concentrations. The stimulatory effect of amino acids on glucagon secretion appears to be mediated, at least in part, by a direct interaction with the alpha cell, as indicated by studies measuring glucagon release from purified alpha-cell preparations. Current evidence suggests

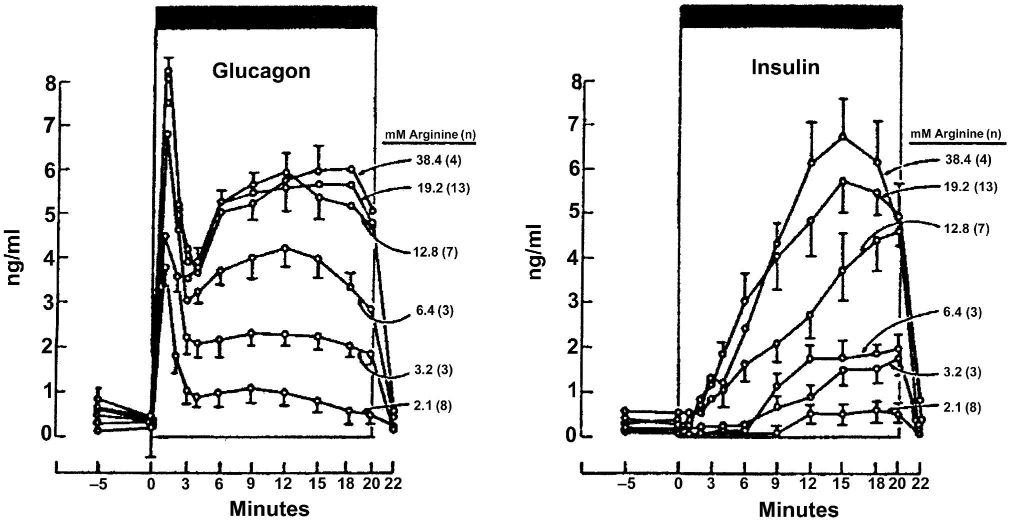

FIGURE 3 Release of glucagon and insulin from the *in vitro* perfused rat pancreas at various arginine concentrations. Reprinted from Gerich *et al.* (1974) *J. Clin. Invest.* 54, 833–841, with permission.

that the release of glucagon is triggered by the binding of amino acids to membrane receptors, a process that is $Ca^{2+}$ dependent. Changes in second messengers, such as diacylglycerol, $Ca^{2+}$, and cAMP, may also be involved.

Arginine is the most potent amino acid stimulator of glucagon and insulin secretion and has proven useful for the experimental study of hormonal relationships in the endocrine pancreas. Arginine increases the frequency of action potentials in alpha cells by a direct effect. Glutamine stimulates glucagon release and is readily metabolized by alpha cells. Regulation of glucagon secretion by glutamine appears to be reciprocally related to factors affecting glucose metabolism and ATP levels in the alpha cell. Leucine, although an effective stimulator of insulin release, does not stimulate glucagon secretion. Other amino acids effective in stimulating the release of glucagon are glycine, homoserine, serine, asparagine, valine, alanine, and glutamic acid. It is noteworthy that amino acids, which themselves have little or no activity in stimulating glucagon secretion, are able to potentiate secretion induced by another amino acid, so that ingestion of a mixture of amino acids produces a stimulatory effect that is greater than would be expected from summation of the separate amino acids' stimulatory capacities. Chronic ingestion of a high-protein diet reportedly results in chronically elevated fasting glucagon levels.

Stimulation of glucagon release during ingestion of a mixed meal—presumably the result of amino acids from the digested protein in the meal—would act to balance the actions of concomitantly released insulin (e.g., suppression of hepatic glucose release) to prevent postprandial hypoglycemia.

## C. Free Fatty Acids and Ketone Bodies

Free fatty acids (FFA) and ketone bodies (β-hydroxybutyrate and acetoacetate), the predominant fuels in human, inhibit glucagon secretion *in vivo* and *in vitro*. It has been suggested that pancreatic alpha-cell function may in fact be more sensitive to changes in FFA than to glucose. Glucagon is an important stimulator of hepatic ketogenesis, and increases in FFA availability augment ketone body production not only by a mass effect but also by the diversion of hepatic fatty acid metabolism away from triglyceride formation and toward ketogenesis. Thus, suppression of glucagon release by FFA and ketone bodies may act as a possible negative feedback system to limit lipolysis and ketogenesis. Free fatty acids may also participate in the feedback regulation of lipolysis in the starvation state by permitting insulin secretion to occur despite a fall in the plasma glucose level, and this feedback system may involve limitation of glucagon secretion by rising plasma FFA levels.

FFA differ in their ability to affect glucagon secretion. Short-chain fatty acids (octanoate, valerate, butyrate, and propionate), long-chain fatty acids (oleate and palmitate), and ketone bodies inhibit glucagon release as a function of their degree of metabolism. Diminution of FFA oxidation by means of various metabolic inhibitors augments glucagon release from intact guinea pig islets.

Polyunsaturated fatty acids (PUFA) are precursors for eicosanoids including prostaglandins, leukotrienes, and thromboxanes, which have hormone-like activities. Thus, certain FFA may indirectly influence glucagon release. For example, PUFA increase insulin release.

## III. NEURAL EFFECTS ON GLUCAGON SECRETION

The sympathetic and parasympathetic nervous systems are important modulators of both islet alpha- and beta-cell function and therefore their effects on glucagon secretion are complex. The abundant nervous innervation of the islets has long been recognized. Nerves to the pancreas contain fibers from the greater and middle splanchnic nerves (originating in the celiac and superior mesenteric plexuses), visceral afferent fibers (which mediate pain due to distention), and parasympathetic fibers from the vagus nerve. There is reportedly no obvious relationship between the type of neuronal terminal and the type of islet cell innervated (alpha, beta, and delta cells are all associated with each neuron type) and there may be three or more neuron types innervating a single islet cell.

### A. Sympathetic Nervous System

There is considerable evidence that the sympathetic nervous system may modulate islet hormone secretion both neurally (norepinephrine) and by release of adrenomedullary catecholamines. Epinephrine has been shown to stimulate glucagon secretion *in vivo* and *in vitro*. In dogs, electrical stimulation of mixed pancreatic nerves containing cholinergic and adrenergic fibers enhances glucagon secretion while suppressing insulin secretion. Stimulation of splanchnic nerves or the administration of catecholamines increases both insulin and glucagon release.

In humans, epinephrine, a mixed α- and β-adrenergic agonist, affects pancreatic alpha- and beta-cell function reciprocally, augmenting glucagon secretion via its β-adrenergic effects while inhibiting insulin release via its α-adrenergic effects. These actions probably reflect different sensitivities of α- and β-adrenergic receptors on human islet cells. α-Adrenergic receptor antagonists have no effect on basal glucagon release, but exert a potentiating effect (thought to be mediated by an increase in cAMP) on epinephrine-induced glucagon secretion. However, species differences exist; in the rat, for example, epinephrine-induced glucagon secretion may be completely inhibited by an alpha nonselective blocker or an α2 selective blocker (but not an α1 selective blocker), indicating that α modulation may be α2 specific.

Glucagon release is increased during exercise or various stresses. Activation of sympathetic innervation of the pancreas could account for the increased glucagon secretion and decreased insulin secretion during stress. Diabetic patients, having compromised beta-cell function, are prone to exaggerated hyperglycemic episodes during stress because of the inability to counteract the effects of catecholamines and glucagon with additional insulin release.

### B. Parasympathetic Nervous System

The effects of the parasympathetic nervous system on glucagon secretion are purely stimulatory. Stimulation of the vagus nerve at various levels or administration of cholinergic drugs increases insulin and glucagon secretion in human and other species. Stimulation of glucagon and insulin release appears to be mediated by muscarinic receptors since atropine blocks the release of glucagon *in vitro* and lowers basal glucagon levels as well as glucagon responses to intravenous arginine in human.

### C. Central Nervous System Control Sites

Stimulation of the ventromedial hypothalamus (VMH), a sympathetic center, augments glucagon secretion and suppresses basal insulin release, resulting in hyperglycemia due to increased glycogenolysis and plasma FFA levels due to increased lipolysis. The increase in plasma glucagon is not prevented by adrenalectomy, which suggests that glucagon release is due to direct innervation; however, adrenalectomy increases plasma insulin in these circumstances, indicating that insulin release had been under tonic inhibition by adrenomedullary catecholamines. Conversely, lesion of the ventromedial hypothalamus leads to increased plasma insulin and glucagon. It is therefore generally thought that the VMH represents a sympathetic center and the ventrolateral hypothalamus a parasympathetic center. It has been further proposed that the lateral and medial hypothalamus, splanchnic and vagus nerves, and the endocrine pancreas constitute a circuit that mediates the above-mentioned physiological responses.

In rats, intraventricular injection of 2-deoxyglucose, which causes central nervous system neuroglycopenia, leads to hyperglycemia associated with diminished insulin release and increased glucagon release. The latter is augmented by β-adrenergic receptor activation and inhibited by α-adrenergic receptor stimulation.

## IV. HORMONAL EFFECTS ON GLUCAGON SECRETION

### A. Insulin

Insulin suppresses glucagon. Acute withdrawal of insulin from patients with type 1 diabetes rapidly results in hyperglucagonemia, which is readily reversed by reinfusion of physiological amounts of insulin (Fig. 4). Excessive glucagon responses to arginine and after ingestion of balanced meals found in patients with diabetes can be reduced by physiologic quantities of insulin. Permissive low levels of insulin appear to be important for optimization of the response of alpha cells to glucose. The hyperglucagonemia seen in diabetic ketoacidosis, despite the hyperglycemia characteristic of the state, is consistent with this theory. One must consider, however, the relative influence of arterial insulin versus intraislet insulin, which is still poorly understood. The suppressive effects of insulin on glucagon secretion are mediated at least in part by increased intracellular ATP resulting from increased glucose metabolism. As discussed earlier, increased energy directed (portioned) to compartments limiting glucagon release could be involved.

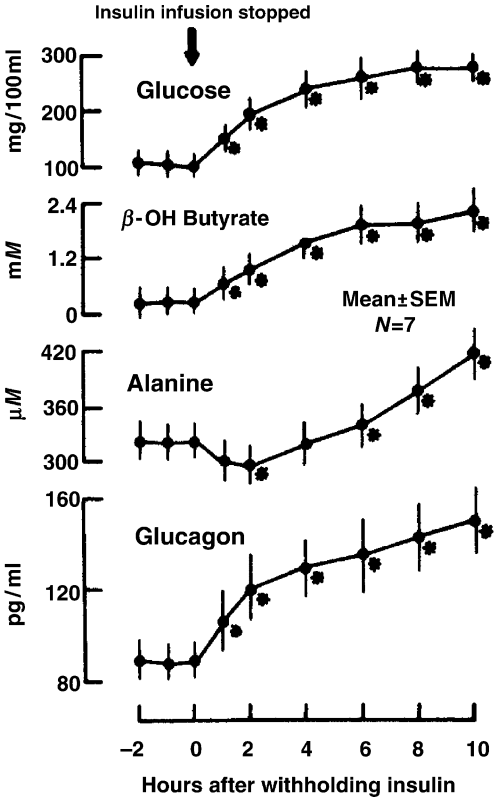

FIGURE 4 Effect of acute withdrawal of on plasma glucagon levels insulin in patients with type 1 diabetes. $^{*}P \leq 0.01$. Reprinted from Gerich, J. E., Tsalikian, E., Lorenzi, M., Karam, J. H., and Bier, D. M. Plasma glucagon and alanine responses to acute insulin deficiency in man. *J. Clin. Endocrinol. Metab.* 40, 526–529 (1975), with permission. Copyright The Endocrine Society.

### B. Somatostatin

Somatostatin is a 14-amino-acid polypeptide synthesized and released from pancreatic islet delta cells; it inhibits insulin and glucagon. Studies in rats in which administration of anti-somatostatin gamma globulin was observed to augment insulin and glucagon secretion indicate that somatostatin acts as a physiologic local regulator of both insulin and glucagon secretion. Its mode of action remains speculative.

There are five known somatostatin receptor subtypes, and inhibition of glucagon release is mediated via the sst1 subtype. The mechanism of transduction of the somatostatin signal has not been entirely elucidated. There is evidence that somatostatin, after binding to an external membrane receptor, is internalized via a calmodulin-dependent process. This interaction was further postulated to result in inhibition of adenylate cyclase, calcium channel opening, and phosphoinositide turnover. The inhibitory effect on the cyclase is reinforced by concomitant stimulation of soluble phosphodiesterase. The net effect is a reduction of cytosolic $Ca^{2+}$ and inhibition of adenylate cyclase (and hence cAMP production). The inhibition produced by somatostatin is noncompetitive and therefore independent of the strength of stimulation for glucagon secretion.

### C. Growth Hormone

The effects of growth hormone (GH) on glucagon secretion appear to be indirect, in that it increases the sensitivity of alpha cells to other primary regulators. When administered in pharmacological doses over the course of several days, GH elevates both basal and evoked levels of glucagon.

### D. Glucocorticoids

In Cushing's syndrome and during glucocorticoid treatment, basal levels of glucagon and insulin are increased. This may be at least partially due to islet adaptation to insulin resistance and also to elevated plasma amino acid levels accompanying glucocorticoid excess. Glucocorticoids exert a direct inhibitory effect on insulin release that could secondarily increase glucagon release.

### E. Catecholamines

The effects of catecholamines on glucagon secretion were discussed in Section III. A.

## V. OTHER REGULATORY FACTORS

### A. Pituitary Adenylate Cyclase-Activating Polypeptide

Pituitary adenylate-cyclase activating polypeptide (PACAP), originally isolated from ovine hypothalamus, exists in two forms: PACAP38, a 39-amino-acid peptide, and PACAP27, which is composed of the first 27 amino acids found in PACAP38. The peptide derives its name from its effectiveness in stimulating adenylate cyclase activity in the rat anterior pituitary. PACAP has been localized to nerves of the exocrine parenchyma, perivascular nerves, intrapancreatic nerve ganglia, and the nerve endings associated with islets (indicating neurotransmitter-like activity). PACAP shares considerable homology with various peptides from the secretin/glucagon/vasoactive intestinal peptide (VIP) family. PACAP has been shown to stimulate the secretion of glucagon from the rat pancreas *in vivo* and *in vitro* and more recently to stimulate both insulin and glucagon secretion in humans, indicating a role for this peptide in the regulation of islet cell function. The actions of PACAP are believed to be mediated through two receptor types: (1) a type 1 receptor with a relatively higher affinity for PACAP38 and PACAP27 than for VIP and (2) a VIP–PACAP common receptor that shows similar affinities for VIP, PACAP38, and PACAP27.

### B. Serotonin and Dopamine

Islets of several species contain serotonin and dopamine, suggesting that these amines may influence islet function. Conflicting results, which may be due to different intra- and extracellular actions of these agents, leave their precise roles unclear. Serotonin diminishes the insulin response to glucose in the golden hamster, mouse, and rabbit, but increases it or has no effect in the rat, dog, and human. Serotonin antagonists (e.g., methysergide maleate) increase insulin release. Reserpine, which depletes catecholamine stores, reverses serotonin inhibition of insulin release, suggesting that serotonin may act indirectly by releasing catecholamines. Dopamine—or the administration of <E5 > L < /E5 > -3,4-dihydroxyphenylalanine, which is subsequently converted to dopamine—has been reported to diminish insulin release *in vitro* and to stimulate glucagon release *in vivo*. Whether dopamine acts by releasing catecholamines from local nerve endings, by a central nervous system action, or by a direct dopaminergic effect or other effect on islets is unresolved.

### C. Gamma-aminobutyric Acid

GABA has recently been co-localized with insulin (in beta cells) and may mediate part of the inhibitory action of glucose on glucagon secretion by activating $\mathrm{GABA_A}$-receptor $\mathrm{Cl^-}$ channels in alpha 2 cells.

## VI. DEVELOPMENTAL AND NUTRITIONAL ASPECTS OF GLUCAGON SECRETION

Alpha cells are present early in fetal development, but functional maturity does not occur until after birth. Fetal islets are responsive to amino acids and to certain neurotransmitters (e.g., epinephrine and acetylcholine) but not to changes in glucose concentration. The large number of amino acids that can influence glucagon and insulin release from the pancreas of fetal and newborn lambs suggests that physiological changes in plasma amino acid concentration may be the primary regulators of glucagon and insulin release *in utero*. Plasma glucagon levels rise in the rat and human coincident with a fall in blood glucose levels at birth; however, this response is impaired in children of diabetic mothers. This may be due to hyperglycemia and hyperinsulinemia occurring *in utero* as a result of poor glycemic control in the mother.

Changes in glucagon secretion occur during pregnancy, including a reduced glucagon response to protein ingestion, which may contribute to the facilitated anabolism of late pregnancy. Changes in glucagon secretion also occur with the estrous cycle and after the administration of oral contraceptives.

### A. Obese State

Islets in obese animals are frequently hypertrophied and hyperplastic. Basal glucagon levels are generally normal in obesity, but responses to arginine and hypoglycemia are excessive. There is evidence, however, that glucagon levels are elevated in grossly obese persons, particularly in persons with impaired glucose tolerance.

## VII. GLUCAGON SECRETION IN DIABETES MELLITUS

Diabetes mellitus is the most common condition associated with abnormal glucagon secretion. In both type 1 and type 2 diabetes, fasting plasma glucagon levels are elevated in an absolute sense or are clearly inappropriate for the prevailing hyperglycemia. Since the withdrawal of insulin in euglycemic type 1 diabetic patients is associated with an increase in plasma glucagon that can be reversed by infusion of insulin, this hyperglucagonemia is at least in part secondary to insulin deficiency and/or insulin resistance. Responses to arginine infusion and to meal ingestion are also increased in both forms of diabetes. Since insulin responses are reduced in such individuals, the resultant increased glucagon:insulin ratio would contribute to hyperglycemia.

Curiously, although glucagon responses to nonglucose stimuli are increased in patients with diabetes, their responses to increases (suppression) and decreases (stimulation) of plasma glucose levels are impaired. This has led to the hypothesis that diabetes mellitus may be a bihormonal disorder due to a primary effect on “blindness” to glucose in both alpha and beta cells. Certainly this cannot be the case for patients with type 1 diabetes, in which defects in glucagon secretion appear to be primarily due to the lack of insulin release but the situation is less clear for patients with type 2 diabetes.

The diminished increase in glucagon release during hypoglycemia in people with type 1 and type 2 diabetes has been identified as a risk factor for severe hypoglycemia. To some extent this is secondary to repetitive episodes of hypoglycemia, which will reduce glucagon responses as demonstrated by patients with insulin-producing tumors of the pancreas. The mechanism for this phenomenon is unclear. It has been suggested that the glucagon response to hypoglycemia is dependent on a reduction in insulin release, which results in the deinhibition of glucagon release.

At the present time, the abnormal glucagon secretion in patients with type 1 diabetes, which is due to autoimmune destruction of islet beta cells, can be best explained on the basis of insulin deficiency. In patients with type 2 diabetes, the situation may be more complex and may involve resistance to insulin as well as a genetic defect in the alpha and beta cells of the pancreas.

## VIII. SUMMARY AND CONCLUSIONS

Over the past 3 decades, much has been learned about the regulation of glucagon secretion. Like the regulation of insulin, it is complex and involves numerous factors, the most important of which are the prevailing plasma glucose concentration, the intraislet insulin concentration, and the sympathetic nervous system. However, much more needs to be known regarding the molecular mechanisms by which these regulators affect pancreatic alpha-cell function and its abnormalities in diabetes mellitus.

## Glossary

**adipocytes** Large cells that are specialized for fat storage.

**Golgi apparatus** A complex cytoplasmic organelle consisting of a series of layered cisternae and associated small vesicles; it is involved in terminal glycosylation, membrane flow, secretion, and delivery of cellular products either to the cell surface or to the appropriate intracellular destination.

**islets of Langerhans** The irregular microscopic masses of cells that constitute the endocrine pancreas. Of the three cell types that form the islets, the alpha cells produce glucagon, the beta cells produce insulin, and the delta cells produce somatostatin.

**ketone bodies** Any of three compounds that arise from acetyl coenzyme A and that may accumulate in excess amounts as a result of starvation, diabetes mellitus, or other defects in carbohydrate metabolism.

## See Also the Following Articles

**Diabetes Type 1 (Insulin-Dependent Diabetes Mellitus) • Diabetes Type 2 (Non-Insulin-Dependent Diabetes Mellitus) • Glucagon Action • Glucagon Gene Expression • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagonoma Syndrome • Glucagon Processing**

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# Glucocorticoid Biosynthesis: Role of StAR Protein

Douglas M. Stocco
*Texas Tech University Health Sciences Center*

I. INTRODUCTION
II. REGULATION OF GLUCOCORTICOID SYNTHESIS
III. THE STEROIDOGENIC ACUTE REGULATORY PROTEIN
IV. CONSEQUENCES OF MUTATIONS IN THE StAR GENE
V. SUMMARY

**Steroid hormones make up a very important class of cholesterol-derived regulatory molecules that are synthesized mainly in the adrenal glands, ovary, placenta, testis, and brain. Steroids of one class, the glucocorticoids, are synthesized specifically in the fasciculata cells of the adrenal cortex. The mechanisms of steroidogenesis, the series of reactions leading to production of a steroid hormone, have long been of interest. The steroidogenic acute regulatory protein is a key regulator in glucocorticoid synthesis.**

## I. INTRODUCTION

In most cases, steroids are synthesized in response to steroidogenic stimuli. All of the steroid hormones are of great physiological importance. Glucocorticoids, produced in the adrenal glands, regulate carbohydrate metabolism and stress responses; the adrenal glands also synthesize the mineralocorticoids, which are necessary to regulate salt balance and maintain blood pressure. The sex steroid hormones, estrogen and progesterone, synthesized in the ovary and placenta in the female, are essential for reproductive function and for maintaining secondary sex characteristics. These same functions are mediated in the male by androgens synthesized in testicular Leydig cells. Neurosteroids, another class of steroids, are synthesized in the brain and their functions include both stimulation and inhibition of γ-aminobutyric acid (GABA)ergic responses, modulation of the response of specific brain cells to excitatory amino acids, and enhancement of memory function. Thus, the steroid hormones have very diverse and important physiological functions within the body. This article focuses on the synthesis of a specific class of steroids, the glucocorticoids, and primarily on how this synthesis is regulated.

The process by which steroids are synthesized is referred to as steroidogenesis, which comprises a series of reactions that are identical in the initial stages. Steroidogenesis occurs in specialized cells in specialized tissues within an organism and results in the production of tissue- and cell-specific steroids. In all steroidogenic tissues, regardless of the final steroid hormones synthesized, the initial step in steroidogenesis is the conversion of the nonsteroid, cholesterol, to the first steroid formed, pregnenolone. This conversion occurs via the action of the cytochrome P450 side chain cleavage enzyme (P450scc), which resides in the inner mitochondrial membrane in all steroidogenic cells. Pregnenolone then exits

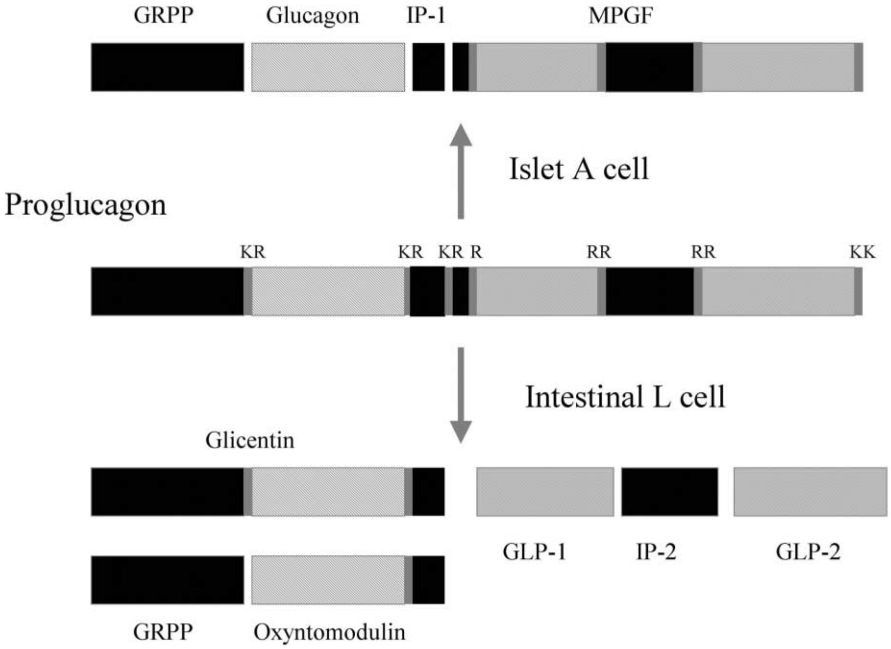

**FIGURE 1** Schematic representation of the structure and tissue-specific processing of the proglucagon molecule in islet alpha cells and intestinal L cells. In the alpha cell, proglucagon processing leads to glicentin-related pancreatic polypeptide (GRPP, proglucagon 1–30), glucagon (proglucagon 33–61), the intervening peptide-1 (IP-1, proglucagon 64–69), and the major proglucagon-derived fragment (MPGF, proglucagon 72–158); in intestinal L cells, proglucagon processing liberates glicentin (proglucagon 1–69), glucagon-like peptide-1 (GLP-1, proglucagon 78–107), IP-2 (proglucagon 111–122), and GLP-2 (proglucagon 126–158). Glicentin is also partially processed to GRPP and oxyntomodulin (proglucagon 33–69).

in the crypts but a few can also be observed in the intestinal villi.

## II. REGULATION OF GLP SECRETION

The GLPs are synthesized and secreted in a nutrient-dependent manner. Before the development of specific GLP-1 and GLP-2 radioimmunoassays, L cell secretion was quantified as gut glucagon-like immunoreactivity (GLI), which measured the levels of oxyntomodulin and glicentin. Because the production of GLI is correlated with that of GLP-1 and GLP-2, studies reporting secretion of GLI are assumed to reflect that of GLP-1 and GLP-2.

To investigate the direct effects of nutrients and endocrine, paracrine, and neural mediators at the level of the L cell, models consisting of primary cultures of fetal rat intestinal cells (FRICs) or canine ileal mucosal cells have been successfully developed. The limited number and viability of cells obtained by these techniques in addition to the heterogeneity of the isolated cells prevent extensive analysis of proglucagon gene regulation. However, the development of tumor-derived cell lines that express the proglucagon gene has aided in this regard. The GLUTag cell line was developed from intestinal tumors in proglucagon–SV40 large T antigen transgenic mice, whereas the STC-1 cell line was derived from a proximal intestinal endocrine tumor that developed in mice carrying the transgene for the rat insulin promoter linked to SV40 large T antigen and the polyoma virus small T antigen. Recently, a human L cell line (NCI-H716) has also been developed from a cecal tumor. The development of *in vitro* models of the L cell has facilitated investigation into the intracellular mechanisms underlying proglucagon gene expression and GLP secretion. Studies using FRIC cultures and the GLUTag cell line have shown that activation of protein kinase A (PKA) stimulates both synthesis and release of the GLPs, whereas activation of protein kinase C (PKC) results in increased secretion of the GLPs without affecting proglucagon gene expression. Calcium has also been shown to play an important role in basal L cell secretion; secretion is abolished when FRIC cultures are incubated with a calcium blocker ($CoCl_2$) and is stimulated by increasing the intracellular calcium concentration.

The release of GLP-1 and GLP-2 after nutrient ingestion is biphasic; an early peak occurs after 15–30 min and a second peak is seen 60 min later. The first peak occurs before the nutrients reach L cells in the distal ileum and colon, through an indirect neural and hormonal mechanism. The later peak is mainly due to direct contact of the nutrients with the L cells. Several studies have identified the mechanisms underlying the rapid release of GLP after food ingestion. Administration of the ganglionic blocker hexamethonium inhibits the release of GLP-1 stimulated by intraduodenal mixed meal or oleic acid *in vivo* in rats, showing the involvement of the nervous system in the control of distal L cell secretion. It has also been shown that bilateral subdiaphragmatic vagotomy completely abolishes duodenal fat-induced GLP-1 release in rats. Interestingly, glucose-dependent insulinotropic polypeptide (GIP), a hormone secreted by endocrine K cells of the upper small intestine, was also shown to stimulate GLP-1 secretion *in vivo* in rats. However, the effect of GIP is abolished by selective hepatic branch vagotomy, suggesting that GIP acts through vagal afferent pathways to stimulate L cells indirectly. This stimulation appears to be transmitted to L cells by the efferent pathway in the celiac branch of the vagus nerve.

Gastrin-releasing peptide (GRP), a neuropeptide, was also shown to stimulate GLP-1 secretion *in vitro* as well as *in vivo* in humans and several animal species. Mice lacking the GRP receptor have reduced GLP-1 and insulin responses as well as oral glucose intolerance, showing that intact GRP receptors are required for normal GLP-1 release. However, whether GRP is involved in the vagal regulation of the L cell is not known.

Acetylcholine and muscarinic receptors appear to be involved in the regulation of L cell secretion in both rats and humans, and they likely mediate the effect of the vagus on GLP secretion. Cholinergic muscarinic agonists stimulate GLI and GLP-1 release from the NCI-H716, STC-1, and GLUTag cell lines, as well as from FRIC cultures. In humans, the infusion of atropine (a nonspecific muscarinic antagonist) reduces the secretion of GLP-1 in response to oral glucose, indicating the involvement of muscarinic receptors in GLP-1 secretion. Recent data implicate the muscarinic m1 receptor subtype in this phenomenon in the rat. Epinephrine and the β-adrenergic agonist isopropanol stimulate GLP-1 secretion in isolated rat ileum or colon but have no effect on GLP-1 secretion by GLUTag and FRIC cells. Thus, the role of the sympathetic nervous system in the regulation of the L cell remains unclear.

Finally, the neuropeptide galanin was shown to inhibit both basal and GRP-stimulated GLP-1 secretion from isolated rat ileal L cells, and insulin and somatostatin-28 have been reported to inhibit GLP-1 release both *in vivo* and *in vitro*. Collectively, these findings underscore the complexity of the neural and endocrine mechanisms regulating GLP release from distal L cells in response to nutrients in the proximal duodenum (Fig. 2).

In humans and different animal species, food ingestion promotes secretion of GLP-1 and GLP-2 into the circulation; the most important nutrients inducing GLP secretion are carbohydrate and fat. Oral glucose administration stimulates GLP-1 and GLP-2 release in humans, rats, and pigs, but systemic administration of glucose has no effect on GLP-1 release, indicating that the glucose sensors are localized on the intestinal lumen. These observations are consistent with the role of GLP-1 as an important incretin hormone acting on the pancreatic beta cells to stimulate insulin release after glucose absorption. However, the majority of glucose ingested is absorbed into the jejunum, and the rapid release of GLP-1 after an oral glucose dose indicates that glucose does not directly stimulate the L cell. Although high concentrations of glucose (28 m*M*) have been shown to

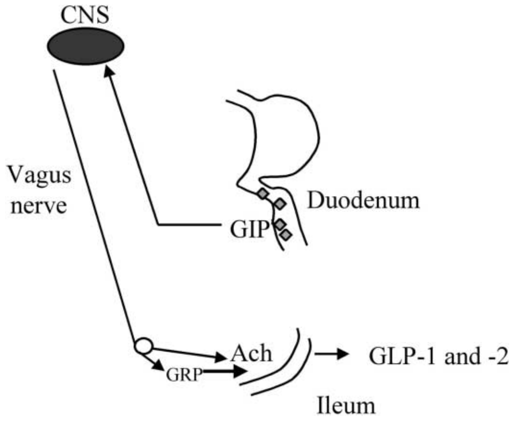

**FIGURE 2** Model of the indirect mechanism underlying glucagon-like peptide-1 (GLP-1) and GLP-2 secretion from L cells after food ingestion. Entry of chyme into the duodenum induces the release of glucose-dependent insulinotropic polypeptide (GIP) from duodenal endocrine K cells. GIP stimulates the afferent branch of the vagus nerve to the vagus center, which sends a stimulatory signal via the efferent branch of the vagus nerve to L cells through muscarinic receptors and/or by stimulating the release of gastrin-releasing peptide (GRP) by myenteric neurons to stimulate GLP secretion by the L cell. Ach, Acetylcholine; CNS, central nervous system.

stimulate GLI secretion from FRIC cultures, the consensus opinion is that glucose has little direct effect on the L cell *in vivo*. By contrast, fat appears to be the most important nutrient inducing GLP secretion. Ingestion of mixed fat or triglycerides stimulates GLP-1 and GLP-2 release in humans and pigs. Intraduodenal fat stimulates GLP-1 secretion independently of contact with L cells, suggesting an indirect effect of fat to stimulate GLP-1 release. However, fatty acids can also stimulate GLP secretion from FRIC cultures and GLUTag cells, suggesting that fatty acids can act directly on L cells. Both the length and the degree of saturation of the fatty acids affect the ability of fats to stimulate GLP secretion; monounsaturated long-chain fatty acids are more potent stimulators of GLP release than are short- or medium-chain, saturated or polyunsaturated fatty acids.

Neither amino acids nor protein alone consistently increase GLP-1 release in humans, dogs, and rats. In contrast, peptone (a protein hydrolysate) has been shown to stimulate GLP-1 secretion from the isolated vascularly perfused rat intestine. Peptone also stimulates proglucagon mRNA expression in the STC-1 cell line. However, although peptone is more representative of the proteins found in the lumen, compared to simple amino acids or intact protein, most proteins are digested and absorbed in the jejunum. Thus, the role of proteins in modulating the L cell remains unclear.

## III. GLP METABOLISM

Degradation of both GLP-1 and GLP-2 in the circulation appears to occur initially through the actions of dipeptidyl peptidase IV (DPP IV), which cleaves the amino terminus (His$^1$-Ala$^2$) of both peptides, resulting in GLP-1(9–36 $NH_2$) and GLP-2(3–33). These two fragments are the major metabolites of GLP-1 and GLP-2 in the circulation, and both are biologically inactive.

In normal rats, 50% of a bolus of GLP-1 is cleaved within 2 min, whereas GLP-1 remains intact for at least 10 min in DPP IV-deficient rats. Similarly, the half-life of GLP-2 in normal rats is approximately 6 min, but greater than 60 min in DPP IV-deficient animals. A recent study has shown high levels of DPP IV in the capillaries surrounding the L cells, and DPP IV is also present in the circulation. These findings have opened new avenues for developing GLP analogues that are resistant to DPP IV degradation, thereby enhancing the half-lives of these peptides. An alternative approach is the oral administration of a DPP IV inhibitor that, in rats, potentiates the actions of GLP-1 and GLP-2. These findings also indicate that the intact N-terminus of these peptides is important to their biological activity.

## IV. GLP RECEPTORS

The receptors for GLP-1 and GLP-2 are both members of the glucagon receptor subfamily of the G-protein-coupled superfamily of receptors. The gene encoding the human GLP-1 receptor is localized to chromosome 6p21. The encoded 463-amino-acid protein has a high affinity for GLP-1 but can also bind glucagon with a 100- to 1000-fold reduced affinity. Interestingly a 39-amino-acid peptide (exendin4) isolated from the venom of the lizard *Heloderma suspectum* (gila monster) exhibits high sequence homology with GLP-1 and was shown to act as a full GLP-1 agonist at the GLP-1 receptor. The amino-terminally truncated form of exendin4 [exendin4(9–39)] is a potent antagonist of the GLP-1 receptor *in vitro* and *in vivo*. GLP-1 acts through its receptor by activating the adenylyl cyclase pathway. GLP-1 may also increase intracellular calcium concentrations by mobilizing $Ca^{2+}$ from intracellular stores, both by activation of a phospholipase C pathway and through cAMP-dependent $Ca^{2+}$-induced $Ca^{2+}$ release from ryanodine-sensitive $Ca^{2+}$ stores. Finally, GLP-1 can also exert a direct stimulatory influence on voltage-dependent calcium channels in the pancreatic beta cell and stimulates the opening of $Ca^{2+}$-activated nonselective cation channels that are permeable to $Ca^{2+}$ and $Na^+$.

The GLP-1 receptor is widely distributed in different tissues in the body, including the pancreas (primarily the beta and delta cells), brain, lung, stomach, kidney, and intestine. Biological activities of GLP-1 have also been detected in liver, muscle, and adipose tissue, but the presence of the GLP-1 receptor in these tissues remains controversial. *In vitro* studies show that the GLP-1 receptor is susceptible to rapid desensitization after 5 min of ligand binding and this is reversed within 10–20 min after removal of the ligand. GLP-1 receptor desensitization is correlated with the phosphorylation of serine residues in the C-terminal tail of the receptor. The GLP-1 receptor has also been shown to internalize. However, no tachyphylaxis to GLP-1 administration has been observed *in vivo*, thus the significance of this finding remains unclear.

It was only in 1999 that the rat and human GLP-2 receptors were cloned. The GLP-2 receptor is a 550-amino-acid protein, and the gene is localized on chromosome 17p33.3 in humans. Like the related GLP-1 receptor, the GLP-2 receptor is coupled to the adenylyl cyclase pathway and is highly expressed throughout the gastrointestinal tract and the brain. Interestingly, recent studies have shown that the GLP-2 receptor is expressed in endocrine cells of the human gut, which suggests that some of the effects of GLP-2 are mediated through other intestinal hormones, growth factors, and/or neurotransmitters.

## V. PHYSIOLOGICAL ACTIVITIES OF GLPs

### A. Physiological Activities of GLP-1

The biological actions of GLP-1 are reflected by the functions of the organs expressing the GLP-1 receptor.

#### 1. *Effect of GLP-1 on Pancreatic Islets*

The first biological effect of GLP-1 described was its ability to stimulate insulin secretion from pancreatic beta cells. GLP-1 was shown to increase levels of beta cell cAMP and insulin gene transcription, and to stimulate glucose-dependent insulin release. *In vivo* studies using exendin4(9–39), the GLP-1 receptor antagonist, have clearly shown that GLP-1 is an incretin hormone, mediating postprandial release of insulin from pancreatic beta cells. Consistent with these findings, elimination of GLP-1 receptor signaling in beta cells is associated with reduced intracellular cAMP and defective glucose-stimulated calcium influx. Mice with a null mutation in the GLP-1 receptor are also glucose intolerant.

Entry of glucose into beta cells induces generation of ATP, increasing the ATP/ADP ratio and closing the ATP-sensitive potassium channels. The closure of these channels results in a rise in resting potential (depolarization) of the beta cell, leading to opening of voltage-dependent calcium channels (L-type VDCCs). The influx of $Ca^{2+}$ through these channels then triggers vesicular insulin secretion through the process of exocytosis. Repolarization of the beta cell is achieved by opening of delayed-rectifyer and calcium-sensitive potassium channels. Binding of GLP-1 to its receptor triggers signaling pathways that can affect each of these steps of glucose-dependent insulin secretion, including enhancement of the sensitivity of the K-ATP channel to ATP, and increased flux of $Ca^{2+}$ through the VDCCs. Phosphorylation of secretion granule proteins by PKA may also trigger insulin secretion. It is believed that these actions are mediated through GLP-1-induced activation of PKA-dependent or related pathways (Fig. 3).

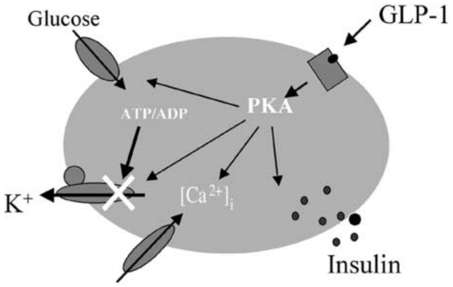

**FIGURE 3** Model of the signal transduction pathways involved in the mechanism of insulin secretion in response to glucose and glucagon-like peptide-1 (GLP-1) in the beta cell. Glucose entry into the beta cell increases the ATP/ADP ratio, inducing closure of the potassium channels. This causes depolarization of the beta cell, leading to the opening of voltage-dependent calcium channels. The influx of $Ca^{2+}$ triggers vesicular insulin secretion by exocytosis. Binding of GLP-1 to its receptor activates a protein kinase A (PKA)-dependent pathway, and, likely, other signaling pathways that can enhance each step of glucose-dependent insulin secretion.

Excitingly, recent studies have shown that GLP-1 can also stimulate islet cell proliferation *in vitro* and *in vivo*. Administration of GLP-1 or exendin4 for 10 days to neonatal diabetic rats following partial pancreatectomy or to prediabetic db/db mice stimulates expansion of the beta cell mass via induction of islet proliferation and neogenesis. GLP-1 has also been shown to induce differentiation of the acinar-like AR42J cells to endocrine cells containing immunoreactive insulin and glucagon. These effects are believed to occur through enhancement of ductal pdx-1 expression, a beta cell transcription factor. When taken together, these finding indicate that GLP-1 acts as a growth factor for the pancreatic islets.

#### 2. *Extrapancreatic Effects of GLP-1*

GLP-1 is part of the so-called ileal brake, i.e., factors secreted by the distal intestine that inhibit gastric emptying and upper intestinal motility. GLP-1 has been shown to inhibit pentagastrin and meal-induced gastric acid secretion, and gastric emptying at physiological concentrations. In humans, the effect of GLP-1 on gastric emptying is mediated by the nervous system (e.g., requires intact vagal innervation). A recent study has further shown that the effect of GLP-1 on gastric emptying occurs before its effect on beta cells to stimulate insulin secretion, and it is hypothesized that the early peak of GLP-1 secretion may be responsible for inhibiting gastric emptying and the later peak may be involved in the regulation of insulin secretion.

GLP-1 has also been shown to inhibit food intake *in vivo* in rodents after intracerebroventricular administration. However, mice lacking GLP-1 receptor signaling are lean with normal food intake even after several months of high-fat feeding. These data show that GLP-1 is not essential for physiological control of nutrient intake and body weight regulation *in vivo*. However, one explanation for the anorexic effects of GLP-1 derives from studies demonstrating that neurons expressing the GLP-1 receptor are activated as part of the stress response, such as following lithium chloride administration.

Finally, as described earlier, GLP-1 is rapidly inactivated by the enzyme DPP IV, which is highly expressed in the capillaries surrounding the L cells. The inactivation of GLP-1 by DPP IV is very rapid, leading to a 1- to 1.5-min half-life for GLP-1(7–36) amide. Furthermore, it has recently been demonstrated that 50% of the GLP-1(7–36) amide that is released from the intestinal L cells is degraded in the surrounding capillaries; thus, biologically active GLP-1(7–36) amide may never reach the pancreas. However, a recent study has shown that co-infusion of GLP-1 and glucose into the portal vein increases insulin secretion through activation of a neural mechanism. Other studies show that portal vein infusion of glucose in mice lacking the GLP-1 receptor fails to increase the glucose clearance rate. These findings indicate that the GLP-1 receptor is part of the hepatoportal glucose sensor and suggest that basal fasting GLP-1 levels are sufficient to confer maximum glucose competence to the sensor. The hepatoportal system therefore constitutes an important extrapancreatic target for GLP-1 in the control of glucose homeostasis.

#### *3. GLP-1 and Treatment of Type 2 Diabetes*

In many patients with type 2 diabetes, insulin resistance ultimately compromises the ability of the beta cell to maintain an adequate level of insulin biosynthesis and secretion over a prolonged time. GLP-1 has emerged as a promising therapeutic for type 2 diabetes, because GLP-1 stimulates insulin secretion and insulin gene expression in a glucose-dependent manner; GLP-1 also lowers glucagon secretion, delays gastric emptying, reduces food intake, and may enhance insulin sensitivity and stimulate beta cell neogenesis. These combined effects of GLP-1 thereby improve glucose tolerance. Indeed, long-term infusion of GLP-1 into diabetic patients reduces fasting and fed plasma glucose levels, as well as body weight. The ideal mode and frequency of GLP-1 (or GLP-1 analogue) administration are still under investigation. Nonetheless, GLP-1 remains a promising therapeutic approach for the treatment of hyperglycemia in patients with type 2 diabetes.

### B. Biological Activities of GLP-2

Although it has been suspected for many years that one of the proglucagon-derived peptides plays a role in adaptive bowel growth, it was only in 1996 that GLP-2 was shown to act as an intestinal growth factor. Exogenous GLP-2 induces crypt cell proliferation and inhibits villus tip apoptosis, thereby increasing villus height and small and large bowel weights within 6 days of twice-daily administration. Furthermore, GLP-2 also increases bowel function, increasing enzyme activity and enhancing nutrient absorption via stimulation of apical sodium-dependent glucose transporter (SGLT-1) and of basolateral glucose transporter-2 in rats. GLP-2 also enhances barrier function in the murine intestinal epithelium via effects on both the transcellular and paracellular pathways.

GLP-2, like GLP-1, also seems to be part of the so-called ileal brake, because GLP-2 can inhibit meal-induced gastric acid secretion and gastric motility. GLP-2 also appears to be involved in the central regulation of feeding behavior, although the physiological relevance of these findings remains unclear. Although all of the studies have been conducted in normal rodents, GLP-2 has also been shown to enhance bowel mass and function in several models of intestinal damage. These include atrophy caused by total parenteral nutrition and intestinal damage such as in dextran sulfate-induced colitis and both indomethacin- and chemotherapy-induced enteritis. In several of these models, GLP-2 has been shown to suppress the inflammatory response, possibly due to enhancement of intestinal barrier functions.

The intestinotropic effect of GLP-2 in the small and large bowels has paved the way for the use of GLP-2 as a treatment for intestinal insufficiency in humans. Indeed, administration of GLP-2 to patients with short bowel syndrome for 35 days produces increases in villus height concomitant with enhanced energy retention and weight gain. Whether GLP-2 can also be used to reduce inflammation in inflammatory bowel disease remains to be established.

## VI. SUMMARY

GLP-1 and GLP-2 are intestinal peptide hormones with strong therapeutic potential for the treatment of patients with type 2 diabetes and intestinal insufficiency, respectively. Future studies into the physiology and pathophysiology of these peptides will further establish the potential of both GLP-1 and GLP-2 for the treatment of human disease.

## Acknowledgments

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## Glossary

**glucagon-like peptide-1** A 30-amino-acid antidiabetic hormone that is found in endocrine cells of the gastrointestinal tract and in the brain.

**glucagon-like peptide-2** A 33-amino-acid intestinotropic hormone that is found in endocrine cells of the gastrointestinal tract and in the brain.

**G-protein-coupled receptor** A type of receptor that contains seven transmembrane domains and that is noncovalently linked to signalling pathways via G proteins.

**L cells** Endocrine cells localized in the intestine; produce and secrete GLP-1 and GLP-2 and are categorized as "open" cells because the apical microvilli are in contact with the intestinal lumen.

**proglucagon** A 160-amino-acid peptide processed in a tissue-specific manner. In the pancreatic alpha cells, proglucagon is processed to glucagon, whereas in intestinal endocrine L cells, proglucagon is processed to several biologically active peptides, including glucagon-like peptide-1 and glucagon-like peptide-2.

## See Also the Following Articles

**Glucagon Action • Glucagon Gene Expression • Glucagonoma Syndrome • Glucagon Processing • Glucagon Secretion, Regulation of • Glucose-Dependent Insulinotropic Polypeptide (GIP)**

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# Glucagonoma Syndrome

NICHOLAS CLARKE AND UDAYA M. KABADI
*University of Iowa*

I. OVERVIEW
II. CLINICAL FEATURES
III. LABORATORY FINDINGS
IV. TUMOR LOCALIZATION
V. TREATMENT

**Glucagonoma syndrome is a rare disorder characterized by necrolytic migratory erythema, cheilosis, diabetes mellitus or impaired glucose tolerance, normocytic normochromic anemia, venous thrombosis, and psychiatric symptoms.**

## I. OVERVIEW

The incidence of glucagonoma syndrome is one in 20 million and there are 200 reported cases in the literature. Glucagonoma, a tumor of the pancreatic alpha cell, is frequently malignant. Hepatic metastases are seen in two-thirds of patients at presentation. An elevated plasma glucagon in the setting of other findings associated with the syndrome is diagnostic. Most tumors are detected by computer tomography (CT) images of the abdomen or with selective arteriography of the pancreatic bed. Benign disease is curable through resection of the primary tumor. Tumor debulking in patients with metastatic disease often improves symptoms. Hepatic embolization may induce symptomatic relief, although almost half the patients manifest recurrent symptoms by 6 months. Somatostatin analogues may provide successful remission of the initial stage of the disease, although a progressive increase in dosage is often required to maintain efficacy.

## II. CLINICAL FEATURES

Necrolytic migratory erythema (NME) is the characteristic cutaneous rash seen in patients with glucagonoma syndrome (Fig. 1). The rash starts as small erythematous macules and papules in the lower extremity, perineum, and perioral areas. The lesions blister, leaving central erosions and necrosis in the upper third of the epidermis. The lesions coalesce as they heal and become hyperpigmented. They are very pruritic and painful. Frequently the course of the dermopathy waxes and wanes, with exacerbations and spontaneous remissions occurring without treatment. The lesions often become secondarily infected with *Candida* or *Staphylococcus* species, leading to much of the morbidity and mortality of this disease. Diagnosis is difficult, often requiring multiple biopsies from an edge of a characteristic lesion. A characteristic lesion shows dermal perivascular lymphocytic infiltrate with associated edema, pallor, necrosis, and bullous disruption of the upper third of the epidermis. Other diseases associated with NME include liver disease, pellagra, kwashiorkor, and epidermal necrolysis.

The pathophysiology of NME remains elusive and controversial. Amino acid deficiency, zinc deficiency, and liver impairment have all been postulated as causes for NME. Some patients with glucagonoma syndrome and NME showed improvement in the rash on amino acid supplementation with total parenteral nutrition. In these patients, glucagon levels remained elevated, suggesting that glucagon is not a causative factor for the rash. However, not all patients receiving amino acid supplementation manifested an improvement of their rash.

Another major feature of glucagonoma syndrome is impaired glucose tolerance or diabetes mellitus. Diabetes in most patients is mild to moderate if it occurs. The patients are not prone to ketoacidosis and do not manifest microvascular complications of diabetes, but often require insulin for corrections of their hyperglycemia. Weight loss, noted frequently, occurs because of anorexia from the tumor burden and increased caloric expenditure caused by gluconeogenesis, resulting in the loss of both adipose tissue and muscle mass. Diarrhea is the most frequent gastrointestinal symptom, occurring in 15–29% of patients. Patients may also present with a sore mouth and a smooth and shiny beefy red tongue tender to palpation. Painful oral erosions and angular stomatitis may also be present.

Neurological and psychiatric symptoms are highly variable in patients with glucagonoma syndrome.

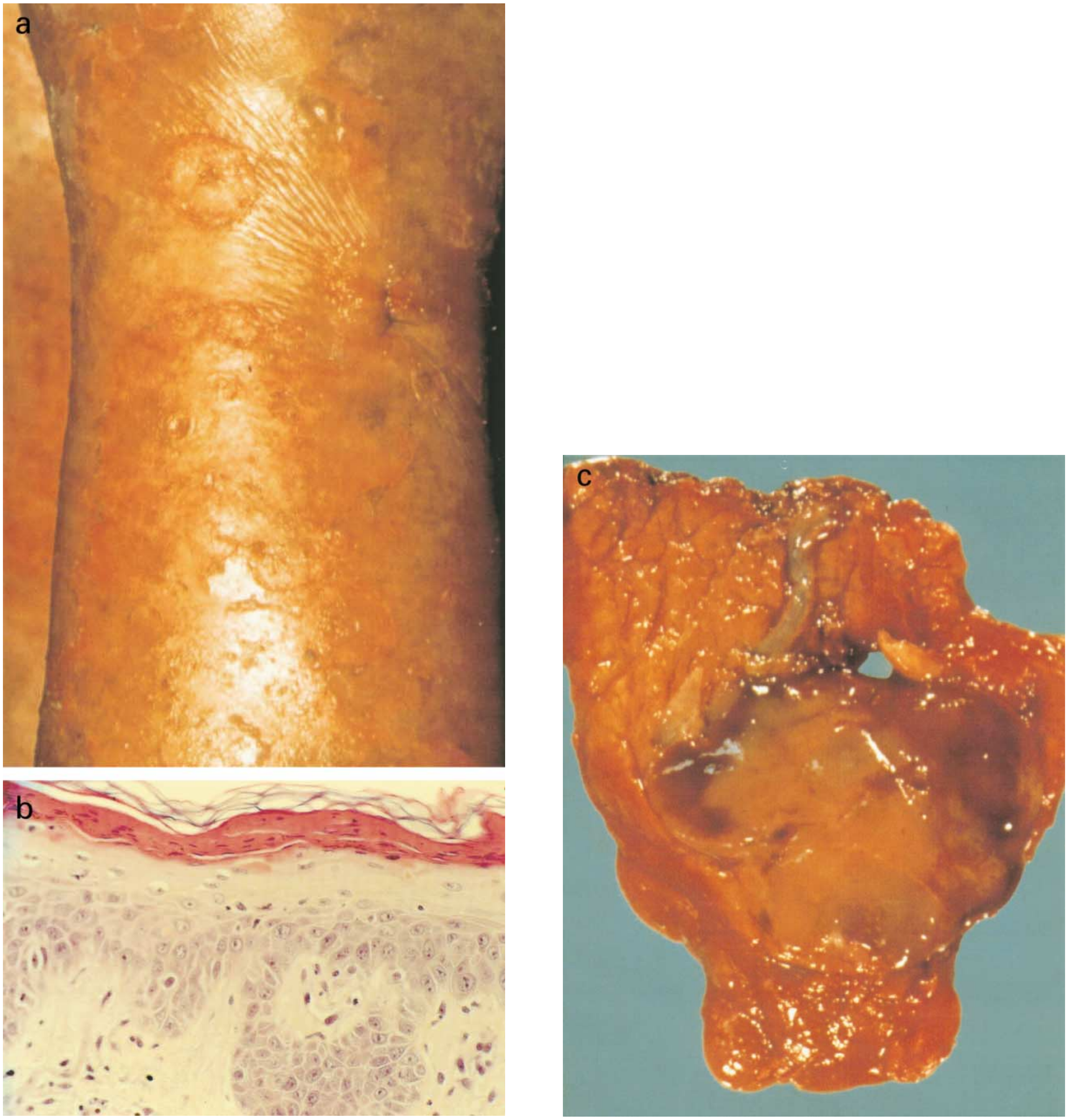

**FIGURE 1** Characteristic rash on upper "necrolytic erythema" (a) with skin biopsy (b) and excised tumor (c).

These symptoms include depression, dementia, psychosis, decreased cognitive function, agitation, nervousness, insomnia, ataxia, muscle weakness, hyperreflexia, and fecal and urinary incontinence.

Thromboembolic events occur frequently in patients with glucagonoma syndrome, with 11–14% of patients manifesting these events at diagnosis and up to 30% being observed during the course of the disease. These events consist of deep venous thromboses and pulmonary emboli and account for half of all deaths attributed to glucagonoma syndrome. Other common causes of death include secondary infections of the skin lesions and gastrointestinal bleeding.

## III. LABORATORY FINDINGS

All patients with glucagonoma syndrome have elevated plasma glucagon levels, usually between 1000 and 5000 pg/ml. Hyperglucagonemia has been seen in other conditions, including uncontrolled diabetes mellitus, with the highest levels being noted in diabetic ketoacidosis and hyperglycemic nonketotic hyperosmolar coma, strenuous exercise, burns, acute trauma, bacteremia, cirrhosis, starvation, renal failure, and Cushing's syndrome. However, in these conditions, fasting glucagon levels rarely rise above 500 pg/ml. A normocytic normochromic anemia is seen in patients with glucagonoma syndrome, possibly due to chronic disease. Serum iron, vitamin B12, folic acid, and bone marrow studies are usually normal. Panhypoaminoacidemia is a universal finding in patients with glucagonoma syndrome. Glucagon stimulates hepatic gluconeogenesis, leading to a decrease in plasma amino acid levels, especially alanine and glutamine. Therefore, in patients with glucagonoma syndrome, hepatic amino acid extraction is increased in response to elevated glucagon levels, causing gluconeogenesis with a decrease in protein synthesis, leading to hypoaminoacidemia. Once glucagon levels are lowered in patients with glucagonoma syndrome by medical or surgical means, a prompt increase in amino acid levels ensues. Hypoproteinemia, hypoalbuminemia, and hypocholesterolemia are frequently found in these patients as well. Elevated glucagon levels also promotes lipolysis, causing a rise in free fatty acids. Liver enzymes are usually normal, even in the presence of liver metastasis.

## IV. TUMOR LOCALIZATION

The glucagonoma tumor is usually single and large with the presence of metastasis, usually in the liver in 50% of the subjects, at the time of diagnosis. Multiple imaging modalities assist in localizing the tumor, including CT or magnetic resonance imaging (MRI) of the abdomen as well as pancreatic arteriography and ultrasound. CT scanning is the most reliable initial test for localizing the tumor as well as metastatic lesions. Usually, the tumor is greater than 3 cm in diameter and may be located in any part of the pancreas, the tail being the most frequent pancreatic site. If a tumor is not detected by abdominal CT or MRI and the clinical suspicion is high, selective pancreatic angiography may be effective in the detection with the finding of the characteristic tumor "blush" because of high vascularity associated with the tumor.

## V. TREATMENT

Treatment options include surgical excision, tumor debulking, hepatic artery embolization, somatostatin analogues, and anti-tumor chemotherapy. If the tumor is located exclusively in the pancreas, surgical excision may result in a cure. With a rapid fall in serum glucagon levels, improvement in the rash occurs within 24–48 h and resolution of the anemia, diabetes, and hypoproteinemia follows. Unfortunately, in two-thirds of patients, the tumors are malignant with the presence of hepatic metastases at the time of diagnosis. Nevertheless, surgical intervention in these patients may be a reasonable approach since it may alleviate the symptoms because of the lowering of the glucagon level. Procedures include a distal localized pancreatectomy or subtotal or 95% pancreatectomy and occasionally a Whipple's procedure, depending on the extent of the disease involvement.

These patients are high operative risks because of their weight loss, hypoproteinemia, skin rash, and tendency to develop venous thromboses. Perioperative management therefore includes transfusions, hyperalimentation or nasogastric feeding with amino acid supplements, topical and oral zinc supplements, administration of subcutaneous somatostatin analogues, and low-dose heparin for several weeks.

Somatostatin inhibits glucagon release. A long-acting analogue of somatostatin (octreotide acetate) has provided symptom relief for days to a month. It is most effective in amelioration of the skin rash with a lesser efficacy in preventing or reversing weight loss and lowering hyperglycemia. It is most useful in controlling symptoms at an early stage of disease. However, the dosage rises progressively in order to be effective in maintaining symptomatic relief.

Hepatic metastases are supplied mainly by the hepatic artery, whereas the blood supply to the surrounding liver tissue derives from both the hepatic artery and the portal vein. Therefore, hepatic artery embolization may be effective at inducing a regression of these metastases with the blood supply to normal liver tissue remaining adequate if the portal vein is patent. Therefore, prior to embolization of the hepatic artery, the patency of the portal vein is demonstrated by ultrasound and confirmed by angiography. Eighty percent of embolizations result in symptom relief, associated with lowering or normalization of glucagon levels. Unfortunately, in 50% of subjects, symptoms recur within 6 months either from revascularization of the tumor or from natural progression of the disease, requiring further embolizations if surgery is contraindicated. Treatment with streptozotocin and 5-fluorouracil has neither yielded symptom relief nor induced normalization of glucagon levels.

Zinc therapy, applied topically or administered orally (e.g., 200 mg zinc sulfate daily), as well as amino acid infusions may alleviate cutaneous manifestations. Hyperglycemia may be controlled with a high-protein diet and insulin. However, despite several therapeutic approaches, weight loss frequently persists and the prognosis remains grave with a 5-year survival rate of 25–30% and a mean survival period of between 3 and 7 years.

## Glossary

**glucagonoma syndrome** A disorder caused by elevated glucagon levels from a pancreatic alpha-cell tumor, leading to necrolytic migratory erythema, cheilosis, diabetes mellitus, impaired glucose tolerance, anemia, venous thrombosis, and psychiatric symptoms.

**impaired glucose tolerance** An early aspect of diabetes mellitus when the subject is unable to secrete sufficient insulin for a given carbohydrate load and leading to higher than normal plasma glucagon levels.

**necrolytic migratory erythema** The characteristic rash seen in glucogonoma syndrome, consisting of macules and papules in the lower extremities, perineum, and perioral areas, which blister, leaving central erosions and necrosis. They are often pruritic and painful.

**somatostatin** A peptide that inhibits glucagon release. A long-acting analogue of somatostatin is octreotide acetate, which is most effective with the skin rash in the early stages of the disease.

**thromboembolic events** Blood clots in the venous system leading to pulmonary embolism and possibly death.

## See Also the Following Articles

**Glucagon Action • Glucagon Gene Expression • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagon Processing • Glucagon Secretion, Regulation of • Somatostatin**

## Further Reading

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# Glucagon Processing

Donald F. Steiner
*University of Chicago, Illinois*

I. INTRODUCTION
II. BIOSYNTHESIS OF GLUCAGON
III. THE PROHORMONE CONVERTASES
IV. DIFFERENTIAL PROCESSING OF PROGLUCAGON BY PC2 AND PC1/3

**Glucagon processing refers to the proteolytic cleavage of proglucagon, a large glucagon-containing precursor protein, by specific neuroendocrine prohormone convertases (PC2 and/or PC1/PC3); this processing releases glucagon and/or other biologically active peptides also contained within the proglucagon molecule, i.e., the glucagon-like peptides GLP-1 and GLP-2. Proglucagon is expressed in the body in several locations, including the alpha cells of the pancreatic islets, where proglucagon is processed to release only glucagon, and in the L cells of the intestinal tract, where proglucagon is processed differently to release mainly GLP-1 and GLP-2. This "differential processing" of the proglucagon molecule provides an example of the regulation of specific hormone production from multifunctional precursors via differential tissue expression of prohormone convertases PC1/PC3 and PC2.**

## I. INTRODUCTION

The existence of a pancreatic hypoglycemic factor was first postulated in the early 1920s, but it was not isolated and sequenced until the mid-1950s. Glucagon, the hyperglycemic hormone, is a 29-amino-acid peptide (Fig. 1) that is secreted primarily by the alpha cells of the islets of Langerhans, but in some species it is also secreted in small amounts from cells in the stomach and intestine. Its actions are the mitochondria and is converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) in the microsomal compartment and subsequently to a variety of other steroids, the final product resulting from the specific complement of steroidogenic enzymes present within those tissues.

In glucocorticoid biosynthesis, cholesterol is first converted to pregnenolone in the mitochondria by P450scc in adrenal fasciculata cells. The next step in the major pathway for the further metabolism of pregnenolone is the hydroxylation of pregnenolone by 17α-hydroxylase to form 17α-hydroxypregnenolone. 17α-Hydroxypregnenolone is then converted to 17α-hydroxyprogesterone by the 3β-HSD enzyme in a manner analogous to the conversion of pregnenolone to progesterone. Then, two subsequent hydroxylations of 17α-hydroxyprogesterone occur, yielding cortisol. The first hydroxylation occurs at position 21 as a result of the action of the microsomal enzyme cytochrome P450 21-hydroxylase, yielding 11-deoxycortisol. This compound then re-enters the mitochondria, where, by the action of the mitochondrial enzyme cytochrome P450 11β-hydroxylase, which hydroxylates the molecule at the 11 position, it is converted to cortisol. The pathways involved in the synthesis of glucocorticoids in the adrenal cortex and the enzymes utilized within these pathway are illustrated in Fig. 1.

## II. REGULATION OF GLUCOCORTICOID SYNTHESIS

The biosynthesis of glucocorticoids is regulated mainly by the pituitary tropic hormone, adrenocorticotropic hormone (ACTH), and usually occurs in two phases. The acute phase occurs on the order of minutes and is responsible for the rapid production of steroids in response to immediate need. The rapid biosynthesis of glucocorticoids to combat stressful situations comes to mind in this type of regulation. A more chronic form of regulation also occurs and encompasses the longer term expression of the mRNAs and proteins for the steroidogenic pathway enzymes to assure long-term steroidogenic capacity in the cells. The events involved in the chronic regulation of steroid hormone biosynthesis are not discussed further here; the focus here is on those events that result in the rapid biosynthesis of glucocorticoids in response to steroidogenic stimuli, i.e., the acute phase.

Like most biosynthetic pathways, the steroidogenic pathway has a rate-limiting step. For a long period of time, the rate-limiting step in this pathway was believed to be the activation of the P450scc enzyme, the first enzyme in the pathway, which converts cholesterol to pregnenolone. However, it soon became clear that this was not the case and it was demonstrated that the P450scc enzyme was active even in unstimulated cells. The search for the true rate-limiting step in steroid hormone biosynthesis was ongoing for many years and culminated in the finding that the regulated and rate-limiting step was the delivery of the substrate cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where the P450scc enzyme is located. This step was rate limiting because the hydrophobic nature of cholesterol would not allow it to traverse the aqueous intermembrane space of the mitochondria by simple diffusion and reach

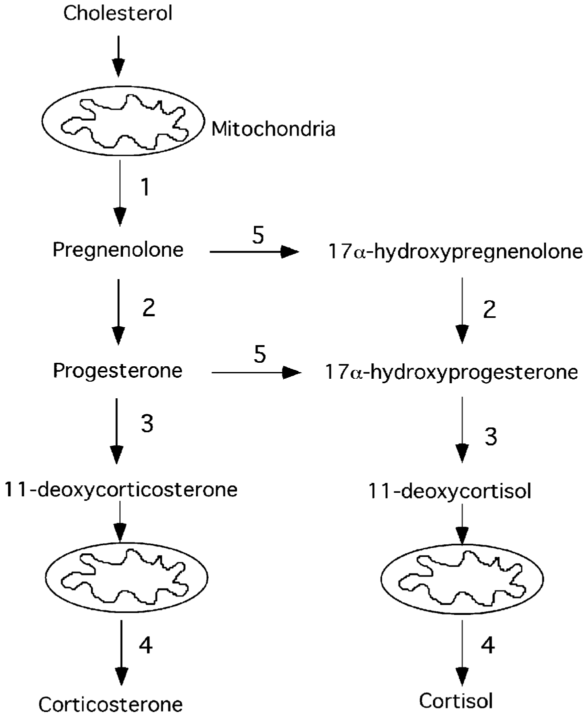

**FIGURE 1** The biosynthetic pathway of glucocorticoids. Biosynthetic pathways lead from cholesterol to the major glucocorticoid hormones produced in the adrenal glands, cortisol and corticosterone. Conversions of cholesterol to pregnenolone, and 11-deoxycortisol to cortisol, occur in the mitochondria; the remaining reactions occur in the microsomal compartment. The numbers represent the enzymes involved in synthesis of the glucocorticoids: (1) cytochrome P450 side chain cleavage (mitochondrial); (2) 3β-hydroxysteroid dehydrogenase/$\Delta^5$, $\Delta^4$-isomerase (microsomal); (3) cytochrome P450 21-hydroxylase (microsomal); (4) cytochrome P450 11β-hydroxylase (mitochondrial); (5) cytochrome P450 17α-hydroxylase/C17-20 lyase (microsomal).

the P450scc enzyme within the time frame observed for acute synthesis. Therefore, the acute synthesis of glucocorticoids is regulated by events that rapidly mediate the transfer of cholesterol from the outer to the inner mitochondrial membrane in response to ACTH.

With this knowledge came a great deal of investigation to determine the nature of the acutely regulated step. Initial studies were performed in the adrenal gland, because it had been observed that ACTH could stimulate the biosynthesis of steroids in incubated adrenal tissue slices. One of the first, and perhaps most important, observations concerning steroidogenesis was that acutely stimulated steroid production had an absolute requirement for the synthesis of new proteins. This was determined in studies that demonstrated that ACTH-stimulated corticoid synthesis in adrenal glands was sensitive to inhibitors of protein synthesis, an observation that has been corroborated many times since then. The importance of these observations can be seen when it is considered that the search for the putative regulator then had a potential target on which to focus, namely, a hormone-induced newly synthesized protein.

An important observation indicated that the protein-sensitive step was located in the mitochondria but had no effect on the activity of the P450scc. In similar studies, it was determined that inhibition of protein synthesis had no effect on the increased delivery of cholesterol to the outer mitochondrial membrane, but that the transfer of this substrate from the outer to the inner membrane was completely inhibited by cycloheximide. These observations indicated that the unknown protein(s) functioned at the level of the delivery of cholesterol to the P450scc enzyme, and thus the precise site of the cycloheximide-inhibited regulation had been determined. Powerful proof of this hypothesis was added when it was demonstrated that incubation of intact cells or isolated mitochondria with hydroxylated, and thus hydrophilic, cholesterol analogues that could freely diffuse to the P450scc enzyme resulted in full steroid biosynthesis.

In summary, many observations resulted in the characterization of the acute regulation of steroidogenesis and indicated that the production of steroids was dependent on a hormone-stimulated, rapidly synthesized, cycloheximide-sensitive protein, the function of which was to mediate the transfer of the hydrophobic substrate, cholesterol, from the outer to the inner mitochondrial membrane and the P450scc enzyme. The effort to identify and characterize this acute regulatory protein has been ongoing for approximately four decades. Although several candidates have emerged from these efforts, only the observations made for the steroidogenic acute regulatory (StAR) protein will be summarized here.

## III. THE STEROIDOGENIC ACUTE REGULATORY PROTEIN

A candidate protein for the acute regulator of steroidogenesis was first described in the mid-1980s as an ACTH-induced, 30-kDa phosphoprotein in hormone-treated rat and mouse adrenocortical cells, and as a luteinizing hormone-induced protein in rat corpus luteum cells and mouse Leydig cells. These studies indicated that a close relationship between the 30-kDa proteins and steroid hormone biosynthesis existed in these tissues and that their synthesis, as was steroidogenesis, was sensitive to cycloheximide. Our laboratory was engaged in similar studies and described, in hormone-stimulated MA-10 mouse Leydig tumor cells, a protein family that was identical to proteins that had been previously described. In both laboratories, these proteins were found localized to the mitochondria and consisted of several forms of a newly synthesized 30-kDa protein. Later studies determined that the 30-kDa mitochondrial proteins were processed from a 37-kDa precursor form with an N-terminus containing a mitochondrial signaling sequence. Subsequent to those initial observations, many studies then went on to document tight correlations between the synthesis of steroids and the synthesis of the 30-kDa mitochondrial proteins. However, even as the correlations grew, a direct cause-and-effect relationship between 30-kDa protein expression and steroidogenesis was lacking, and it became necessary to clone the 30-kDa protein to prove or disprove unequivocally its function in steroidogenesis.

The 30-kDa protein was purified from hormone-stimulated MA-10 Leydig tumor cells, a cell line that is highly steroidogenic in response to stimulation. Peptide sequence information was obtained from the purified protein and this sequence was utilized to design degenerate oligonucleotides that were in turn used to screen a cDNA library generated from hormone-stimulated MA-10 cells. A full-length 1456-bp cDNA that had an open reading frame of 852 nucleotides was subsequently obtained for the precursor 37-kDa protein. This open reading frame coded for a protein that was 284 amino acids long. The nucleic acid sequence of the cDNA and the amino acid sequence of the 37-kDa protein were analyzed

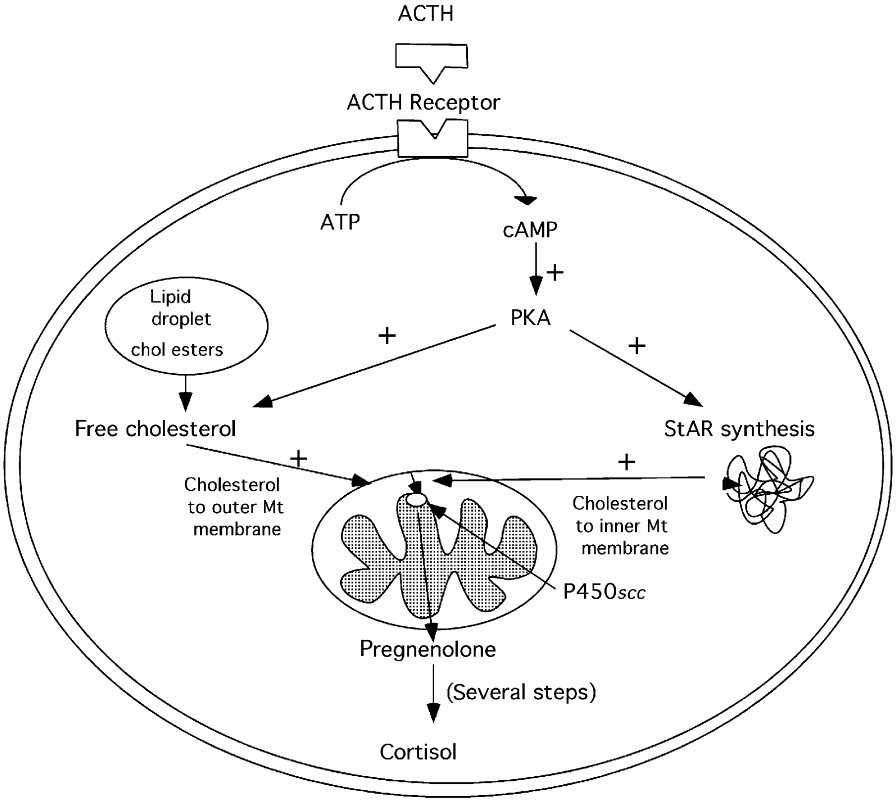

**FIGURE 2** Regulation of glucocorticoid synthesis: cellular response to adrenocorticotropic hormone (ACTH) stimulation in adrenal cells. Tropic hormone stimulation of adrenal cells results in an increase in intracellular cAMP. Free cholesterol is mobilized to the outer mitochondrial membrane by mechanisms still not well understood. These steps are necessary to assure an adequate supply of this precursor for sustained steroid biosynthesis. Precursors for steroid biosynthesis can be supplied from *de novo* synthesis of cholesterol, from uptake of low-density and/or high-density lipoprotein cholesterol, or from plasma membrane cholesterol, as occurs in some cell types. In addition, the *de novo* synthesis of the steroidogenic acute regulatory (StAR) protein is also required to obtain full steroidogenic capacity in response to hormone stimulation. The function of StAR is to mediate the transfer of cholesterol from the outer mitochondrial (Mt) membrane to the inner mitochondrial membrane, where the cytochrome P450 side chain cleavage enzyme system (P450scc)resides. Although the exact mechanism of action of StAR has thus far escaped characterization, in its absence normal steroidogenesis is not possible. Cholesterol is converted to pregnenolone by P450scc, and then to glucocorticoids by microsomal and mitochondrial enzymes. PKA, Protein kinase A.

and found to be unique, indicating that the cDNA represented a novel protein. Most importantly, expression of the cDNA-derived protein in both a steroidogenic cell line (in the absence of stimulation) and in a nonsteroidogenic cell line (rendered steroidogenic by transfection with the appropriate enzymes) resulted in significant increases in steroid production, indicating a direct role for the 37- to 30-kDa proteins in steroid production. As a result of these studies, the newly characterized protein was named the steroidogenic acute regulatory (StAR) protein. Many studies rapidly followed the initial cloning of StAR and demonstrated that the StAR protein was acutely regulated by tropic hormone stimulation in many steroidogenic tissues and that its localization was essentially confined to steroidogenic tissues. To date, many observations have been recorded for the StAR protein and all indications are that it satisfies all of the criteria listed for the acute regulator. A summary of the cellular events that regulate hormone-stimulated glucocorticoid synthesis is shown in Fig. 2.

## IV. CONSEQUENCES OF MUTATIONS IN THE StAR GENE

Closely following the cloning of the StAR cDNA, a series of investigations were conducted, the results of which would emphatically underscore the importance of StAR in the regulation of steroid hormone biosynthesis. Congenital lipoid adrenal hyperplasia (lipoid CAH) is a rare, autosomal recessive, and potentially lethal condition in humans resulting from an almost complete inability of the newborn child to synthesize steroids. Due to the lack of testosterone biosynthesis *in utero*, individuals with this condition are born with female external genitalia regardless of genotype. This condition is manifested by the presence of large adrenals containing very high levels of cholesterol and cholesterol esters and also by a lesser, but increased amount of, lipid accumulation in the steroidogenic cells of the testis, the Leydig cells. Unless timely diagnosis of lipoid CAH is made and appropriate mineralocorticoid and glucocorticoid hormone replacement therapy is administered, death will result in days to weeks as a result of adrenocortical hormone deficiency, the most critical steroid required being the mineralocorticoid, aldosterone. Observations from nearly three decades ago demonstrated that tissue or isolated mitochondria from afflicted patients were unable to convert cholesterol to pregnenolone, and thus this disease was originally thought to be due to mutations in the P450scc gene. This belief had persisted until relatively recent times, when studies on P450scc genes, amplified from lipoid CAH patients, were shown to be normal. Further studies deduced that the defect was upstream of P450scc at the point of cholesterol delivery to the enzyme. In fact, it was subsequently, and correctly, argued that embryonic homozygous mutations in the P450scc enzyme would be incompatible with life in humans because the placenta is required to produce progesterone after the first trimester, and the placenta is derived from embryonic tissue. In addition to the P450scc gene and its accompanying electron transfer proteins, other proteins that had been reported to be involved in cholesterol transfer in the mitochondria were also found to be normal in lipoid CAH patients.

When StAR was cloned in 1994, it immediately became an excellent candidate for the causative agent in lipoid CAH. In studies designed to determine if StAR may be involved in lipoid CAH, StAR cDNA was prepared by reverse transcription and polymerase chain reaction (RT-PCR) using RNA isolated from the tissue of patients who had this disease. Careful examination of the PCR products revealed mutations in the StAR cDNA sequences of the first four patients examined, and these mutations were confirmed in the genomic DNA. Importantly, whereas expression of the normal human StAR protein in COS-1 cells (again rendered steroidogenic by transfection) resulted in significant increases in steroid production, expression of the mutant StAR cDNAs from these patients indicated that although the proteins were indeed expressed, they were completely inactive in promoting steroidogenesis. The results of these studies indicated that expression of normal StAR protein was an indispensable requirement in promoting cholesterol transfer to the P450scc enzyme, that lipoid CAH represented a natural knockout of StAR, and that the consequences of this knockout were totally in keeping with the hypothesized function of StAR. In addition to the original report indicating that StAR mutations caused lipoid CAH, many additional examples of mutations in StAR resulting in this disease have now been documented. To date, there have been 28 different mutations in the StAR gene that have been shown to result in lipoid CAH. It also appears that the greatest prevalence of this disease occurs in Japanese and Korean individuals. In summary, the potentially lethal condition in humans known as lipoid CAH can be considered as a naturally occurring knockout of the StAR gene, and its consequences have dramatically demonstrated the indispensable role of StAR in hormone-stimulated steroidogenesis. Indeed, the absence of its activity in the adrenal results in the loss of steroid hormones that are essential for life.

That mutations in the StAR gene resulted in lipoid CAH produced compelling evidence for the essential role of this protein in the acute regulation of adrenal steroidogenesis. Therefore, with the goal of having a model system to study its role in steroidogenic tissues and the effects of its loss on other tissues, a knockout of the StAR gene was produced in mice. All pups appeared normal at birth but all had female external genitalia regardless of genotype, as seen in the human condition. Following birth, all animals failed to grow normally and death occurred within a short period of time, presumably as a result of adrenocortical insufficiency. This was confirmed by the observation that serum levels of corticosterone and aldosterone were depressed but that levels of ACTH and corticotropin-releasing hormone (CRH) were elevated. These observations indicated an impairment in the production of adrenal steroids with an accompanying loss of feedback regulation at the level of the hypothalamus or pituitary. Inspection of the adrenal gland revealed a normal medulla but an abnormal cortex, having a disrupted fascicular zone replete with vacuolated areas. Specific staining procedures revealed that the vacuoles observed in the adrenal cortex region of the StAR knockout mouse contained highly elevated levels of lipid. This lipid consisted of cholesterol and cholesterol esters that accumulated as a result of the inability of the cells to metabolize cholesterol. Subsequent studies extended the original findings in the newborn StAR knockout mice to include observations in animals that were kept alive with corticosteroid replacement therapy. The temporal effects of the StAR knockout on the adrenals were assessed. At birth, the adrenal glands were profoundly affected and contained high levels of lipid deposits within the cortical region. With progressing time after birth, the adrenals demonstrated increasing elevation in lipid deposition, particularly in the zona glomerulosa and zona fasciculata. Eventually, the entire cortical layer appeared to be filled with lipid, thus obliterating the cellular architecture of the cortex. In summary, StAR knockout mice demonstrated a phenotype that is essentially identical to lipoid CAH in humans. With specific regard to the adrenal gland, the inability to deliver cholesterol to the mitochondrial P450scc enzyme for synthesis of adrenocortical steroids was manifested by greatly increased lipid deposits in this gland and ultimately in death. Therefore, using observations obtained from both the human disease and the mouse knockout, the indispensable role of StAR in supporting normal adrenal steroidogenesis can readily be seen.

## V. SUMMARY

The acute regulation of hormone-stimulated glucocorticoid synthesis in the fasciculata cells of the adrenal cortex, as in all steroid-synthesizing cells, requires the transfer of cholesterol from cellular stores and the outer mitochondrial membrane to the inner mitochondrial membrane. This cholesterol is utilized as substrate by the P450scc enzyme and is converted to pregnenolone, the first steroid synthesized in this pathway. This is the rate-limiting step in hormone-regulated steroidogenesis. An early observation demonstrated that hormonally regulated steroid biosynthesis required the synthesis of new proteins. The search for the putative regulatory protein has been ongoing for almost four decades and has resulted in several candidates. The steroidogenic acute regulatory protein appears to satisfy all of the criteria for the acute regulator. It is rapidly synthesized in steroidogenic cells in response to tropic hormone stimulation and appears to be specifically located in those cells. Its expression in both steroidogenic and nonsteroidogenic cells can result in the induction of steroid biosynthesis in the absence of tropic hormone treatment. Perhaps the most compelling example of the indispensable role of StAR in regulating steroid biosynthesis comes from the observation that mutations in its gene that render it inactive result in the disease congenital lipoid adrenal hyperplasia, a disease in which the afflicted patient can synthesize virtually no steroids. StAR knockout mice display a phenotype that is essentially identical to the human condition.

## Acknowledgments

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## Glossary

**cholesterol side chain cleavage enzyme** A P450 enzyme (P450scc) located on the matrix side of the inner mitochondrial membrane in all steroidogenic cells studied to date. It is the rate-limiting enzymatic step in steroidogenesis and utilizes the substrate cholesterol to form pregnenolone, the first steroid synthesized in the steroidogenesis pathway.

**cholesterol transfer** The transfer of cholesterol to the inner mitochondrial membrane and to the P450scc enzyme constitutes the true regulated and rate-limiting step in the process of steroid hormone biosynthesis. Therefore, the mechanism of transferring cholesterol to P450scc is of critical importance.

**glucocorticoids** Steroids (cortisol and corticosterone) synthesized in the fasciculata cells of the adrenal cortex. Regulation of glucocorticoid synthesis utilizes mechanisms that are similar to those used for the synthesis of all steroids. Functions of the glucocorticoids include fostering vascular sensitivity to catecholamines, modulating immune responses, and promoting gluconeogenesis to maintain blood glucose levels and thus to preserve brain function.

**mitochondria** Intracellular organelles usually associated with energy production. In the steroidogenic cell, they are also the sites of the cytochrome P450 side chain cleavage enzyme system, which catalyzes the first step in the steroid biosynthetic cascade and is essential for the biosynthesis of steroids.

**steroidogenic acute regulatory protein** A protein that, to date, appears to possess all of the characteristics of the putative regulator protein. Hormone-stimulated steroid biosynthesis requires *de novo* protein synthesis, thus it is of critical importance to determine which proteins are involved in this regulation.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects on Physiology**

**and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids, Pharmacology of • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Stress**

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# Glucocorticoid Drugs, Evolution of

Christopher J. Larson, Jon Rosen, and
Jeffrey N. Miner
*Ligand Pharmaceuticals, San Diego, California*

I. INTRODUCTION
II. PHYSIOLOGY OF GLUCOCORTICOIDS
III. PHARMACOLOGY OF GLUCOCORTICOIDS
IV. MECHANISM OF ACTION OF GLUCOCORTICOIDS
V. GLUCOCORTICOID THERAPY
VI. HISTORY OF SELECTIVE GLUCOCORTICOID RECEPTOR MODULATORS
VII. CURRENT AND FUTURE RESEARCH AND DEVELOPMENT
VIII. CONCLUSIONS

**Glucocorticoids have broad clinical applications due to the anti-inflammatory, immunosuppressive, and cytostatic effects they elicit. Therapies for rheumatoid arthritis, collagen diseases, lymphatic leukemias and lymphomas, and asthma rely on the actions of glucocorticoids, making them one of the most widely employed classes of drugs.**

## I. INTRODUCTION

Glucocorticoids have a wide range of clinical applications, but unfortunately, their importance and utility are limited by serious side effects. Surprisingly, given their long history of use in the clinic, the molecular mechanisms of their therapeutic and adverse effects are poorly understood. This article will review the present understanding of glucocorticoid action in the body, summarize the historical evolution of glucocorticoid drugs to date, and then speculate as to how current research into the molecular mechanisms that generate the effects of glucocorticoids could drive the development of a future generation of drugs that mimic the therapeutic efficacy of glucocorticoids while minimizing some of their more serious side effects.

## II. PHYSIOLOGY OF GLUCOCORTICOIDS

Human physiology is controlled by the four major classes of hormones: amines, prostaglandins, polypeptides, and steroids. Glucocorticoid steroid hormones such as cortisol are synthesized from cholesterol in the adrenal cortex and target most cell types to regulate the basic functions of homeostasis and metabolism. Glucocorticoids stimulate mobilization of amino acids from muscle, mobilization of free fatty acids from adipose tissue, and deamination of amino acids and gluconeogenesis in the liver, all of which produce elevated blood glucose. Other physiologic actions of these steroid hormones include retarding inflammation, immunosuppression, and alteration of mood and cognition. Given their broad range of functions in many tissues, glucocorticoid levels are tightly regulated by a hormonal cascade within the hypothalamic–pituitary–adrenal (HPA) axis. Physiological stress also induces glucocorticoid production. Stress or circadian rhythms generate neural stimuli that induce corticotropin-releasing hormone (CRH) release by hypothalamic secretory cells. CRH and another factor, arginine vasopressin, cause the release of corticotropin (ACTH) by the anterior pituitary, which stimulates the secretion of glucocorticoids by the adrenal cortex. Glucocorticoids decrease the production of CRH in the hypothalamus and ACTH in the pituitary in a negative feedback loop.

## III. PHARMACOLOGY OF GLUCOCORTICOIDS

Glucocorticoids govern carbohydrate, lipid, and protein metabolism to effect homeostasis. They induce the production of glucose in the liver by increasing the levels of a number of gluconeogenic enzymes and by providing substrates for those enzymes through catabolic action in skeletal muscle and adipose tissue. Glucose uptake in muscle and adipose is inhibited by glucocorticoids, resulting in increased circulating glucose, which in turn blocks protein synthesis and lipogenesis, so ongoing proteolysis and lipolysis lead to increased levels of amino and free fatty acids that become available for further gluconeogenesis. These permissive actions on gluconeogenesis and adipose tissue metabolism contribute to the maintenance of body temperature. Glucocorticoids inhibit the synthesis of collagen, fibronectin, and glycosaminoglycan and prevent normal function of the intercellular matrix; thus, glucocorticoid excess contributes to poor wound healing and facile bruising. Glucocorticoids modulate the inorganic as well as organic aspects of bone metabolism by various mechanisms, thereby contributing to osteoporosis under conditions of long-term excess. Glucocorticoids affect the cardiovascular and renal systems directly through their actions on heart and smooth muscle, enhancement of catecholamine synthesis, antagonism of vasodilators such as prostaglandins, activation of the angiotensin system, and regulation of epithelial sodium channel expression in kidney and colon. Glucocorticoids not only act directly on the kidney to affect sodium retention and volume control, they also act indirectly through their crossover effects in the mineralocorticoid receptor pathway. Glucocorticoids also modulate inflammatory and allergic reactions and probably decrease the immune response to prevent autoimmune diseases. Glucocorticoids also show effects in central nervous system (CNS) imprinting and general CNS function.

## IV. MECHANISM OF ACTION OF GLUCOCORTICOIDS

Hormones such as glucocorticoids cause their effects in the body by binding to and activating intracellular receptors that in turn modulate gene expression. There is evidence that the steroids themselves or, more recently, the liganded receptors are capable of having very rapid nongenomic effects on the membrane of specific cell types (neural-derived cells in particular). These signaling pathways are only now being elucidated. The glucocorticoid receptor protein itself has separate domains dedicated to transcriptional activation, DNA binding, nuclear localization, and hormone binding. Compounds that bind and induce the activities of the natural hormone are termed agonists; compounds that bind but do not induce the activities of the natural hormone are termed antagonists. Compounds that fully induce or fully inhibit the activities of the natural hormone are considered full agonists and full antagonists, respectively. These classes lie at two ends of a continuum of partial or selective agonists, some of which may bind efficiently yet fail to induce all activities of the natural hormone, resulting in the regulation of a subset of the normal cadre of glucocorticoid-regulated genes.

The glucocorticoid receptor (GR) not only activates gene transcription when bound by glucocorticoid hormone, it represses the expression of a number of genes (Fig. 1). Gene activation by the GR proceeds by a widely accepted mechanism in which hormone diffuses across the cell membrane into the cytoplasm to where the receptor is held in an inactive complex by heat-shock proteins. Hormone binding induces a conformational change in the receptor that releases the heat-shock proteins and permits translocation of the receptor into the nucleus. The conformational changes induced upon hormone binding also allow dimerization of GR monomers, DNA binding at

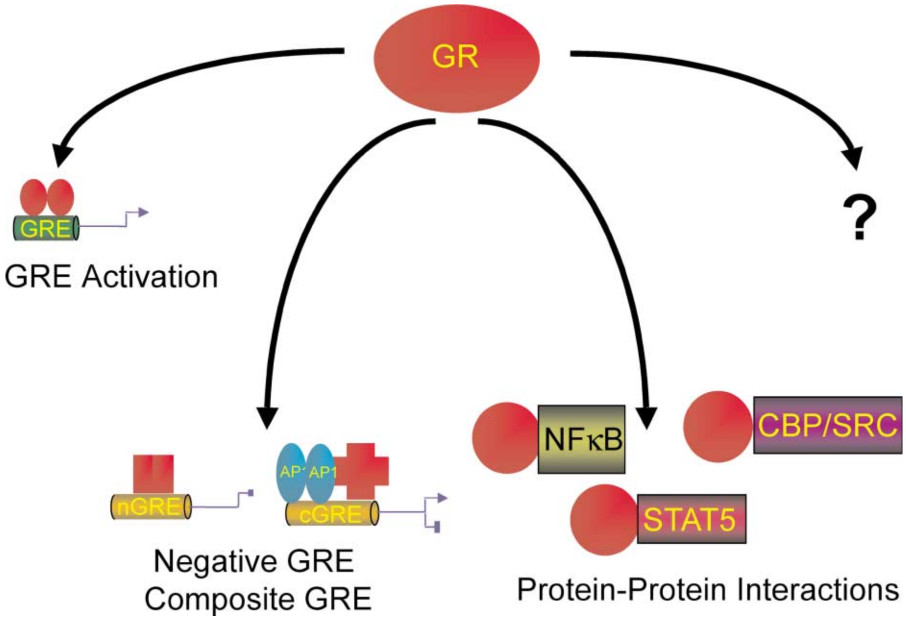

**FIGURE 1** Gene regulation by the GR occurs by several mechanisms. The GR is depicted as the oval. From left to right, a series of possible receptor interactions is shown. The receptor can bind to GREs and activate transcription or it can bind to negative GREs (nGREs) and repress transcription. It can also bind to so-called "composite" GREs (cGREs), where its transcriptional activity can be increased or decreased by other nonreceptor factors bound in the same region. The GR can also regulate transcription by interacting with specific transcription factors [nuclear factor κB (NF-κB); signal transducers and activators of transcription 5 (STAT5)] and co-activators [CREB-binding protein (CBP); steroid receptor co-activator (SRC)]. These interactions can indirectly regulate gene expression by either reducing or enhancing the activity of the associated proteins. There are likely other potential interactions as yet undiscovered, depicted by the question mark.

glucocorticoid-response elements (GREs) by GR dimers, and interaction of GR dimers with various ancillary proteins that regulate transcription, all of which lead to activation of gene transcription. Genes activated by GR include gluconeogenic enzymes such as phosphosenolpyruvate carboxykinase, glucose-6-phosphatase, serine dehydratase, and tyrosine aminotransferase.

The mechanisms by which the GR represses gene transcription are less clearly understood, although again they probably involve protein–protein interactions. The two cases in which the actions of the GR have been the most extensively studied are the AP-1 and nuclear factor κB (NF-κB) transcription factor systems, although the GR also interferes with the transcriptional activities of Sp-1, $Ca^{2+}$/cAMP-response element-binding protein (CREB), GATA-1, and Oct-1. Glucocorticoids probably repress NF-κB transcriptional activation through some combination of (1) up-regulation of the inhibitor κB protein that sequesters NF-κB in the cytoplasm, thereby abrogating NF-κB-dependent gene transcription, (2) indirect or direct protein–protein interactions between the GR and NF-κB that either mask the transactivation domains of NF-κB or disrupt its interactions with the basal transcription machinery, and (3) squelching or competing for interactions with critical transcriptional co-activator proteins. Glucocorticoid repression of AP-1 transcriptional activation does not appear to involve inhibition of DNA binding by AP-1 due to direct protein–protein interactions but may include GR inhibition of a phosphorylation event required for AP-1 activation. Genes repressed by glucocorticoids acting through the GR include prolactin, proliferin, proopiomelancortin, chorionic gonadotropin α-subunit, collagenase type I, collagenase-3, *POMC*, and phorbol ester induction of stromelysin and collagenase. Interestingly, a protein normally thought to be involved in transcriptional activation by GR (glucocorticoid receptor-interacting protein 1) was recently shown to be involved in transcriptional repression. Clearly, more work is needed to understand the process of repression. Glucocorticoids also inhibit the production of a range of proinflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-5, IL-6, IL-8, IL-12, interferon-γ, tumor necrosis factor α, and granulocyte/macrophage colony-stimulating factor.

## V. GLUCOCORTICOID THERAPY

Abnormal levels of glucocorticoid production by the body can lead to several pathophysiologies. Overproduction of glucocorticoids driven by adrenocortical adenoma or carcinoma leads to excess cortisol and the condition known as Cushing's syndrome. In addition to Cushing's disease of primary origin in which excess cortisol secretion is due to overproduction, a defect in the control of cortisol secretion, typically caused by bilateral adrenal hyperplasia, can secondarily produce cortisol hypersecretion and manifest as Cushing's disease. Clinical symptoms of cortisol excess include hypertension, glucose intolerance, hirsutism, trunkal obesity, and osteoporosis. Additionally, the prolonged hyperglycemia generated by excess cortisol can exhaust insulin-producing pancreatic beta cells, converting mere insulin resistance to outright diabetes mellitus. At the other end of the spectrum, glucocorticoid insufficiency causes Addison's disease, in which outright destruction of the adrenal glands, glandular atrophy, or reduced adrenocortical secretion due to secondary causes produces cortical insufficiency and results in weakness, weight loss, hypoglycemia, and other symptoms.

Exogenous glucocorticoid therapy is therapeutically efficacious for numerous indications (Fig. 2), the majority of which follow from the strong anti-inflammatory actions of glucocorticoid-liganded glucocorticoid receptor. Asthma and allergies are inflammatory conditions in which treatment with glucocorticoids has proven useful. The immunosuppressive activity of glucocorticoids has proven useful in the treatment of rheumatoid arthritis and inflammatory bowel disease and also in reducing the chances of implant rejection after organ transplantation. The combined anti-inflammatory and immunosuppressive actions of glucocorticoids presumably underlie the effectiveness of glucocorticoids in the treatment of a variety of autoimmune- and allergen-mediated skin diseases such as psoriasis, atopic dermatitis, and seborrheic dermatitis. In addition, glucocorticoids inhibit proliferation and induce apoptosis in lymphocytes, which underlies their widespread application in the treatment of malignant proliferative disorders of the lymphatic system.

Glucocorticoids have broad physiological effects across many systems. The side effects of glucocorticoid therapy are extensive. They occur in both acute and chronic dosing regimens, are dosage-dependent, and ultimately limit the clinical utility of glucocorticoids as drugs (Fig. 3). The most common side effects of glucocorticoid treatment include osteoporosis, diabetes, myopathy, impaired wound healing, fat redistribution, suppression of the HPA axis, disease flare upon withdrawal, and behavioral changes.

Glucocorticoids cause bone loss by at least three mechanisms. First, they increase calcium excretion and inhibit calcium absorption, leading to lower serum calcium levels. Second, glucocorticoids decrease the activity of osteoblasts, the cells responsible for laying down new bone. Finally, glucocorticoids decrease testosterone and estrogen levels, hormones that maintain bone density. The result of these various molecular effects is increased risk of fracture and aseptic necrosis of the femoral head, increased risk of degradation of trabecular bone, and increased fracture rate. Several bone-protective

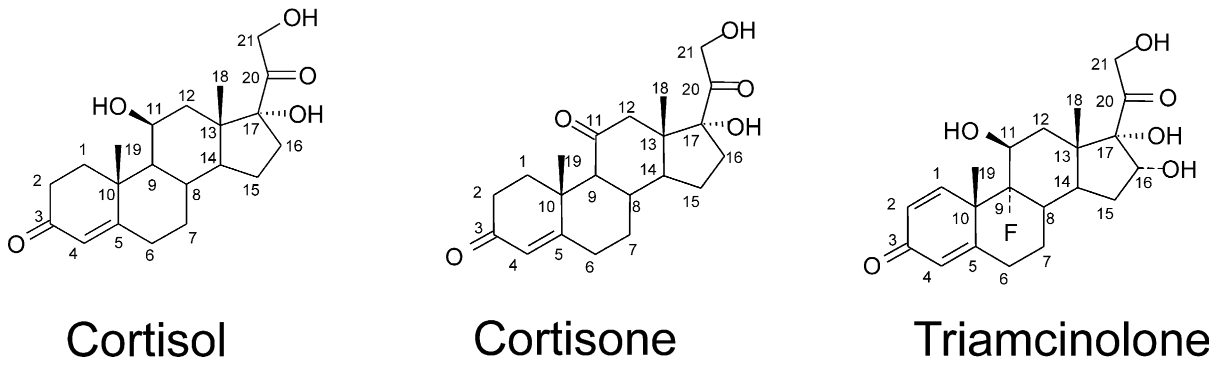

**FIGURE 2** The structures of three glucocorticoids are shown together with the numbering system identifying carbons in the molecule. Cortisol, the endogenous glucocorticoid, is characterized by a hydroxyl at the 11 position. Cortisone has a double-bond oxygen at the same position. Triamcinolone is cortisol with a fluorine at the 9 position and a hydroxyl at the 16 position.

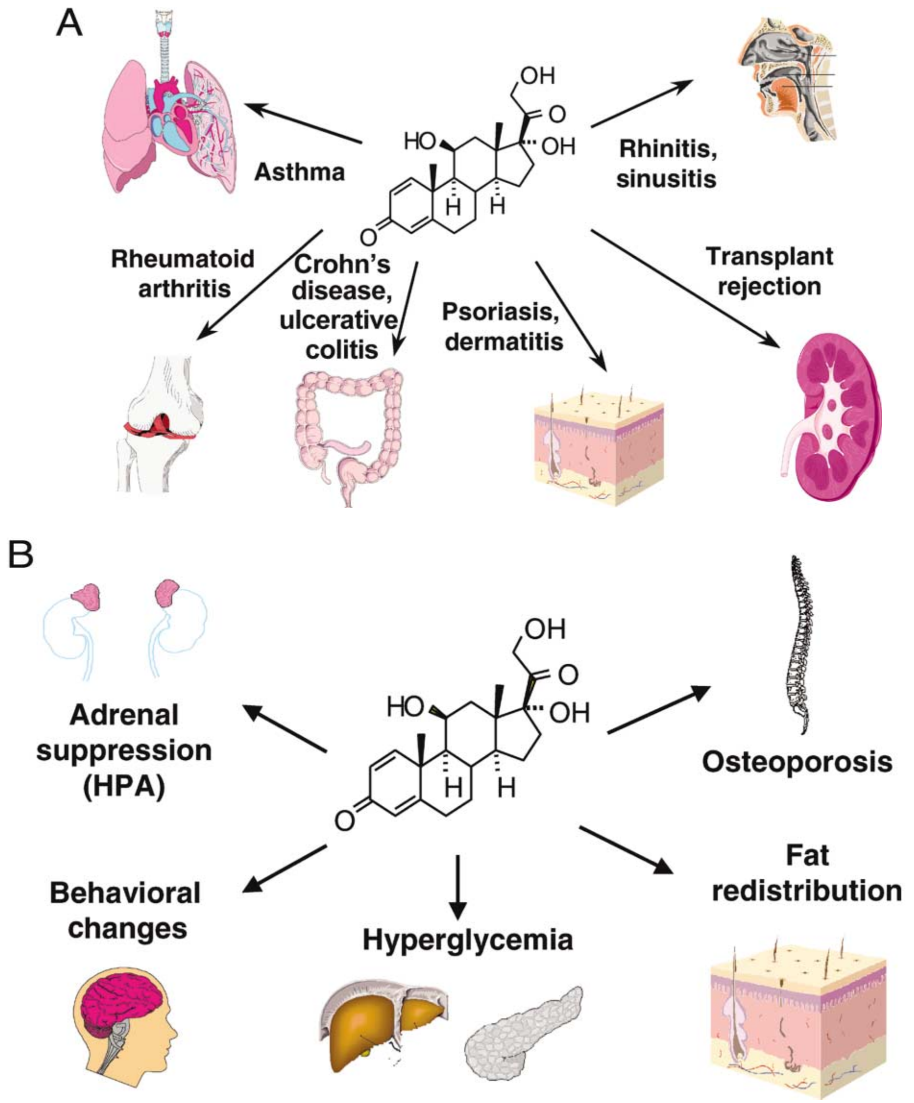
A
Asthma
Rhinitis, sinusitis
Rheumatoid arthritis
Crohn's disease, ulcerative colitis
Psoriasis, dermatitis
Transplant rejection
HO
OH
O
OH
H
H
O
B
Adrenal suppression (HPA)
Osteoporosis
Behavioral changes
Hyperglycemia
Fat redistribution

**FIGURE 3** (A and B) Glucocorticoid therapy is both effective and destructive to patients. (A) Anti-inflammatory therapy with glucocorticoids is effective against a wide variety of inflammatory conditions. The steroid prednisolone is shown in the center. (B) The side effects of steroids are problematic and include adrenal suppression, behavioral changes, hyperglycemia, fat redistribution, and osteoporosis.

therapies such as reduced duration and dosage of glucocorticoids, anabolic steroids in men, hormone replacement therapy in women, and bisphosphonate treatment can mitigate these effects. Additionally, inhaled steroids have a reduced though detectable effect on bone. Administration of anti-osteoporosis agents from the bisphosphonate class (Alendronate) will likely reduce the negative effects of glucocorticoids on bone.

Glucocorticoids (GCs) can induce insulin resistance and even outright diabetes through long-term and/or high-dose application. This adverse consequence of glucocorticoid therapy probably results from three facets of glucocorticoid action that together increase circulating glucose levels. First, GCs suppress the levels of important insulin receptor signaling molecules, thus dampening the uptake of glucose from serum in response to elevated glucose levels. Second, GCs raise hepatic levels of phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis, thereby increasing liver glucose output and further elevating glucose levels in the blood. Third, GCs inhibit insulin stimulation of blood flow to skeletal muscle, the major site of insulin-mediated glucose uptake in the body, reducing the opportunity to reduce glucose levels in the bloodstream through normal physiological mechanisms. Combination therapy with insulin-sensitizing agents potentially could reduce this side effect.

High-dose glucorticoid treatment induces various states of myopathy, disorders of muscle tissue or muscle. Atrophy of fast-twitch muscle fibers has been observed histologically in several studies of human and animal tissues. GCs repress gonadal production of testosterone, a hormone that acts to maintain muscle tissue, highlighting the problem of loss of muscle mass and ensuing increased risk of dangerous falls in patients who are already at risk for fractures from GC-induced osteoporosis. These effects are the result of the normal catabolic action of GCs: they induce the breakdown of fats and muscle proteins to generate substrates for gluconeogenesis in the liver.

Glucocorticoids impair wound healing by several mechanisms. First, the anti-inflammatory effects of GCs inhibit the inflammation required for the wound healing process. Second, epithelialization and wound contracture are diminished, perhaps due to inhibition by GCs of growth factor secretion and keratinocyte proliferation. Third, GCs inhibit collagen synthesis and cross-linking, which reduces the extent and strength of regenerative tissue at the wound site. Thus, glucocorticoids can impair wound healing and increase the risk of infection several fold.

High-dose and/or long-term glucocorticoid treatment can induce a distinctive accumulation and redistribution of fat in which fat is depleted in limbs and deposited in visceral and trunkal areas. In particular, glucocorticoid treatment stimulates fat accumulation in depots in the face, supraclavical, and visceral areas, leading to the moon face and buffalo hump phenotypes characteristic of long-term glucocorticoid therapy. Additionally, individuals on long-term GCs can experience weight gain and loss of lean body mass, although the former may be due to an improvement in the underlying disease condition. These actions of glucocortiocoids are probably due to effects on lipid metabolism and insulin resistance.

Prolonged glucocorticoid therapy may lead to suppression of the HPA axis and thereby increase the risk of developing adrenal insufficiency during periods of stress. Until recovery of the axis is total, patients may need regular physiologic replacement doses administered in a tapering format; high-dose supplemental therapy may be required during a major illness or surgery.

The disease condition of some patients will flare upon the withdrawal of glucocorticoid therapy. This has been observed during treatments for inflammatory bowel diseases such as moderate to severe Crohn's disease, severe ulcerative colitis, and moderate ulcerative colitis.

High doses of glucocorticoid have been shown to induce euphoria or psychosis in a small fraction of patients, perhaps driven by cross-reactivity of GCs with the mineralocorticoid receptor (MR). The MR has been shown in rodents to be involved in a number of behaviors associated with exogenous treatment by glucocorticoids, and MR and GR levels are particularly high in the hippocampus, a region of the brain that is critical for the regulation of behavior.

## VI. HISTORY OF SELECTIVE GLUCOCORTICOID RECEPTOR MODULATORS

Limited amounts of cortisone were first purified from animal adrenals in 1935–1936. A route of production was developed, involving synthesis from a bile acid, and in 1949, cortisone was first offered commercially. In 1948, at the Mayo Clinic, daily injections of a corticosteroid were administered to a cohort of arthritis patients. The results were so dramatic and the improvement was so significant that it was originally held that the cure for arthritis had been discovered. As the use of corticosteroids increased over subsequent years, side effects appeared and it was realized that large doses, given over prolonged periods of time, yielded significant adverse effects. Patients began to decline treatment because of the potential problems, and the use of corticosteroids became more conservative. Since that time organic chemists have synthesized a vast array of modified corticosteroids, with the goal of maintaining efficacy while reducing side effects.

Early approaches concentrated on finding compounds with greater anti-inflammatory activity than the natural steroid, cortisol. Introduction of a double bond at the 1,2 position of cortisol yields the well-known anti-inflammatory drugs prednisone and prednisolone. Prednisolone is the active form and is formed from prednisone by first-pass enzymatic conversion in the liver to the 11-hydroxyl form. These compounds have a four- to fivefold increase in anti-inflammatory activity with somewhat reduced sodium retention relative to cortisol. In addition, prednisolone has an increased duration of action relative to cortisone due to its slower metabolism. Combining the 1,2 double bond with the introduction of fluorines at the 6,6-methyl or 9 positions further potentiated anti-inflammatory activity. These compounds, however, led to much greater increases in sodium and water retention than cortisol relative to anti-inflammatory activity due to increased mineralocorticoid activity. Further introduction of a methyl group at the 16 position combined with the 1,2 double bond and 9-fluoro substitution forming, for example, the potent anti-inflammatory dexamethasone led to further increases in anti-inflammatory activity while greatly reducing the mineralocorticoid activity. Hydroxylation at the 16 position had an effect similar to that of methylation (triamcinolone). For all of the systemic steroids in current use, although the anti-inflammatory potency is high and problems with sodium and water retention are somewhat lower, all of the other systemic problems caused by cortisol remain.

In order to use these glucocorticoids as topical agents, it was highly desirable to increase lipophilicity to improve penetration. For example, masking the 16- and 17-hydroxyls of triamcinolone as acetonides proved very effective. Numerous other strategies also proved very effective, especially for use on the skin. However, in the treatment of asthma, even the small amount of systemic activity seen with a variety of potent compounds could be problematic. In order to further reduce the systemic effects, several strategies were used. For example, fluticasone propionate has the same activating substitutions previously used (1,2 double bond, 6-fluoro, 9-fluoro, 16-methyl) but also two substitutions at the 17 position to enhance topical activity, a 17β-fluoromethyl carbothioate and 17α-propionate. This yields not only an exceedingly potent compound but also one that is likely to be highly metabolized at the 17β position to the inactive carboxylic acid. This is clearly a potential improvement in topical steroids for asthma, but for systemic administration there is clearly room for significant improvement in the development of more useful anti-inflammatory agents. Along these lines, the steroid deflazacort was originally believed to have less impact on bone while maintaining efficacy as an anti-inflammatory agent. This compound is a D-ring-substituted steroid that is otherwise similar to cortisol. The initial clinical data on deflazacort were quite encouraging, suggesting a decreased impact on both bone and glucose metabolism. Multiple randomized clinical trials appeared to indicate that deflazacort indeed has less severe side effects. The difficulty lies in demonstrating equivalent anti-inflammatory efficacy between prednisone and deflazacort. As described above, clear equivalence trials require large numbers of patients to ensure that a small but significant difference could be detected. Many of these clinical trials relied on the original determination of a 1:1.2 relative potency ratio described by the manufacturer (5 mg of prednisone = 6 mg of deflazacort). Subsequent trials that adjusted the steroid dose to maintain equivalent anti-inflammatory efficacy usually needed higher levels of deflazacort than the ratio of 1.2. Thus, trials comparing side effects may not have used biologically equivalent doses of deflazacort. Unfortunately, at these higher doses, the advantages of deflazacort vanished. In 1997, a group from Roussell Uclaf published the second attempt at a selective glucocorticoid receptor modulator after deflazacort. These compounds were again steroids, but these authors had used the molecular hypothesis described above and found several steroidal structures that were capable of separating transcriptional activation from repression (RU24858, RU40066, and RU24782). This group was able to show significant differences between their compounds and commonly used steroids. These molecules were very efficient inhibitors of both AP-1- and NF-κB-mediated gene induction and were strong anti-inflammatory agents *in vivo*. They also appeared to have reduced ability to activate gene expression in some contexts. Subsequent *in vivo* experiments from another group have not shown a therapeutic advantage of these types of molecules for body weight, thymic involution, and inhibitory effects at the growth plate of the femoral head, but the jury is still out. The results with these molecules have been utilized to suggest that the transcriptional repression/activation hypothesis fails to translate into therapeutic benefit; this view remains controversial.

## VII. CURRENT AND FUTURE RESEARCH AND DEVELOPMENT

Since current formulations of glucocorticoids provide clear therapeutic value at existing levels of efficacy, new drug discovery in the area of selective glucocorticoid receptor modulators is and will be aimed at reducing the side effects that currently limit the clinical application of glucocorticoids. Although the specific side effects that could be targeted may vary depending on the disease target and patient population, a decrease in the rate and/or amount of bone loss induced by glucocorticoid therapy would represent a very significant improvement in the treatments available to patients on both anti-cancer and anti-inflammatory courses. Reduction in behavioral side effects and dysfunctional fat metabolism also would provide major clinical benefits. The therapeutic efficacy and side effects of glucocorticoids are

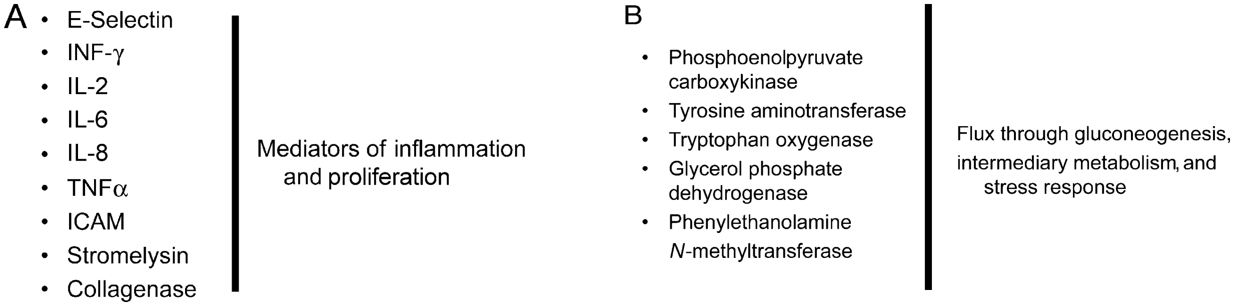
A
E-Selectin
INF-γ
IL-2
IL-6
IL-8
TNFα
ICAM
Stromelysin
Collagenase
Mediators of inflammation and proliferation
B
Phosphoenolpyruvate carboxykinase
Tyrosine aminotransferase
Tryptophan oxygenase
Glycerol phosphate dehydrogenase
Phenylethanolamine N-methyltransferase
Flux through gluconeogenesis, intermediary metabolism, and stress response

**FIGURE 4** (A and B) Glucocorticoid receptor gene targets. (A) GC-mediated transcriptional repression; a large number of genes are involved in mediating inflammation and proliferation. (B) GC-mediated transcriptional activation; a number of genes are involved in the metabolic regulation of glucose homeostasis, fatty acid metabolism, and muscle metabolism.

mediated by the glucocorticoid receptor liganded to the glucocorticoid agent, and thus current and future work in pharmaceutical and academic laboratories is focused on identifying receptor modulators, compounds that possess only a portion of the activities of the natural steroids when bound to the receptor. With the publication of the crystal structures of the steroid- or ligand-binding domains of several steroid receptors, it has become apparent that the ligand-binding domain of intracellular receptors such as the glucocorticoid receptor transmits information from the ligand buried in its binding pocket out to the surface of the receptor. This information transfer allosterically contributes to the structure of the protein–protein interaction surfaces of the receptor, and changes in the structure of these surfaces lead to altered interactions with the panoply of proteins with which the glucocorticoid receptor cooperates to effect changes in gene expression. There are at least two general strategies for drug discovery that exploit these ligand-driven changes in the structure of interaction surfaces of the receptor. One approach is to ascribe the beneficial effects of glucocorticoids to one set of molecular functions of the glucocorticoid receptor and the side effects to another, distinct set molecular functions of the receptor and then attempt to discover ligands for the receptor that cause the receptor to retain the molecular functions leading to the beneficial actions but not the functions leading to the side effects. A separate approach follows the experience of the estrogen receptor field and the discovery of selective estrogen receptor modulators (SERMs), in which the beneficial effects of the steroid are assigned to receptor action in certain tissues in the body and the side effects are assigned to the actions of the receptor in other tissues; then, attempts are undertaken to discover ligands for the receptor that cause the receptor to be active in the tissues leading to the beneficial actions but not in the tissues leading to the side effects.

The first approach, based on the hypothesis that certain cellular functions of the glucocorticoid receptor are associated with the beneficial effects of a ligand and that other functions are associated with side effects, derives partially from the body of work demonstrating that transcriptional repression of specific pro-inflammatory genes by the glucocorticoid receptor may be involved in the anti-inflammatory efficacy of glucocorticoids (Fig. 4). Another body of work supports the argument that at least some of the side effects can be associated with transcriptional activation of certain metabolic genes in liver and fat (Fig. 5). In particular, genetic experiments in mice have provided powerful evidence for the separation of transcriptional activation from repression. Mutations in the dimerization domain of the glucocorticoid receptor that abrogate its DNA-binding ability and thus eliminate its transcriptional activation from some GREs have been created. Importantly, these mutations had little or no effect on transcriptional repression, which generally does not require association with canonical GREs, yet glucocorticoids retain their anti-inflammatory activity in these animals and can still down-regulate various pro-inflammatory cytokines. However, in response to glucocorticoids, these mice do not activate the transcription of liver enzymes that mediate glucose production. Following this hypothesis, selection of compounds that demonstrate an enhanced repression:activation ratio over steroids should yield a more beneficial therapeutic ratio than current glucocorticoids. However, the activation/repression hypothesis is debatable. For example, Lipocortin, a protein that supposedly mediates some anti-inflammatory activity, is up-regulated by the transcriptional activation of glucocorticoids. Thus, compounds that retain their transcriptional repression abilities but lose the transcriptional activation that induces Lipocortin may have reduced anti-inflammatory properties. Furthermore, both osteocalcin and osteoprotegerin, important molecules in bone remodeling, are repressed by glucocorticoids; this repression could contribute to the osteoporosis associated with steroid use, and accordingly any compound that represses transcription may cause negative side effects in bone. Obviously, then, it will be difficult to predict the *in vivo* activities of compounds that favor transcriptional repression over activation, and only their synthesis and testing will determine whether the activation/repression hypothesis is useful for drug discovery.

The second approach follows the experience of the estrogen receptor field and the discovery of SERMs, in which beneficial effects of the steroid are assigned to receptor action in certain tissues in the body and the side effects are assigned to the actions of the receptor in other tissues, and then one attempts to discover ligands for the receptor that cause the receptor to be active in the tissues leading to the beneficial actions but not in the tissues leading to the side effects. One possibility in this model would be to use molecular and cellular assays to screen for compounds with reduced activity on the receptor in liver, fat, bone, and muscle—tissues in which glucocorticoid receptor-mediated side effects predominate—but that retain the desired anti-inflammatory activity. This strategy has been successfully employed in the estrogen receptor field to find selective estrogen receptor modulators that have reduced side effects in breast and uterus compared to estrogen but retain therapeutic efficacy in bone and heart.

A "third way" that combines tissue selectivity and selectivity with respect to the molecular functions activated in the glucocorticoid receptor by its ligands relies on the fact that there are individual promoters and tissues that require and express different sets of transcriptional co-activators and co-repressors for the function of the glucocorticoid and other steroid receptors. This approach relies on the accepted wisdom in the field that ligand binding determines steroid receptor conformation, steroid receptor conformation determines what subset of the set of transcriptional co-activators and co-repressors present in any given tissue is recruited to the steroid receptor in the presence of the ligand, and the set of transcriptional co-activators and co-repressors recruited determines the level and gene selectivity of transcriptional activation that is called the agonism, partial agonism, or antagonism of the ligand. Theoretically, a drug discovery program could be organized through this paradigm to develop novel glucocorticoid receptor modulators. The effort could be based on identifying ligands of the glucocorticoid receptor that alter the set of co-activators and co-repressors with which the liganded receptor interacts. In general, a ligand that permits only a subset of co-factor interactions should induce only a subset of the activities of the steroidal glucocorticoid ligands, and when combined with the differential representation of co-factors in various tissues in the body, this restricted co-factor recruitment profile should predict restricted *in vivo* activities in terms of both molecular functional specificity and tissue specificity (Fig. 5).

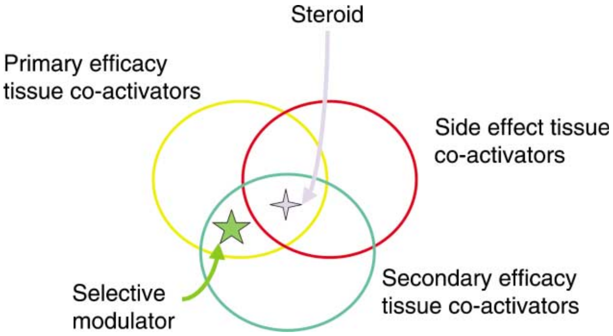
Steroid
Primary efficacy tissue co-activators
Side effect tissue co-activators
Selective modulator
Secondary efficacy tissue co-activators

**FIGURE 5** Selective co-activator interactions. Individual promoters and tissues are known that require and express different sets of transcriptional co-activators and co-repressors for the function of the glucocorticoid and other steroid receptors. It has been well established for numerous nuclear receptors that ligand binding determines steroid receptor conformation, steroid receptor conformation determines what subset of the set of transcriptional co-activators and co-repressors present in any given tissue is recruited to the steroid receptor in the presence of the ligand, and the set of transcriptional co-activators and co-repressors recruited determines the level and gene selectivity of transcription. Gene activation by glucocorticoids in various tissues in the body results in both therapeutic efficacy and detrimental side effects. Glucocorticoids and other steroids recruit distinct sets of co-factors in each of the tissues in which therapeutic effects and side effects emerge. A novel drug discovery paradigm for the identification of selective glucocorticoid receptor modulators would be to select for ligands that recruit to the receptor the same co-factors as glucocorticoids in the tissues in which efficacious results are desired, yet do not recruit to the receptor the same co-factors as glucocorticoids in the tissues in which side effects would be generated. This should generate full gene activation leading to therapeutic efficacy and reduced gene activation leading to side effects.

## VIII. CONCLUSIONS

The identification of a tissue- or a function-selective glucocorticoid modulator has yet to be achieved, and though compounds with suggestive *in vitro* activities have been found, it is not yet clear that these compounds have the necessary profile *in vivo* to continue to provide the anti-inflammatory and anticancer activities of existing glucocorticoids while avoiding their most serious side effects. However, recent and ongoing research into the molecular and cellular actions of the glucocorticoid receptor when bound by glucocorticoids and their synthetic analogues provides both starting points for further research and hope that new drugs will be developed to address this large and unmet medical need.

### Acknowledgments

We thank Robin Chedester for preparation of the manuscript, Mark Chapman and Mike Stallcup for critical comments, and Ben Lane and Bob Higuchi for providing figures.

### Glossary

**glucocorticoid** Any of a group of hormones having the characteristic ring structure of steroids (such as hydrocortisone or dexamethasone) that are involved in carbohydrate, protein, and fat metabolism, that tend to increase liver glycogen and blood sugar, that are anti-inflammatory and immunosuppressive, and that are used widely in medicine (as in the alleviation of the symptoms of rheumatoid arthritis).

**intracellular receptors** A family of related transcription factors, some of which have affinity for specific hormones. These proteins mediate a wide range of physiological processes and are directly responsible for the effect of retinoids, estrogens, glucocorticoids, progestins, androgens, and thiazolidinediones, among others.

**modulators** Small-molecule drugs that mimic endogenous steroids by binding to intracellular steroid hormone receptors, but on binding allow only a subset of the molecular, cellular, and physiological responses that would be induced by the native steroid hormone to occur, thus effecting a desired pharmaceutical outcome.

### See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids and Autoimmune Diseases • Glucocorticoids, Pharmacology of**

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# Glucocorticoid Effects on Physiology and Gene Expression

Ian M. Adcock
*Imperial College of Science, Technology, and Medicine, London*

I. INTRODUCTION
II. EFFECTS ON CARBOHYDRATE AND PROTEIN METABOLISM
III. ANTI-INFLAMMATORY/IMMUNOSUPPRESSIVE EFFECTS
IV. OTHER ACTIONS
V. CONTROL OF CORTISOL RELEASE
VI. MECHANISM OF CORTISOL ACTION
VII. DISEASE STATES

**The major glucocorticoid in most mammals is cortisol. Cortisol secretion increases in response to any stress in the body, whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological. However, this hormone is more than a simple marker of stress levels—it is necessary for the correct**

**functioning of almost every part of the body. Excesses or deficiencies of this crucial hormone also lead to various physical symptoms and disease states.**

## I. INTRODUCTION

The vast majority of glucocorticoid activity in most mammals is from cortisol, also known as hydrocortisone. Corticosterone is the major glucocorticoid in rodents. Although cortisol is not essential for life per se, it helps an organism to cope more efficiently with its environment. Nevertheless, loss or profound diminishment of cortisol secretion leads to a state of abnormal metabolism and an inability to deal with stressors, which, if untreated, may be fatal.

Cortisol is made in the zona fasciculata of the adrenal glands (Fig. 1). Its important functions in the body include roles in the regulation of blood pressure and cardiovascular function as well as regulation of the body's use of proteins, carbohydrates, and fats. Ninety percent of the secreted cortisol in circulation is bound to protein, mainly to cortisol-binding globulin. This protein binding probably decreases the metabolic clearance rate of glucocorticoids and, because the bound steroid is not biologically active, tends to act as a buffer and blunts wild fluctuations in cortisol concentration.

The body's level of cortisol in the bloodstream displays a diurnal variation; that is, normal concentrations of cortisol vary throughout a 24 h period. Cortisol levels in normal individuals are highest in the early morning at approximately 8 AM and are lowest just after midnight. This early morning dip in cortisol level often corresponds to increased symptoms of inflammatory disease in human.

## II. EFFECTS ON CARBOHYDRATE AND PROTEIN METABOLISM

The name glucocorticoid derives from early observations that these hormones were involved in glucose metabolism. This role is most apparent in the fasted state, in which cortisol stimulates several processes that collectively serve to increase and maintain normal concentrations of glucose in blood. When cortisol is secreted, it causes a net reduction of extrahepatic protein stores, including skeletal muscle, leading to the release of amino acids into the bloodstream. This is due to both protein catabolism and reduced protein synthesis, possibly as a result of reduced amino acid transport into muscle cells. These amino acids are then transported to the liver, where they are used to synthesize glucose, which is required for energy production, in a process called gluconeogenesis (Fig. 2). Enhancing the expression of key

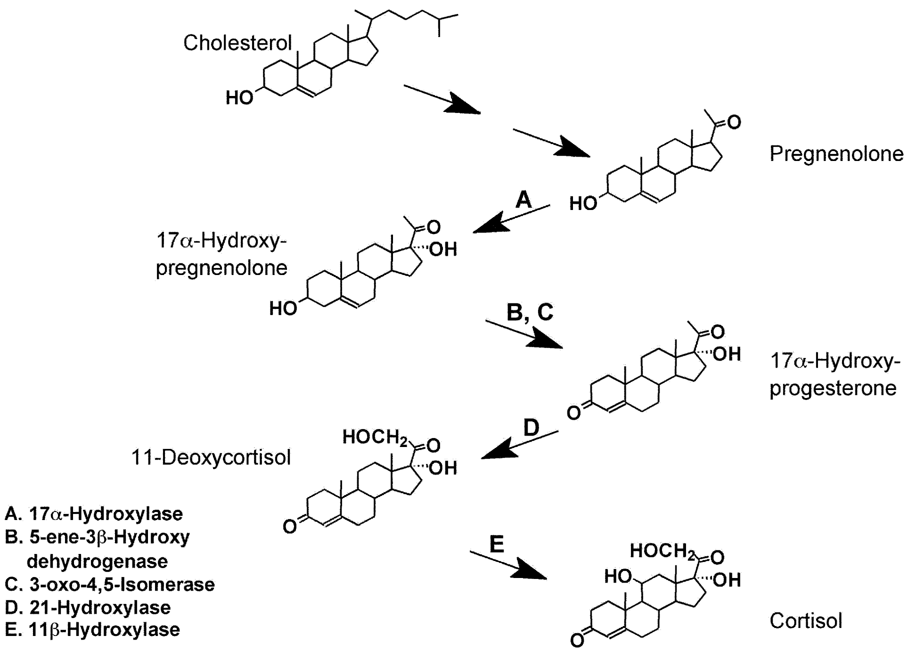

FIGURE 1 Pathway of cortisol synthesis from cholesterol in the liver. Pregnenolone is converted to 17α-hydroxyprogesterone before subsequent conversion to cortisol.

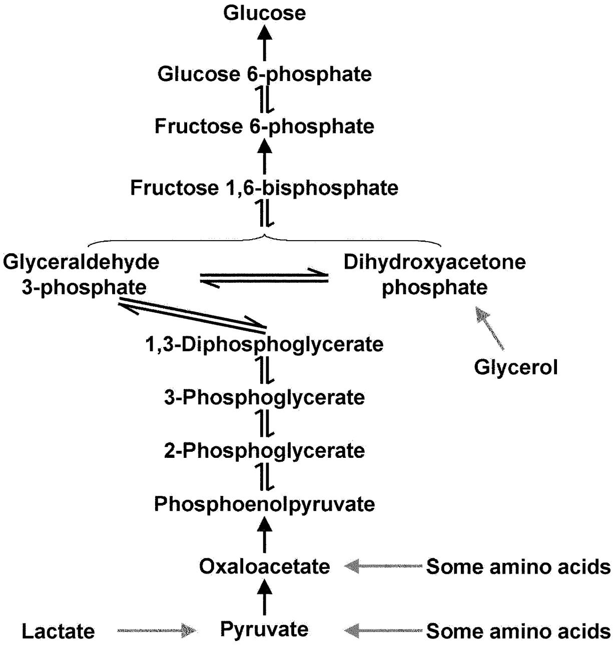

FIGURE 2 Pathway of gluconeogenesis. The distinctive reactions are the conversion of pyruvate to oxaloacetate and oxaloacetate conversion to phosphoenolpyruvate. The other reactions are common to glycolysis. The entry points for lactate, glycerol, and amino acids are shown.

enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphate, and glycogen synthase, involved in gluconeogenesis is probably the best known metabolic function of glucocorticoids. The gene for PEPCK is acutely upregulated by cortisol in the liver and kidney, whereas insulin inhibits this process. PEPCK is absent in fetal liver but appears at birth, concomitant with the capacity for gluconeogenesis.

Gluconeogenesis is crucially important since the brain uses glucose as its primary energy source. Typically, the brain requires 120 g glucose per day and the body has reserves of 190 g available from glycogen stores. Therefore, during fasting and extreme exercise, glucose must be formed from noncarbohydrate sources to supplement cortisol-induced glycogen breakdown. At the same time, the other tissues of the body, including muscle and adipose tissue, but not the brain, decrease their use of glucose as fuel. As such, the actions of cortisol are to oppose those of insulin, generally by reversing the effects of insulin on enzyme expression.

Cortisol also stimulates the breakdown of fat in adipose tissue. The lipolytic activity of cortisol itself is not high but it enhances the actions of other lipolytic agents such as adrenaline and growth hormone. The fatty acids released by lipolysis are used for production of energy in tissues such as muscle, and the released glycerol provides another substrate for gluconeogenesis. Cortisol, in addition, moderately enhances the oxidation of cellular fatty acids secondary to reduced availability of glycolytic products. This process becomes increasingly important in times of starvation. Taken together, these energy-directing processes prepare the individual to deal with stressors and ensure that the brain receives adequate energy sources.

## III. ANTI-INFLAMMATORY/ IMMUNOSUPPRESSIVE EFFECTS

Separate from its metabolic function, increased levels of glucocorticoids serve as potent suppressors of the immune and inflammatory systems. This is particularly evident when they are administered at pharmacologic doses but also is important in normal immune responses. As a consequence, glucocorticoids are widely used as drugs to treat inflammatory conditions such as arthritis, asthma, or dermatitis and as adjunct therapy for conditions such as autoimmune diseases. Synthetic glucocorticoids may also be used in organ transplantation to reduce the chance of rejection. Thus, although the early effects of cortisol are to stimulate the immune system, cortisol and synthetic glucocorticoids predominantly repress the inflammatory response by decreasing the activity and production of immunomodulatory and inflammatory cells.

The usefulness of glucocorticoids in treating inflammatory diseases was exemplified by the early work of Kendall and Hench. In a classic experiment, 100 mg of cortisone was injected into the muscle of a patient (Mrs. G.) suffering from chronic rheumatoid arthritis on September 21, 1948. Seven days later, the patient was able to walk to the shops for the first time in years. Kendall and Hench were awarded the Nobel prize for this work in 1950 and it represented a new approach to therapy with natural hormones by utilizing pharmacological, rather than physiological, doses.

There are five main aspects of inflammation: (1) the release of inflammatory mediators, such as histamine, prostaglandins, leukotrienes, cytokines, and chemokines; (2) increased blood flow in the inflamed area (erythema) caused by some of the released factors; (3) leakage of plasma from the vasculature into the damaged area (edema) due to increased capillary permeability; (4) cellular infiltration signaled by chemoattractants; and (5) repair processes such as fibrosis. Glucocorticoids can modify all of these processes.

Glucocorticoids act on numerous inflammatory and immunomodulatory cells, including leukocytes, leading to a reduction in their localization at inflammatory sites. This is achieved by reducing the expression of adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1), particularly on endothelial cells, and consequently decreasing the movement of cells (T cells, monocytes, macrophages, and eosinophils) from the vasculature to the inflamed tissue. Glucocorticoids can also affect cell survival and death; as such, they can induce eosinophil and T-cell apoptosis and promote neutrophil survival. Inflammatory cells release a host of chemical mediators that enhance and perpetuate the inflammatory response. These mediators include cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), and chemokines, such as eotaxin and IL-8. Glucocorticoids inhibit leukocyte function and responsiveness to inflammatory signals by suppressing the expression and release of all these factors. In addition, glucocorticoids reduce the expression of the receptors for these mediators along with the products of arachadonic acid metabolism (prostaglandins and leukotrienes).

## IV. OTHER ACTIONS

The loss of the placenta at birth importantly deprives the fetus of a source of oxygen, glucose, and heat. In anticipation of these requirements, several organs, including the lungs, the gastrointestinal tract, the skin, the retina, and the central nervous system, undergo maturational changes in late pregnancy aimed at meeting these needs. For example, the lungs mature structurally and functionally, becoming distensible and capable of coping with high surface tension when air enters the alveoli with the first breath. Cortisol increases the rate of alveolar development, flattening of the lining cells, and thinning of the lung septa. In addition, cortisol stimulates the production of the phospholipid surfactant protein A. Mice with homozygous disruptions in the corticotropin-releasing hormone gene (CRH; see below) die at birth due to pulmonary immaturity. In addition, just before birth, cortisol induces a number of enzymes that are essential for survival postpartum. Thus, cortisol also increases the production of triiodothyronine and catecholamines in preparation for the sharp increase in metabolic rate and thermogenesis associated with breathing and the cold environment encountered by the newborn. In human, if birth is expected before 34 weeks gestation, maternal glucocorticoid supplementation reduces the likelihood of respiratory distress syndrome.

Cortisol maintains the contractility and work performance of skeletal and cardiac muscle (Fig. 3). This inotropic action may result from increased acetylcholine release or induction of the $Na^+$,$K^+$-ATPase and β-adrenergic receptors. However, to counterbalance this effect, cortisol decreases muscle protein synthesis, increases catabolism, and reduces muscle mass. This process accounts for the peripheral muscle loss seen in Cushing's disease (see below).

Another important role of cortisol is in the maintenance of normal blood pressure, an effect due primarily to modulating arteriole responsiveness to catecholamines and angiotensin II. Cortisol also

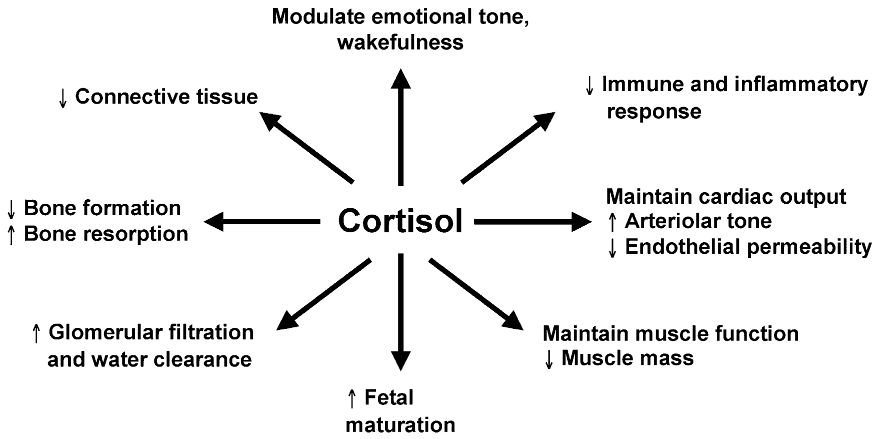

FIGURE 3 Overview of the major actions of cortisol on various tissues, organs, and systems other than its effects on carbohydrate metabolism.

decreases the synthesis of prostaglandins, which are important vasodilators and decrease endothelial cell permeability.

Cortisol also has important actions on bone formation. Cortisol reduces type 1 collagen synthesis, the fundamental component of bone matrix, and decreases osteoblast activity as detected by reduced osteocalcin release. Exacerbating these effects, cortisol also decreases the absorption of calcium from the intestinal tract by antagonizing 1,25-$(OH)_2$-vitamin $D_3$ synthesis and actions. These effects, in concert with an increase in the rate of bone resorption, promote an overall reduction in bone mass (osteoporosis). Cortisol also inhibits the synthesis of other collagens, resulting in skin thinning and capillary fragility.

Without adequate cortisol in the body, renal function is impaired, suggesting an important role in kidney function. Cortisol increases glomerular filtration by a combination of enhanced glomerular plasma flow and decreased preglomerular resistance. An additional action of cortisol is to inhibit antidiuretic hormone release from the hypothalamus by feedback inhibition, thereby further increasing kidney function.

Receptors for cortisol are fairly abundant in the brain, particularly within the limbic system and the hippocampus, and not surprisingly, cortisol is also known to alter mood, behavior, and sleeping patterns although the mechanisms for these effects are unknown. Long-term high-dose glucocorticoid therapy for immune and inflammatory diseases has a number of well-known side effects on the central nervous system.

## V. CONTROL OF CORTISOL RELEASE

The body possesses an elaborate feedback system for controlling cortisol secretion and regulating the amount of cortisol in the bloodstream (Fig. 4). Cortisol is secreted in response to a single stimulator, ACTH, which is synthesized and released from the anterior pituitary gland, a small organ at the base of the brain, and is derived from the precursor molecule proopiomelanocortin (POMC). ACTH binds to receptors in the plasma membrane of cells in the zona fasciculata and reticularis of the adrenal. ACTH-receptor engagement stimulates G-protein activation, leading to enhanced adenyl cyclase activity and elevated intracellular levels of cyclic AMP. This ultimately leads to activation of the enzyme systems involved in the biosynthesis of cortisol from cholesterol (Fig. 1). ACTH is itself secreted under the control of the hypothalamic peptide CRH. The central nervous system thus controls glucocorticoid responses, providing an excellent example of close integration between the nervous system and the endocrine system.

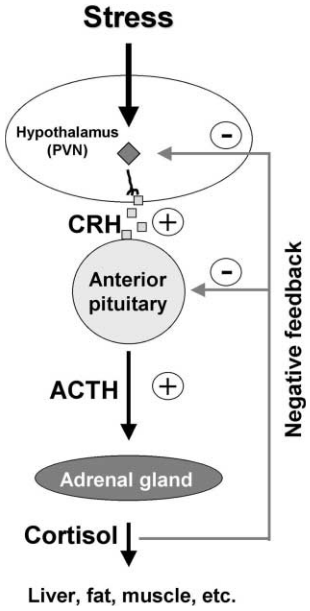

FIGURE 4 Feedback inhibition of stress-induced corticotropin-releasing hormone (CRH)- and adrenocorticotropic hormone (ACTH)-mediated production of cortisol. CRH released by the paraventricular nucleus (PVN) within the hypothalamus travels via the portal vessel to the anterior pituitary, where it stimulates ACTH production. ACTH, in turn, is released and stimulates adrenal cortisol synthesis and release. To maintain homeostasis, cortisol feeds back on this pathway to attenuate cortisol release.

Almost immediately after a stressful event, the levels of the regulatory hormones ACTH and CRH increase, causing an immediate rise in cortisol levels. When cortisol is present in adequate (or excess) amounts, a negative feedback system operates on the pituitary gland and hypothalamus, alerting these areas to reduce the output of ACTH and CRH, respectively, in order to reduce cortisol secretion to normal homeostatic levels. This is mediated by cortisol acting on glucocorticoid receptors (GRs) within the hypothalamus and pituitary gland that act to suppress the expression and release of both ACTH and CRH. This combination of positive and negative control on CRH secretion results in pulsatile secretion of cortisol. Typically, pulse amplitude and frequency are highest in the morning and lowest at night, accounting for the diurnal rhythm of cortisol.

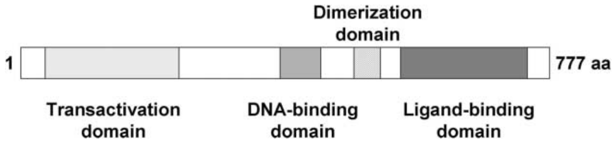
Dimerization domain
1
777 aa
Transactivation domain
DNA-binding domain
Ligand-binding domain

**FIGURE 5** Modular structure of the human 777-amino-acid glucocorticoid receptor.

## VI. MECHANISM OF CORTISOL ACTION

Cortisol and other synthetic glucocorticoids exert their effects by binding to a single 777-amino-acid GR that is predominantly localized to the cytoplasm of target cells. GRs are expressed in almost all cell types and the density of GRs varies from 200 to 30,000 binding sites per cell. The affinity of the GR for cortisol is approximately 30 nM, which falls within the normal range for plasma concentrations of free hormone.

The GR has several functional domains (Fig. 5). The glucocorticoid ligand-binding domain is at the carboxyl-terminus of the molecule and is separated from the DNA-binding domain by a hinge region. There is an N-terminal transactivation domain that is involved in the activation of genes once binding to DNA has occurred. This region may also be involved in binding to other transcription factors. The inactive GR is part of a large protein complex (~300 kDa) that includes two subunits of the heat-shock protein Hsp 90, which blocks the nuclear localization of GR.

Cortisol can freely diffuse from the circulation into cells across the cell membrane and bind to the GR (Fig. 6A). Once cortisol binds to the GR, Hsp 90 dissociates, allowing the nuclear localization of the activated GR–cortisol complex and its binding to DNA. The GR combines with another GR to form a dimer at consensus DNA sites, termed glucocorticoid-response elements (GREs), in the regulating regions of steroid-responsive genes. The GRE is composed of two palindromic half-sites (AGAACA) separated by three nucleotides. This interaction allows the GR to associate with a complex of DNA-modifying and -remodeling proteins including steroid receptor co-activator-1 (SRC-1) and CREB-binding protein (CBP), which produce a DNA–protein structure that allows enhanced gene transcription. The number of GREs and their position relative to the transcriptional start site may be an important determinant of the magnitude of the transcriptional response to steroids.

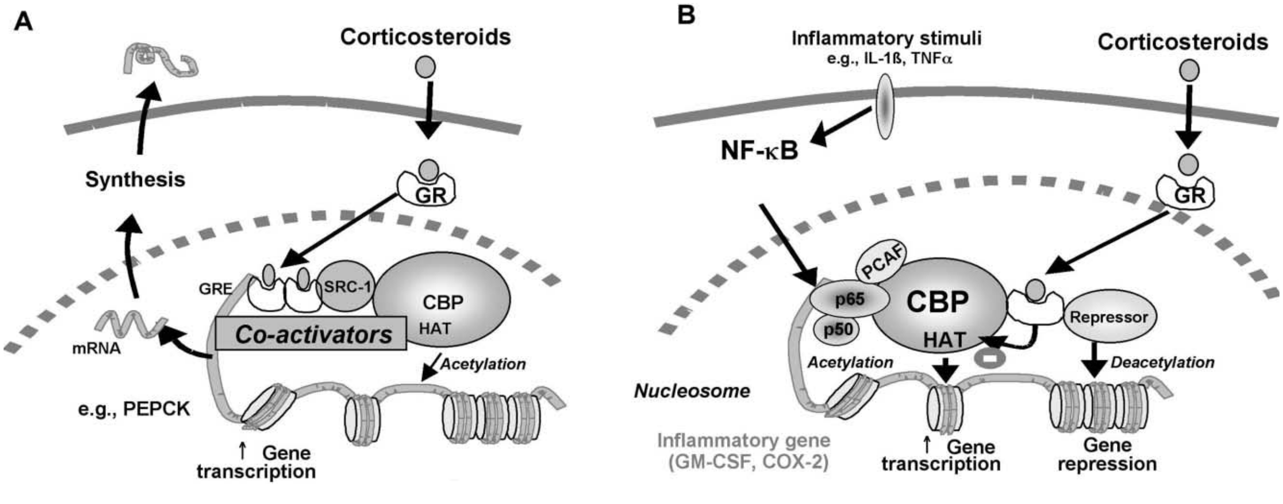
A
Corticosteroids
Synthesis
GR
GRE
SRC-1
CBP
HAT
Co-activators
Acetylation
mRNA
e.g., PEPCK
↑ Gene transcription
B
Inflammatory stimuli
e.g., IL-1ß, TNFα
Corticosteroids
NF-κB
GR
PCAF
p65
p50
CBP
HAT
Repressor
Acetylation
Deacetylation
Nucleosome
Inflammatory gene (GM-CSF, COX-2)
↑ Gene transcription
Gene repression

**FIGURE 6** Molecular mechanism of glucocorticoid actions. Glucocorticoids freely diffuse across the plasma membrane and bind to a glucocorticoid receptor (GR). This complex translocates to the nucleus and, acting as a dimer (A), binds to a glucocorticoid-response element (GRE), resulting in binding to CREB-binding protein (CBP) and other co-activators, such as steroid receptor co-activator-1 (SRC-1). This activates the histone acetyl transferase (HAT) activity in CBP, resulting in histone acetylation and increased gene transcription of genes, such as phosphoenolpyruvate kinase (PEPCK). (B) Inflammatory mediators can stimulate granulocyte/macrophage colony-stimulating factor (GM-CSF) and cyclooxygenase 2 (COX-2) gene expression via nuclear factor κB (NF-κB) activation, leading to CBP and p300/CBP-associated factor (PCAF)-regulated chromatin remodeling. GR monomers can associate with CBP-associated HATs and reverse the histone acetylation induced by inflammatory stimuli by a direct effect and by recruiting repressor proteins such as histone deacetylases, thus leading to repression (silencing) of inflammatory genes, such as GM-CSF and COX-2, induced by NF-κB.

The PEPCK gene has two potential GRE half-sites within its promoter region (−395 to −349) but it must also associate with tissue-specific factors since glucocorticoids are positive regulators of PEPCK in the liver and negative regulators within adipose tissue. Accessory factor 1 (AF1) and AF2, binding to adjacent sites, act with the GR to form a glucocorticoid-response unit, and it is this unit that ultimately mediates cortisol stimulation of PEPCK gene transcription.

The mechanisms involved in gene repression are less well understood since most genes that are switched off by cortisol or synthetic glucocorticoids do not possess negative GREs (nGREs). One gene that does possess a nGRE is that for POMC. The GR binding to the nGRE blocks the action of Nur77, a transcription factor that mediates CRH stimulation of POMC expression.

GRs may form complexes with other transcription factors, such as activator protein 1 (AP-1) and nuclear factor κB (NF-κB), and co-factors within the nucleus that are involved in activating inflammatory gene expression without the requirement for DNA binding (Fig. 6B). This was first demonstrated by an inhibitory action of corticosteroids on the collagenase gene and shown to be due to a protein–protein interaction between the activated GR and AP-1. This has subsequently been demonstrated for other transcription factors, including NF-κB and nuclear factor of activated T-cells (NF-AT). NF-κB is an attractive target for corticosteroids since it regulates the expression of many inflammatory genes including granulocyte/macrophage colony-stimulating factor (GM-CSF), eotaxin, RANTES, IL-1β, TNFα, inducible nitric oxide synthase, cyclooxygenase 2, and ICAM-1. However, this cannot account for all of the anti-inflammatory effects of corticosteroids, since some inflammatory genes that are potently repressed by corticosteroids, such as IL-5, are not regulated by NF-κB. This suggests that reduction of gene expression by the GR may involve interference with transactivation mediated by recruitment of co-factors such as CBP and p300/CBP-associated factor (PCAF) and modulation of the nucleosomal structure.

Due to the differential effects of various ligands on GR gene induction and repression, it was postulated that the GR could recruit distinct sets of co-factors depending upon the context in which the GR was acting. One possible explanation for this effect proposed that the GR acts as a dimer bound to DNA or as a monomer when repressing AP-1 or NF-κB. In a series of recent experiments using mice expressing mutated GRs unable to dimerize and subsequently bind to DNA, Schutz and colleagues confirmed a role for GR DNA binding in the control of POMC expression but not in corticotropin-releasing factor expression or in that of inflammatory genes regulated by AP-1 or NF-κB.

## VII. DISEASE STATES

The action of cortisol has many significant medical implications. For diabetics, more insulin may be required when they are under stress to cope with the increased blood glucose levels. Surgeons need to be aware that the protein catabolism of their patients may increase due to the stressfulness of their situation.

The most prevalent disorder involving glucocorticoids in humans and animals is Cushing's disease or hyperadrenocorticism. Excessive levels of endogenous glucocorticoids are generally seen as a result of a primary adrenal defect (adrenal neoplasm) or from excessive secretion of ACTH (pituitary adenoma). Alternatively, administration of excessive glucocorticoids for therapeutic purposes may lead to cushingoid symptoms. This condition generally affects adults, and approximately 10–15 people per million will develop this condition each year. Signs and symptoms of Cushing's syndrome include elevated blood pressure, development of diabetes, pink-to-purple stretch marks on the abdominal skin, fatigue, depression, moodiness, and accentuated fatty tissue on the face and upper back (buffalo hump). Women with Cushing's syndrome often have irregular menstrual periods and develop new facial hair growth. Men may show a decrease in sex drive. Treatment options are varied and depend on the cause of the excess cortisol.

Insufficient production of cortisol, often accompanied by an aldosterone deficiency, is called Addison's disease or hypoadrenocorticism. Most commonly, this disease is a result of infectious disease (e.g., tuberculosis in humans) or autoimmune destruction of the adrenal cortex. Usually more than 90% of the adrenal cortex must be destroyed before symptoms of adrenal insufficiency appear. This condition occurs in persons of all ages and affects approximately 1 in 100,000 people per year, affecting both sexes equally. Symptoms include fatigue, low blood pressure, weight loss, weakness, loss of appetite, moodiness, nausea, vomiting, and diarrhea. The production of other hormones by the adrenal is also often affected, with reduced levels of the hormone aldosterone, which is important for body salt and water balance, often accompanying the reduction in cortisol. This condition can be treated by the administration of synthetic steroid hormone preparations.

## Glossary

**adrenocorticotropic hormone** A hormone released by the anterior pituitary that stimulates the production of glucocorticoids and catecholamines by the adrenal gland.

**cortisol** The major glucocorticoid that is produced by the adrenal cortex in humans.

**glucocorticoid-response element** The DNA sequence to which the glucocorticoid receptor binds, thereby regulating gene expression.

**glucose** $C_6H_{12}O_6$, a 6-carbon aldose that is the major sugar in the blood and a key intermediate in metabolism. Used as a fluid and nutrient replenisher, usually given intravenously. The major energy source for the brain.

**inflammatory mediators** The endogenous compounds that mediate inflammation (autocoids) and related exogenous compounds including the synthetic prostaglandins.

**nuclear co-factor** A protein that enables a transcription factor to modify nucleosomal structure and/or gene transcription.

**nuclear factor κB** A pro-inflammatory transcription factor that is responsible for the activation of many inflammatory genes.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids and Autoimmune Diseases • Glucocorticoids, Pharmacology of**

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# Glucocorticoid Receptor, Natural Mutations of

Jeffrey M. Harmon

*Uniformed Services University of the Health Sciences, Maryland*

- I. INTRODUCTION
- II. GLUCOCORTICOID RECEPTOR STRUCTURE AND ORGANIZATION
- III. GERM-LINE MUTATIONS IN THE GR-CODING REGION
- IV. SOMATIC MUTATIONS IN THE GR-CODING REGION
- V. GENETIC POLYMORPHISMS

**The glucocorticoid receptor (GR) is a ligand-dependent transcription factor essential for the proper function of the hypothalamic–pituitary–adrenal axis and a host of other homeostatic processes. It also mediates the pharmacologic actions of therapeutic corticosteroids. Naturally occurring mutations in the GR can result in clinically important phenotypes.**

## I. INTRODUCTION

The glucocorticoid receptor (GR) mediates the physiologic action of endogenous corticosteroids and the pharmacologic action of therapeutic corticosteroids. Given its physiologic significance, it is not surprising that receptor knockouts in mice result in nonviable offspring and that relatively few

## II. GLUCOCORTICOID RECEPTOR α AND β

There are two highly homologous human GR isoforms, hGRα and hGRβ. The hGR gene, which is located on chromosome 5, consists of nine exons (Fig. 1). Exon 1 and the first part of exon 2 contain 5′-untranslated region (5′-UTR), and exon 2 contains the translation start site. Exon 9 contains the translation stop site and a large 3′-untranslated region (3′-UTR). The GR gene is transcribed into multiple GRα mRNA messages. The two predominant GRα transcripts observed on Northern blots of human, rat, and mouse tissues are 7.0 and 5.5 kb mRNAs that are produced by alternative polyadenylation in the 3′-UTR. The 7.0 kb mRNA contains the full-length 3′-UTR (approximately 4.0 kb), and the 5.5 kb mRNA contains only about 2.4 kb 3′-UTR. Alternative splicing in exon 9 of the human GR primary transcript produces two variants, hGRα and hGRβ. In the default splicing pathway, the end of exon 8 is linked to the beginning of exon 9, resulting in the hGRα mRNA transcripts. The hGRβ mRNA transcript is generated by the alternative splicing pathway, which links the end of exon 8 to downstream sequences in exon 9. Thus, translation of hGRα and hGRβ mRNAs produces two proteins that are identical through amino acid 727, but diverge in the carboxy-terminus, with hGRα having an additional 50 amino acids and hGRβ having an additional, nonhomologous 15 amino acids. The difference in the carboxy-terminal sequences of hGRα and hGRβ results in different ligand-binding patterns. hGRα binds glucocorticoids with high affinity and regulates the transcription of glucocorticoid-response genes. However, hGRβ does not bind glucocorticoid and is transcriptionally inactive. No potential ligands for hGRβ have been identified.

Recently, the co-existence of two forms of GRα, GR-A (molecular mass = 94 kDa) and GR-B (molecular mass = 91 kDa), has been demonstrated in various mammalian tissues and cell lines. GR-A and GR-B are products of alternative translation initiation with the longer form, GR-A, initiated from the normal start codon ATG corresponding to methionine 1 and the shorter protein, GR-B, initiated from a downstream ATG codon corresponding to methionine 27 in hGRα. Interestingly, the Kozak sequence adjacent to the upstream ATG is weak and mutagenesis studies indicate that the GR-A and GR-B forms are generated by a "leaky" ribosomal scanning mechanism. Both GR-A and GR-B exhibit similar subcellular localization and nuclear translocation after ligand activation and are transcriptionally active upon hormone binding. GR-B appears to be more effective than the GR-A form in glucocorticoid-induced reporter gene transactivation assays.

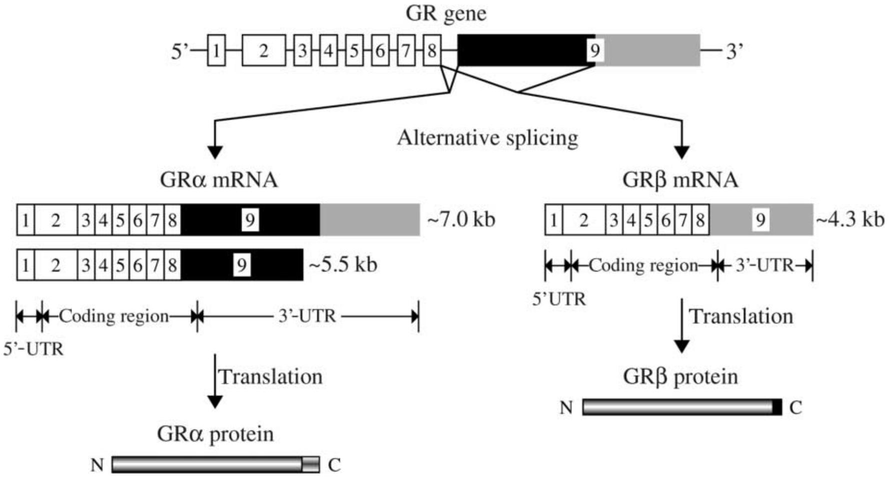

**FIGURE 1** Expression of human GRα and GRβ. The top of the figure shows the DNA structure of the human GR gene. The exons are shown as boxes and numbered 1 to 9; the introns are shown as lines. A pre-mRNA is transcribed from this DNA and spliced in two different ways. On the left, the default splicing pathway includes exons 1 to 8 and links the end of exon 8 to the beginning of exon 9, generating different sizes of GRα mRNAs due to alternative polyadenylation. The default splicing pathway generates the GRα protein. On the right is shown the alternative splicing pathway, which includes exons 1 to 8 and links the end of exon 8 to downstream exon 9. This form of mRNA is translated into GRβ protein. Reproduced with permission, from The glucocorticoid receptor: Expression, function, and regulation of glucocorticoid responsiveness. R. H. Oakley and J. A. Cidlowski, "Glucocorticoids (Milestones in Drug Therapy)" (N. J. Goulding and R. J. Flower, eds.), pp. 55–80, ©2001 by Birkhäuser, Basel.

The expression regulation, tissue distributions, and physiological significance of these two GRα isoforms remain to be elucidated. Preliminary findings suggest that hGRβ also has A and B isoforms.

## III. STRUCTURAL FEATURES OF GLUCOCORTICOID RECEPTOR α

In common with other nuclear receptors, the GR has a modular structure of three functionally separable domains (Fig. 2): (1) the amino-terminal domain, which has a constitutively active transcriptional activation function (AF-1); (2) the central DNA-binding domain (DBD), which contains two zinc-fingers responsible for interacting with DNA as well as forming homodimers; and (3) the carboxy-terminal ligand-binding domain (LBD), which is involved in hormone binding and contains a ligand-dependent transcriptional activation function (AF-2). There is a linker between the DNA-binding domain and the ligand-binding domain called the hinge region, which appears to be structurally flexible. As with other nuclear receptors, the structures of the three different domains of GR have been studied separately. It is poorly understood how these domains are organized and interact with one another within the context of a full-length receptor.

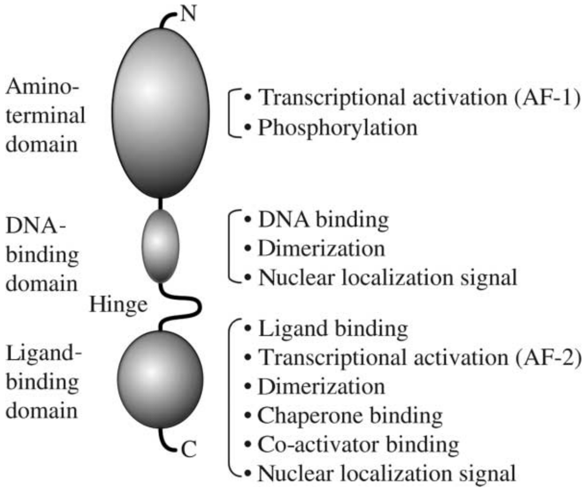

**FIGURE 2** Modular structure of GRα. The GRα is composed of three functional domains. The amino-terminal domain contains a constitutive transcriptional activation function (AF-1) and all known amino acid phosphorylation sites. The central DNA-binding domain is involved in binding to a specific DNA-response element, the GRE, and also contains a dimerization surface and nuclear localization signal. The carboxy-terminal ligand-binding domain has multiple activities that include a ligand-binding activity, a ligand-dependent transcriptional activation function (AF-2), and a nuclear localization signal. In addition, it contains surfaces for dimerization and the binding of co-activators and molecular chaperones. A hinge region is shown between the DNA-binding domain and the ligand-binding domain.

### A. The Amino-Terminal Domain

The amino-terminal domain of GR is the least conserved portion of the protein among different species and contains an amino acid sequence important for constitutive transactivation, called tau1 (τ1) or AF-1 (residues 77–262 in human GR). A smaller fragment that represents the activation core of the τ1, called τ1c, has been localized to residues 187–244 in hGR. The τ1c is almost as active as the intact τ1 and represents the only autonomous transactivation activity within the larger fragment.

The three-dimensional structure is not available for the amino-terminal domain of GR. It has been determined that purified τ1 and τ1c, which are enriched in acidic amino acid residues, are poorly structured in aqueous solution by circular dichroism and nuclear magnetic resonance (NMR) spectroscopy studies. In more hydrophobic solvents, three segments display a propensity to form α-helical conformations. There is a correlation between transcriptional activity and the propensity for α-helix formation, as suggested by proline substitution studies within these segments, indicating that the putative helices may be important for the GR function *in vivo*. It has also been reported that τ1 interacts with basal transcriptional machinery and other transcriptional co-factors. However, the mechanism by which the amino-terminal domain is involved in the constitutive transcriptional activity of the receptor is not clear.

Like other members of nuclear receptor family, GR is a phosphoprotein. The identified phosphorylated sites are located in the amino-terminal domain. Upon hormone binding, basally phosphorylated GR becomes hyperphosphorylated. Although the exact role of GR phosphorylation is not completely clear, it is generally believed that phosphorylation status plays an important role in receptor turnover and may account for the short half-life of the protein in the presence of ligand.

### B. The DNA-Binding Domain

The glucocorticoid receptor can homodimerize and bind to the cognate glucocorticoid-response element (GRE) in the promoter region of target genes with a high affinity. The consensus sequence of the GRE is the palindromic 15 bp sequence 5′GGTACAnnnTGTTC- T 3′ (where n is any nucleotide). The GR DBD contains eight cysteines, which can coordinate $Zn^{2+}$ ions to form characteristic DNA-binding zinc-fingers. The GR DBD contains protein motifs that allow the receptor to bind to DNA and homodimerize.

The crystal structure of rat GR DBD in complex with DNA has been solved by X-ray diffraction analysis. The DBD contains two zinc-finger motifs that fold together into a globular domain. Each zinc-finger is formed by four cysteines chelated to a single $Zn^{2+}$ ion (Fig. 3). The DBD can be divided into two modules, each consisting of a zinc-finger followed by an amphipathic α-helix. The two modules differ both structurally and functionally and fold together mainly through the interaction of the aromatic side chains of their amphipathic helices. The main loop of the amino-terminal zinc-finger has a short segment of anti-parallel β-sheet, and the carboxy-terminal zinc-finger has a distorted α-helix. The amino-terminal zinc-finger controls the DNA-binding specificity through amino acid residues in the P-box that contact bases in the major groove of the GRE. The carboxy-terminal zinc-finger provides the homodimerization interface, which is composed of amino acids in the D-box, to form a head-to-head symmetric dimer. Two structures of DBD–GRE complexes were determined by crystallographic analysis using the DBD in complex with different GRE sequences; one had a consensus GRE with three bases between two half-sites, and the other had a modified GRE with four bases between the two half-sites. The results suggested that the receptor adjusts to interaction with different GRE sequences by making different types of DNA contacts instead of by changing its homodimeric structure. The structure of the GR DBD in solution has been determined by NMR studies. It indicates that the GR DBD is a monomer in the absence of GRE.

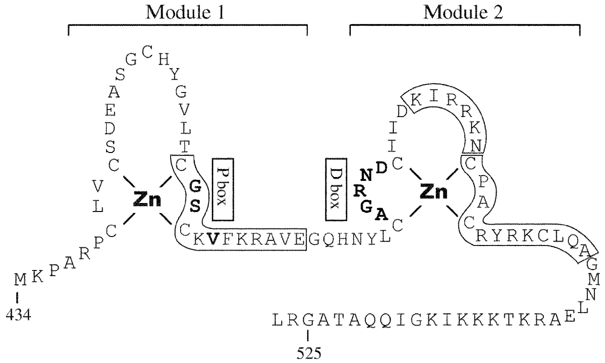

**FIGURE 3** Zinc-finger motifs in the DNA-binding domain of GRα. Amino acid sequence from rat GRα is shown as in the crystallographic analysis and is numbered based on the full-length receptor. Three α-helical segments are shown in the boxes. Each of the structurally and functionally different modules, 1 and 2, contains a zinc-finger followed by an α-helix. The highlighted amino acids in module 1 form the P-box, which is involved in DNA binding. The highlighted amino acids in module 2 form the D-box, which provides the dimerization interface to form a symmetric homodimer. Reprinted by permission from *Nature*, Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. B. F. Luisi, W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler, Volume 352, pp. 497–505. Copyright ©1991 Macmillan Magazines Ltd.

Both X-ray and NMR studies have shown that the amino acid residues of the carboxy-terminus of the DBD, residues 512 to 525 in rat GR, are poorly structured. This region is rich in basic amino acids and is partially responsible for the nuclear localization of the receptor, called the nuclear localization signal (NLS).

## C. The Ligand-Binding Domain

The ability of GR to activate transcription of target genes requires the binding of glucocorticoids to its LBD. In addition to ligand binding, the GR LBD is required for nuclear translocation, the binding of heat shock proteins and co-activators, and homodimerization, and most importantly, it harbors a ligand-dependent transcriptional activation function (AF-2). The X-ray structure of the GR LBD has not been reported. Information has resulted from studies of LBD deletions and mutations. Most mutations of the GR LBD have led to a decrease or loss of ligand binding. The liganded structures of thyroid receptor α (TRα), retinoic acid receptor γ (RARγ), estrogen receptor α (ERα), progesterone receptor (PR), androgen receptor (AR), and peroxisome proliferator-activated receptor γ (PPARγ) have been solved. Unliganded structures have also been determined for LBDs of retinoid X receptor α and PPARγ. Despite substantial sequence divergence, the three-dimensional structures of these LBDs display a conserved helical arrangement and a similar overall protein folding. The LBDs determined contain 11 or 12 α-helices (H1–H12) and two short β-strands (s1, s2). The α-helices are arranged into a three-layer, anti-parallel sandwich structure forming a compact globular domain. A general arrangement of the α helical sandwich fold of nuclear receptors' LBD is illustrated in Fig. 4. It has been suggested that the apo-receptor undergoes conformational changes

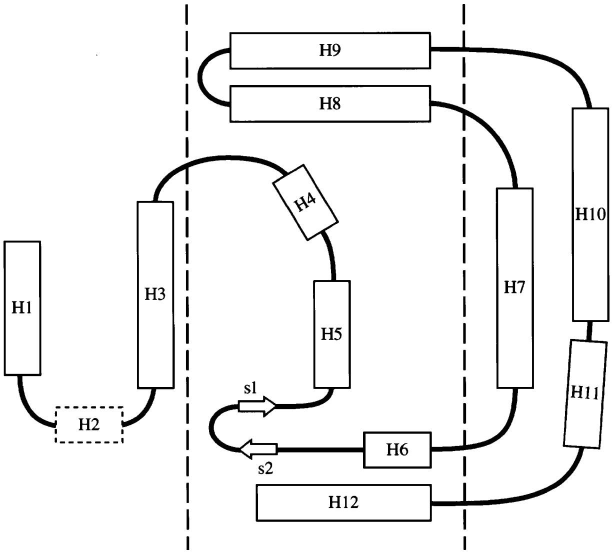

**FIGURE 4** Topology scheme of the ligand-binding domain of nuclear receptors. A general helical arrangement and protein folding of solved nuclear receptor LBDs are shown. Boxed H1–H12 are α-helices; H2 is not in all LBDs. Arrowed s1 and s2 are β-sheets. Some LBDs have more than two β-sheets. Dashed lines separate the overall LBD into three layers. Reproduced with permission, from Steinmetz, A. C. U., Renaud, J.-P., and Moras, D. (2001). Binding of ligands and activation of transcription by nuclear receptors. *Annual Review of Biophysics and Biomolecular Structure* 30, 329–359. ©2001 by Annual Reviews: www.AnnualReviews.org.

upon ligand binding, resulting in a more compact structure. Ligand binding may induce a major rearrangement of helix 12, which contains the core of ligand-dependent AF-2, holding back against the body of the receptor to close the entrance of ligand-binding pocket and generating the binding site for the co-activators.

A homology model of liganded hGR LBD can be developed based on known liganded hPR LBD crystal structure since among previously solved nuclear receptor LBDs, it shares the best pairwise sequence identity (54.183%) with hPR LBD. The secondary structure of holo-hPR LBD contains 10 α-helices without helix 2, and helices 10 and 11 are contiguous. Helix 12 in the hPR LBD is longer than in RAR, TR, and ER and spans all three layers of the "sandwich" enclosing the hormone-binding pocket. Following helix 12, the PR LBD has a 12-residue carboxy-terminal extension that is also present in GR and AR. This 12 amino acid residues form a β-sheet (s4) that is stabilized by an anti-parallel β-sheet interaction with another β-sheet (s3), between helix 8 and helix 9, located in the middle layer of the overall three-layered sandwich. This structure is thought to be important for ligand binding. The bound hormone contacts amino acids from helices 3, 5, 7, 11, and 12 and the β-turn (s1) between helices 5 and 6. In the crystal structure, the liganded PR LBD forms a dyad-symmetric homodimer in the asymmetric unit, with an interface that includes helix 12.

The amino acid sequence of hGRβ is identical to that of hGRα through amino acid 727. Thus, hGRβ does not contain helix 12. The last 15 nonhomologous amino acids in hGRβ, which correspond to the carboxy-terminus of helix 10 and the entire helix 11 in hGRα, are not helical as analyzed by several different secondary structure prediction schemes. These structural differences in hGRα and hGRβ may determine the functional differences between them.

The ligand binding domain of GR has recently been crystalized. It has an overall structure similar to other nuclear receptors although its dimerization interface may vary.

## IV. THE FUNCTIONS OF GLUCOCORTICOID RECEPTOR α

In the absence of hormone, GRα is inactive and exists as a large protein complex that includes the receptor, two molecules of hsp90, and other associated proteins in the cytoplasm (Fig. 5). The association of GRα with hsp90, which functions as a molecular chaperone, is necessary for high-affinity hormone binding to the receptor. Hsp90 also keeps GRα in a conformation masking the NLSs, thus maintaining cytoplasmic localization. The GRα ligand-binding domain is involved in the interaction between GRα and hsp90 molecules. Two regions of GRα contain a NLS; the first NLS is identified in the carboxy-terminus of the DNA-binding domain and the second is located in the ligand-binding domain. Once lipophilic glucocorticoid molecules diffuse across the plasma membrane into the cytoplasm, they bind to the ligand-binding domain of GRα, resulting in a conformational change of the receptor and dissociation of hsp90 and other associated proteins. This process is referred to as receptor activation. Upon receptor activation, GRα becomes hyperphosphorylated. The sites of both basal phosphorylation and hormone-induced hyperphosphorylation have been identified in the amino-terminal domain of GRα. It has been suggested that receptor phosphorylation may regulate the interaction of GRα with other transcription factors important for gene transactivation and the stability of the receptor.

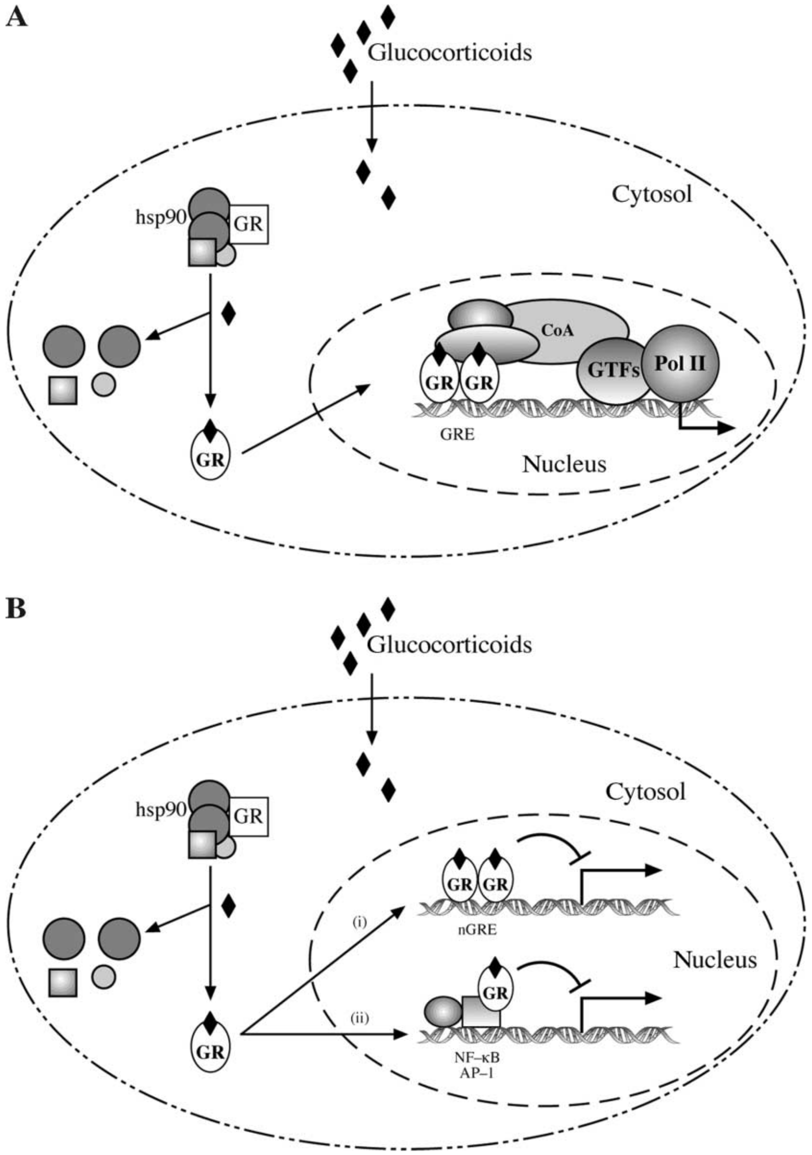

**FIGURE 5** Regulation of gene expression by glucocorticoid receptor α. Lipophilic glucocorticoid molecules are able to diffuse easily across the cell membrane into the cytoplasm. Upon ligand binding, the glucocorticoid receptor (GR) is activated by releasing the inhibitory hsp90 and other associated proteins. (A) Activation of gene expression by GRα. Activated GR translocates into the nucleus and binds to the glucocorticoid-response element (GRE) as a homodimer where it interacts with co-activators (CoA). Co-activators bridge the GR with general transcription factors (GTFs) and RNA polymerase II (Pol II) and stimulate the transcription of GR target genes. (B) Repression of gene expression by GRα. Glucocorticoids induce GR dissociation from hsp90 and its translocation into the nucleus where GR can repress gene expression by (i) binding to the negative GRE (nGRE) or (ii) interacting with other transcription factors such as AP-1 and NF-κB.

Liganded GRα rapidly translocates into the nucleus, where it binds its cognate GRE in the regulatory region of the target gene as a homodimer. Regions participating in homodimerization include the D-box in the DNA-binding domain and a region within the ligand-binding domain that has not been precisely determined. The formation of GRα homodimers is required for both high-affinity binding to the GRE and modulation of target gene transcription by GRα. This type of action has been shown to regulate many genes such as the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and tyrosine aminotransferase. Once bound to the GRE, GR stimulates the transcription of target genes by interacting directly or indirectly through intermediary proteins, known as co-activators, with the basal transcription machinery (Fig. 5A). The mechanisms of co-activator action have been studied extensively. It has been revealed that co-activators regulate nuclear receptor-induced transactivation by remodeling chromatin or by facilitating the recruitment of basal transcriptional machinery. For example, the p160 family co-activator SRC-1 (steroid receptor co-activator-1) and p300/CBP [CREB (cyclic AMP-response element-binding protein) binding protein] are histone acetyltransferases (HATs), which stimulate transcription by modifying and destabilizing chromatin structure. The co-activator p/CAF (p300/CBP-associated factor) contains HAT activity, as well as TATA-box-binding protein (TBP)-associated factors, which facilitate the recruitment of the basal transcriptional machinery in order to initiate transcription. At least two regions in the GR are required for transcriptional activation function including ligand-independent AF-1 in the amino-terminal domain and ligand-dependent AF-2 in the carboxy-terminus of the ligand-binding domain. Most known co-activators primarily interact with the AF-2 surface in the presence of ligand.

Glucocorticoids can also inhibit gene expression via GRα binding to a negative response element, termed nGRE [Fig. 5B, (i)]. The nGRE has a more variable sequence and is different from the classic GRE. Examples of GRα-mediated transcription repression through an nGRE include genes encoding pro-opiomelanocortin, osteocalcin, and α-fetoprotein. The exact mechanisms involved in the repression are unclear. It is generally suggested that binding of GR to the nGRE may prevent the binding of essential transcription factors to their DNA cognate sites. For example, in the osteocalcin promoter, an nGRE site overlaps the TATA-box. Therefore, GRα and the basal transcription factor, TBP, may compete for the binding of the same site.

In addition to direct DNA binding, GRα can also repress gene transcription by negative cross talk with other transcription factors, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [Fig. 5B, (ii)]. GRα directly interacts with these transcription factors and inhibits their transcriptional activities. However, the mechanisms of transrepression of AP-1 and NF-κB by GRα remain to be determined. The interaction surfaces between GRα and AP-1 or NF-κB have not been clearly localized. NF-κB and AP-1 are important regulators in mediating the transcriptional activation of many genes involved in the immune system. The negative cross talk between GRα and these transcription factors appears to be the potential mechanism underlying the important anti-inflammatory and immunosuppressive functions of glucocorticoids. Interestingly, in contrast to GR knockout mice, which die shortly after birth, knock-in mice containing a mutant GR that fails to homodimerize and subsequently does not bind DNA survive to adulthood. This suggests that transrepression is a critical function of the GR and that transactivation is dispensable, but these results have not yet been confirmed.

## V. THE FUNCTIONS OF GLUCOCORTICOID RECEPTOR β

Both mRNA transcripts encoding the hGRα and hGRβ proteins have been detected in many human tissues, with hGRβ expressed at a considerably lower level than hGRα. In contrast to hGRα, hGRβ does not bind glucocorticoids or anti-glucocorticoids and does not transactivate glucocorticoid-responsive genes. In addition, hGRβ does not repress the transcriptional activity of other transcription factors, such as NF-κB. The biological function of hGRβ is not understood as yet; however, experimental evidence has shown that hGRβ can exert a dominant negative effect on transactivation by hGRα. Several possible mechanisms responsible for this dominant negative effect have been suggested, including the following: (1) hGRβ competes with hGRα for GRE binding, (2) hGRβ forms transcriptionally inactive heterodimers with hGRα, and (3) hGRβ competes to bind to co-activators needed by hGRα for transcriptional activity. In addition, an abnormal level of hGRβ, or the hGRα to hGRβ ratio, has been found in glucocorticoid-resistant patients. Interestingly, mice totally lack GRβ. Nevertheless, the biological significance of the GRβ isoform is still controversial.

## VI. SUMMARY AND FUTURE PERSPECTIVES

The GR modulates gene expression by a number of different molecular mechanisms. There are two main modes of gene regulation, including transactivation by direct binding of the receptor to its cognate DNA-response elements and repression of gene expression through binding to nGRE or via cross talk with other transcription factors such as AP-1 and NF-κB. However, the mechanisms of both transactivation and transrepression are not completely understood. Future studies on the GR structure in the amino-terminal domain and the ligand-binding domain, as well as the interactions of each structural domain within the context of the full-length protein, will aid in the elucidation of the function of this important nuclear receptor. Furthermore, knowledge of the relationship between the structure and the transcriptional regulation of GR may eventually lead to the development of new therapeutic strategies that selectively retain the potent anti-inflammatory and immunosuppressive effects of glucocorticoids while minimizing their negative side effects.

## Glossary

**alternative splicing** A pre-mRNA processing pathway in which a single gene gives rise to different versions of the mRNA and, ultimately, to different proteins.

**glucocorticoid receptor** An intracellular protein that specifically binds glucocorticoids and functions as a transcription factor by activating or repressing the transcription of target genes. This receptor is a member of the nuclear hormone receptor family of ligand-inducible transcription factors.

**glucocorticoid-response element** A specific gluococorticoid receptor-binding DNA element that is located in the promoter regions of glucocorticoid target genes.

**glucocorticoids** Steroid hormones that are produced predominantly in the adrenal cortex from cholesterol; they regulate protein and carbohydrate metabolism and also inhibit the inflammatory response.

**heat shock proteins** Proteins that are synthesized by cells in response to increased temperature and function mainly as molecular chaperones protecting cellular proteins by aiding in protein folding.

**nuclear receptors** Ligand-inducible transcription factors that modulate target gene expression by binding to specific DNA sequences, termed hormone-response elements.

**transactivation** Stimulation of transcription by a transcription factor binding to DNA.

**transcription factors** Proteins that either are required for initiating RNA synthesis from gene promoters and together with RNA polymerase constitute the basal transcription machinery or recognize specific short consensus elements located upstream of the transcription starting point and regulate the transcription.

**transrepression** Inhibition of the transcriptional activity of a transcription factor.

**zinc-finger** A motif of amino acids with a characteristic spacing of cysteines that may be involved in binding zinc and that is also characteristic of some proteins that bind DNA.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids and Autoimmune Diseases • Glucocorticoids, Pharmacology of**

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TABLE 1 Mutations and Polymorphisms in the GR Gene

| | | Effect on receptor function | | | |
|---|---|---|---|---|---|
| | **Mutation** | **Ligand-binding activity** | **Transactivation** | **Transrepression** | **Additional phenotype** |
| Germ-line mutations (primary cortisol resistance) | R447H | → | ↓ ↓ ↓ | n.d. | |
| | I559N | ↓ ↓ ↓ | ↓ ↓ ↓ | ↓ ↓ ↓ | Dominant negative |
| | D641V | ↓ | ↓ ↓ | ↓ → | |
| | G679S | ↓ | ↓ ↓ | n.d. | |
| | V729I | ↓ | ↓ ↓ | ↓ → | |
| | I747M | ↓ | ↓ ↓ | | Dominant negative |
| | 4 bp deletion in exon 6 | ↓ ↓ ↓ | ↓ ↓ ↓ | ↓ ↓ ↓ | Putative NMD of mRNA |
| Somatic mutations | 1 bp insertion in exon 2 | ↓ ↓ ↓ | ↓ ↓ ↓ | ↓ ↓ ↓ | Putative NMD of mRNA |
| | L753F | ↓ | ↓ ↓ | | |
| Nonsynonymous SNPs and RFLPs | R23K | → | → | → | |
| | F29L | n.d. | n.d. | n.d. | |
| | F65V | n.d. | n.d. | n.d. | |
| | L112F | n.d. | n.d. | n.d. | |
| | D233N | n.d. | n.d. | n.d. | |
| | N363S | → | → | n.d. | |
| | D517K | n.d. | n.d. | n.d. | |
| | *Bcl* I | → | → | → | |
| | *Tth* III1 | → | → | → | |

*Note*. n.d., not determined; NMD, nonsense-mediated decay; ↓ →, loss of transrepression on some but not all responsive promoters.

naturally occurring mutations in the GR-coding region have been described. This article is intended to introduce the structure and function of the GR and to provide an overview of those naturally occurring genetic alterations in the GR gene that have significant effects on receptor function and/or result in a clinically relevant phenotype (see Table 1). Those readers interested in an in-depth review of all mutations in the GR gene are referred to the Glucocorticoid Receptor Resource (http://biochem1.basic-sci.georgetown.edu/grr/grr.html).

## II. GLUCOCORTICOID RECEPTOR STRUCTURE AND ORGANIZATION

The GR (NR3C1) is a member of the nuclear receptor family of ligand-regulated transcription factors. In the absence of ligand, the receptor resides in the cytoplasm in a high-molecular-weight complex that includes heat-shock protein 90 (hsp90) and which maintains the receptor in a form able to bind ligand with high affinity. Ligand binding promotes dissociation of this complex and movement of the GR into the nucleus, where it regulates the transcriptional activity of hormonally responsive genes. Depending on the specific gene, this regulation can be positive or negative and is accomplished through a variety of mechanisms. Positive transcriptional regulation most often involves binding of a receptor homodimer to a glucocorticoid-response element (GRE) and the recruitment of transcriptional coactivators whose activities alter chromatin structure and activate the basal transcription apparatus. Negative regulation of gene expression can also involve the binding of the receptor to *cis*-acting promoter regulatory elements (negative GREs). However, negative regulation can be the result of protein–protein interactions with other transcription factors, such as activator protein-1 (AP-1), nuclear factor κB (NF-κB), and Oct-1, that can inhibit their ability to bind to DNA or to activate the basal transcription apparatus.

The GR, like all other members of the nuclear receptor family, is composed of distinct structural and functional domains. The N-terminal domain contains at least one transactivating function (AF-1) that interacts with a variety of co-regulatory and transcription factors, resulting in a complex network of communication between multiple signaling pathways. The centrally located DNA-binding domain contains two zinc-fingers. The distal knuckle of the N-terminal finger is part of an α-helix that binds in the major groove of the DNA and determines the specificity of binding to the GRE. The C-terminal finger stabilizes the interaction of the receptor with DNA and its proximal portion participates in the formation of the receptor homodimers necessary for transcriptional activation from a canonical GRE. The C-terminally located ligand-binding domain (LBD) is composed of 11 α-helices and 4 short β-strands. In addition to forming a three-dimensional structure for selective, high-affinity binding of ligand, the LBD contains the region that binds to hsp90 as well as a transactivation function (AF-2) located in the C-terminal most α-helix that binds the p160 coactivator protein TIF (or GRIP-1). It is noteworthy that, unlike other members of the nuclear receptor superfamily and, in particular, other steroid hormone receptors, when co-crystallized with ligand and a co-activator peptide, the GR LBD is configured as a homodimer, whose formation appears to be required for efficient nuclear translocation and transcriptional transactivation.

The organization of the human GR gene is highly complex, with multiple promoters controlling transcription, and both alternative splicing and alternative use of translation initiation codons resulting in at least three physiologically relevant isoforms. GRα is a 777-amino-acid protein. It is the most abundant GR isoform and is capable of both transactivation and transrepression. The mRNA encoding GRα contains nine exons, a 5′-noncoding exon that can be derived from any of at least three promoters, and eight coding exons. The entire N-terminal domain is encoded within exon 2. The DNA-binding domain is encoded by exons 3 and 4, each of which codes for a single zinc-finger, and the ligand-binding domain is encoded by exons 5–9α. A shorter form of this protein (GR-B), presumably resulting from translation initiation at a downstream AUG in exon 2 and therefore lacking the N-terminal 26 amino acids, has also been routinely observed in immunoblots. This form of the receptor is more effective in transactivation than its full-length counterpart, but appears to be no more effective in transrepression.

Use of an alternative splice site in exon 9 results in the synthesis of the GRβ isoform, a 742-amino-acid protein whose C-terminal 15 amino acids are GRβ-specific. This isoform does not bind ligand. In addition, it appears to have dominant negative activity in some cells, and it has been proposed that an elevated ratio of the β/α isoform is responsible for the steroid resistance seen in some asthmatic patients. Alternative splicing generates two other GR isoforms, GRδ; and GRγ. GRδ is the result of failure to correctly remove intron G, resulting in the introduction of a premature stop codon. However, since the GR mRNA is subject to nonsense-mediated decay, it is unclear whether a truncated protein is actually synthesized. The GRγ isoform results from the use of an alternative splice site, leading to an additional arginine codon in the GR mRNA between exons 3 and 4 and the insertion of an arginine residue between G451 and Q452 in the interfinger region of the DNA-binding domain. GRγ proteins containing this additional residue are less potent activators of transcription than is the GRα isoform.

## III. GERM-LINE MUTATIONS IN THE GR-CODING REGION

The GR is expressed in virtually all tissues and cells, and its proper function is required to support a large number of homeostatic processes. It is therefore not surprising that viable germ-line mutations in the coding region are quite rare. Nevertheless, a limited number of such mutations have been observed, most often associated with the clinical syndrome of primary cortisol resistance. This inherited disorder is characterized by inappropriately high levels of circulating cortisol, without the accompanying cushingoid body habitus that such levels would normally elicit. Mutations responsible for primary cortisol resistance have been described in the DNA-binding domain of the GR as well as at several positions in the LBD. Although the limited number of mutations makes it difficult to generalize, it appears that all such mutations result in decreased receptor transactivating activity and/or decreased affinity for ligand. It also seems probable that at least part of the variation in the clinical manifestation seen among patients with this disorder is a consequence of subtle differences in receptor phenotype associated with each specific mutation. However, with a single exception, all of the affected individuals are heterozygous. Thus, in individuals functionally hemizygous for the GR gene, there appears to be no compensatory increase in expression of the wild-type allele.

Only one mutation in the DNA-binding domain that can unambiguously be associated with primary cortisol resistance has been identified. This mutation in exon 4, R477H, results in the complete loss of transactivating activity, presumably as a result of the loss of DNA-binding activity to a canonical GRE. Mutations in the LBD responsible for primary cortisol resistance have been identified in exons 5, 7, 8, and 9α. The mutation in exon 5, I559N, results in the complete loss of ligand-binding activity, and consequently this GR mutant lacks both transactivation and transrepression activity. Since I559 is involved in hydrophobic interactions within the LBD, and since the adjacent residue M560 has hydrophobic interactions with both the C-17 and the C-21 hydroxyl groups of the ligand, it is probable that the I559N mutation disrupts the internal structure of the LBD. This mutant also behaves as a dominant negative inhibitor of nuclear translocation of the wild-type GR. Thus, the phenotype associated with the I559N mutation is likely to reflect more than simple reduction of hormone-binding activity. However, primary cortisol resistance is observed even when a heterozygous mutation is not dominant negative. Patients with a 4 bp deletion resulting in the loss of a splice donor site at the $3'$-end of exon 6 fail to express mRNA from the mutant allele, presumably as a result of the fact that GR mRNAs containing premature translation stop codons are subject to nonsense-mediated decay. Thus, simple reduction of GR expression is sufficient to cause primary cortisol resistance.

A D641V mutation in exon 7, a G679S mutation in exon 8, and V729I and I747M mutations in exon 9α are all associated with cortisol resistance. In contrast to receptors containing the I559N mutation, all of these mutants bind ligand, albeit with reduced affinity. All four mutants have substantially reduced transactivating activity but retain varying abilities to repress the activities of promoters regulated by negative GREs or which are NF-κB or AP-1 responsive. In addition, the I747M mutation, which disrupts a hydrophobic interaction of the LBD with the C-21 hydroxyl of the ligand and which fails to interact with the p160 coactivator GRIP-1, behaves as a dominant negative inhibitor of wild-type receptor transactivation.

## IV. SOMATIC MUTATIONS IN THE GR-CODING REGION

The mutations responsible for primary cortisol resistance are inherited by the affected individuals. Somatic mutations in the coding region of the GR can also result in clinically important phenotypes. A single base insertion in GR exon 2 was identified in genomic DNA isolated from the pituitary adenomas of one of four patients examined. This mutation, which like the 4 bp deletion described above would probably result in nonsense-mediated decay of the mutant GR mRNA and loss of expression of the mutant allele, was not seen in DNA isolated from leukocytes obtained from the same patient, indicating that the origin of the mutation was somatic. However, since neither this nor any other mutation was identified in the tumor DNA of three other patients with such adenomas, the role of this insertion in the etiology and/or phenotype of the disease is unclear.

A missense mutation in exon 9α of the GR (L753F), originally identified in the human leukemic cell line CCRF-CEM, has also been shown to be present in archival biopsy material obtained from the patient from whom the cell line was established. This mutation results in an increased rate of ligand dissociation, consistent with the hydrophobic interaction between L753 and the C-11 carbonyl of the steroid molecule. In addition, this mutation results in substantial loss of transactivating activity as well as loss of the ability to repress the activity of NF-κB. However, repression of AP-1 activity is unaffected, reinforcing the observation that individual mutations in the GR gene can result in dissociation of various receptor functions. It is unlikely that the L753F mutation was responsible for the occurrence of the leukemia from which the CCRF-CEM cell line was established, since it was identified in only a fraction of the biopsy samples examined. Nevertheless, it is possible that the L753F mutation decreased the sensitivity of leukemic cells to glucocorticoid therapy, since even a twofold increase in GR concentration is sufficient to render otherwise steroid-resistant cells sensitive to the cytolytic activity of corticosteroids.

Using single-nucleotide polymorphisms (SNPs) in the GR-coding region (see below), loss of heterozygosity (LOH) at the GR locus has been detected in a significant fraction of corticotropinomas obtained from patients with pituitary-dependent Cushing's disease. In three of the six patients whose tumors exhibited LOH at the GR locus, at least two GR exons were undetectable, providing evidence for extensive deletion in the GR gene and presumably loss of expression of the mutated allele. Nevertheless, since more than half of the corticotropinomas examined had no apparent GR defect, the relationship between loss of GR expression and the clinical presentation of the disease is uncertain.

## V. GENETIC POLYMORPHISMS

A rapidly increasing number of SNPs are being identified in the GR gene. For a current compilation of GR SNPs, the reader is referred to the National Center for Biotechnology Information SNP database at http://www.ncbi.nlm.nih.gov/SNP. Most of the reported polymorphisms are in introns or are synonymous and therefore have no effect on the receptor protein. Nonsynonymous polymorphisms in exon 2 result in the amino acid substitutions R23K, F29L F65V, L112F, D233N, and N363S. With the exception of the N363S variant, which has been associated with a modest increase in steroid sensitivity, and for which an association with obesity in men has been proposed, none of these appears to result in a physiologically or clinically relevant phenotype. There is also a nonsynonymous polymorphism in exon 5, resulting in the substitution of lysine for asparagine at residue 517 in the portion of the GR between the nuclear localization sequence and the LBD. No alteration of receptor function has yet been associated with this polymorphism.

Among the SNPs outside of the GR-coding region, a polymorphism in the 3′-untranslated region of exon 9β that increases the stability of the GRβ mRNA has been described and was present in increased frequency in patients with rheumatoid arthritis. In addition, a *Tth*III1 restriction fragment length polymorphism (RFLP) in the GR promoter has been reported to be associated with elevated basal cortisol secretion in men. A *Bcl*I RFLP, also in a noncoding region of the GR gene, has been statistically associated with a variety of clinical phenotypes, including central obesity and dysregulation of the hypothalamic–pituitary–adrenal axis. Given the increasing pace with which SNPs and other polymorphisms are being identified and mapped in the human genome, and the level of interest in the association between such polymorphisms and human disease, it is certain that a substantial number of additional polymorphisms with clinically relevant phenotypes will be identified in the GR gene.

## Glossary

**genetic polymorphism** A heritable genetic difference between two individuals at a specific location in the genome that is present in the general population.

**germ-line mutation** A heritable genetic alteration not present in the general population.

**glucocorticoid receptor** The intracellular receptor that mediates the response to the physiologic adrenal steroid cortisol and to pharmacologic corticosteroids, such as dexamethasone.

**nonsense-mediated decay** The process whereby cells degrade mRNAs containing premature stop codons to prevent the synthesis of truncated proteins.

**somatic mutation** An induced or spontaneous genetic alteration in a differentiated cell that is not transmitted to the next generation.

## See Also the Following Articles

**Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids and Autoimmune Diseases • Glucocorticoids, Pharmacology of**

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# Glucocorticoid Receptor Structure and Function

Diane Dong and John A. Cidlowski

*National Institute of Environmental Health Sciences, North Carolina*

I. INTRODUCTION
II. GLUCOCORTICOID RECEPTOR α AND β
III. STRUCTURAL FEATURES OF GLUCOCORTICOID RECEPTOR α
IV. THE FUNCTIONS OF GLUCOCORTICOID RECEPTOR α
V. THE FUNCTIONS OF GLUCOCORTICOID RECEPTOR β
VI. SUMMARY AND FUTURE PERSPECTIVES

**The glucocorticoid receptor (GR) is an intracellular protein that specifically binds glucocorticoids, steroid hormones produced primarily in the adrenal cortex and necessary for many physiological processes. The GR functions as a transcription factor by activating or repressing the transcription of target genes. It is a member of the nuclear hormone receptor family of ligand-inducible transcription factors and, like other members of this family, is a phosphoprotein. Two splicing variants of a single gene have been described, GRα and GRβ. These two isoforms are highly homologous. The GRα isoform is the primary receptor in the glucocorticoid signal transduction pathway. GRα is crucial for survival and is found in almost all human cells. The biological function of human GRβ has not yet been elucidated, but experimental results indicate that human GRβ can exert a dominant negative effect on transactivation by human GRα.**

## I. INTRODUCTION

Glucocorticoids are steroid hormones derived from the metabolic conversion of cholesterol in the adrenal cortex. They are required for many different physiological processes, including the development of organs, carbohydrate homeostasis, protein and fat metabolism, stress adaptation, and immune surveillance. These diverse actions have made glucocorticoids attractive for therapeutic development, and, as a class of compounds, they are among the most prescribed drugs in the world. Clinically, because of their immunosuppressive and anti-inflammatory activity, glucocorticoids are widely used in the treatment of chronic autoimmune/inflammatory and allergic diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, and skin disorders. At the cellular level, glucocorticoids exert most of their known effects by binding to an intracellular protein, termed the glucocorticoid receptor (GR), which, upon activation by glucocorticoids, activates or represses the transcription of certain target genes.

Glucocorticoid receptors belong to the nuclear receptor superfamily, members of which are ligand-inducible transcription factors that modulate transcription through binding to specific DNA sequences. This family consists of receptors with identified ligands, including receptors for small hydrophobic molecules such as steroid hormones, thyroid hormone, vitamin D, retinoic acids, and metabolites of long-chain fatty acid, as well as a number of orphan receptors, for which no endogenous ligands have been identified.

The glucocorticoid receptor was one of the first nuclear receptors cloned from mammalian cells. The human, rat, and mouse GR cDNAs were isolated in the mid-1980s. Two alternative splicing variants of a single gene have been described, GRα and GRβ, where GRα is the primary receptor in the glucocorticoid signal transduction pathway. Thus, the GRα isoform is commonly referred to as the GR. Consistent with the widespread physiological functions of glucocorticoids, GRα is distributed in almost all human cells and tissues with an expression level on the order of 3000–30,000 receptor molecules per cell. GRα is essential for survival. Transgenic mice devoid of GRα exhibit impaired lung development, reduced liver gluconeogenic enzyme expression, impaired proliferation, and elevated adrenocorticotropic hormone and corticosterone levels. The newborn mice died minutes after birth due to respiratory failure.

and function as ligand-activated transcription factors. Within this superfamily of intracellular receptors, there is a distinct subfamily consisting of the intracellular receptors for steroid hormones including glucocorticoids, mineralocorticoids, progesterone, and androgens. These related receptor proteins share some striking homologies within their DNA-binding domains and regulate the expression of specific target genes and gene networks.

The glucocorticoid receptor, which is shown schematically in Fig. 1, has three basic domains similar to the other members of this subfamily. It contains a variable and poorly conserved amino-terminal domain that is sometimes referred to as the immunogenic domain, since several monoclonal antibodies recognize epitopes located within this region of the protein. This domain is quite long (roughly the amino-terminal half of the polypeptide) and contains a strong independent transactivation domain (AF1) that is important for adding specificity to GR action. In the mouse GR protein, seven phosphorylation sites have been identified in the AF1-transactivating domain. This posttranslational modification increases the acidity of this region and by so doing may modulate the transactivation function. The GR protein also contains a highly conserved and more centrally located DNA-binding domain (DBD). Of a total of 9 cysteine residues in this domain, 8 are involved in complexing two zinc ions in tetrahedral arrangements. These "zinc-fingers" contribute to the folding of this domain as it interacts with DNA sequences and contacts specific nucleotides in the glucocorticoid-response elements (GREs). This DBD also contains a dimerization subdomain and a nuclear localization subdomain (NLS1). The carboxy-terminal ligand-binding domain comprises approximately 250 amino acids and contains a number of subdomains specifying not only the steroid-binding region, but also other functions including heat shock protein 90 (hsp90) binding, a second transactivation subdomain (AF2), a second nuclear localization subdomain (NLS2), and subdomains that interact with other transcription factors such as c-jun or c-fos and the nuclear NF-κB heterodimer. This domain seems to have a fairly complex structure, since some essential residues are quite remote from one another in the primary sequence. Alternative splicing of the primary GR gene transcript can generate a polypeptide in which the carboxy-terminal 50 amino acid residues are exchanged for an unrelated 15-amino-acid sequence originating from an alternative exon encoded by the same gene. This GR variant, which is referred to as GR-β to differentiate it from wild-type GR-α, is thus unable to bind ligand. Despite its inability to bind steroid, GR-β has been shown in transfection studies to block the transcriptional response mediated by hormone-bound GR-α. Whether endogenous GR-β levels are high enough to render a target cell glucocorticoid resistant by functioning as a dominant-negative inhibitor of GR-α is not currently known.

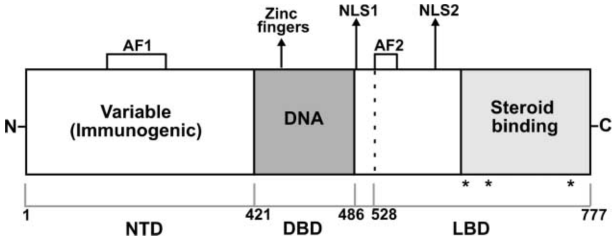

FIGURE 1 Domain structure of the human glucocorticoid receptor. Although not depicted, two dimerization domains have been identified. One dimerization domain is located in the DNA-binding domain (DBD) within the second zinc-finger; the other is located near the carboxy-terminal end of the ligand-binding domain (LBD). The location of the second nuclear localization signal (NLS2) has not been precisely mapped but does appear to involve a large portion of the carboxy-terminal domain. This suggests that NLS2 may actually be "assembled" from nonadjacent amino acid residues that are juxtaposed only after the conformational change induced by hormone binding. Binding of the hsp90 dimer requires a specific 7-amino-acid sequence at the amino-terminus of the LBD. The asterisks indicate 3 amino acid residues (604, 638, and 736) affinity-labeled by covalent binding of a glucocorticoid analogue. NTD, amino-terminal domain, AF1 (77–262) and AF2 (526–556), sequences important for transactivation of gene transcription; NLS1, nuclear localization signal 1.

To fully appreciate the molecular basis for the potential mechanisms underlying glucocorticoid resistance, it is important to understand the basic model that details the interaction of glucocorticoid hormones with their target cells found throughout the body. This model is depicted in Fig. 2 and will be briefly reviewed here. Clearly a defect or abnormality in any one of the steps depicted in this diagram could result in glucocorticoid resistance. As shown in this figure, cortisol (the primary glucocorticoid in humans) circulates in the plasma loosely bound to a transport protein called corticosteroid-binding globulin or CBG. Once delivered to the target cell, the lipid-soluble cortisol dissociates from the CBG and diffuses across the plasma membrane. Once inside the target cell, cortisol binds with high affinity and specificity to the unactivated/untransformed (non-DNA-binding form) GR protein. This unactivated

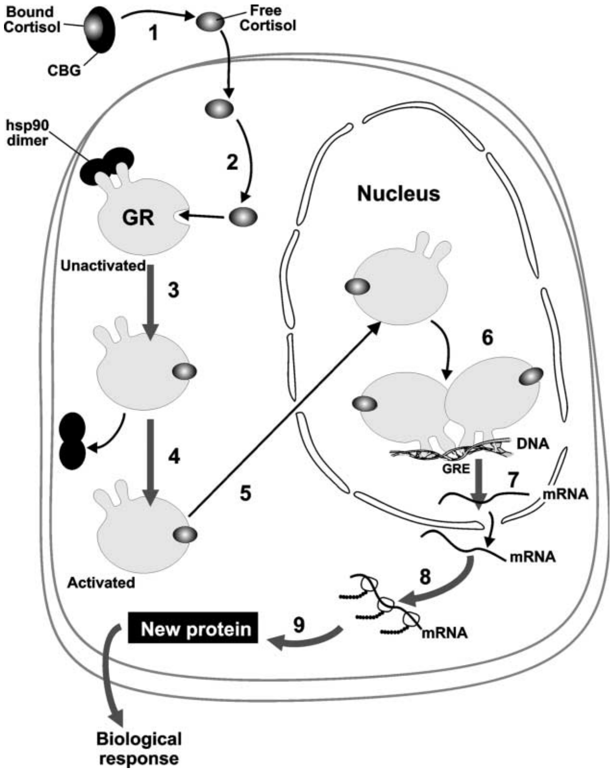

FIGURE 2 Steps involved in the interaction of cortisol with a glucocorticoid target cell. (Step 1) Cortisol molecule dissociates from its transport protein, CBG. (Step 2) After diffusing across the plasma membrane, cortisol binds to its unactivated, cytoplasmic GR with high affinity and specificity. (Step 3) Binding of cortisol to the GR induces a conformational change and dissociation of the hsp90 dimer. (Step 4) The DNA-binding domain (zinc-finger) is uncovered in the activated GR complex. (Step 5) The activated GR complex is translocated into the nucleus. (Step 6) The activated GR complex dimerizes and binds reversibly to GRE sequences. (Step 7) Gene transcription is enhanced, and mature messenger RNA species are generated. (Step 8) Translation of induced messenger RNAs occurs in the cytoplasm. (Step 9) Translated proteins mediate specific biological responses.

cytoplasmic GR is heteromeric and has a molecular weight of approximately 300 kDa because it is associated in a complex with other non-hormone-binding proteins. A dimer of the 90 kDa heat shock protein, which is induced when cells are stressed but is also expressed constitutively in nonstressed cells, is associated with the ligand-binding domain of the unactivated GR. In the properly folded GR, this hsp90 dimer occludes, or masks, the DNA-binding domain (zinc-fingers) of the receptor. Thus, in the absence of ligand, the unactivated GR is incapable of binding to DNA. In addition to blocking the DNA-binding domain, hsp90 also ensures that the 94 kDa GR protein will be in the proper conformation for subsequent ligand binding. Other non-hormone-binding proteins are also included in this heteromeric complex. For example, another heat shock protein, designated hsp56, is also located in this complex. This heat shock protein also functions as an immunophilin, since it binds a number of potent immunosuppressive drugs. The chaperone heat shock protein designated hsp70 also appears to associate with the GR and promote the assembly of this heteromeric complex. Binding of cortisol to the 94 kDa GR induces a conformational change in the protein and dissociation of the hsp90 dimer. Following this activation/transformation step and the exposure of the DNA-binding domain, the receptor translocates into the nucleus and binds to specific DNA sequences termed GREs. The GR binds as a homodimer to these GREs and either induces (increases) or represses (decreases) the rate of transcription of glucocorticoid-response genes. The induced messenger RNA molecules are then translocated into the cytoplasm and assembled into translation complexes for the synthesis of new proteins that alter the metabolism and/or function of the target cell.

The model presented in Fig. 2 is clearly an oversimplification of how activated GR complexes transactivate gene expression. For example, this model does not show how other proteins, referred to as co-activators and co-repressors, regulate the transactivational functions of the nuclear GR complexes. Binding of co-repressors to DNA appears to cause deacetylation of histones, producing condensation of chromatin and repression of transcription. Binding of activated GRs to GREs may displace such co-repressor molecules and recruit co-activator molecules. The co-activators then catalyze the acetylation of histones and decondensation of DNA. This allows for the further recruitment of other proteins required for the activation of RNA polymerase II and a subsequent increase in the rate of transcription. A family of steroid receptor co-activators has been described and these transcription-activating proteins appear to interact with these receptors through a conserved 5-amino-acid motif within the AF2 site and possibly within the AF1 site. The first functional co-activator cloned and identified was SRC-1 (steroid receptor co-activator-1). This co-activator was shown to interact with, and stimulate the activity of, both Type I receptors (estrogen, progesterone, androgen, mineralocorticoid, and glucocorticoid) and Type II receptors (thyroid hormone, Vitamin D3, retinoic acid, and orphan receptors). This molecule has been shown to be functional *in vivo*, since SRC-1 knockout mice exhibit partial resistance to several hormones. Finally, SRC-1 has been shown to belong to a family of three co-activators that also includes TIF-2 and PCIP.

## III. CLASSES OF GLUCOCORTICOID RESISTANCE SYNDROME

As summarized in Table 1, glucocorticoid resistance can be classified into four major subtypes including generalized inherited glucocorticoid resistance, pharmacologically induced glucocorticoid resistance, acquired or tissue-specific glucocorticoid resistance, and physiological resistance to glucocorticoids including genetic variations in sensitivity. It has been known for many years that within the normal population there is wide variability in the sensitivity to glucocorticoid hormones. This variability is clearly reflected in the wide range of responses to the same relative dose of exogenous glucocorticoids used in the treatment of various diseases. Published studies demonstrate that primary or hereditary abnormalities in the glucocorticoid receptor gene make 6.6% of the normal population relatively hypersensitive to glucocorticoids, whereas 2.3% are relatively resistant. This variability helps to explain why some individuals develop severe adverse effects during hormonal therapy with a low dose of glucocorticoids, yet others do not develop side effects even during long-term therapy with a much higher dose of glucocorticoids.

In the primary or hereditary abnormalities just mentioned, mutations in the GR gene have been shown to make individuals resistant or hypersensitive to the feedback effects of synthetic glucocorticoids such as dexamethasone. These mutations result in elevated (glucocorticoid resistance) or lowered (glucocorticoid hypersensitivity) circulating cortisol levels. These altered cortisol levels usually do not result in any specific signs or symptoms because they represent an adaptation or resetting of the hypothalamic–pituitary–adrenal axis at a higher (resistance) or lower (hypersensitivity) level in order to overcome the function of abnormal GRs in the target tissues. The symptoms associated with glucocorticoid resistance are the consequence of overstimulation of

**TABLE 1 Classification of Human Glucocorticoid Resistance Syndromes**

| Type of resistance | Underlying mechanism |
|---|---|
| I. Generalized inherited (familial) glucocorticoid resistance (GIGR) | Mutations in GR gene |
| II. Pharmacologically induced glucocorticoid resistance | |
| A. Administration of GR antagonist RU486 | Blocks ligand binding to GR protein and activates HPA by preventing negative feedback mediated by glucocorticoids |
| B. Treatment of leukemic cell line with chemotherapeutic drugs | Deletion of GR gene |
| C. Treatment of leukemic cell line with chemical mutagens | Deletion of GR gene |
| III. Acquired glucocorticoid resistance | |
| A. Neoplastic | |
| 1. Ectopic ACTH syndrome | Decreased GR number, truncated GR, aberrant splicing and mutation of GR |
| 2. Pituitary tumors (Nelson's syndrome) | Mutation of GR |
| 3. Hematological malignancies | Mutations and aberrant splicing of GR |
| B. Transient | |
| 1. Depression | Decreased number of GR |
| 2. AIDS | Increased number of GR and reduced ligand affinity |
| 3. Steroid-resistant asthma, rheumatoid arthritis, systemic lupus erythematosus | Number of GR abnormalities reported |
| IV. Physiological resistance to glucocorticoids and variations in sensitivity | |
| A. Receptor down-regulation | Glucocorticoids decrease rate of transcription of GR gene and decrease sensitivity |
| B. Distal nephron of kidney | 11β-Hydroxysteroid dehydrogenase catalyzes conversion of cortisol to cortisone, which is an inactive glucocorticoid |
| C. Individual differences in glucocorticoid sensitivity | Genetic variation in control of cortisol secretion, regulation of HPA axis, and GR expression |

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the synthesis and secretion of ACTH by the anterior pituitary gland. Elevated ACTH secretion in turn stimulates increased production of adrenocortical androgens, which can cause acne and hirsutism, and mineralocorticoids, which can cause hypokalemia and hypertension. Several different GR abnormalities have been reported for primary glucocorticoid resistance. Missense mutations in the ligand-binding domain of the GR resulting in a decrease in ligand-binding affinity have been reported in two different families. A deletion of 4 bp in the intron close to exon 6 of the GR has been shown to result in a splice site deletion. This deletion results in the expression of only one allele and a 50% decrease in the GR protein level in the affected members of another family. Finally, a third GR gene mutation in the proximal region of the ligand-binding domain (in exon 5) has been shown to result in a functional loss of the mutant GR protein in other patients.

## IV. GLUCOCORTICOID RESISTANCE IN DISEASES AND SPECIFIC PHYSIOLOGICAL STATES

As already mentioned, two major forms of glucocorticoid resistance include generalized inherited glucocorticoid resistance, in which all tissues are partially glucocorticoid resistant, and local (tissue-specific) or acquired glucocorticoid resistance, in which only the affected tissues are resistant to glucocorticoids. Generalized glucocorticoid resistance, in which most patients appear to be basically asymptomatic despite elevated cortisol levels, has been reported in some families. In this situation, the high cortisol levels are due to the glucocorticoid resistance of the anterior pituitary gland to cortisol-mediated negative feedback, leading to an elevation of ACTH levels. In patients with generalized glucocorticoid resistance, the elevated cortisol level exists without the signs and symptoms of hypercortisolism detected in Cushing's syndrome, where the patient is glucocorticoid sensitive. Clearly, complete or absolute glucocorticoid resistance would not be compatible with life, and therefore patients with generalized glucocorticoid resistance do develop some signs of Cushing's syndrome. As mentioned previously, an aberrant GR gene appears to be the cause of this syndrome.

What about glucocorticoid resistance that occurs in a tissue-specific manner? Nelson's syndrome is caused by an aggressive fast-growing ACTH-producing tumor that is highly resistant to inhibition by synthetic glucocorticoids. This syndrome occurs in approximately 10–30% of Cushing's patients who have had both of their adrenal glands removed. Nelson's syndrome in one patient has been shown to be associated with a heterozygous frameshift mutation in the GR gene. This frameshift results in premature termination of translation and low levels of the GR protein, which accounted for the decreased glucocorticoid sensitivity of the tumor. This specific mutation was not detected in the GR gene obtained from the patient's leukocyte DNA, demonstrating that it was of somatic origin.

In addition to GR defects resulting from GR gene mutations, glucocorticoid resistance is actually very dynamic and can vary in relation to a variety of physiological situations. For example, following physical exercise, a decreased ability of dexamethasone to inhibit interleukin-6 production by leukocytes has been reported. However, the precise mechanism underlying this acute glucocorticoid resistance is not known. More prolonged acquired glucocorticoid resistance may also play an important role in the origin of several diseases. For example, in rheumatoid arthritis and systemic lupus erythematosus, a decreased number of systemic lymphocyte GRs have been reported. In systemic lupus erythematosus, this decreased number of lymphocyte GRs was correlated with poor response to glucocorticoid therapy and, importantly, with a poor prognosis. Asthma is another interesting example of a disease in which a small percentage of patients are insensitive to glucocorticoid treatment. Studies indicate that steroid-resistant asthma is associated with a failure of glucocorticoids to inhibit the *in vitro* proliferation and cytokine secretion from T-lymphocytes obtained from these patients. In these resistant patients, both a reduced ligand-binding affinity of the GR and a reduced binding of the ligand–GR complex to specific DNA sequences (GREs) on the genome have been reported. This resistance to glucocorticoids could affect the immune system in two distinct ways. First, it could directly result in reduced control of inflammatory processes. Second, it could also result in altered cytokine patterns. Resistance to glucocorticoid treatment would probably slow the recovery of the patient in both of these situations.

Acquired glucocorticoid resistance can be induced by a number of mechanisms. Published data suggest that cytokines released during immune activation can actually alter the characteristics of the GR. For example, interleukin-1 alters GR function in cultured hepatoma cells, as evidenced by a decrease in GR-binding levels. Data such as these suggest that during acute inflammation, cytokines may alter metabolic pathways in the liver by interfering with glucocorticoid action. A combination of interleukin-2 and interleukin-4 *in vitro* renders lymphocytes relatively resistant to dexamethasone-mediated inhibition and lowers the GR-binding affinity for ligand. Therefore, during any prolonged immune activation resulting in elevated concentrations of local or circulating cytokines, glucocorticoid resistance in other tissues and cell types could very well be induced.

Several specific cell types are rendered relatively resistant to endogenous glucocorticoids as a consequence of their metabolism in that tissue. Cells such as those lining the renal cortical collecting duct are targets for mineralocorticoids such as aldosterone and hence express intracellular mineralocorticoid receptors (MRs). These MRs share structural homologies with the GRs, especially when one compares the DNA- and ligand-binding domains. The endogenous glucocorticoid in humans, which is cortisol, actually binds to the MR with the same relative affinity as aldosterone. However, the normal circulating cortisol level is approximately 100-fold higher than the circulating aldosterone level. Despite this situation, these cells actually respond to aldosterone because they express an enzyme, Type II 11-β-hydroxysteroid dehydrogenase, which catalyzes the metabolism of cortisol to inactive cortisone. Since cortisone fails to bind to the MR, this mineralocorticoid receptor can now respond to the much lower circulating levels of aldosterone. This metabolism of cortisol to cortisone thus renders these mineralocorticoid target tissues less responsive, or partially resistant, to cortisol. It is important to note that this generated cortisone is then transported to the liver, where it can be converted back into active cortisol via the activity of Type I 11-β-hydroxysteroid dehydrogenase.

The possibility that glucocorticoid resistance may be associated with obesity has also received considerable attention. For several years, it was speculated that defects in hypothalamic–pituitary–adrenal (HPA) axis function might result in changes in energy metabolism, which could contribute to changes in total body fat. Altered HPA axis activity in obesity has in fact been reported, but the precise effect of this alteration on circulating cortisol levels was unclear. Deficiencies in GR feedback regulation in obesity were proposed based on resistance to administered dexamethasone, a potent glucocorticoid agonist. Recent studies have focused on the mechanism(s) underlying this resistance to glucocorticoid feedback in obesity. Obese subjects were found to have significantly higher basal ACTH levels but lower cortisol levels, suggesting that their adrenal glands are relatively insensitive to ACTH. When physiological doses of hydrocortisone (the same as cortisol) were infused into obese individuals, basal ACTH levels were decreased during the day, but not during the night as they were in control subjects. These data thus showed that obesity is associated with a relative insensitivity to glucocorticoid feedback and that this defect is most pronounced at night. The relatively low doses of hydrocortisone that were administered to these obese individuals would partially saturate their brain MRs and not GRs, since cortisol binds to MRs with an affinity that is 10-fold higher than its affinity for GRs. Thus, it was concluded that this insensitivity to glucocorticoids results from defects in the MR, rather than in the GR. This conclusion is consistent with the facts that MRs are expressed at high concentrations in the hippocampus and that this region of the brain has been strongly implicated in the negative feedback control of the HPA axis. Thus, this tissue-specific glucocorticoid resistance associated with obesity does not appear to be a consequence of GR defects like those reported in other types of glucocorticoid resistance.

## V. GLUCOCORTICOID RESISTANCE IN HEMATOLOGIC MALIGNANCIES

The ability of glucocorticoids to inhibit the growth of, and subsequently lyse, immature lymphoid cells provides the rationale for their therapeutic utilization in the treatment of various human leukemias, lymphomas, and multiple myelomas. However, prolonged treatment with glucocorticoids often results in the emergence of resistant clones. Understanding the mechanism(s) underlying this resistance has been aided by the availability of a number of glucocorticoid-sensitive human cell lines. Analysis of numerous glucocorticoid-resistant clones isolated from these sensitive cell lines has revealed that mutations in the human GR gene are the principal cause of steroid resistance *in vitro*. Analysis of glucocorticoid resistance in the human leukemic CEM-C7 cell line suggests that these leukemic cells escape the cytolytic effects of glucocorticoids by mutating their GR genes at functionally important sites. Data demonstrate that the wild-type, glucocorticoid-sensitive CEM-C7 cells express two alleles of GR, the normal GR and an abnormal GR, which has a $\mathrm{Leu}^{753} \rightarrow \mathrm{Phe}^{753}$ mutation in the ligand-binding domain. In a spontaneously arising glucocorticoid-resistant clone, only this abnormal GR is expressed, and the normal GR gene is deleted. This abnormal GR has been characterized as an "activation-labile" receptor. Although this mutated GR binds dexamethasone with the same relative affinity as the wild-type GR, activation or transformation of this dexamethasone–receptor complex to a DNA-binding form results in the dissociation of ligand (hence, the designation as activation-labile) and loss of transactivation capacity. Clearly, mechanisms other than mutations in the GR gene can contribute to *in vitro* glucocorticoid resistance in this human leukemic cell line. For instance, an endogenous modulator molecule appears to inhibit both nuclear translocation of the GR and glucocorticoid-induced apoptosis in this human leukemic cell line. Treatment of this sensitive CEM-C7 clone with glucocorticoid hormones also results in suppression of c-Myc and this response may play a key role in the hormone-induced apoptosis of these cells. This conclusion is demonstrated by the fact that sustained expression of c-Myc blocks glucocorticoid-induced apoptosis and antisense c-Myc oligomers induce cell lysis. These data are thus consistent with the theory that the lytic effects of glucocorticoids in CEM-C7 cells may involve reduction of c-Myc below the minimum levels required to maintain normal growth and viability. In multiple myeloma, it has been shown that aberrant GRs with deletions in their hormone-binding domains are generated by alternative splicing and that these abnormal GRs may contribute to glucocorticoid resistance in this hematological cancer. Whether these same mechanisms also occur *in vivo* during the development of glucocorticoid resistance in leukemias and multiple myeloma, and at what frequency, is not known. However, it is interesting to note that receptor-negative cell lines with GR gene deletions have been detected after exposure of CEM-C7 leukemic cells to chemical mutagens and cancer chemotherapeutic drugs. In contrast, spontaneously arising resistant cells are predominantly receptor positive but activation labile, suggesting that spontaneous and induced mutations may occur at different loci in human leukemic cells.

An association between a *Bcl*I restriction fragment length polymorphism in the human GR gene and hyperinsulinemia in obese women suggests that mutations in the GR gene may cause alterations in the regulation of the hypothalamic–pituitary axis and in target tissue sensitivity to glucocorticoid hormones. It has been reported that obese individuals have decreased glucocorticoid sensitivity in their adipose tissue and a decreased central sensitivity to glucocorticoids with a blunted negative feedback mechanism. In CEM-C7 glucocorticoid-sensitive leukemic cells, this polymorphic *Bcl*I fragment has actually been shown to contain a sequence from exon 2, which is deleted from glucocorticoid-resistant CEM clones.

## VI. GLUCOCORTICOID RESISTANCE IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

A major question concerning the clinical manifestations of human immunodeficiency virus (HIV) infection is that some of the symptoms and signs seen in the earlier stages of this disease cannot be easily explained by the relatively small proportion of infected CD4$^+$ T-lymphoid cells. One possible explanation for this observation is that there may be altered responses to hormones and hormone-like substances in HIV-infected cells. Several neuroendocrine orders have been described in HIV-infected individuals, and these include alterations in the hypothalamic–pituitary–adrenal axis. Several studies of adrenal function in HIV-infected patients have revealed high circulating levels of cortisol, as well as ACTH, and it has been speculated that this hypercortisolism may be associated with the immunodysfunction, neurological deterioration, and weight loss often seen in patients with acquired immunodeficiency syndrome (AIDS). It is well known that the physiological effects of glucocorticoid hormones include catabolic responses like those just mentioned. However, when a group of AIDS patients with high serum and urinary cortisol levels were studied, they exhibited a number of symptoms and signs associated with adrenal insufficiency, rather than hypercortisolism. Thus, the clinical picture associated with AIDS appears to be consistent with peripheral resistance to cortisol. The possibility that a GR abnormality is responsible for this glucocorticoid resistance was therefore investigated. Since the ability of glucocorticoid hormones to induce apoptosis of T-lymphocytes is well documented, and since HIV infection has also been reported to induce this programmed cell death, it was speculated that the hormone and virus might synergize to cause even greater cell death. However, when this hypothesis was directly tested in human CEM cells, an HIV-sensitive CD4$^+$ cell line, antagonism between HIV infection and glucocorticoids was actually detected. *In vitro* chronically infected CEM cells thus appear to be resistant to glucocorticoid-induced cell lysis. These infected CEM cells exhibit reduced numbers of GR-binding sites with little or no change in the affinity between the glucocorticoid hormone and its receptor. Of course, the obvious question to ask was whether this same GR abnormality is detected in the CD4$^+$ lymphocytes from HIV-infected patients. A published study of GR in HIV-infected homosexual men reported changes in the dissociation constant of the GR in these individuals. In more than half of the patients studied, the GR–ligand affinity was reduced. In another clinical study, GRs were analyzed in intravenous drug users with AIDS. These patients exhibited hypercortisolism and the clinical features associated with peripheral resistance to glucocorticoids. Again, the GRs expressed in the lymphocytes from these patients were not reduced in number (they actually increased in number based on binding of [$^3$H]dexamethasone), but did show a significantly reduced affinity for ligand. This reduction in GR-binding affinity was reflected by the fact that these lymphocytes were less sensitive to dexamethasone-induced inhibition of thymidine incorporation than lymphocytes from control subjects. Thus, the mechanisms underlying *in vitro* versus *in vivo* glucocorticoid resistance in HIV-infected cells may differ somewhat. Clearly, the elevated cortisol levels detected in HIV-infected individuals may have serious consequences. For example, if the HIV-infected CD4$^+$ T-cells in these patients are less sensitive to the lytic effects of cortisol than the uninfected T-cells, then the infected T-cells may survive longer in the presence of elevated cortisol. This theoretically would allow the HIV$^+$ cells to preferentially expand and hasten the onset of acquired immunodeficiency syndrome.

## VII. HUMAN MULTIPLE HORMONE RESISTANCE SYNDROME

Several years ago, the first report of human multiple steroid-resistance syndrome was published. A 14-year-old Native American girl presented with the syndrome of apparent mineralocorticoid excess. However, further evaluation revealed that she was resistant to glucocorticoids, mineralocorticoids, and androgens, but not to Vitamin D or thyroid hormones. Despite the fact that her circulating cortisol level was high, she did not exhibit any Cushingoid features such as obesity (primarily in the trunk), buffalo hump, moon face, or bruising. Her menstruation was regular, and there was no clinical evidence of masculinization (acne, hirsutism, enlargement of clitoris), despite the fact that her serum androgen levels were very high. This patient's younger sister showed similar clinical features. Both girls were partially resistant to exogenous glucocorticoids and mineralocorticoids.

Obviously, the mechanism(s) underlying this resistance to multiple steroid hormones could have been caused by defects at various points in the cascade of hormone–receptor DNA interactions. Mutations resulting in reduced expression of receptor proteins, reduced ligand-binding affinity of receptors, decreased nuclear translocation of activated steroid–receptor complexes, or reduced transactivating capability of nuclear receptor complexes could potentially explain this resistance. However, since each individual steroid hormone receptor is encoded by a different gene on a separate chromosome, it seemed very unlikely that simultaneous genetic mutations of each of these steroid receptors was the cause of this multiple hormone resistance in these two sisters. In fact, the involvement of several receptor pathways suggested a shared defect involving multiple steroid receptors. Therefore, a co-activator defect was deemed to be the most likely mechanism underlying this partial multiple hormone resistance. Although deletion analysis of several co-activators was performed, no deletions or major rearrangements were detected. However, since deletion analysis detects only large gene arrangements, other defects in these steroid receptor co-activators could not be totally ruled out. It was thus concluded that these two patients might represent the first cases of partial resistance to multiple steroids due to a co-activator defect. The phenotypic features of knockout mice lacking SRC-1 are apparently very similar to those reported in these two patients. The partial multiple hormone resistance reported in these sisters resembles the multiple hormone resistance syndrome that has been well described in New World primates.

## VIII. GLUCOCORTICOID RESISTANCE IN OTHER SPECIES

Although resistance to cortisol as a clinical syndrome in humans is fairly uncommon, this resistance is characteristic of several species, including New World monkeys, guinea pigs, and the prairie vole. By thoroughly analyzing the GRs in these species in which physiological anomalies exist in glucocorticoid-regulated responses, it has been possible to increase our understanding of how the different GR domains function to mediate a hormonal response. In both the New World primates and the guinea pig, which is a New World hystricomorph rodent, the circulating levels of endogenous glucocorticoids (cortisol) are high. Both species express GRs with a significantly lower affinity for ligand than the human GR.

Although the nucleotide sequences of the GR genes in these species are similar to those of the human gene, several amino acid substitutions in the GR proteins from these species have been reported. These amino acid substitutions, located primarily in the ligand-binding domains of these GR proteins, are the basis for the cortisol resistance observed *in vivo* in these species.

Analysis of the guinea pig GR ligand-binding domain has revealed that in the region reported to mediate association with a dimer of hsp90, there are 11 changes at residues that are completely conserved in the human, rat, and mouse GRs. The most important of these substitutions is a tryptophan at position 632 within this domain. The equivalent position in the human, rat, and mouse GRs has a conserved cysteine. This specific cysteine residue has been shown to be one of three sites of covalent attachment of the synthetic ligand, dexamethasone 21-mesylate, and therefore is most likely very important for steroid binding. In fact, mutation of this tryptophan to a cysteine in the ligand-binding domain of the guinea pig GR increases the sensitivity to dexamethasone by an order of magnitude. Another critical region involved in ligand binding and the interaction of hsp90 lies between residues 550 and 626 of the ligand-binding domain. The guinea pig sequence differs from the human GR by seven amino acids within this region. These substitutions may provide additional evidence for the molecular basis of glucocorticoid resistance in this species. In summary, in comparing the ligand-binding domain of the guinea pig GR with that of the human GR sequence, 10 of the 24 nucleotide differences result in amino acid substitutions. Finally, two additional amino acid substitutions, Pro112 and Asn140, for serine residues in the N-terminal domain of the guinea pig GR may also underlie this glucocorticoid resistance. These serine residues have been shown to be important phosphorylation sites, and their elimination may affect (decrease) transactivation by agonist-GR complexes. The marmoset (*Saguinus oedipus*), a tropical New World monkey, also exhibits high circulating levels of endogenous glucocorticoids and an altered GR ligand-binding domain when compared with the human GR. The marmoset GR is also one amino acid longer than the human GR, and this results from the insertion of a codon for arginine (Arg452) in the interfinger region of the DNA-binding domain. This amino acid insertion influences the affinity of the receptor for GREs and could potentially contribute to the end-organ resistance phenotype. The majority of amino acid substitutions in the marmoset GR are located within the amino-terminus of the receptor. More precisely, they are located within subdomains critical for binding to glucocorticoid-response elements and transactivation. These substitutions may well affect the capacity of the GR to regulate gene transcription. Of the 22 nucleotide substitutions in the marmoset GR ligand-binding domain, only 4 result in amino acid substitutions. Two of these amino acid substitutions are located within the site (amino acids 532–697) that has been shown to be crucial for association of the human GR with hsp90.

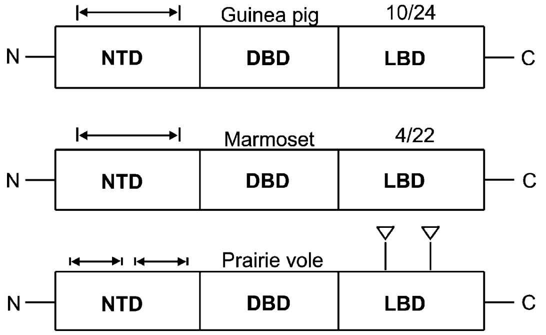

**FIGURE 3** Glucocorticoid receptor mutations in New World species clustering within specific regions of the receptor gene. Arrows and ▽ indicate positions of amino acid variations compared with the human receptor. The numbers of nucleotide substitutions in the ligand-binding domain resulting in codon differences that change the primary amino sequence are indicated. NTD, amino-terminal domain, DBD, DNA-binding domain, LBD, ligand-binding domain. Reprinted from *Steroids*, **61**, S. Werner and M. Bronnegard, Molecular basis of glucocorticoid-resistant syndromes, pp. 216–221. Copyright 1996, with permission from Elsevier Science.

The prairie vole (*Microtus ochrogaster*), a New World rodent, is also very resistant to glucocorticoids. Although plasma corticosterone levels are very high in this species, these animals do not exhibit the signs of hypercortisolism and thus are a novel rodent model for tissue resistance to glucocorticoids. As seen in Fig. 3, the vole GR contains several nonconservative amino acid substitutions in the N-terminal domain and two in the ligand-binding domain. These substitutions clearly have the potential to alter ligand-binding affinity as well as transactivation of gene expression. Binding assays have in fact revealed a significantly lowered dexamethasone affinity for brain and liver GRs in the prairie vole compared with rat GRs in the same tissues.

## IX. SUMMARY

Resistance to glucocorticoid hormones, such as cortisol, occurs when these adrenal steroids are unable to mediate their full physiological effects on specific target tissues and cells. Expression of this phenotype has numerous consequences, since endogenous glucocorticoids regulate important metabolic pathways and exogenous glucocorticoids are used therapeutically to treat a wide range of nonmalignant as well as malignant diseases. This resistant phenotype may be inherited, as is the case with generalized glucocorticoid resistance. This type of resistance is characterized clinically by elevated ACTH and cortisol levels, both of which occur because glucocorticoids are unable to exert their normal negative feedback at the level of the hypothalamus and anterior pituitary gland. Because of the generalized nature of this form of glucocorticoid resistance, the clinical signs and symptoms normally associated with Cushing's syndrome are not detected. Alternatively, glucocorticoid resistance may be acquired or, in some situations, pharmacologically induced. In some situations, such as steroid-resistant asthma and rheumatoid arthritis, the clinical symptoms are transient and tissue-specific. Most of these forms of resistance are the result of specific defects in the intracellular GR that mediate glucocorticoid responses within a target cell. However, it has been speculated that the glucocorticoid resistance that occurs as part of the human multiple hormone resistance syndrome may result from a defective co-activator molecule (transcriptional-activating protein) that is shared by multiple steroid receptor pathways. Finally, although resistance to glucocorticoids is fairly rare in humans, partial resistance to these hormones is characteristic of several species, including New World monkeys and rodents. Analysis of the specific GR defects in these species has shed light on how the different domains of this protein function to mediate a glucocorticoid response.

## Glossary

**generalized inherited (familial) glucocorticoid resistance** A rare inherited syndrome characterized by elevated plasma cortisol levels but lacking the symptoms characteristic of Cushing's syndrome because of general target tissue resistance to glucocorticoids.

**glucocorticoid receptor** The intracellular receptor protein that binds glucocorticoid hormones, such as cortisol, with high affinity and specificity and subsequently functions as a ligand-activated transcription factor in glucocorticoid target cells.

**glucocorticoid-response elements** Specific DNA sequences (15 nucleotides) that are located in proximity to hormone-responsive gene promoters and reversibly bind activated glucocorticoid receptor complexes.

**multiple hormone resistance syndrome** This rare syndrome characterized by partial resistance to several steroid hormones including glucocorticoids, mineralocorticoids, and androgens may be caused by a defect in a co-activator molecule that is shared by multiple steroid receptor pathways.

**receptor domains** The three major functional regions of the glucocorticoid receptor include the N-terminal, or variable, domain that contains the epitopes recognized by several monoclonal antibodies; the central DNA-binding domain containing two zinc-fingers required for binding to DNA; and the carboxy-terminal ligand-binding domain that is involved in steroid binding.

## See Also the Following Articles

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# Glucocorticoids and Asthma

Peter J. Barnes
*Imperial College, London*

I. MOLECULAR MECHANISMS
II. EFFECTS ON AIRWAY INFLAMMATION
III. CLINICAL EFFICACY IN ASTHMA
IV. ADD-ON THERAPIES
V. PHARMACOKINETICS
VI. SIDE EFFECTS OF INHALED GCs
VII. SYSTEMIC GCs
VIII. STEROID-RESISTANT ASTHMA

**Glucocorticoids (GCs), or corticosteroids, are by far the most effective therapy currently available for the management of asthma, and clinical improvement with GC is one of the hallmarks of asthma. Inhaled GCs have revolutionized asthma treatment and have become the mainstay of therapy for patients with persistent disease. We now have a much better understanding of the molecular mechanisms whereby GCs suppress inflammation in asthma and why they may be ineffective in rare patients who are steroid resistant. There is now a much better understanding of how to use inhaled GCs in asthma treatment and of the safety of these drugs in the long-term management of adults and children with asthma. Systemic GCs are used to treat exacerbations of asthma, but their use in chronic management has been superceded by that of inhaled GCs in all but the most severe cases of asthma.**

## I. MOLECULAR MECHANISMS

Glucocorticoids (GCs) constitute a highly effective anti-inflammatory therapy in asthma and the molecular mechanisms involved in the suppression of airway inflammation in asthma are now better understood. GCs are extremely effective in asthma because they block many of the inflammatory pathways that are abnormally activated in asthma and they have a wide spectrum of anti-inflammatory actions.

### A. Glucocorticoid Receptors

GCs bind to members of a single class of glucocorticoid receptor (GR) that are localized in the cytoplasm of target cells. GCs bind at the C-terminal end of the receptor, whereas the N-terminal end of the receptor is involved in gene transcription. Between these domains is the DNA-binding domain, which has two finger-like projections formed by a zinc molecule bound to four cysteine residues that bind to the DNA double helix. The inactive GR is bound to a protein complex that includes two molecules of the 90 kDa heat shock protein (hsp90) and various other proteins that act as "molecular chaperones," preventing the unoccupied GR from moving into the nuclear compartment. Once a GC binds to a GR, conformational changes in the receptor structure result in the dissociation of these chaperone molecules, thereby exposing nuclear localization signals on the GR, resulting in rapid nuclear localization of the activated GR–GC complex and its binding to DNA (Fig. 1). Two GR molecules bind to DNA as a dimer, resulting in changed transcription. A splice variant of the GR, termed GR-β, that does not bind to GCs but does bind to DNA and that may theoretically interfere with the action of GCs has been identified.

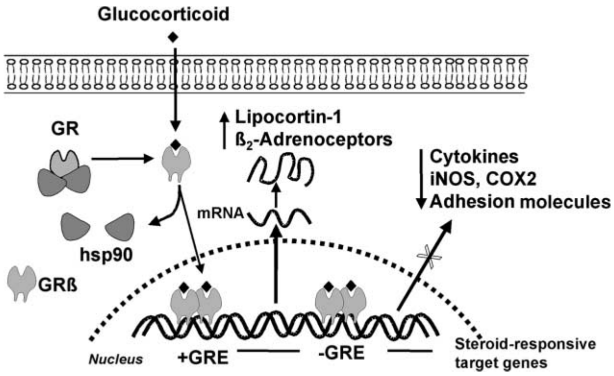

**FIGURE 1** Classical model of corticosteroid action. GCs enter the cell and bind to cytoplasmic GRs that are complexed with two molecules of hsp90. GR translocates to the nucleus, where, as a dimer, it binds to a GRE on the 5′-upstream promoter sequence of steroid-responsive genes. GREs increase transcription, whereas negative GREs may decrease transcription, resulting in increased or decreased mRNA and protein synthesis. An isoform of GR, GR-β, binds to DNA but is not activated by GCs.

## B. Increased Gene Transcription

GCs produce their effect on responsive cells by activating GRs to directly or indirectly regulate the transcription of certain target genes. The number of genes per cell directly regulated by GCs is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with other transcription factors. GR dimers bind to DNA at consensus sites termed glucocorticoid-response elements (GREs) in the $5'$-upstream promoter region of steroid-responsive genes. This interaction changes the rate of transcription, resulting in either induction or repression of the gene. Interaction of the activated GR homodimer with the GRE usually increases transcription, resulting in increased protein synthesis. GR may increase transcription by interacting with a large co-activator molecule, CREB (cyclic AMP response element binding protein) binding protein (CBP), which is bound at the start site of transcription and switches on RNA polymerase, resulting in the formation of messenger RNA (mRNA) and the subsequent synthesis of protein. Binding of activated GR to CBP results in increased acetylation of core histones around which DNA is wound within the chromosomal structure. This results in the binding and activation of RNA polymerase, which then results in mRNA formation.

## C. Decreased Gene Transcription

In controlling inflammation, the major effect of GCs is to inhibit the synthesis of inflammatory proteins, such as cytokines. This was originally believed to occur through the interaction of GRs with negative GREs, resulting in repression of transcription. However, negative GREs have rarely been demonstrated. GRs may also affect the synthesis of some proteins by reducing the stability of mRNA, through effects on ribonucleases that break down mRNA.

## D. Interaction with Transcription Factors

Activated GRs may bind directly with several other activated transcription factors as a protein–protein interaction. Most of the inflammatory genes that are activated in asthma do not appear to have GREs in their promoter regions yet are repressed by GCs. GCs inhibit the effects of transcription factors that regulate the expression of genes that code for inflammatory proteins, such as cytokines, inflammatory enzymes, adhesion molecules, and inflammatory receptors. These "inflammatory" transcription factors include activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), which may regulate many of the inflammatory genes that are switched on in asthmatic airways.

## E. Effects on Chromatin Structure

There is increasing evidence that GCs may have effects on the structure of chromatin. DNA in chromosomes is wound around histone molecules in the form of nucleosomes. Several transcription factors interact with large co-activator molecules, such as CBP, that bind to the basal transcription factor apparatus. Several transcription factors bind directly to CBP, including AP-1, NF-κB, and GR. At the microscopic level, chromatin may become dense or opaque due to the winding or unwinding of DNA around the histone core. CBP has histone acetylation activity, which is activated by the binding of transcription factors, such as AP-1 and NF-κB. Acetylation of histone residues results in the unwinding of DNA coiled around the histone core, thus opening up the chromatin structure, which allows transcription factors to bind more readily, thereby increasing transcription. Repression of genes follows the reversal of this process by histone deacetylation. Deacetylation of histone increases the winding of DNA around histone residues, resulting in a dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of inflammatory genes. Activated GRs may bind to several transcription co-repressor molecules that associate with histone deacetylases (HDACs), leading to repression of inflammatory genes. In addition, activated GRs recruit HDACs to the transcription start site, resulting in deacetylation of histones and a further decrease in inflammatory gene transcription.

## F. Target Genes in Inflammation Control

GCs may control inflammation by inhibiting many aspects of the inflammatory process in asthma; this is achieved by increasing the transcription of anti-inflammatory genes and decreasing the transcription of inflammatory genes (Table 1).

Anti-inflammatory proteins that are increased by corticosteroids include lipocortin-1, interleukin-1 (IL-1) receptor antagonist, IL-10, and secretory leukoprotease inhibitor. In addition, corticosteroids increase the expression of $\beta_2$-adrenoceptors and this may prevent the down-regulation of these receptors that may occur with regular dosing with $\beta_2$-agonist bronchodilators.

**TABLE 1** Effect of GCs on Gene Transcription

| |
|---|
| ***Increased transcription*** |
| Lipocortin-1 (phospholipase $A_2$ inhibitor) |
| $\beta_2$-Adrenoceptor |
| Secretory leukoprotease inhibitor |
| Clara cell protein (CC10, phospholipase $A_2$ inhibitor) |
| IL-1 receptor antagonist |
| IL-1R2 (decoy receptor) |
| IκB-α (inhibitor of NF-κB) |
| ***Decreased transcription*** |
| Cytokines |
| (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-11, IL-12, IL-13, IL-16, IL-17, IL-18, TNF-α, GM-CSF, SCF) |
| Chemokines |
| (IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxins) |
| Inducible nitric oxide synthase |
| Inducible cyclo-oxygenase (COX-2) |
| Cytoplasmic phospholipase $A_2$ |
| Endothelin-1 |
| $NK_1$ receptors, $NK_2$ receptors |
| Bradykinin $B_1$ and $B_2$ receptors |
| Adhesion molecules (ICAM-1, E-selectin) |

Corticosteroids inhibit all of the inflammatory genes that are abnormally expressed in asthmatic airways, including cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors for inflammatory mediators.

### G. Effects on Cell Function

GCs may have direct inhibitory actions on several inflammatory cells and structural cells that are implicated in asthma, including macrophages, eosinophils, T-lymphocytes, and dendritic cells (Fig. 2). Corticosteroids reduce the number of mast cells in the airway mucosa and this may account for the marked inhibitory effects of corticosteroids on allergen- and exercise-induced asthma. A striking effect of corticosteroid therapy is a reduction in the number of eosinophils in the airways, due to (1) an inhibitory effect on the release of eosinophil chemotactic chemokines, such as eotaxin, (2) inhibition of eosinophil differentiation resulting from a reduction in IL-5, and (3) a direct increase in apoptosis.

It is now apparent that corticosteroids also have an important inhibitory effect on the expression of inflammatory genes by structural cells of the airway, such as epithelial, endothelial, and airway smooth muscle cells, which may be the major source of inflammatory mediators in chronic asthma. Of particular importance for inhaled corticosteroids are airway epithelial cells, which are exposed to the highest concentration of steroids. Corticosteroids also suppress mucus hypersecretion, which is another characteristic of asthmatic inflammation.

## II. EFFECTS ON AIRWAY INFLAMMATION

GCs are remarkably effective at suppressing inflammation in asthmatic airways. Biopsy studies in patients with asthma show that inhaled GCs reduce the number and activation of inflammatory cells in the airway mucosa and in bronchoalveolar lavage. The disrupted epithelium is restored and the ciliated cell to goblet cell ratio is normalized after 3 months of therapy with inhaled GCs.

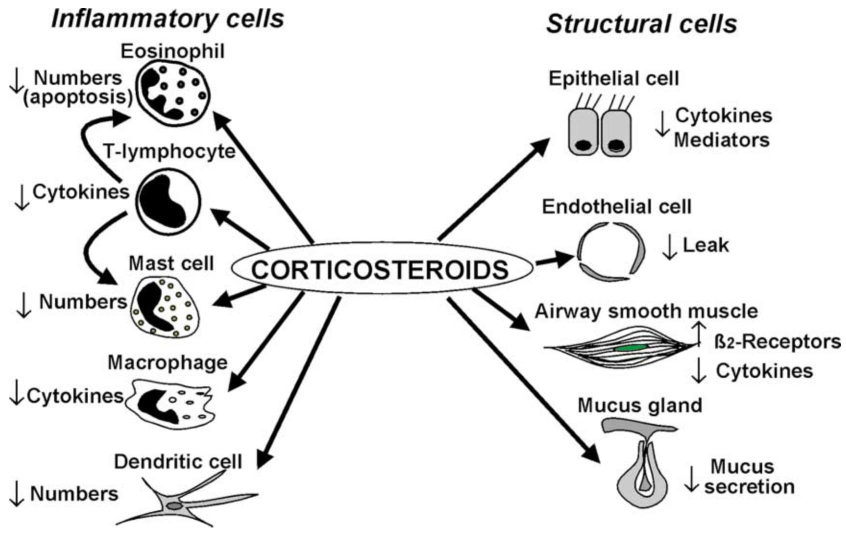
Inflammatory cells
Eosinophil
↓ Numbers (apoptosis)
T-lymphocyte
↓ Cytokines
Mast cell
↓ Numbers
Macrophage
↓ Cytokines
Dendritic cell
↓ Numbers
CORTICOSTEROIDS
Structural cells
Epithelial cell
↓ Cytokines Mediators
Endothelial cell
↓ Leak
Airway smooth muscle
↑ ß2-Receptors
↓ Cytokines
Mucus gland
↓ Mucus secretion

**FIGURE 2** Cellular effect of GCs.

By reducing airway inflammation, inhaled GCs consistently reduce airway hyperresponsiveness (AHR) in asthmatic adults and children. Chronic treatment with inhaled GCs reduces responsiveness to histamine, cholinergic agonists, allergen (early and late responses), exercise, fog, cold air, bradykinin, adenosine, and irritants (such as sulfur dioxide). The reduction in AHR takes place over several weeks and may not be maximal until several months of therapy have been completed. In many patients, AHR does not return to normal and this may reflect suppression of the inflammation but persistence of structural changes that cannot be reversed by GCs.

## III. CLINICAL EFFICACY IN ASTHMA

Inhaled GCs are very effective at controlling asthma symptoms in asthmatic patients of all ages and at all levels of disease severity. Inhaled GCs improve the quality of life of patients with asthma and allow many patients to lead normal lives; they improve lung function, reduce the frequency of exacerbations, and may prevent irreversible airway changes. They were first introduced to reduce the requirement for oral GCs in patients with severe asthma, and many studies have confirmed that the majority of patients can be weaned off oral GCs. But as experience was gained with inhaled GCs, they were introduced in patients with milder asthma, with the recognition that inflammation is present even in patients with mild asthma. Inhaled anti-inflammatory drugs have now become the first-line therapy in any patient who needs to use a $\beta_2$-agonist inhaler more than once a day and this is reflected in national and international guidelines for the management of chronic asthma. Inhaled GCs reduce asthma symptoms and the need for rescue $\beta_2$-agonists and they also significantly reduce exacerbations and the need for hospitalization.

Inhaled GCs are used to treat mild, moderate, and severe persistent asthma, with dosage increasing as asthma severity increases. A fourfold increase in dosage controls exacerbations of asthma. However, when inhaled GCs are discontinued, there is usually a gradual increase in symptoms and airway responsiveness back to pretreatment values.

Inhaled GCs are equally effective in children at all grades of asthma severity and are effective in young children. Nebulized GCs reduce the need for oral GCs and also improve lung function in children under the age of 3 years.

### A. Dose–Response Studies

Surprisingly, the dose–response curve for the clinical efficacy of inhaled GCs is relatively flat and, although all studies have demonstrated a clinical benefit of inhaled GCs, it has been difficult to demonstrate differences between doses, with the most benefit obtained at the lowest doses used. This is in contrast to the steeper dose response for systemic effects, indicating that although there is little clinical benefit from increasing doses of inhaled GCs, the risk of adverse effects is increased. However, the dose–response effect of inhaled GCs may depend on the parameters measured and, although it is difficult to discern a dose response when traditional lung function parameters are measured, there may be a dose–response effect in prevention of asthma exacerbations.

### B. Prevention of Irreversible Airway Changes

Some patients with asthma develop irreversible airflow obstruction, presumably due to structural changes in the airways resulting from uncontrolled chronic inflammation. There is some evidence that the annual decline in lung function may be slowed by the introduction of inhaled GCs. Increasing evidence also suggests that a delay in starting inhaled GCs may result in less overall improvement in lung function in both adults and children. These studies suggest that introduction of inhaled GCs at the time of diagnosis is likely to have the greatest impact, and extensive studies are now under way to assess the benefit of very early introduction of inhaled GCs in children and adults. Thus far, there is no evidence that early use of inhaled GCs is curative, and even when inhaled GCs are introduced at the time of diagnosis, symptoms and lung function revert to pretreatment levels when GCs are withdrawn.

### C. Reduction in Mortality

Inhaled GCs may reduce the mortality from asthma but it is not possible to conduct prospective studies. In a retrospective review of the risk of mortality and prescribed anti-asthma medication, there was a significant protection provided by regular inhaled corticosteroid therapy.

### D. Cost Effectiveness

Although inhaled GCs may be more expensive than short-acting inhaled $\beta_2$-agonists, they are the most cost-effective way of controlling asthma, since reducing the frequency of asthma attacks will save on total costs. Inhaled GCs also improve the quality of life of patients with asthma and allow many patients to lead a normal lifestyle, thus saving costs indirectly.

## IV. ADD-ON THERAPIES

It was previously recommended that if asthma was not controlled, there should be a stepwise increase in the dose of inhaled GCs until symptom control was achieved. However, many studies have now demonstrated that adding another class of anti-asthma therapy gives better control than increasing the dose of inhaled GCs. This partly reflects the flatness of the dose response to inhaled GCs but may also reflect the complementary actions of the add-on therapy.

Inhaled long-acting $\beta_2$-agonists (LABAs), such as salmeterol and formoterol, appear to be the most effective add-on therapies and provide improved symptom control and reduced exacerbations. This has led to the introduction of fixed combination inhalers that contain a LABA and a GC (salmeterol/fluticasone, formoterol/budesonide). These inhalers provide the most effective and convenient means to control asthma.

Low-dose theophylline is an alternative add-on therapy, which is more effective than doubling the dose of inhaled corticosteroids but less effective than adding a LABA. Anti-leukotrienes, another oral add-on therapy, are also less effective than LABA.

### A. Steroid-Sparing Therapy

In patients who have serious side effects with maintenance GC therapy, there are several treatments that have been shown to reduce the requirement for oral GCs. These treatments are commonly termed corticosteroid sparing, although this is a misleading description that could be applied to any additional asthma therapy (including bronchodilators). The amount of GC sparing with these therapies is not impressive. Several immunosuppressive agents have been shown to have GC-sparing effects in asthma, including methotrexate, oral gold, and cyclosporin A. These therapies all have side effects that may be more troublesome than those of oral GCs and are therefore indicated only as an additional therapy to reduce the requirement of oral GCs. None of these treatments is very effective, but there are occasional patients who appear to show a good response. Because of side effects, these treatments cannot be considered as a way to reduce the requirement for inhaled GCs. Several other therapies, including azathioprine, dapsone, and hydroxychloroquine, have not been found to be beneficial. The macrolide antibiotic troleandomycin is also reported to have corticosteroid-sparing effects, but this is seen only with methylprednisolone and is due to reduced metabolism of this corticosteroid, so that there is little therapeutic gain.

## V. PHARMACOKINETICS

The pharmacokinetics of inhaled GCs is important in determining the concentration of drug reaching the target cells in the airways and in the fraction of drug reaching the systemic circulation and therefore causing side effects. Beneficial properties in an inhaled corticosteroid are a high topical potency, a low systemic bioavailability of the swallowed portion of the dose, and rapid metabolic clearance of any corticosteroid reaching the systemic circulation. After inhalation, a large proportion of the inhaled dose (80–90%) is deposited on the oropharynx and is then swallowed and therefore available for absorption via the liver into the systemic circulation (Fig. 3). This fraction is markedly reduced by using a large-volume spacer device with a metered dose inhaler (MDI) or by using mouthwash and discarding the mouthwash with dry powder inhalers. Between 10 and 20% of inhaled drug enters the respiratory tract, where it is deposited in the airways, and this fraction is available for absorption into the systemic circulation. Most of the early studies on the distribution of inhaled GCs were conducted in healthy volunteers, and it is not certain what effect inflammatory disease, airway obstruction, age of the patient, or concomitant medication may have on the disposition of the inhaled dose. There may be important differences in the metabolism of different inhaled GCs. Beclomethasone dipropionate is metabolized to its more active metabolite, beclomethasone monopropionate, in many tissues including lung, but there is no information about the absorption or metabolism of this metabolite in humans. Flunisolide and budesonide are subject to extensive first-pass metabolism in the liver so that less reaches the systemic circulation. Little is known about the distribution of triamcinolone. Fluticasone propionate is almost completely metabolized by first-pass metabolism, which reduces systemic effects.

When inhaled GCs were first introduced, it was recommended that they be administered four times daily, but several studies have now demonstrated that twice daily administration gives comparable control, although four times daily administration may preferable in patients with more severe asthma.

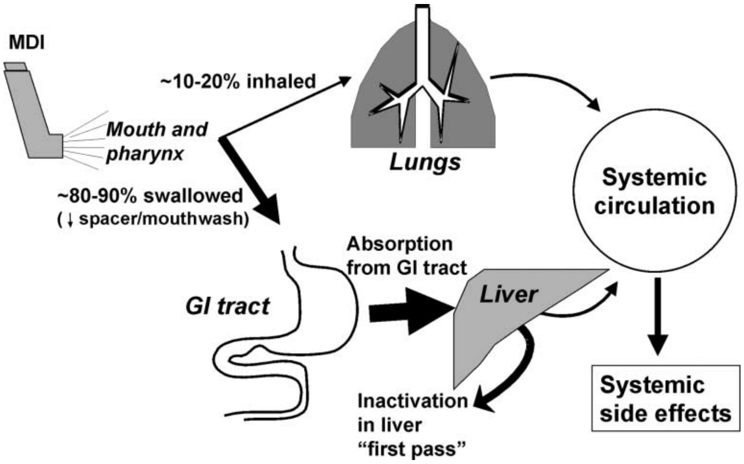

**FIGURE 3** Pharmacokinetics of inhaled GCs.

However, patients may find it difficult to comply with such frequent administration unless they have troublesome symptoms. For patients with mild asthma who require ≤400 μg/day, once daily therapy may be sufficient.

## VI. SIDE EFFECTS OF INHALED GCs

The efficacy of inhaled GCs is now established in short- and long-term studies in adults and children, but there are still concerns about their side effects, particularly in children and when high doses are needed. Several side effects have been recognized (Table 2).

### A. Local Side Effects

Side effects due to the local deposition of the inhaled corticosteroid in the oropharynx may occur with inhaled GCs, but the frequency of complaints depends on the dose and frequency of administration and on the delivery system used. The most common complaint is hoarseness of the voice (dysphonia) and this may occur in over 50% of patients using MDI. Dysphonia is not appreciably reduced by using spacers, but may be reduced with dry powder devices. Dysphonia may be due to myopathy of laryngeal muscles and is reversible when treatment is withdrawn. Oropharyngeal candidiasis (thrush) may be a problem in some patients, particularly in the elderly, with concomitant oral GCs and more than twice daily administration. Large-volume spacer devices protect against this local side effect by reducing the dose of inhaled corticosteroid that is deposited in the oropharynx.

**TABLE 2 Side Effects of Inhaled GCs**

- Local side effects
  - Dysphonia
  - Oropharyngeal candidiasis
  - Cough
- Systemic side effects
  - Adrenal suppression
  - Growth suppression
  - Bruising
  - Osteoporosis
  - Cataracts
  - Glaucoma
  - Metabolic abnormalities (glucose, insulin, triglycerides)
  - Psychiatric disturbances

### B. Systemic Side Effects

The efficacy of inhaled GCs in the control of asthma is undisputed, but there are concerns about the systemic effects of inhaled GCs, particularly because they are likely to be used over long periods and in children of all ages. The safety of inhaled GCs has been extensively investigated since their introduction 30 years ago. One of the major problems is deciding whether a measurable systemic effect has any significant clinical consequence, and this necessitates careful long-term follow-up studies. As biochemical markers of systemic corticosteroid effects become more sensitive, systemic effects may be seen more often, but this does not mean that these effects are clinically relevant. The systemic effect of an inhaled corticosteroid will depend on several factors, including the dose delivered to the patient, the site of delivery (gastrointestinal tract and lung), the delivery system used, and individual differences in the patient's response to the corticosteroid. Recent studies suggest that systemic effects of inhaled corticosteroids are reduced in patients with more severe asthma, presumably as less drug reaches the lung periphery.

The systemic side effects of inhaled GCs that have been studied most extensively are their effects on growth in children and the development of osteoporosis in adults. Recent careful studies in children with long-term follow-up have shown that inhaled GCs do not affect final adult height, although there may be some initial slowing of growth. Studies of bone density are complicated by the fact that patients with more severe asthma who are on higher doses of inhaled GCs are also treated with courses of oral GCs that are known to have a prolonged effect on bone metabolism. Thus far, no studies have shown a convincing effect on bone density and there is no evidence that inhaled GCs increase the risk of fracture. Other systemic effects attributed to inhaled GCs include cataracts and glaucoma, but this has not been convincingly demonstrated in controlled studies.

Based on extensive clinical experience, inhaled GCs appear to be safe in pregnancy, although no controlled studies have been performed. There is no evidence for any adverse effects of inhaled GCs on the pregnancy, the delivery, or the fetus. It is important to recognize that poorly controlled asthma may increase the incidence of perinatal mortality and retard intrauterine growth, so that more effective control of asthma with inhaled GCs may reduce these problems.

## VII. SYSTEMIC GCs

Oral or intravenous GCs may be indicated in several situations. Prednisolone, rather than prednisone, is the preferred oral corticosteroid, as prednisone must be converted in the liver to the active prednisolone. Enteric-coated preparations of prednisolone are used to reduce side effects (particularly gastric side effects) and give delayed and reduced peak plasma concentrations, although the bioavailability and therapeutic efficacy of these preparations are similar to those of uncoated tablets. Prednisolone and prednisone are preferable to dexamethasone, betamethasone, or triamcinolone, which have longer plasma half-lives and therefore an increased frequency of adverse effects.

Short courses of oral GCs (30–40 mg prednisolone daily for 1–2 weeks or until the peak flow values return to best attainable) are indicated for exacerbations of asthma, and the dose may be tailed off over 1 week once the exacerbation is resolved. The tailing-off period is not strictly necessary, but some patients find it reassuring. Maintenance oral GCs are needed in only a small proportion (approximately 1%) of asthmatic patients with the most severe asthma that cannot be controlled with maximal doses of inhaled GCs (2 mg daily) and additional bronchodilators. The minimal dose of oral corticosteroid needed for control should be used, and reductions in the dose should be made slowly in patients who have been on oral GCs for long periods (e.g., reduce by 2.5 mg prednisolone per month for doses down to 10 mg daily and thereafter by reduce by 1 mg per month). Oral GCs are usually given as a single morning dose, as this reduces the risk of adverse effects since it coincides with the peak diurnal concentrations of GCs. Alternate-day administration may also reduce adverse effects, but control of asthma may not be as good on the day when the oral dose is omitted in some patients.

Systemic GCs are given for acute severe asthma, and they speed the resolution of exacerbations and reduce the rate of readmission. Oral prednisolone is as effective as intravenous hydrocortisone or methylprednisolone and there is no evidence that high intravenous doses have any advantage. There is some evidence that high doses of nebulized GCs may also be effective in acute exacerbations of asthma, with a more rapid onset of action.

## VIII. STEROID-RESISTANT ASTHMA

Although GCs are highly effective in the control of asthma and other chronic inflammatory or immune diseases, a small proportion of patients with asthma fail to respond even to high doses of oral GCs. Resistance to the therapeutic effects of GCs is also recognized in other inflammatory and immune diseases, including rheumatoid arthritis and inflammatory bowel disease. Steroid-resistant patients, although uncommon, present considerable management problems. Corticosteroid-resistant asthma is defined as a failure to improve $FEV_1$ (forced expiratory volume in 1s) by $>15\%$ after treatment with oral prednisolone at a dose of 30–40 mg daily for 2 weeks, provided that the oral steroid is taken (verified by plasma prednisolone level or a reduction in early morning cortisol levels). These patients are not Addisonian and they do not suffer from the abnormalities in sex hormones described in the very rare familial glucocorticoid resistance. Plasma cortisol and adrenal suppression in response to exogenous cortisol are normal in these patients, so they suffer from side effects of GCs. Complete corticosteroid resistance in asthma is very rare, with a prevalence of

<1:1000 asthmatic patients. Much more common is a reduced responsiveness to GCs, so that large inhaled or oral doses are needed to control asthma adequately (steroid-dependent asthma). It is likely that there is a range of responsiveness to GCs and that steroid resistance is at one extreme of this range.

It is important to establish that the patient has asthma, rather than chronic obstructive pulmonary disease (COPD), "pseudo-asthma" (a hysterical conversion syndrome involving vocal cord dysfunction), left ventricular failure, or cystic fibrosis, which do not respond to GCs. Asthmatic patients are characterized by a variability in peak expiratory flow (PEF) and, in particular, a diurnal variability of >15% and episodic symptoms. Biopsy studies have demonstrated the typical eosinophilic inflammation of asthma in these patients.

The molecular mechanisms of steroid resistance are still not understood, but there are likely to be several mechanisms. GRs appear to function normally, but there are defects in the interactions of GRs with other transcription factors. Certain cytokines are able to reduce steroid responsiveness by enhancing the activity of transcription factors, such as AP-1, which then combine with and divert GRs. Other patients appear to have a defect in nuclear localization of the GR.

## Glossary

**asthma** Chronic inflammatory disease of the airways, resulting in variable airflow obstruction manifested as intermittent wheezing and shortness of breath.

**glucocorticoid receptor** Specific receptor for glucocorticoids that acts as a transcription factor on ligand activation.

**glucocorticoid-response element** Specific consensus DNA-binding site of the glucocorticoid receptor in the promoter region of steroid-sensitive genes.

**histone deacetylases (HDACs)** Proteins that deacetylase core histones around which DNA is wound, resulting in inflammatory gene suppression. Glucocorticoids recruit HDACs to activated inflammatory genes and thereby switch off inflammation.

**nuclear factor-κB** Pro-inflammatory transcription factor that regulates many of the inflammatory genes overexpressed in asthmatic airways and whose effects are inhibited by glucocorticoids.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Autoimmune Diseases • Glucocorticoids, Pharmacology of**

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# Glucocorticoids and Autoimmune Diseases

J. Bruce Smith, Mark K. Haynes,
John L. Abruzzo, and Ralphael J. DeHoratius
*Thomas Jefferson University, Pennsylvania*

I. INTRODUCTION
II. ANTI-INFLAMMATORY MECHANISMS
III. CLINICAL USE IN AUTOIMMUNE DISEASES
IV. ADVERSE REACTIONS TO STEROID TREATMENT
V. SUMMARY

**Cortisol and its several synthetic derivatives are widely employed in the treatment of systemic autoimmune conditions. They have a well-established place in the therapy of inflammatory and autoimmune conditions because of their profound anti-inflammatory and immune suppressive effects. However, their use is not without danger or controversy. While glucocorticoid treatment may allow patients to lead normal or near normal lifestyles, there are often concomitant undesirable and sometimes serious side effects and physicians must be vigilant regarding these.**

## I. INTRODUCTION

The anti-inflammatory effects of glucocorticoids were first investigated at the Mayo Clinic in 1948 by Hench and colleagues in patients with rheumatoid arthritis (RA). The history dates to 1929 when Hench noted that a patient who developed the sudden onset of jaundice experienced remission of RA symptoms. This was felt to be due to increased plasma cortisol because of the inability of the failing liver to metabolize it. These observations were later extended to pregnancy, also associated with increased cortisol levels. In 1938, Hench and co-workers reported pregnancy-related remission of arthritis in two patients with RA and one with psoriatic arthritis. These observations led to widespread use of cortisol and the development of more potent synthetic analogues (Table 1) Although the clinical results were dramatic, it was soon realized that significant toxicity was associated with glucocorticoid use. This article will address the mechanisms of action of glucocorticoids in systemic inflammatory processes, the clinical preparations available, their use in systemic autoimmune diseases, and their side effects.

## II. ANTI-INFLAMMATORY MECHANISMS

The inflammatory process is complex and includes activation of T and B lymphocytes, activation of cells of the monocyte/macrophage series, production of numerous cytokines and chemical mediators, alterations in leukocyte trafficking, and production of substances that result in vascular permeability. The anti-inflammatory effects of glucocorticoids are a result of their down-regulation of most of these processes.

Approximately 90% of glucocorticoid hormones are transported in the plasma bound to transcortin, an α-2 globulin that is produced by hepatocytes. The remaining 10% of glucocorticoid hormones are loosely bound to albumin and are able to enter the cytoplasm of cells. Once in the cytoplasm, they attach to high-affinity glucocorticoid receptor elements (primarily heat-shock protein 90), and these complexes are then transported to the cell nucleus where they bind to DNA (mostly in interphase cells) and modulate the transcription of DNA by competing with transcription factors such as activator protein-1 and nuclear factor κB. Thus, the subsequent production of messenger RNA and new protein synthesis are reduced. These transcription factors are particularly active in the production of tumor necrosis factor α (TNFα) and interferon-γ (IFN-γ), both important cytokines in the inflammatory process. Glucocorticoid receptors in the cytoplasm of cells exist in α and β

TABLE 1 Glucocorticoid Preparations and Their Potency

| Drug | Anti-inflammatory properties | Salt-retaining properties | Equivalent oral dose (mg) |
|---|---|---|---|
| Short-to-medium acting | | | |
| Hydrocortisone | 1 | 1 | 20 |
| Cortisone | 0.8 | 0.8 | 25 |
| Prednisone | 4 | 0.3 | 5 |
| Prednisolone | 5 | 0.3 | 5 |
| Methylprednisolone | 5 | 0 | 4 |
| Medium-to-long acting | | | |
| Triamcinolone | 5 | 0 | 5 |
| Fluprednisolone | 15 | 0 | 4 |
| Long acting | | | |
| Dexamethasone | 30 | 0 | 0.75 |

*Note.* All preparations are compared to 20 mg hydrocortisone with its anti-inflammatory and salt-retaining properties set to 1 by convention.

isoforms. Only the α isoform binds glucocorticoids. The β isoform acts as a glucocorticoid inhibitor and is found to be increased in blood lymphocytes of patients with RA who may exhibit resistance to the anti-inflammatory effects of glucocorticoid treatment. The α isoform of glucocorticoid receptor is highly expressed in monocytes, neutrophils, eosinophils, and most subpopulations of lymphocytes.

Transcortin becomes saturated when plasma cortisol levels are over 20–30 μg/dl. Thus, immune suppression and other clinical effects of steroid hormones occur in situations in which plasma glucocorticoid levels are elevated, as a result of either a disease state or exogenous administration. Via that mechanism, glucocorticoids profoundly inhibit the production of mediators involved in immune responses and inflammation (Fig. 1). These include the suppression of synthesis of nearly all cytokines, inhibition of phospholipase $A_2$, thus decreasing production of prostaglandin and leukotriene, and inhibition of histamine and bradykinin production. Glucocorticoids also reduce the expression of cyclooxygenase 2 (COX-2). Additional effects include direct cytotoxic activity for lymphocytes and it also seems likely that other direct effects of high-dose glucocorticoids occur. The fundamental mechanism of action is probably related to glucocorticoid suppression of transcription factors involved in the production of the various mediators mentioned above. The main effects of glucocorticoids on inflammatory processes are listed in Table 2.

The effects of glucocorticoid treatment on most immune functions is profound. Many functions of T lymphocytes are affected, especially delayed-type hypersensitivity and cytokine production. Within a few weeks of beginning glucocorticoid treatment, serum levels of immunoglobulins (Ig), particularly IgG, decrease. This is most likely related to the decreased synthesis of Ig by B cells. However, glucocorticoid treatment does not suppress all immune functions. For example, glucocorticoid treatment does not alter the function of natural killer (NK) cells or cells mediating antibody-dependent cellular cytotoxicity although these cells express the cytoplasmic receptors to approximately the same degree as monocytes and neutrophils and probably mediate their cytotoxic effects ultimately via production of TNFα.

Probably the most immunologically powerful effect of high-dose glucocorticoid treatment is inhibition of production of interleukin-1 (IL-1) by monocytes. IL-1 is a pivotal player in the cytokine cascade. It induces the production of IL-2 and IFN-γ, both highly pro-inflammatory. Increased IL-2 levels stimulate the activation of both T and B lymphocytes and the subsequent production of numerous other cytokines including TNFα, transforming growth factor-β, IL-6, IL-8, and others. Some of the biological effects of these cytokines are listed in Table 3.

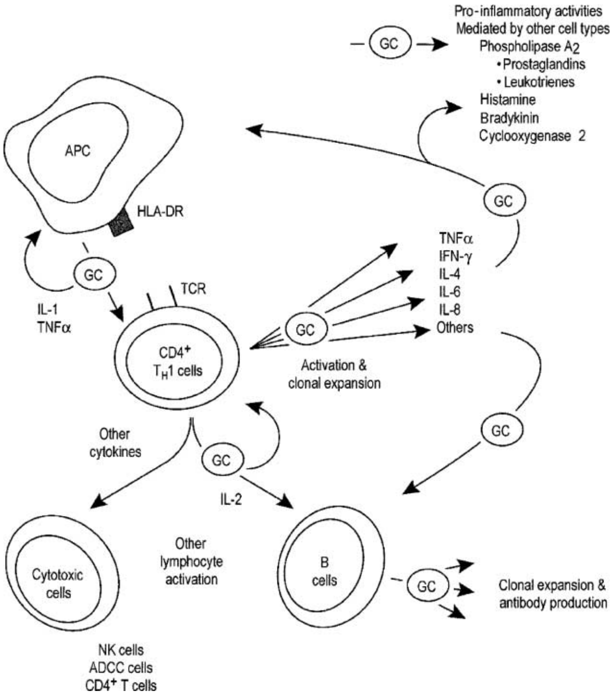

**FIGURE 1** Schematic of immune responses and cytokine production indicating where glucocorticoids intervene or suppress. The immune response results from interaction of $CD4^+$ T cells with environmental or autoantigens processed and presented to them by antigen-presenting cells (APC) in conjunction with histocompatibility class II molecules represented by human leukocyte antigen (HLA) class II in the diagram. The antigen is associated with HLA class II on the APC surface and interacts with the T-cell receptor (TCR) expressed on $CD4^+$ T cells. These T cells become activated and expand as clones of cells that specifically recognize the relevant antigen. Cytokines as indicated in the diagram mediate a variety of immune responses as noted in Table 3. These include further clonal expansion of T cells, enhancement of other immune cell activities, and activation and clonal expansion of B cells that produce specific antibodies. Glucocorticoids (GC) inhibit most of these responses via suppression of transcription factors as noted in the text. The cytotoxic functions of natural killer (NK) cells and cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC cells) are not affected by GC. $T_H1$, T helper cell type 1; $T_H2$, T helper cell type 2; TNFα, tumor necrosis factor α; IFN-γ, interferon-γ; IL-4, IL-6, and IL-8, interleukin-4, -6, and -8.

TABLE 2 Cellular Targets of Glucocorticoids

| Cell target | Effect |
|---|---|
| Lymphocytes | Short-term lymphopenia |
| | Depletion of recirculating lymphocytes |
| | Decreased production of IL-2 resulting in decreased clonal expansion of T and B lymphocytes |
| | Decreased production of chemoattractants |
| | Decreased proliferative response to antigens |
| | Decreased immunoglobulin synthesis in general |
| Monocytes/ macrophages | Transient monocytopenia |
| | Decreased delayed-type hypersensitivity |
| | Inhibition of Fc receptor binding |
| | Decreased chemotaxis |
| | Decreased bacteriocidal activity |
| Neutrophils | Increased in peripheral blood |
| | Slight decrease in chemotaxis but not much effect on function |
| | Decreased egress from vascular space |
| Eosinophils | Decreased in peripheral blood |
| | Decreased movement to immediate hypersensitivity test sites |

## III. CLINICAL USE IN AUTOIMMUNE DISEASES

A detailed review of the clinical characteristics of the autoimmune diseases in which glucocorticoids are prescribed is beyond the scope of this article, and readers are referred to current specialty textbooks for information. Diseases in which glucocorticoids are commonly employed include systemic lupus erythematosus (SLE), RA, multiple sclerosis (MS), inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease, various syndromes associated with vasculitis such as giant cell arteritis (GCA and polyarteritis nodosa), and other systemic diseases thought to have an immune component.

The rationale for treatment with glucocorticoids includes quick onset of action, their potency as anti-inflammatory agents and analgesics, and their ability to modify the clinical course of certain diseases. There are several studies that show a favorable disease-modifying effect of long-term low-dose prednisone in patients with RA. Also, early intervention with high oral doses (60–80 mg) of prednisone clearly prevents ocular complications including blindness in patients with GCA (also known as temporal arteritis). Although the side effects discussed below are often severe, treatment with glucocorticoids may permit patients to lead a normal daily existence and on some occasions can be life-saving. In addition, inhibition of inflammation may in some cases modify the disease process.

Glucocorticoids can be given orally, by intramuscular or intra-articular injection, or by intravenous infusion. The object of therapy is to prevent serious autoimmune damage to organs while adjusting the dose downward in an attempt to prevent serious adverse reactions (Table 4).

There are various glucocorticoid preparations in use. These preparations and their relative strengths compared to cortisone are presented in Table 1. In this article, oral glucocorticoid use generally refers to the use of prednisone and intravenous (iv) glucocorticoid usually refers to methylprednisolone (Solumedrol) since these are the preparations most commonly used in the treatment of patients.

### A. Low Dose Glucocorticoids

While it is true that most serious adverse reactions seen with glucocorticoid use occur with daily doses of 15–20 mg prednisone or greater, low oral doses generally mean less than 10 mg daily of prednisone or equivalent. Even so, most clinicians attempt to use 5 mg daily or less, often prescribing pills of 1 mg strength in order to be able to titrate to the lowest possible dose that alleviates symptoms. Dosing schedules include once a day, divided daily doses, and every other day. It is convenient and easy to prescribe prednisone in a once daily dose. It allows for more convenient manipulation of dosage in 1 mg increments and a single daily dose, as with other medications, provides better patient compliance. Dosing on a twice daily schedule is sometimes prescribed but it is key to remember that the clinical effect of this schedule is different from that of a single daily dose. For example, because of more sustained blood levels, 5 mg of prednisone twice a day provides a steroid effect that is more equivalent to 12 or 13 mg as a single daily dose than to 10 mg once a day. An every other day schedule is often prescribed because of the decrease in unwanted side effects. However, again there are clinical differences. For example, a single dose of 20 mg every other day provides a clinical effect that is somewhat less than that seen with 10 mg daily. In addition, patients with rheumatologic diseases often experience "breakthrough" symptoms on the days they do not take the medication. Often dosing schedules must be tailored to the individual patient. For example, some patients with polymyalgia rheumatica may require small maintenance doses of prednisone given twice daily

TABLE 3 **Biological Effects of Selected Cytokines**

| Cytokine | Activity |
|---|---|
| Primarily pro-inflammatory cytokines | |
| Interleukin-1 | Stimulates proliferation of T and B lymphocytes; activates T cells; activates metalloproteinases; increases production of ACTH and other pituitary hormones; has endogenous pyrogen activity; increases production of colony-stimulating factors (G-CSF, M-CSF, GM-CSF); increases expression of adhesion molecules; promotes thrombosis; chemoattractant for leukocytes; stimulates production of TNFα and additional IL-1 |
| Interleukin-2 | Stimulates proliferation of T and B lymphocytes; activates T lymphocytes; induces progression of cell cycle phases in resting cells; stimulates production of numerous other cytokines, especially IFN-γ and TNFα; induces proliferation of lymphokine-activated killer (LAK) cells |
| Interferon-γ | Enhances IL-1 production; enhances expression of cell surface HLA class II molecules on antigen-presenting cells and other cell types; stimulates expression of intercellular adhesion molecules; stimulates release of reactive oxygen species; stimulates production of TNFα and other $T_H1$-related lymphocyte functions |
| Tumor necrosis factor-α | Enhances IL-1 production; enhances expression of cell surface HLA class II molecules on antigen-presenting and other cells; enhances expression of intracellular adhesion molecules; stimulates additional production of TNFα |
| Interleukin-5 | Hematopoetic growth factor; promotes proliferation and activation of eosinophils; promotes generation of cytotoxic T cells |
| Interleukin-6 | Stimulates B-cell differentiation to plasma cells; increases antibody production; stimulates production of acute-phase reactants by hepatocytes; increases platelet counts; is prothrombotic; induces resting T cells to become cytotoxic |
| Interleukin-8 | Chemotactic factor for neutrophils and other migratory cell types; activates neutrophils; increases expression of adhesion molecules; increases angiogenesis |
| Interleukin-12 | NK-cell growth factor; promotes proliferation of LAK cells; enhances activation and proliferation of $T_H1$ lymphocytes |
| Primarily anti-inflammatory cytokines | |
| Interleukin-4 | Down-regulates $T_H1$-mediated immune functions; down-regulates IL-6 synthesis; decreases production of IL-1; stimulates B-cell differentiation and antibody production |
| Transforming growth factor-β | Inhibits many IL-1, IL-2, IFN-γ and TNFα activities; blocks activity of cytotoxic cells (LAK and T cells); pro-fibrotic; enhances matrix protein secretion; enhances production of tissue inhibitor of metalloproteinases inhibitor; chemoattractant for neutrophils |
| Interleukin-10 | Inhibits proliferation of cytokines produced by $T_H1$ and $T_H2$ cells; inhibits IL-1 production; promotes differentiation of cytotoxic T lymphocytes |

because they experience breakthrough symptoms as soon as 12 h after a dose.

Low-dose oral glucocorticoid treatment is indicated in systemic autoimmune disease patients who have mild to moderate symptoms despite prescription of nonsteroidal anti-inflammatory drugs (NSAIDs) or other indicated immune-suppressive treatments such as hydroxychloroquine or methotrexate. It should also be noted that patients with systemic autoimmune diseases who are being treated with glucocorticoids may benefit from adding an immune-suppressant drug as this may allow a reduction in the dose of glucocorticoid—the so-called "steroid-sparing effect."

Higher doses of orally administered glucocorticoids are indicated for acute flares of autoimmune diseases. For relatively mild flares, a "minipulse" with prednisone may be sufficient. It could be prescribed

TABLE 4 Adverse Reactions Associated with Glucocorticoid Treatment

| Common | Uncommon | Rare |
|---|---|---|
| Hypertension | Metabolic acidosis | Congestive heart failure |
| Osteoporosis | Diabetic ketoacidosis | Arrhythmias |
| Azotemia | Peptic ulcer disease | Hirsutism |
| Secondary Cushing's disease | Glaucoma | Fatty liver |
| Impaired wound healing | Pseudotumor cerebri | Pancreatitis |
| Increased susceptibility to infections | Spontaneous fractures | Convulsions |
| Elevated blood glucose | Psychosis | |
| Atherosclerosis/hyperlipidemia | | |
| Myopathy | | |
| Osteonecrosis | | |
| Mood alterations | | |
| Posterior subcapsular cataracts | | |

for 6 days as 30, 25, 20, 15, 10, and 5 mg and then discontinued or continued when the patient has reached his or her usual dose. For more severe flares, doses in the 60 mg range may be required and it may be necessary to continue at the high dose until symptoms abate before tapering to the usual dose.

## B. Intravenous Glucocorticoids

Intravenous glucocorticoids, usually Solumedrol, are administered for serious or life-threatening clinical situations. Common scenarios include flares in lupus involving vital organs (brain, kidney), vasculitis, and severe flares of rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and others. They are also used in conjunction with starting other immune-suppressive treatment. For example, a patient with dermatomyositis or vasculitis who is being started on methotrexate or cyclophosphamide will benefit from the anti-inflammatory and immune suppressive effects of iv methylprednisolone with rapid symptomatic improvement (over a period of days) while the more profound immune suppressive effects of methotrexate or cyclophosphamide will take weeks to develop.

A typical regimen for iv glucocorticoid administration in a patient with one of the rheumatic diseases is 1 g of methylprednisolone in 250 or 500 ml iv saline given over a period of 2–3 h on 3 consecutive days. Neurologists treating MS may use 1 g daily for a week or more. These regimens can be repeated on a periodic basis dictated by the clinical condition of the patient. When treating with monthly iv methylprednisolone, low doses of oral preparations are generally prescribed. Gastroenterologists, when they employ iv steroids for treating IBD, usually use hydrocortisone at 100 mg or methylprednisolone at 60 mg three times daily. In steroid-naive patients, some gastroenterologists use adrenocorticotropic hormone to stimulate endogenous glucocorticoid production.

## IV. ADVERSE REACTIONS TO STEROID TREATMENT

The immune and anti-inflammatory effects of glucocorticoids are highly potent and desirable in the treatment of autoimmune diseases. However the metabolic effects are also profound and account for the wide variety of side effects. The best way to prevent side effects is to keep the dose of glucocorticoid as low as possible while still maintaining the desired clinical effect. As noted above, strategies such as every-other-day dosing may be effective in preventing or minimizing side effects but may not provide the desired clinical effect. There are also medical interventions that serve to minimize potential side effects. Concomitant treatment with an immunosuppressive drug, most commonly Methotrexate, will often allow a reduction in the glucocorticoid dose without compromising the beneficial clinical response. Other measures are discussed in the individual sections that follow.

Many side effects are not particularly serious but are disconcerting to the patient. These include physical changes similar to Cushing's disease, such as centripetal obesity, "moon" facies, hirsutism, and striae. Other usually nonserious side effects include thinning of the skin, acne, alopecia, and purpura. More serious side effects and their possible prevention are discussed below with respect to the various target organ systems.

## A. Musculoskeletal System

Osteoporosis is one of the most serious side effects of glucocorticoid treatment. The mechanism appears to be a combination of inhibition of osteoblast function, increased bone resorption, increased urinary calcium loss, decreased calcium absorption, and decreased sex hormone production. Osteoporosis may develop after short term-low dose glucocorticoid treatment and it may occur in as many as 50% of patients receiving long-term glucocorticoid treatment. Osteoporosis occurs primarily in trabecular bone but cortical bone may also be a target. Preventive measures may be taken in patients who are being treated with glucocorticoids. This includes prescribing adequate doses of calcium and vitamin D along with an exercise program. Treatment with a bisphosphonate may also be considered unless there are contraindications. One is cautioned against using bisphosphonates in women of child-bearing potential as the effects on developing bone could be profound.

Osteonecrosis (avascular necrosis) occurs in patients receiving long-term glucocorticoid treatment and it is particularly common in patients with SLE. In SLE, osteonecrosis may occur with or without glucocorticoid treatment but it is certainly most commonly found in patients receiving greater than 20 mg daily of prednisone or equivalent. Osteonecrosis should be considered in at-risk patients who complain of sudden onset of joint pain when they are otherwise doing well. Diagnosis by typical X-ray findings can be accomplished in the later stages of osteonecrosis but magnetic resonance imaging may be required to see early changes in at-risk patients. Osteonecrosis is most commonly seen in the weight bearing joints, notably the hip, knee, and ankle. However, it can occur in non-weight-bearing joints such as the shoulder and it has been seen in the wrists and elbows of at-risk patients who stress these joints using canes or walkers. Growth retardation may be seen in children receiving systemic glucocorticoids.

Myopathy clinically presenting as muscle weakness is a common complication of glucocorticoid use. It may prove to be a diagnostic dilemma when treating patients with immune inflammatory diseases of muscle such as polymyositis or dermatomyositis. Onset of proximal muscle weakness is insidious and may occur at any time after starting glucocorticoid treatment. Myopathy appears to be dose-related and is generally not seen in patients receiving less than 20 mg prednisone or equivalent daily. Diagnosis is by combined electromyogram (EMG) and muscle biopsy. The EMG may or may not show myopathic changes and the biopsy usually shows dropout of type II muscle fibers.

Paradoxically, especially when higher doses are employed in the treatment of patients with rheumatoid arthritis, too rapid tapering of the glucocorticoid dose may result in diffuse polyarticular pain.

## B. Ocular

Posterior subcapsular cataracts are commonly seen in patients receiving long-term glucocorticoid treatment. These cataracts develop slowly and often occur bilaterally. Children may be more susceptible than adults to cataract formation. Increased intraocular pressure is also a common side effect. This side effect is most common after steroid eyedrop use but it can occur with systemic administration. Glaucoma usually occurs in patients who are otherwise predisposed by family history and in those receiving more than 10 mg daily over long time periods. Periodic ophthalmology examinations—at least once a year—are recommended.

## C. Gastrointestinal System

While glucocorticoids alone are not usually associated with peptic ulcer disease, concomitant use of glucocorticoids and traditional NSAIDs appears to be synergistic with respect to the development of gastritis, peptic ulcer disease, and gastrointestinal (GI) bleeding. It is not currently known whether glucocorticoids and COX-2 inhibitors will demonstrate the same synergy with respect to GI adverse events. Glucocorticoid use is also associated with pancreatitis and fatty infiltration of the liver but these are less common.

Patients being treated with glucocorticoids often exhibit increased blood sugar but usually do not become ketotic. Hepatic gluconeogenesis, decreased peripheral sugar utilization, and suppression of insulin secretion by pancreatic islet cells all contribute to the elevated blood sugar. Development of overt diabetes mellitus in a patient with a negative family history and a normal glucose tolerance test is unusual but the relative risk rises with the glucocorticoid dose.

## D. Cardiovascular and Renal

Hypertension is one of the most common cardiovascular effects and azotemia is the most common renal side effect. Hypertension is related in part to salt and fluid retention but other factors are also important.

These include higher baseline blood pressure, age, and enhanced activity of the renin–angiotensin system. Azotemia can occur due to the catabolic effect of glucocorticoids on muscle. Glucocorticoid treatment elevates levels of lipoproteins and that probably represents a mechanism for an increased incidence of atherosclerosis along with increased peripheral vascular calcifications found in patients being treated with these drugs. This side effect is not likely to be found in patients receiving less than 20 mg daily unless treatment has been long-term (years versus weeks or months). Acute cardiac death has been reported in patients receiving high-dose pulse iv methylprednisolone. Arrhythmias and electrolyte balance disturbances in patients with concomitant cardiac disease were probably factors regarding these adverse events.

### E. Central Nervous System

Euphoria is a common side effect of high-dose glucocorticoid treatment. Memory loss has been reported and insomnia may occur, particularly when patients take their glucocorticoid medications in the evening or at bedtime. Psychiatric states such as depression and mania are also observed. Overt psychosis may be seen in patients receiving more than 20 mg prednisone for extended periods. This may be diagnostically confusing in patients who also have central nervous system involvement from SLE or in MS patients.

### F. Immune System

In addition to the desired effects regarding immune suppression, there is a concomitant undesirable dose-related effect regarding infections. These include reactivation of latent tuberculosis (TB) and increased susceptibility to bacterial, viral, and fungal infections. Herpes zoster is common and should be treated with an anti-herpes agent as soon as it is clinically suspected. Infections with opportunistic organisms are vastly increased in patients receiving glucocorticoids compared to patients not receiving these medications. Close monitoring for symptoms of infectious processes and other precautions are warranted. Skin testing for TB and a screening chest X ray should be performed in patients who are going to be treated with high-dose or long-term glucocorticoids. Individuals with a positive result in either test should receive TB prophylaxis prior to starting glucocorticoid therapy. Vaccination against *Pneumococcus* and influenza should also be recommended to patients who are going to be treated with glucocorticoids or who are already on long-term treatment.

## V. SUMMARY

Glucocorticoids are commonly employed in the treatment of systemic autoimmune diseases. Clinical use may be required to manage unacceptable day-to-day symptoms (general quality of life issues), for maintenance of the patient at an acceptable disease activity level, for treating acute flares of diseases, for treatment of vital organ involvement, or occasionally as a life-saving measure such as in multiple organ failure. The effects of glucocorticoids on the immune response and on the production of a variety of mediators of inflammation are profound and the desired immune suppression may be attended by a number of unwanted side effects such as susceptibility to opportunistic infections. In addition, no organ system is exempt from the metabolic side effects of these drugs. Since unwanted side effects are generally dose-related, one should attempt to use the lowest dose required to obtain the desired clinical effect. Measures to prevent side effects in both acute and chronic use situations are discussed.

## Glossary

**antigen-presenting cells** Cells such as macrophages and dendritic cells that can bind and engulf foreign substances or "antigens," break them down into smaller units, and insert them into their cell surface membranes where they are "presented" for interaction with T lymphocytes.

**arteritis** A condition in which the walls of arteries are infiltrated with inflammatory cells, causing damage to the arterial walls and often leading to compromised blood flow and local clotting.

**cytokine** Intercellular hormone-like messenger proteins that are usually active within a small radius of the cells producing them. They are products of many different cell types but particularly of activated cells of the immune system.

**immune response** Series of events that occurs after T and B cells are exposed to antigens for which they have a specific receptor. T cells that are thus activated produce cytokines that foster the proliferation of activated cells (clonal expansion) plus the recruitment and activation of other cells of the immune system. B cells also proliferate as a result of the actions of cytokines and secrete the antibody specific for the antigen.

**myositis** Inflammation of muscle.

**osteonecrosis** A condition in which there is decreased blood supply to bones—usually weight-bearing bones near joints. It is also known as avascular necrosis.

**osteoporosis** A condition in which there is decreased bone density as a result of loss of calcium. It commonly occurs after menopause, after long periods of inactivity, and as a result of drug treatment, particularly with glucocorticoids.

**T cells and B cells** Lymphocytes in general are divided into T and B cells. Subgroups of T cells exhibit diverse activities including production of cytokines, mediation of delayed-type hypersensitivity, regulation of on-going immune responses, cytotoxic functions, and "help" for the activation of B cells. T cells interact with APCs via an antigen-binding molecule, the T-cell receptor (TCR). The general T-cell population has TCRs for all the antigens that they would encounter in an individual's lifetime. B cells produce antibodies. The B-cell surface receptor molecule is an antibody and when it binds to its specific antigen, activated B cells secrete antibodies of the same specificity.

## See Also the Following Articles

**Corticotropin-Releasing Hormone, Stress, and the Immune System • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids, Pharmacology of • Osteoporosis: Pathophysiology**

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# Glucocorticoids, Pharmacology of

Isabelle Bourdeau and
Constantine A. Stratakis
*National Institute of Child Health and Human Development, National Institutes of Health, Maryland*

I. INTRODUCTION
II. STEROID BIOSYNTHESIS
III. REGULATION OF STEROID SECRETION
IV. MECHANISMS OF ACTION OF GLUCOCORTICOID RECEPTORS
V. SYNTHETIC STEROIDS
VI. STEROID-BINDING PLASMA PROTEIN AND METABOLISM
VII. THERAPEUTIC USES OF GLUCOCORTICOIDS AND MINERALOCORTICOIDS
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IX. SUMMARY

**Normal levels of glucocorticoids are essential for the maintenance of physiologic homeostasis and for an adequate response to stress. Both the lack of glucocorticoids (adrenal insufficiency) and excess glucocorticoids (as seen in Cushing syndrome) lead to life-threatening conditions.**

## I. INTRODUCTION

The adult human adrenal gland is composed of two parts: the cortex (outer part), which originates from mesenchymal cells, and the catecholamine-producing medulla (inner part), which is derived from neuroectodermal cells. The cortex, which represents 90% of the weight of the gland, is made of three zones: the zona glomerulosa (outermost, just under the adrenal capsule), the zona fasciculata (middle), and the zona reticularis (innermost, next to the medulla). Each cortical zone has the capacity to secrete its own specific steroids. The fasciculata and reticularis zonae synthesize glucocorticoids (in humans, cortisol in the fasciculata and androgenic steroids in the reticularis); the zona glomerulosa synthesizes aldosterone.

## II. STEROID BIOSYNTHESIS

All adrenal steroid hormones have a 21-carbon structure containing a four-ring nucleus derived from cholesterol (Fig. 1). Cholesterol is converted to steroid hormones after specific cytochrome P450

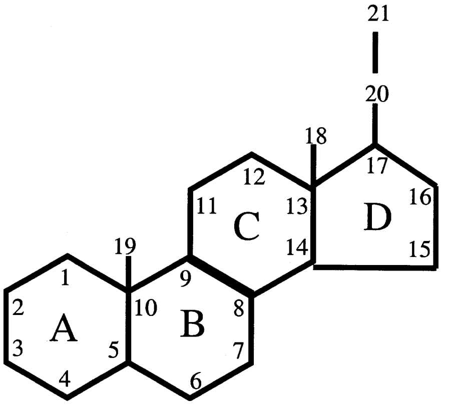

**FIGURE 1** All adrenal steroid hormones have 21 carbons distributed mainly in a four-ring nucleus (rings A, B, C, and D) derived from the cholesterol molecule.

(CYP) enzymes act on its different ring carbons. The CYPs are oxidative enzymes localized in the mitochondria or in the endoplasmic reticulum. They act on steroids by oxygen insertion (hydroxylation) reactions or by oxidative carbon–carbon bond cleavage reactions. There are five CYP enzymes involved in adrenal steroid biosynthesis; CYP11A1 (cholesterol side chain cleavage enzyme), CYP17 (17α-hydroxylase enzyme), CYP21A2 (21-hydroxylase enzyme), CYP11B1 (11β-hydroxylase enzyme), and CYP11B2 (aldosterone synthetase enzyme). Although the cortex has the ability to synthesize cholesterol *de novo*, most of the cholesterol utilized in adrenal steroid biosynthesis is taken up from the blood. In humans, the major source of adrenal cholesterol is carried in blood by circulating plasma low-density lipoproteins (LDLs). The adrenal cortex, which contains cell surface receptors for LDLs, may then take up the cholesterol. Cholesterol is transported into the mitochondria from the outer membrane to the inner membrane. This transport is facilitated by steroidogenic acute regulatory protein (StAR), which plays a rate-limiting role in steroid synthesis. Cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme (CYP11A1) at the mitochondrial level. In the zona fasciculata, pregnenolone is transferred from the mitochondria to the smooth endoplasmic reticulum, where most is hydroxylated by the 17α-hydroxylase enzyme to 17α-hydroxypregnenolone, which, in turn, is converted to 17α-hydroxyprogesterone. The latter reaction is catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) (type 2), one of the few non-P450 enzymes involved in steroidogenesis. 17α-Hydroxyprogesterone then undergoes two successive hydroxylations: first, it is hydroxylated at position 21 by the 21-hydroxylase enzyme, resulting in 11-deoxycortisol (also called compound S); second, compound S is hydroxylated at position 11 by the 11β-hydroxylase enzyme to cortisol (also called compound F).

In the zona fasciculata, a percentage of pregnenolone is directly converted by 3β-HSD to progesterone; the latter is then hydroxylated at the 17 position to 17α-hydroxyprogesterone. In the zona reticularis, 17,20-lyase removes the two-carbon side chain at C-17, the first step in the pathway leading to the synthesis of two adrenal androgens, dehydroepiandrosterone (DHEA) from 17-hydroxypregnenolone and $\Delta^4$-androstenedione from 17α-hydroxyprogesterone. DHEA may be converted to $\Delta^4$-androstenedione by 3β-HSD; testosterone is then produced from $\Delta^4$-androstenedione by the action of 17-ketosteroid reductase, another non-P450 enzyme. Aromatization to estrogens by P450 aromatase both in the adrenal as well as in other steroidogenic and peripheral tissues may follow for both testosterone and/or $\Delta^4$-androstenedione (which is aromatized directly to estrone).

In contrast to the other two zones, the zona glomerulosa has no 17α-hydroxylase enzyme and thus all pregnenolone present in glomerulosa cells is transformed to progesterone. Progesterone is hydroxylated at C-21 by 21-hydroxylase to 11-deoxycorticosterone (DOC), which, in turn, follows the pathway for the synthesis of aldosterone. Aldosterone synthase, which is expressed only in glomerulosa cells, catalyzes the last three steps of aldosterone synthesis: the 11β-hydroxylation of deoxycorticosterone to corticosterone, the 18-hydroxylation of corticosterone, and the 18-methyl oxidation of 18α-hydroxycorticosterone to aldosterone. These biosynthetic pathways are outlined in Fig. 2.

## III. REGULATION OF STEROID SECRETION

The regulation of cortisol synthesis and secretion is mediated by the hypothalamus, the pituitary, and the adrenal glands. Adrenocorticotropic hormone (ACTH; corticotropin) is synthesized and secreted by the corticotrophs of the adenohypophysis, which are regulated mainly by two hypothalamic hormones: the corticotropin-releasing hormone (CRH) and arginine vasopressin. Pituitary ACTH is secreted in the peripheral circulation and reaches ACTH receptors [the product of the melanocortin-2 receptor (MC2R) gene] located on the surface of the adrenocortical cells. ACTH binds to these cell surface receptors and initiates a cyclic adenosine monophos-

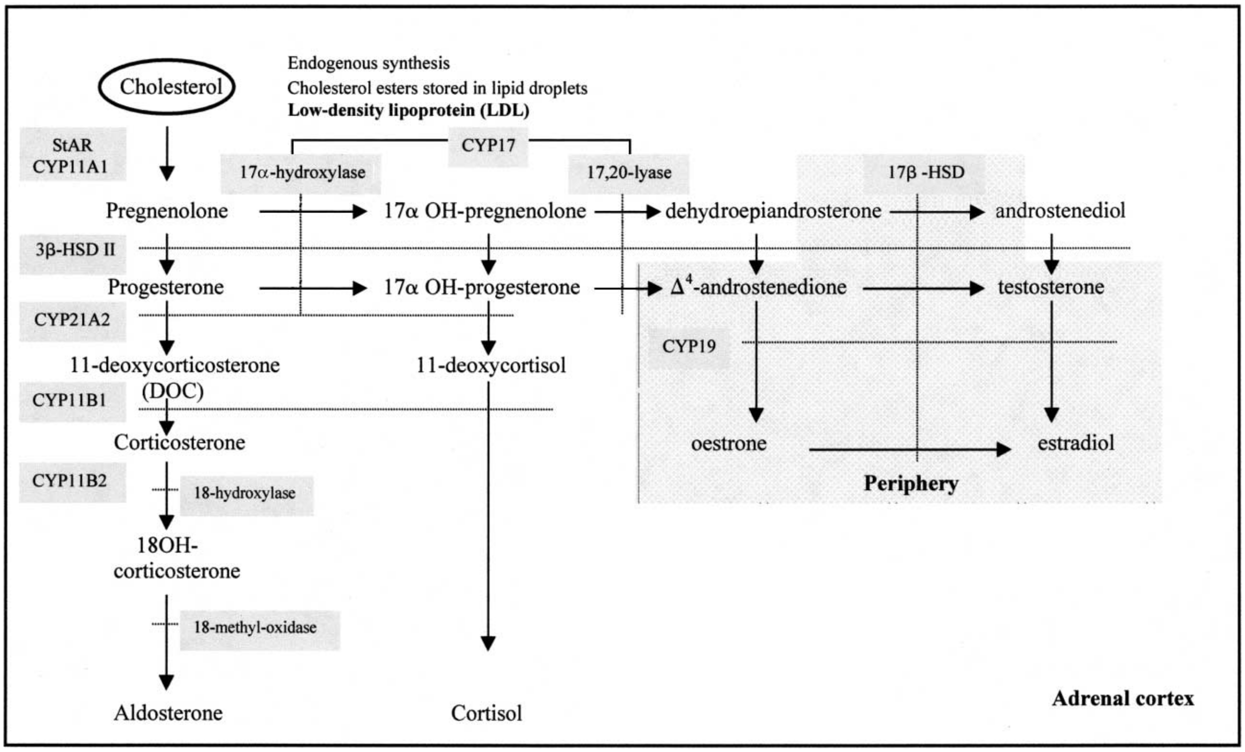
Cholesterol
Endogenous synthesis
Cholesterol esters stored in lipid droplets
Low-density lipoprotein (LDL)
StAR
CYP11A1
CYP17
17α-hydroxylase
17,20-lyase
17β -HSD
Pregnenolone
17α OH-pregnenolone
dehydroepiandrosterone
androstenediol
3β-HSD II
Progesterone
17α OH-progesterone
Δ4-androstenedione
testosterone
CYP21A2
11-deoxycorticosterone (DOC)
11-deoxycortisol
CYP19
CYP11B1
Corticosterone
oestrone
estradiol
CYP11B2
18-hydroxylase
Periphery
18OH-corticosterone
18-methyl-oxidase
Aldosterone
Cortisol
Adrenal cortex

FIGURE 2 Biosynthesis of steroids in the adrenal cortex and conversion in the periphery from substrates originating from the adrenal glands. Abbreviations: StAR, steroidogenic acute regulatory protein; 3β-HSD II, 3β-hydroxysteroid dehydrogenase enzyme II; CYP11A1, cholesterol side chain cleavage enzyme; CYP17, 17α-hydroxylase; CYP21A2, 21-hydroxylase; CYP11B1, 11β-hydroxylase; CYP11B2, aldosterone synthetase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase.

phate (cAMP)-dependent protein kinase pathway, increasing the conversion of cholesterol to pregnenolone, the initial step in the cortisol biosynthesis, which results in the acute increase of cortisol production. In addition, the chronic effects of ACTH, which occur in hours or days, include increased synthesis of most of the enzymes of the steroidogenic pathway and its role as a growth factor on the cortical zone. Thus, a decrease of plasma ACTH may lead to gradual atrophy of the adrenal cortex. Most ACTH is released in a series of secretory episodes followed by an equal number of bursts in plasma cortisol concentration. These secretory episodes of ACTH and cortisol are characterized by a sharp rise of cortisol levels followed by a slower decline, due to its relatively long plasma half-life of about 80 min. In a 24-h period, ACTH peaks occur in highest amplitude in the second half of the night; consequently, plasma cortisol levels are maximal in the early morning hours around the time of awakening. Plasma cortisol levels decline throughout the morning and reach nadir values late in the evening. This diurnal variation of ACTH and cortisol values constitutes the normal circadian rhythm of endogenous glucocorticoid synthesis that is mimicked pharmacologically when exogenous glucocorticoids are given as replacement therapy.

The key mineralocorticoid aldosterone that serves as a primary regulator of extracellular fluid volume and as a major determinant of potassium metabolism is mainly regulated by the renin–angiotensin system and by potassium ion. Renin, a serine protease enzyme that is produced by the kidney, cleaves the angiotensinogen and $\alpha_2$-globulin (synthesized by the liver) to angiotensin I. Angiotensin I is then converted to angiotensin II by the angiotensin-converting enzyme, which originates in part from the lungs. Angiotensin II stimulates aldosterone secretion.

## IV. MECHANISMS OF ACTION OF GLUCOCORTICOID RECEPTORS

All natural and synthetic glucocorticoids act by binding to a specific cytoplasmic glucocorticoid receptor. There are two isoforms of the glucocorticoid receptor; the α-isoform is a 777-amino-acid protein and the β-isoform has 742 amino acids. The α-isoform is the most abundant and represents the functional receptor that can be divided into three domains. The glucocorticoid-binding domain is located near the carboxy-terminal region of the molecule and is where the ligand binds. The DNA-binding domain is located in the middle of the proteins and is a cysteine-rich region that binds the molecule to DNA sequences in the promoter regions of target genes; these sequences (which have a specific motif) are called glucocorticoid response elements. This region folds into a two-finger structure stabilized by zinc ions, connected to cysteines to form two tetrahedrons. The zinc fingers represent the basic structure by which the DNA-binding domain recognizes specific nucleic acid sequences. The third domain, the amino-terminus domain of the receptor, is highly antigenic and contains a ligand-independent transcriptional activation function (AF-1).

The inactive forms of the glucocorticoid receptors are located in the cytoplasm as polymers bound with other proteins, one of which is the heat-shock protein Hsp 90. This heteromeric protein complex has no metabolic effect. Binding of a glucocorticoid to its receptor activates the receptor by changing the spatial conformation of the inactive protein. Activation includes dissociation of the receptor from the heat-shock proteins and a change in conformation of the hormone-binding domain. The activated glucocorticoid ligand–receptor complex then enters the nucleus (translocation), where its hormone-binding domain binds to DNA glucocorticoid-responsive elements and alters the transcription of specific genes.

## V. SYNTHETIC STEROIDS

Synthetic glucocorticoids have related structural properties to endogenous glucocorticoids. However, cortisol and prednisolone possess a hydroxyl group at C-11, which provides their glucocorticoid activity. Thus, exogenous cortisone and prednisone, which have a keto group at C-11, must undergo *in vivo* (mainly hepatic) hydroxylation to cortisol and prednisolone, respectively, to become active (Fig. 3).

Each glucocorticoid has a specific chemical structure that imparts a particular glucocorticoid and mineralocorticoid potency. For example, prednisolone has the same structure as hydrocortisone (cortisol), but also has a double bond between the C-1 and C-2, and thus has about four times more glucocorticoid activity compared to cortisol. Also, the addition of a fluoro atom in the 9α position of hydrocortisone produces fludrocortisone. This molecular change provides fludrocortisone with 10 times more potent glucocorticoid activity and 125 times

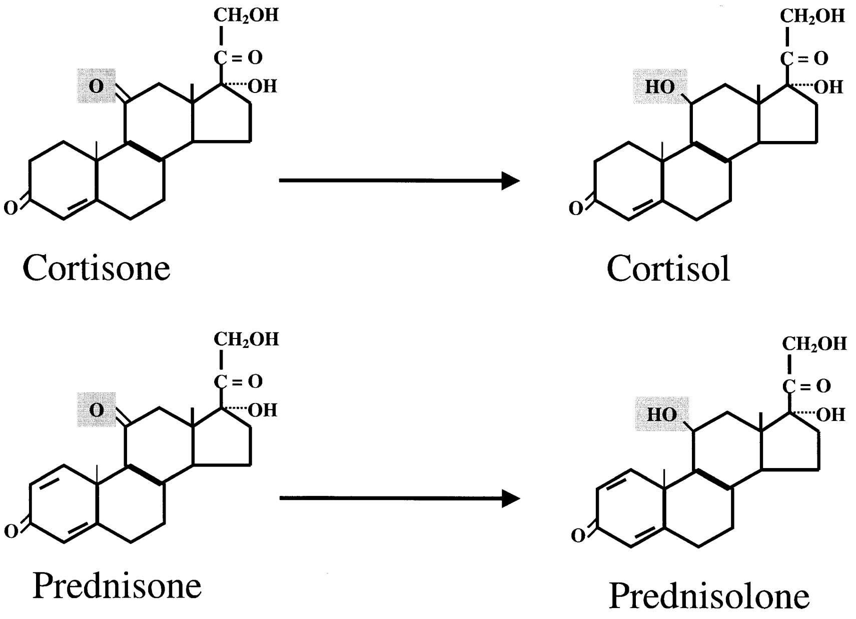

FIGURE 3 Cortisone and prednisone, which have a keto group at C-11, must undergo *in vivo* hydroxylation to cortisol and prednisolone, respectively, to become active.

TABLE 1 Comparison of the Most Commonly Used Synthetic Corticosteroids[a]

| Steroids | Half-life (minutes) | Duration of action (hours) | Glucocorticoid potency | Mineralocorticoid potency | Replacement dose (mg) |
|---|---|---|---|---|---|
| **Glucocorticoids** | | | | | |
| Hydrocortisone (cortisol) | 80 | 8–12 | 1.0 | 1.0 | 20 |
| Cortisone | 30 | 8–12 | 0.8 | 0.8 | 25 |
| Prednisone | 60 | 12–36 | 4.0 | 0.3 | 5 |
| Prednisolone | 200 | 12–36 | 4.0 | 0.3 | 5 |
| Methylprednisolone | 200 | 12–36 | 5.0 | 0.0 | 4 |
| Triamcinolone | 200 | 12–36 | 5.0 | 0.0 | 4 |
| Betamethasone | 300 | 36–72 | 25.0 | 0.0 | 0.6 |
| Dexamethasone | 300 | 36–72 | 30.0 | 0.0 | 0.75 |
| **Mineralocorticoids** | | | | | |
| Fludrocortisone | 240 | 12–24 | 10.0 | 125.0 | 2 |

[a] Cortisol is arbitrarily assigned a value of 1.0.

more mineralocorticoid activity compared to hydrocortisone. Thus, fludrocortisone is used mainly for mineralocorticoid replacement therapy. The clinically used glucocorticoids and mineralocorticoids and their pharmacology are listed in Table 1.

Various factors affecting the therapeutic activity of the various synthetic glucocorticoids should be taken into account. Different formulations of glucocorticoids are available for systemic therapy (intravenous or oral) or nonsystemic therapy. Nonsystemic therapy includes local applications as topicals, aerosols (lungs, sinus), enemas, and intra-articulars, which may decrease the occurrence of systemic side effects (see later). However, high doses and long-term administration of local therapy may also lead to significant absorption and thus systemic effects. The glucocorticoid dose may have to be increased in patients receiving other medications, including phenytoin, barbiturates, and rifampin, which induce hepatic microsomal enzyme activity and lead to increased metabolism of glucocorticoids.

## VI. STEROID-BINDING PLASMA PROTEIN AND METABOLISM

In normal conditions, more than 90% of the circulating cortisol is bound to plasma protein; most is bound to cortisol-binding globulin (CBG) and the remaining, to albumin. Thus, approximately less than 10% of cortisol is in the free, unbound form, which is biologically active. Conditions such as pregnancy or estrogen therapy, which may lead to increased synthesis of CBG by the liver, are associated with elevated total plasma cortisol concentrations. In contrast, only 20% of plasma aldosterone is bound to CBG, 40% is bound to albumin, and about 40% is free. Among the synthetic steroids, only prednisone and its metabolite prednisolone may bind to proteins. This means that close to 100% of a given concentration of other synthetic steroids (including dexamethasone and fludrocortisone, which are commonly used) will circulate in the free (and metabolically active) form.

The liver and kidneys are the two major sites of glucocorticoid inactivation by metabolism. In the liver, the pathways leading to inactivation of cortisol include reduction of the double bond between positions 4 and 5 in the A ring, reduction of the keto group of the C-3, hydroxylation at the C-6 position, and subsequent conversion to tetrahydrocortisol and tetrahydrocortisone by 3-hydroxysteroid dehydrogenase. These metabolites of cortisol are then conjugated with glucuronic acid or sulfate and excreted in the urine. This biotransformation is applied to both endogenous and synthetic glucocorticoids.

Perhaps the most (metabolically) important cortisol inactivation step takes place in the kidneys and involves cortisol oxidation to cortisone by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme. The inactive metabolite cortisone does not bind to the kidney mineralocorticoid receptor, which normally can be activated by both cortisol and aldosterone. Thus, 11β-HSD plays a major role in balancing glucocorticoid versus mineralocorticoid activity; indeed, 11β-HSD genetic defects (syndrome of apparent mineralocorticoid excess) or acquired, functional defects (from substances such as licorice) result in cortisol-mediated mineralocorticoid effects and clinical hypertension.

## VII. THERAPEUTIC USES OF GLUCOCORTICOIDS AND MINERALOCORTICOIDS

The primary therapeutic application of glucocorticoids is replacement therapy for adrenal insufficiency with hydrocortisone at a dose of 12–15 mg/m$^2$/day. For this purpose, physiological doses of glucocorticoids are administered in order to mimic adrenal axis secretion under normal and stressful conditions. Synthetic glucocorticoids may also be required at supraphysiological doses (pharmacologic doses) to provide anti-inflammatory and immunosuppressive effects. These therapeutic uses of glucocorticoids apply to a large spectrum of conditions, including pulmonary diseases (asthma), allergic reactions, rheumatoid arthritis, gastrointestinal inflammatory diseases, autoimmune disorders, and vasculitis syndromes; in the field of transplantations, glucocorticoids are administered to avoid graft rejection.

In all applications, the goal is to optimize the therapeutic effect of glucocorticoids using the smallest dose, in order to minimize adverse side effects. In addition, in certain conditions, corticosteroids are used transiently until an alternative therapy can control the disease. Synthetic glucocorticoids, usually dexamethasone, are also useful for the evaluation of the hypothalamic–pituitary–adrenal axis. Mineralocorticoids may be needed to replace aldosterone deficiency in primary adrenal insufficiency or after surgical adrenalectomy at the usual starting dose of fludrocortisone 0.1 mg/day.

## VIII. GLUCOCORTICOID EFFECTS

Glucocorticoids regulate a large number of physiological processes and affect almost every tissue. The side effects of exogenous synthetic glucocorticoids are similar to those found in endogenous oversecretion of glucocorticoids (Cushing syndrome).

### A. Hypothalamic–Pituitary–Adrenal Axis Suppression

The hypothalamic–pituitary–adrenal axis is very sensitive to negative feedback by circulating cortisol or synthetic glucocorticoids. Supraphysiological plasma concentrations of glucocorticoids suppress hypothalamic–pituitary–adrenal activity by decreasing normal endogenous production of cortisol. Thus, an abrupt cessation of a chronically administered glucocorticoid can result in the simultaneous lack of both endogenous and exogenous glucocorticoids. This situation is potentially life threatening because the hypothalamic–pituitary–adrenal axis may need up to a year to adjust and secrete cortisol at a normal rate after the cessation of exogenous glucocorticoids. The suppressive effect of glucocorticoids on the hypothalamic–pituitary–adrenal axis appears within days after starting glucocorticoid treatment. Usually, any patient who has received glucocorticoids equivalent to 10 mg or more of prednisone daily for longer than 2–3 weeks should be considered to have a suppressed hypothalamic–pituitary–adrenal axis. The time needed for the axis to recover depends on the type of glucocorticoid given, the dose and frequency of administration (i.e., daily versus alternate days), the individual metabolism, and the length of treatment. In cases of prolonged glucocorticoid administration, recovery may take 12–24 months. CRH secretion recovers first, then the normal pulsatile ACTH secretion is restored, which then leads to adrenal cortex stimulation and growth and normalization of endogenous cortisol secretion.

### B. Gonadal Axis

Pharmacological doses of glucocorticoids also act on the hypothalamic–pituitary–gonadal axis by inhibiting the secretion of gonadotropins, suppressing the effect of gonadotropins on the gonads, and affecting the action of sex steroids on their target tissues. Chronic treatment with pharmacological doses of glucocorticoids produces hypogonadism associated with erectile dysfunction and infertility in men and menstrual irregularities or even secondary amenorrhea in women.

### C. Glucocorticoid Effects on Catecholamines

Glucocorticoids influence the conversion of norepinephrine to epinephrine, which is carried out in the adrenal medulla. The adrenal cortex surrounds the chromaffin-containing cells in the adrenal medulla. The latter receives the adrenocortical venous effluent and thus is normally exposed to high concentrations of endogenous cortisol. The cortisol in the adrenocortical venous effluent regulates the enzyme phenylethanolamine *N*-methyltransferase, which catalyzes the conversion of norepinephrine to epinephrine and is located exclusively in the adrenal medulla and the organ of Zuckerkandl.

### D. Mineralocorticoid Effects

The various glucocorticoids exhibit different affinities toward the mineralocorticoid receptor.

Glucocorticoid-induced activation of the mineralocorticoid receptor causes elevation of blood pressure, sodium retention, and potassium and hydrogen ion excretion from the kidneys. In severe cases, patients develop hypertension and hypokalemic alkalosis. Among the glucocorticoids, cortisol and, indirectly, cortisone have the highest mineralocorticoid activity. Dexamethasone and betamethasone have minimal mineralocorticoid activity, whereas prednisone and prednisolone have some, but very limited, activity.

## E. Glucocorticoid Effects on the Hematopoietic and Immune Systems

Glucocorticoids, as well as other steroids, have beneficial effects on erythropoiesis and they are being used therapeutically in the treatment of various aplastic anemias. But the main use of glucocorticoids has to do with their effect on the immune system. Glucocorticoids affect the immune system at multiple levels: (1) neutrophil traffic, (2) antigen processing, (3) growth, proliferation, and function of eosinophils and mastocytes, and (4) general inhibitory effects on growth and function of lymphocytes. Within hours after administration of glucocorticoids the, number of circulating neutrophils increases as a result of alterations on their trafficking dynamics. Neutrophilia may be due to glucocorticoid-induced decrease of neutrophil adherence to the vascular endothelium and the inability of neutrophils to egress toward bone marrow or inflammatory sites.

Glucocorticoids inhibit antigen processing by macrophages, suppress T-cell helper function, inhibit synthesis of cellular mediators of the inflammatory response (i.e., interleukins and other cytokines and porstanoids), and inhibit phagocytosis. In addition, glucocorticoids stabilize lysosomal membranes and induce eosinopenia and lymphopenia. These latter effects may be due to modification of production, distribution, and/or cell lysis and are more profound on T lymphocytes as compared to B lymphocytes. Glucocorticoids also inhibit accumulation of neutrophils and monocytes at inflammatory sites. These effects explain the increased susceptibility to opportunistic infections (such as *Pneumocystis carinii* and others) seen in patients exposed to supraphysiological levels of glucocorticoids.

## F. Glucocorticoid Effects on Bone Metabolism

One of the major side effects of glucocorticoids is their detrimental action on bone metabolism, growth, and function. In patients exposed to high levels of glucocorticoids, this is usually seen as low bone mineral density. Glucocorticoids cause osteoporosis by reducing bone formation and increasing bone resorption. Bone formation is decreased at several levels by direct inhibitory effects on osteoblasts, inhibition of production of insulin-like growth factor-I (IGF-I), and an increase in osteoblast and osteocyte apoptosis.

Glucocorticoids may increase bone resorption by acting directly on bone and, indirectly, by reducing androgen and estrogen secretion as a result of inhibition of the hypothalamic–pituitary–gonadal axis, and by causing dysregulation of calcium homeostasis. Glucocorticoids appear to decrease calcium intestinal absorption and increase renal calcium excretion. The combined effect of these actions leads to hypocalcemia. The parathyroid glands respond to hypocalcemia by increasing the secretion of parathyroid hormone, which, in turn, stimulates bone to release calcium into the circulation.

The inhibition of osteoblastic activity may be documented by the low concentrations of osteocalcin, a protein produced by osteoblasts. Bone resorption, on the other hand, is documented by the increased number of osteoclasts (i.e., increasing the transformation of precursor cells to osteoclasts) and increased concentrations of urine hydroxyproline, an index of bone collagen catabolism. Bone density usually increases after discontinuation of exogenous glucocorticoids or treatment of Cushing syndrome. In general, therapy for prevention of bone loss is given to patients who undergo long-term glucocorticoid therapy.

## G. Glucocorticoid Side Effects on the Central Nervous System

Acute exposure to high-dose glucocorticoids may induce psychosis. Chronic exposure is often associated with neuropsychological and emotional alterations. Psychiatric disease is present in up to 70% of patients suffering from Cushing syndrome and is expressed primarily by major, atypical depression in about half of the patients. In addition, apparent cerebral atrophy has been described in patients with endogenous hypercortisolism and who are using pharmacological doses of exogenous glucocorticoids. Recent data suggest that this brain volume loss may be partially reversible following correction of hypercortisolism.

### H. Glucocorticoid Effects on the Gastrointestinal Tract

Chronic administration of high doses of glucocorticoids increases the incidence of peptic ulcers. The exact mechanism is unknown, but glucocorticoids inhibit the synthesis of mucopolysaccharides, which protect the gastric mucosa from acid; glucocorticoids also increase gastric acid output in response to histamine.

### I. Glucocorticoid Effects on Glucose Metabolism

Glucocorticoids were given their name because of the profound role they play in glucose metabolism. Glucocorticoids increase plasma glucose by (1) activating hepatic enzymes involved in gluconeogenesis in liver, (2) decreasing glucose uptake and utilization by peripheral tissues, (3) having a permissive role in the gluconeogenic action of counterregulatory hormones such as glucagon and catecholamines, and (4) increasing the availability of substrates for gluconeogenesis (for example, by increasing muscle proteolysis). Glucocorticoids also maintain hepatic glycogen stores by activating glycogen synthesis and inactivating glycogenolysis.

### J. Glucocorticoid Effects on Lipid Metabolism

Glucocorticoids activate lipolysis in adipose tissue. On a chronic basis, supraphysiological cortisol secretion or glucocorticoid administration may lead to weight gain. The obesity is central and is characterized by moon facies, enlarged supraclavicular fat pads, and a dorsal fat pad.

## IX. SUMMARY

Glucocorticoids are the "hormones of life"—they are important for the maintenance of metabolic homeostasis. It is not surprising that their pharmacological use is riddled with a number of effects, both desired and detrimental. Successful pharmacologic use requires knowledge of glucocorticoid synthesis, action, and metabolism, and complicated physiology.

## Glossary

**Addison's disease** Autoimmune process leading to adrenal hypofunction; may be isolated or associated with polyglandular autoimmune syndrome, including diabetes, hypoparathyroidism, hypothyroidism, and mucocutaneous candidiasis.

**adrenocorticotropic hormone** Peptide synthesized in the pituitary and composed of 39 amino acids; stimulates the adrenal cortex to secrete cortisol and adrenal androgens.

**Cushing syndrome** First described by Harvey Cushing in 1912; clinical symptoms and signs secondary to chronic oversecretion of adrenal glucocorticoids (mainly cortisol); may result from increased secretion of adrenocorticotropic hormone due to pituitary or nonpituitary tumors or to a benign or malignant adrenal tumor.

**cytochrome P450** A family of oxidative enzymes with a characteristic 450-nm absorbance maximum when reduced with carbon monoxide; transfer electrons from NADPH to molecular oxygen. One subgroup of these enzymes is involved in adrenal steroid biosynthesis.

**glucocorticoid response elements** Specific DNA sequences that are activated by the glucocorticoid receptor complex; following activation, transcription of the glucocorticoid target gene is induced.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Glucocorticoid Biosynthesis: Role of StAR Protein • Glucocorticoid Drugs, Evolution of • Glucocorticoid Effects of Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Receptor Structure and Function • Glucocorticoid Resistance • Glucocorticoids and Asthma • Glucocorticoids and Autoimmune Diseases**

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# Glucose-Dependent Insulinotropic Polypeptide (GIP)

TIMOTHY J. KIEFFER
*University of British Columbia, Canada*

I. THE ROLE OF GIP IN FAT METABOLISM
II. THE ROLE OF GIP IN GLUCOSE HOMEOSTASIS
III. GIP IN THE PATHOPHYSIOLOGY OF OBESITY AND DIABETES
IV. CONCLUSION

**Gastric inhibitory polypeptide (GIP) was isolated from intestinal extracts in the early 1970s as an enterogastrone—a hormone secreted in response to fat or its digestive products in the intestinal lumen and that acts to inhibit gastric acid secretion. However, a more definitive physiological role for GIP came in 1973 with the discovery that GIP is a so-called “incretin” hormone—a factor released from the gut during ingestion of glucose in order to potentiate insulin release. Notably, the insulinotropic action of GIP is dependent upon the ambient glucose concentration. Therefore, GIP was given the alternate designation glucose-dependent insulinotropic polypeptide, retaining the same acronym. In addition to the role of GIP in glucose homeostasis, GIP has a recognized role in lipid metabolism. Glucose is a potent stimulator of GIP release, as would be expected for an incretin hormone, and so too is fat. Indeed the release of GIP in response to fat is sustained beyond that which occurs with glucose. Furthermore, GIP release by fat that is not accompanied by glucose will not stimulate the release of insulin, supporting a role for GIP in fat metabolism. In such a circumstance, it is certainly desirable to have a hormone with insulin-like capabilities at the level of adipocytes in order to promote lipid absorption, without any glucose-lowering actions. GIP appears to provide this role, being quite effective at promoting postprandial triglyceride clearance without generating hypoglycemia. This article explores the metabolic roles of GIP in carbohydrate and triglyceride disposal as well as the corresponding possibility that GIP might play a role in the pathophysiology of diabetes mellitus and obesity.**

## I. THE ROLE OF GIP IN FAT METABOLISM

### A. Discovery of GIP as an Enterogastrone

In the early 1900s, Pavlov demonstrated that fat added to a meal fed to dogs inhibited the secretion of gastric acid and pepsin. It was later discovered that this response could be mimicked by injection of extracts of the duodenal mucosa and thus suggested that a hormonal mechanism must be involved. The term “enterogastrone” was proposed to describe a putative hormone that was secreted in response to fat or its digestive products in the intestinal lumen and that inhibited gastric acid secretion. In 1970, GIP was isolated from impure intestinal extracts of cholecystokinin, a potent stimulator of gall bladder contraction. Purified porcine GIP was shown to be a potent inhibitor of gastric acid and pepsin secretion in the dog. Subsequently, it was demonstrated in rodents that this action of GIP appears to be indirectly mediated by GIP-induced release of the potent inhibitory peptide, somatostatin, from the stomach. The relative contribution of GIP as an enterogastrone in humans remains controversial, yet the highly conserved nature of the 42-amino-acid peptide suggests an important physiological function.

### B. GIP Release by Fats

Consistent with the role of GIP as an enterogastrone, immunoreactive GIP (IRGIP) cells are concentrated in the upper small intestine where most fat digestion and absorption occur. Although they are most highly concentrated in the duodenum and jejunum, GIP-producing cells, termed K cells, are scattered as far as the terminal ileum, and GIP expression has been detected in the stomach and submandibular salivary gland. The K cells are typically flask-shaped and pass through adjacent epithelial cells reaching to the lumen of the gut. The basolateral side of the cell contains secretory granules that release GIP into the blood following stimulation of the cell. The cells are believed to last for a few days before they are sloughed off; new K cells are derived from stem cells residing in the crypts of Lieberkühn.

In humans, the constituents of a mixed meal that largely determine the magnitude of GIP secretion are fats and carbohydrates (Fig. 1). While fat ingestion is a potent stimulator for GIP release in humans who typically attain ~40% calories as fat on a Western diet, fat alone is a poor stimulus of GIP secretion in species such as rats and pigs where typical diets usually provide <10% calories as fat. In the upper intestine, triglycerides are converted to free fatty acids and monoglycerides, form micelles, and then diffuse among the microvilli that form the enterocyte brush border. The high lipid solubility of the fatty acids and monoglycerides promotes their diffusion into the enterocytes. Which step in this process evokes GIP release is currently unknown, but the release of GIP in response to fat appears to require hydrolysis. Notably, long-chain fatty acids are much more potent GIP secretagogues than medium- or short-chain fatty acids, with saturated fats preferred over polyunsaturated fats. Generally, the duration of GIP release by fat is greater than that of glucose, likely attributable to the reduced rate of gastric emptying that accompanies fat ingestion and therefore the prolonged exposure of K cells to the stimulus.

## C. Lipid-Lowering Actions of GIP

While fats such as corn oil are potent stimulators of GIP release in humans, fitting with the role of GIP as an enterogastrone, the physiological function of fat-induced GIP release extends beyond that of a simple enterogastrone. There are data suggesting that GIP is an important hormonal regulator of postprandial triglyceride levels. Plasma GIP levels parallel that of triglycerides, and antibody-mediated neutralization of GIP in rats increases the triglyceride increment late in the time course of a fat load. Furthermore, exogenous GIP promotes the clearance of chylomicron triglycerides from the circulation in dogs (Fig. 2) and reduces plasma triglyceride increments following intraduodenal infusion of a lipid test meal in rats. Both inhibition of fat absorption and stimulation of triglyceride uptake by peripheral tissues may be involved in GIP action. The GIP receptor was cloned in 1993 and characterized as a member of the secretin-vasoactive intestinal polypeptide family of seven-transmembrane-spanning G-protein-coupled receptors. In support of a direct action of GIP on adipose tissue, both GIP-binding sites and receptor mRNA have been detected in adipocytes.

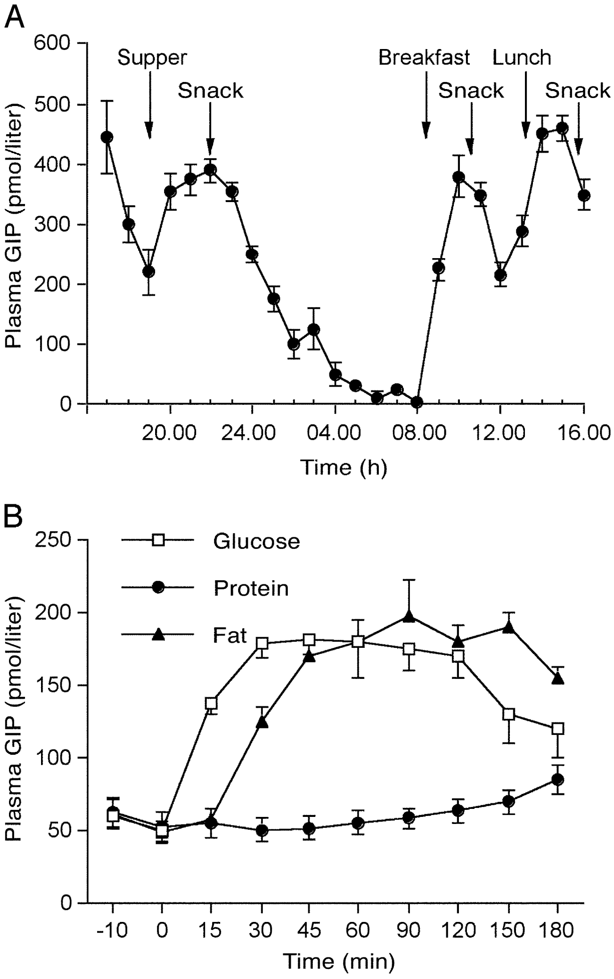

**FIGURE 1** Mean plasma GIP in healthy subjects (A) consuming a normal diet over a 24 h period ($n = 6$) and (B) following ingestion of 375 kcal meals of glucose, protein, or fat ($n = 8$). Adapted from R. M. Elliott *et al.*, Glucagon-like peptide-1(7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: Acute post-prandial and 24-h secretion patterns. *J. Endocrinol.* 138, 159–166, 1993, with permission of the Society for Endocrinology.

One way in which GIP appears to reduce plasma triglycerides is through the activation of lipoprotein lipase (LPL). Adipose tissue LPL plays a key regulatory role in the hydrolysis of circulating triglyceride, liberating nonesterified fatty acids for

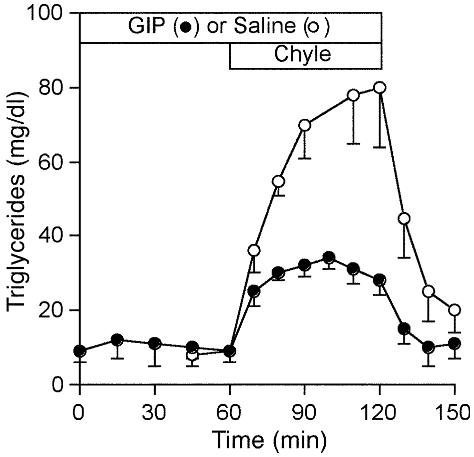

FIGURE 2 Effect of intravenously infused porcine GIP (1 μg/kg/h; filled circles) or saline (open circles) on plasma levels of triglyceride before and during infusion of chyle in normal dogs (*n* = 6 in each group). Chyle was obtained from donor dogs via a thoracic duct fistula and was infused at a rate of 2 ml/min intravenously during the period indicated by the bar. Adapted from T. Wasada *et al.*, Effect of gastric inhibitory polypeptide on plasma levels of chylomicron triglycerides in dogs. *J. Clin. Invest.* **68**, 1106–1107, 1981, with permission.

uptake and storage within the adipocyte. There are progressive parallel increases in postheparin plasma LPL and plasma GIP *in vivo* following meals of increasing fat content, without changes in insulin levels. Therefore, the postprandial lipid responses to meals of increasing fat content could be moderated by stepwise increases in LPL activity, which in turn is mediated by fat-stimulated GIP secretion. In support of this concept, GIP, in common with insulin, stimulates LPL activity in explants of rat epididymal adipose tissue and stimulates both the synthesis and the release of LPL activity in cultured mouse 3T3-L1 preadipocytes. Therefore, GIP may be the major hormonal signal linking meal size to the postprandial LPL activity in the physiological control of blood lipids.

The action of GIP on adipocytes is not limited to LPL activity. GIP has been found to enhance the insulin sensitivity of adipocytes. Interestingly, the anti-lipolytic potency of insulin is increased after oral but not intravenous administration of glucose, an effect perhaps attributable to GIP. In support of this hypothesis, adipocytes incubated with GIP develop increased insulin receptor affinity and sensitivity to insulin-stimulated glucose transport. GIP itself also stimulates glucose uptake in isolated adipocytes as well as the conversion of glucose to extractable lipids. In common with insulin, physiological concentrations of GIP were shown to stimulate fatty acid synthesis in a dose-dependent manner in explants of rat adipose tissue, as determined by measuring the incorporation of [$^{14}$C]acetate into saponifiable fat. Therefore, *in vivo* GIP might contribute to more effective postprandial uptake of glucose and may enhance the effect of insulin on fatty acid synthesis from glucose as a precursor.

Consistent with an overall anabolic role, GIP has been observed to inhibit glucagon-stimulated lipolysis in rat adipocytes. This is somewhat counterintuitive given that GIP itself, like glucagon, increases cAMP levels in adipocytes. One might therefore anticipate that GIP would exhibit lipolytic rather than lipogenic effects. Indeed, GIP is lipolytic in differentiated 3T3-L1 adipocytes. However, studies on the effect of insulin on GIP-stimulated glycerol release revealed that insulin is capable of antagonizing this GIP-induced lipolysis in a phosphatidylinositol 3′-kinase-dependent manner. The physiological significance of this observation remains to be established, but it is possible that the lipolytic action of GIP may normally occur only during periods of fasting when insulin levels are at their lowest. GIP levels are also at a minimum during fasting so this hypothesis requires that basal GIP be sufficient to induce lipolysis. Nevertheless, in light of observations that fatty acids are important in the maintenance of optimal β-cell function, basal GIP-induced elevations in circulating fatty acids during fasting could be important in ensuring efficient insulin secretion upon ingestion of a meal.

## II. THE ROLE OF GIP IN GLUCOSE HOMEOSTASIS

### A. Discovery of GIP as an Incretin

Following the 1960s development of a radioimmunoassay for insulin, the observation was made that absorption of nutrients from the gut seemed to be accompanied by the release of factors that potentiated glucose-stimulated insulin release. Indeed, the insulin response in humans to intravenous glucose was much smaller than to either oral glucose or intrajejunal infusion, even though there was a greater increase in blood glucose levels with intravenous administration. Early estimates suggested that as much as half of the insulin secreted following an oral glucose load was the result of gastrointestinal factors. This connection between the gut and the pancreatic islets was termed the "enteroinsular axis," and the putative gastrointestinal factors that augmented insulin secretion were termed “incretins.” Given that GIP was found in the upper intestine, the possibility that GIP may be an incretin hormone was raised. In 1973, it was demonstrated that a purified preparation of GIP infused intravenously in humans in concert with glucose resulted in the stimulation of insulin release and an improvement in glucose tolerance (Fig. 3). The insulin response was sustained for the duration of the GIP infusion and was not observed in the euglycemic state. With the 1978 development of a radioimmunoassay to quantify plasma GIP levels, the role of GIP as an incretin was fully established because oral glucose was found to be a potent stimulus for GIP release.

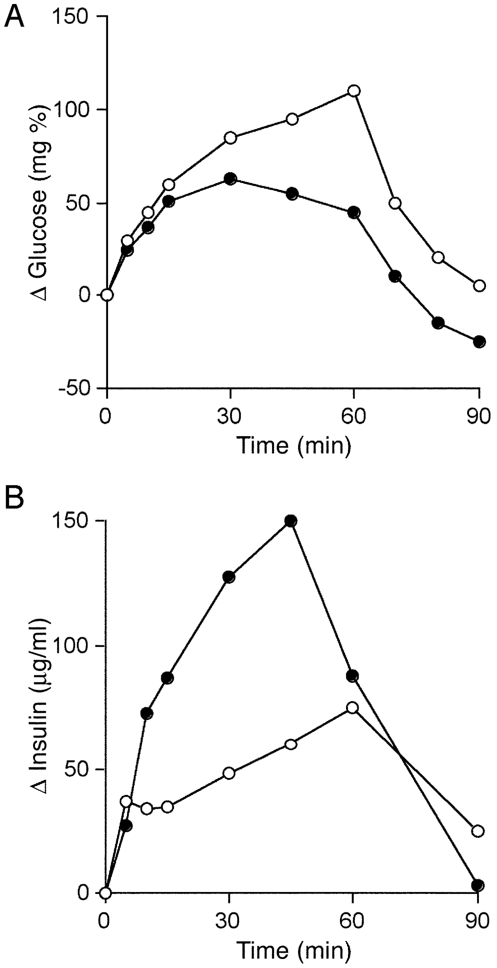

**FIGURE 3** Mean changes in (A) plasma glucose and (B) serum insulin in six subjects receiving intravenous glucose alone (0.5 g/min for 60 min; open circles) or intravenous glucose with porcine GIP (1 µg/min during the first 30 min; filled circles). Adapted from J. Dupre *et al.*, Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J. Clin. Endocrinol. Metab.* 37, 826–828, 1973, ©The Endocrine Society, with permission.

Evidence from patients who had undergone intestinal resection and studies in which the activity of GIP was curtailed with antisera suggested that other incretins exist. Over a decade after the isolation of GIP, analysis of the proglucagon gene revealed that whereas glucagon is the main proglucagon-derived product in pancreatic α-cells, glucagon-like peptides 1 and 2 are liberated by tissue-specific processing of the same precursor protein in endocrine cells located in the distal small intestine. Shortly thereafter, GLP-1, but not GLP-2, was determined to be a potent insulin secretagogue, whose action, like that of GIP, is glucose dependent. Plasma GLP-1 levels increase briskly following oral glucose administration, albeit not nearly as much as the GIP levels increase. The site of release of GLP-1, predominantly the ileum, does not entirely fit with the concept of an incretin hormone, given that the bulk of nutrients are already absorbed in more proximal regions of the gut. Interestingly, however, a proximal-distal loop has been identified in which GIP release from the proximal gut stimulates GLP-1 release from the distal gut, at least in rats. Evidence for a similar pathway in humans is lacking. Nevertheless, GIP and GLP-1 collectively appear to fully account for the intestinally derived hormonal augmentation of insulin secretion following oral nutrients.

## B. GIP Release by Carbohydrates

The concentration of K cells in the duodenum and jejunum fits with the role of GIP as an incretin hormone, as this is where most carbohydrate digestion and absorption occur. Interestingly, IRGIP has been localized to a distinct cell type in the islet organ of elasmobranchs, and evolutionarily it appears that GIP cells are not found in the gut mucosa until a distinct islet organ evolves upon which the peptide may act, i.e., the hagfish. K cells respond relatively poorly to complex carbohydrates but release GIP in response to the digestion products galactose and glucose, with the latter being the most potent. Fructose is a poor GIP secretagogue. There is a direct relationship between the glucose load ingested and the magnitude of GIP release. In general, plasma GIP levels increase several fold following oral glucose administration, slightly preceding insulin release with a peak in peripheral venous blood at 15–30 min and a return to basal levels by 2 h. Notably, the magnitude of the increase is much greater in pigs, monkeys, and humans than it is in rodents. It appears as though the cells respond directly to lumenal glucose and not changes in plasma glucose levels. The mechanism by which K cells "sense" glucose is not entirely clear, but appears to require active transport as opposed to the mere presence of glucose in the lumen. It is possible that they receive a signal from neighboring enterocytes during the transport of glucose from the lumen into the blood. However, K cells express the so-called glucose sensor, glucokinase, and K-cell cultures respond to changes in glucose concentrations, suggesting that the K cells themselves may be directly glucose-sensitive.

### C. Insulinotropic Action of GIP

Once released, GIP stimulates insulin secretion in a dose-dependent manner, provided that there is sufficient plasma glucose. At a fasting concentration of glucose, GIP is not insulinotropic. In the perfused rat pancreas, an ambient glucose concentration of ~5.5 mM is required to see significant potentiation of insulin release by physiological concentrations of GIP. In addition, the insulinotropic action of GIP increases with increasing glucose concentrations, with maximal potentiation at ~16 mM glucose in the perfused rat pancreas. The glucose dependency of GIP-stimulated insulin secretion provides an important safeguard against hypoglycemia by preventing the inappropriate stimulation of insulin release during a high-fat, low-carbohydrate meal. In addition to stimulating insulin release, GIP has been found to stimulate insulin gene expression, perhaps an important mechanism in balancing insulin stores to nutritional status.

The direct action of GIP on pancreatic β-cells is supported by the finding of GIP receptors in pancreatic islets and in membranes from insulin-secreting tumor-derived β-cell lines. In pancreatic β-cells, GIP binding is associated with activation of adenylate cyclase and increases in cAMP content with a resultant increase in intracellular $Ca^{2+}$ concentration that parallels insulin release. In Chinese hamster ovary cells transfected with the GIP receptor, GIP has also been reported to activate mitogen-activated protein kinase through a wortmannin-sensitive pathway and to generate the signaling molecule arachidonic acid through phospholipase $A_2$-mediated hydrolysis of membrane phospholipids. The mechanism of the glucose-dependent nature of GIP-induced insulin secretion is not fully understood.

Although C-terminal truncation of GIP to GIP 1-30 does not impair the ability of GIP to stimulate insulin secretion, an intact N-terminal portion is required for full activity. Following its release, the metabolic action of GIP is curtailed through rapid clearance and degradation. The circulating half-life of GIP in plasma as determined by immunoreactivity is approximately 20 min, with the kidney being the major site of clearance. However, the biological half-life is much shorter, in the range of 1 to 2 min. GIP 1-42 is rapidly cleaved by the ubiquitous protease dipeptidyl peptidase IV (DPPIV), rendering GIP 3-42, which has been found to possess little if any insulinotropic activity. Thus, the biological activity of exogenous or endogenous GIP can be significantly extended with the use of DPPIV inhibitors.

The physiological significance of the insulinotropic action of GIP has been investigated by the use of antagonists and immunoneutralizing antisera. Collectively, these studies confirm that GIP plays an important anticipatory role in promoting brisk insulin secretion following the ingestion of glucose. Thus, when GIP action is impaired, insulin secretion is delayed, resulting in impaired glucose tolerance. Blocking GIP action does not alter basal glucose levels or the glucose excursion following administration of intraperitoneal glucose, suggesting that the role of GIP in glucose control is restricted to functioning as an incretin in the postabsorptive state. These findings have been confirmed by the recent generation of mice harboring a disrupted GIP receptor. These mice have normal fasting blood glucose, but higher glucose levels with an impaired initial insulin response after oral glucose administration (Fig. 4). In contrast, studies with GLP-1 antagonists and GLP-1 receptor knockout mice indicate that GLP-1 signaling appears to be essential for glycemic control both during fasting and after a glucose challenge, independent of the mode of glucose entry. Although most reports indicate that GLP-1 has more potent insulinotropic activity than GIP, it is generally agreed that GIP levels increase more dramatically following a meal. When GIP and GLP-1 are administered at doses chosen to produce plasma levels roughly comparable to values measured after oral glucose in humans (~450 and ~50 pM, respectively), the β-cell response to intravenous glucose is augmented to values not significantly different from those after oral glucose, and GIP appears to make the larger contribution.

## III. GIP IN THE PATHOPHYSIOLOGY OF OBESITY AND DIABETES

### A. Role of GIP in Obesity

Given the clear physiological metabolic roles of GIP in glucose and lipid disposal, there is a possibility that

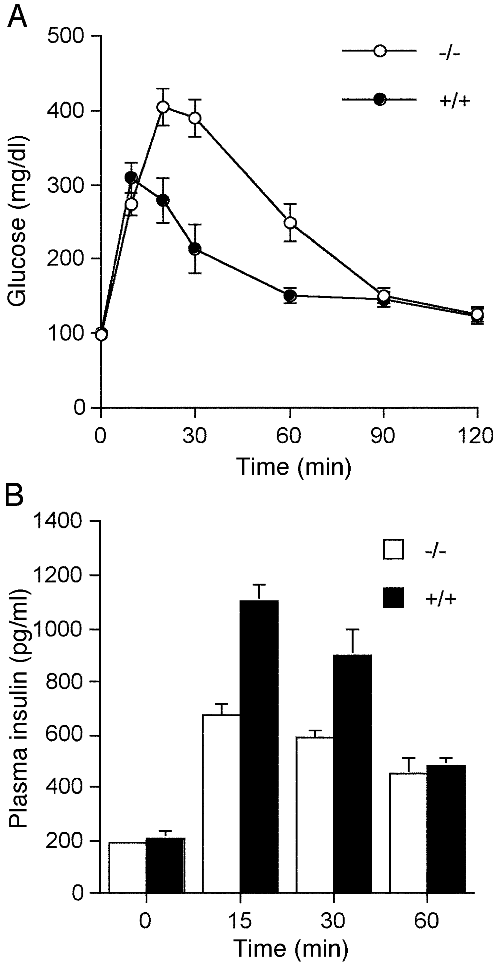

**FIGURE 4** Oral glucose tolerance test in age-matched wild-type mice (+/+, filled symbols, $n = 4$) and GIP receptor knockout mice (−/−, open symbols, $n = 6$). After a 16 h fast, mice were administered glucose (2 g/kg body weight) and blood samples were collected from the tail vein at the indicated times for (A) glucose and (B) insulin determinations. From K. Miyawaki *et al.*, Glucose intolerance caused by a defect in the entero-insular axis: A study in gastric inhibitory polypeptide receptor knockout mice. *Proc. Natl Acad. Sci. USA* **96**, 14843–14847, 1999, with permission. Copyright (1999) National Academy of Sciences, U.S.A.

GIP may be involved in the pathophysiology of obesity and/or diabetes. Some studies have detected elevated fasting and fed plasma GIP levels in obese subjects, which, given the adipogenic nature of GIP combined with the typically elevated insulin levels, could contribute to excess fat mass. Elevated GIP levels might be explained by the observation that the expression of GIP appears to be coordinated with nutritional status. In response to fasting, rodent intestinal GIP mRNA levels decrease. One day after refeeding with either fat or glucose, GIP mRNA levels increase several fold. In response to oral glucose, increases in duodenal GIP mRNA can be detected within a few hours, as can mucosal GIP concentrations. Increased GIP expression in response to glucose seems to involve both pre- and posttranslational mechanisms. In humans, glucose-stimulated GIP secretion is increased following a high-fat diet, and caloric restriction can abolish the exaggerated GIP response to a mixed meal in obese subjects, suggesting that a previously high calorie intake might have been responsible for the excessive GIP production. Even an increased number of K cells themselves could be a contributing factor, as in mice fed high-fat or high-carbohydrate cafeteria diets there is an ~50% increase in the density of K cells in the upper duodenum. Elevated levels of an anabolic hormone such as GIP, especially at a time when food is plentiful, might be a predicted physiological response to efficiently store calories as fat in order to survive a prolonged fast. In this sense, GIP can be considered the product of a so-called "thrifty gene." However, given readily available high-calorie foods and the Western diets rich in fats and ingested in large quantities, combined with the virtually limitless fuel storage capacity of adipocytes, this function may have become maladaptive. Therefore, elevated GIP levels may play a role in the development of obesity, and it is possible that strategies that attenuate GIP action might reduce body weight. However, given the important role of GIP in promoting insulin secretion, therapies involving the use of a GIP antagonist, for example, could have accompanying undesirable effects on glucose homeostasis. In this regard, it is notable that in GIP receptor knockout mice, there is a failure to enhance insulin secretion following a high-fat diet. Thus, while control mice maintain normoglycemia on the diet with elevated insulin levels, the GIP receptor knockout mice gain less weight at the expense of becoming glucose intolerant. These findings underscore the important role that GIP plays in the compensatory enhancement of insulin secretion produced by a high insulin demand and raises the possibility that a defect in this action of GIP may contribute to the pathogenesis of diabetes.

### B. Role of GIP in Diabetes

It has been recognized for some time that subjects with type 2 diabetes mellitus have a severely attenuated incretin response; insulin release is no longer stimulated more by oral glucose than by "isoglycemic" intravenous glucose. Given that diabetes develops only when insulin secretion becomes inadequate, it is of considerable importance to understand the mechanism behind this impaired insulin secretion. In subjects with diabetes, circulating GIP and GLP-1 levels have been reported to be increased, normal, or decreased following administration of nutrients, relative to controls. Thus, there is no clear evidence that the secretion of either GIP or GLP-1 is consistently impaired in subjects with diabetes. However, when one compares the insulinotropic activity of the two hormones in patients with type 2 diabetes, an interesting difference emerges. GLP-1 is typically very potent in these patients. Indeed, the peptide is so effective that numerous studies have now shown that its administration may completely normalize blood glucose levels. In contrast, GIP has little or no effect on insulin secretion in subjects with type 2 diabetes. In parallel studies of GLP-1 and GIP, the full efficacy of GLP-1 and the lack of effect of GIP have been documented in the same patients. Therefore, the possibility has been raised that a defect in the insulinotropic action of GIP could contribute to the impaired secretion of insulin associated with diabetes. However, to date, the reason for the inability of GIP to potentiate insulin secretion in subjects with diabetes has not been identified. GIP receptor mutations do not appear to be responsible. Furthermore, given that both GLP-1 and GIP signal via the same pathways, there does not appear to be a postreceptor defect. Interestingly, in obese *fa*/*fa* rats that are unresponsive to GIP but display normal sensitivity to GLP-1, there is a dramatic reduction in the expression of islet GIP receptors compared to expression in lean controls. Whether the same phenomenon occurs in humans and contributes to the impaired incretin effect associated with diabetes remains to be determined.

## IV. CONCLUSION

Originally identified as an enterogastrone, GIP has emerged as a critical metabolic hormone for the maintenance of normal energy homeostasis. The secretion of GIP from gastrointestinal K cells is acutely regulated by the absorption of glucose and fat and is produced in direct proportion to the quantities of these nutrients ingested. By its glucose-dependent insulinotropic actions on pancreatic β-cells and synergistic effects with insulin on adipocytes, GIP ensures the efficient disposal of the absorbed nutrients. K-cell number and GIP production are also adjusted over the long term in order to accommodate changes in nutritional intake. Although a hyperphagia-induced increase in GIP levels is undoubtedly important in producing compensatory increases in plasma insulin levels, the adipogenic nature of GIP, combined with hyperinsulinemia, could contribute to the development of obesity. It is possible that prolonged hypergipemia followed by eventual GIP desensitization could explain the impaired incretin effect that is associated with type 2 diabetes, itself so often accompanied by obesity. Thus, whereas incretin research has largely focused on GLP-1 as a result of its retained therapeutic potential, a full understanding of the factors contributing to the pathogenesis of diabetes associated with obesity may require a closer look at GIP.

## Glossary

**crypts of Lieberkühn** The base of tubular invaginations in the intestinal mucosa containing rapidly dividing undifferentiated stem cells that continuously produce new epithelial cells.

**enterogastrone** An intestinal hormone that is secreted in response to fat or its digestion products and inhibits gastric acid secretion.

**enteroinsular axis** Nutrient, neural, and hormonal signals arising from the gut that regulate hormone secretion from the endocrine pancreas.

**incretin** A hormone that is released from the intestine in response to the ingestion of glucose and stimulates insulin secretion from pancreatic β-cells.

## See Also the Following Articles

**Diabetes Type 2 • Gastrointestinal Hormone-Releasing Peptides • Insulin Actions • Insulin Gene Regulation • Insulin Processing • Insulin Receptor Signaling • Insulin Secretion**

## Further Reading

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# GnRH

**See *Gonadotropin-Releasing Hormone***

# Gonadotropin-Releasing Hormone (GnRH)

Jon E. Levine
*Northwestern University*

I. INTRODUCTION
II. ANATOMY OF GnRH NEURONS
III. MOLECULAR BIOLOGY OF GnRH
IV. THE GnRH PULSE GENERATOR
V. NEUROENDOCRINE REGULATION OF THE GnRH PULSE GENERATOR
VI. NEUROENDOCRINE REGULATION OF GnRH RELEASE DURING THE OVULATORY CYCLE
VII. PREOVULATORY GnRH SURGES
VIII. HOMEOSTATIC INHIBITION OF GnRH RELEASE
IX. ENVIRONMENTAL AND SOMATIC SIGNALS REGULATING GnRH NEUROSECRETION
X. SUMMARY

**The central nervous system regulates reproductive hormone secretions primarily through the neurosecretion of gonadotropin-releasing hormone. The decapeptide is synthesized in neurons of the basal forebrain, transported intraneuronally to neurovascular junctions in the median eminence of the hypothalamus, and released into the hypothalamic–hypophyseal portal vasculature.**

## I. INTRODUCTION

Following portal transport to the anterior pituitary, gonadotropin-releasing hormone (GnRH) can bind receptors located in the plasma membranes of gonadotropes and activate their associated signal transduction pathways. The major actions of GnRH include stimulation of the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the absence of GnRH release, secretion and synthesis of LH are virtually halted, FSH production is diminished, and gonadal activities are either impaired or completely arrested. Infertility is an invariant consequence of total GnRH deficiency.

Neurosecretion of GnRH is almost invariably intermittent, consisting of pulses of secretion that occur regularly at intervals of 20 min or more, depending on the species and physiological circumstances. The pulsatile GnRH release pattern, moreover, is obligatory for maintaining normal gonadotropin secretion and synthesis. Although control, substantial evidence now exists implicating astrocytes and several of the growth factors they produce as essential components of the facilitatory mechanisms by which the hypothalamus controls GnRH secretion during sexual development. This article develops the concept that the pubertal activation of GnRH secretion is not determined by a single mode of transsynaptic communication but is instead set in motion by at least three interrelated events, two involving transsynaptic pathways (a decrease in GABAergic inhibition and an increase in glutamatergic stimulation) and a third involving the activation of reciprocal glial–neuronal communication mechanisms.

## II. THE TRANSSYNAPTIC CONTROL OF GnRH NEURONS

Studies in rhesus monkeys have demonstrated that the most important inhibitory transsynaptic input controlling GnRH secretion during prepubertal development is provided by GABAergic neurons, which appear to use mainly a type of intrinsic membrane protein known as the $GABA_A$ receptor to exert this effect. The physiological importance of this regulatory mechanism is highlighted by the dramatic advancement of puberty induced in female monkeys by the pharmacological blockade of hypothalamic $GABA_A$ receptors. Whether this inhibition is directly exerted on GnRH neurons, which contain functional $GABA_A$ receptors, or through neurons synaptically connected to the GnRH neuronal network is currently the subject of intense investigation. It has been recently shown by DeFazio and colleagues that GnRH neurons respond with excitation to direct GABA inputs mediated by $GABA_A$ receptors. There is, however, electrophysiological evidence indicating that another type of membrane-embedded GABA recognition molecule, the $GABA_B$ receptor, present on GnRH neurons, mediates a direct and powerful GABAergic inhibitory signal of GnRH neuronal activity. It would appear intuitively reasonable to infer that if GABA utilizes $GABA_B$ receptors to directly inhibit GnRH neurons, then a second inhibitory influence provided by the same neurotransmitter would be exerted on excitatory neuronal subsets connected to GnRH neurons, instead of GnRH neurons themselves. Experimental verification of this idea has not yet been provided.

In opposition to the GABAergic system, enhancement of glutamatergic neurotransmission—the primary mode of transsynaptic excitatory communication used by hypothalamic neurons—accelerates the initiation of puberty. For instance, the premature activation of a class of glutamate recognition molecules present in the cell membrane, and known as *N*-methyl-D-aspartate (NMDA) receptors, has been shown to increase GnRH secretion and advance sexual maturation in both rats and monkeys. During normal sexual development, there is an activation of NMDA receptors at the onset of puberty that occurs even in the absence of the gonads. The physiological importance of this activation was demonstrated by the ability of the pharmacological blockade of NMDA receptors to delay sexual development in female rats. Despite its effectiveness in affecting the timing of puberty, NMDA receptors are not the only class of glutamate recognition molecule involved in stimulating GnRH secretion in developing animals. In fact, only a small subset of GnRH neurons express NMDA receptors during prepubertal development. In contrast, many of them express another class of cell membrane-associated proteins, known as kainate receptors, that also recognize glutamate with high affinity. Stimulation of the hypothalamus with kainate increases both LH and GnRH release, indicating that glutamatergic neurons utilize at least two classes of glutamate receptors to facilitate GnRH secretion: NMDA receptors, located mostly on neurons synaptically connected to GnRH neurons, and kainate receptors, present on GnRH neurons themselves. Using a more sensitive method, Ottern and colleagues showed that up to 80% of GnRH neurons express NMDA receptors. An appropriate strength of the glutamatergic inputs converging onto the GnRH neuronal network appears to be critical for the maintenance of pulsatile GnRH release, as these pulses are obliterated when glutamate synthesis is blocked in the developing hypothalamus.

The above considerations make clear that opposite changes in glutamatergic and GABAergic neurotransmission are critical components of the cell–cell communication process underlying the activation of GnRH release at puberty. They do not, however, tell us which of the two events occurs first. An increase in glutamatergic transmission may precede the pubertal loss in GABAergic inhibitory inputs, as activation of certain glutamate receptors in other brain regions inhibits GABA release. The facts that the primate hypothalamus contains twice as many glutamatergic synapses as GABAergic synapses and that in this species GABAergic neurons receive a strong glutamatergic input suggest that a glutamate-to-GABA hierarchy may, indeed, represent a critical transsynaptic relationship underlying the initiation of puberty. Evidence favoring the alternative possibility, i.e., that a decrease in GABAergic inhibitory transmission precedes the increase in glutamatergic input at the onset of puberty, also exists. For instance, glutamate release into the median eminence of peripubertal monkeys has been shown to increase following an experimental reduction of GABA synthesis, and GABA neurons have been observed to innervate a subset of glutamatergic neurons in the primate hypothalamus.

Other neurotransmitters/neuropeptides, such as norepinephrine and Neuropeptide Y (NPY), contribute to facilitating the advent of puberty, but in a secondary fashion. The role of NPY is, however, controversial, as NPY has been shown to either facilitate or inhibit GnRH secretion during peripubertal development.

## III. THE GLIAL CONTROL OF GnRH neurons

Astroglial cells regulate GnRH secretion via both plastic rearrangements and direct cell–cell communication. Several growth factors, produced by glial cells, have been implicated in the mechanism by which astrocytes facilitate GnRH secretion. The most well studied are transforming growth factor-β (TGF-β), insulin-like growth factor1 (IGF-I) basic fibroblast growth factor, and the family of epidermal growth factor (EGF)-related polypeptides. Nonpeptidergic molecules produced in astrocytes (such as calcium, prostaglandin E2 (PGE2), and glutamate) have also been shown to stimulate GnRH release.

An involvement of TGF-βs in the facilitatory control of GnRH secretion was suggested by studies using a GnRH neuronal cell line and hypothalamic astrocytes in culture. In these experiments, TGF-β1, secreted from astrocytes, was found to stimulate GnRH release directly via receptors located on "immortalized" GnRH neurons (i.e., neurons able to perpetuate themselves in culture). Moreover, TGF-β1 release from hypothalamic astrocytes was enhanced by estradiol, suggesting that astrocytic TGF-β1 may contribute to the mechanism by which the hypothalamus generates the surge of GnRH that, by stimulating the secretion of pituitary gonadotropins, triggers ovulation. Adult GnRH neurons do contain TGF-β receptors, indicating that they—like immortalized GnRH neurons—are also a target for TGF-β1 action. Whether TGF-β1 is physiologically involved in the control of the onset of puberty remains to be determined. Likewise, it is unclear whether the main effect of TGF-β1 on GnRH neurons *in situ* is to facilitate GnRH secretion, because—in contrast to their stimulatory effect on immortalized cells—neither TGF-β1 nor TGF-β2 is able to stimulate GnRH release from the median eminence of the hypothalamus. Perhaps the main role of TGF-β1 is to promote some other aspect of GnRH neuronal function, such as changes in neuronal shape and/or changes in the contact that exists between GnRH nerve terminals and glial cells in the median eminence of the hypothalamus.

In contrast to TGF-β1, a stimulatory effect of IGF-I on GnRH release from the prepubertal hypothalamus is well established. It is also clear that IGF-I is a facilitatory signal for puberty to occur. The central administration of IGF-I advances puberty in both rodents and primates, and the loss of IGF-I in mutant mice lacking growth hormone receptors (which upon growth hormone binding stimulate the production of IGF-I) results in delayed sexual development. Although IGF-I is produced in astrocytes, it appears that the bulk of IGF-I-stimulating GnRH secretion either at puberty or during the estrous cycle derives from a peripheral source(s) (likely the liver) instead of hypothalamic astrocytes. Thus, IGF-I represents one of the metabolic signals long suspected to play a role in facilitating the onset of mammalian puberty.

With regard to the EGF family of growth factors, it is now clear that the EGF relatives TGF-α and neuregulins (NRGs) are produced in hypothalamic astrocytes and stimulate GnRH secretion indirectly, using an astrocyte-to-astrocyte mode of action. To exert this stimulatory effect, they bind to erbB receptors located on the cell membrane of astroglial cells (instead of GnRH neurons) and induce the release of bioactive substances, such as PGE2, that act directly on GnRH neurons to elicit GnRH secretion. TGF-α is recognized by erbB-1 receptors, which are required for normal sexual development. Studies in rodents have shown that if these receptors are blocked in the median eminence, or if their function is diminished by a genetic mutation, female puberty is delayed. Conversely, transgenic mice carrying a molecularly modified TGF-α-producing gene and rats carrying intrahypothalamic grafts of cells genetically engineered to secrete TGF-α, show accelerated sexual development. The results of the latter study led to the notion that activation of a discrete subset of astrocytes near GnRH neurons may suffice to induce an appropriate pattern of GnRH secretion able to set in motion the pubertal process. In support of this concept is the finding that, in humans, some puberty-inducing hypothalamic tumors known as hamartomas contain astroglial cells rich in TGF-α and its receptors.

As indicated above, the hypothalamus of developing female rats also produces NRGs, a group of

TGF-α-related proteins, and two of the three erbB receptors mediating NRG signaling, erbB-2 and erbB-4. Experiments using cell lines have shown that whereas NRGs bind to erbB-4 receptors, erbB-2 functions as an auxiliary subunit, recruited by erbB-4 receptors upon ligand binding. A similar set of interactions occurs in the developing hypothalamus. During female sexual development, the hypothalamic content of erbB-2 and erbB-4 mRNA begins to increase by the end of the juvenile period, when circulating sex steroid levels are still low, suggesting that the increase is independent of an augmentation in gonadal output of sex steroids. The presence of the co-receptor erbB-2 appears to be critical for the timely initiation of female puberty because *in vivo* disruption of erbB-2 synthesis results in delay puberty in female rats. The physiological importance of both the TGF-α- and the NRG-dependent signaling systems for the timely initiation of puberty and subsequent reproductive performance has been demonstrated by recent studies using a genetic approach to disrupt the functional competence of each ligand–receptor complex. Thus, mice carrying a mutated erbB-4 receptor able to block the signaling capability of the intact receptor specifically in astrocytes show delayed female sexual development and compromised fertility, without alterations in erbB-1 function. Breeding these animals to mice carrying a defective erbB-1 receptor aggravated the defect by further delaying the onset of puberty and seriously compromising fertility. As a measure of this defect, litters born to double-mutant mothers were 1–3 pups in size, in marked contrast to the 8–12 pups per litter observed in normal wild-type animals.

## IV. THE NEURONAL CONTROL OF NEUROENDOCRINE GLIAL FUNCTION

An important issue that hitherto had not been resolved is the identity of potential neuron-to-astrocyte communication pathways capable of regulating erbB-dependent astrocytic signaling. Recent studies have implicated excitatory amino acids as participants of one of these communication pathways. Hypothalamic astrocytes contain two classes of glutamate recognition molecules known as metabotropic and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors. They also express several proteins required for these receptors to convey intracellular signals in response to glutamate binding. Upon stimulation with the appropriate receptor ligand, astrocytes respond with functional changes, including mobilization of erbB receptors to the cell surface and association of TGF-α and NRGs with their respective receptors on the cell membrane. As a consequence of this association, erbB receptors are phosphorylated, indicating that excitatory amino acids acting via AMPA and metabotropic receptors activate erbB receptors indirectly by facilitating their interaction with their TGF-α and NRG ligands. These results complement and expand earlier findings showing that activation of AMPA and metabotropic receptors in astrocytes causes PGE2 release, which in turn increases glial glutamate release. Because astrocytic glutamate released in response to PGE2 triggers calcium fluxes in neighboring neurons, it has been postulated that this cell–cell signaling pathway is required for astrocytes to regulate synaptic transmission in the central nervous system. Thus, communication between astrocytes and neuronal networks that use amino acids for neurotransmission appears to represent a fundamental mechanism employed by the neuroendocrine brain to control the advent and progression of mammalian sexual maturation.

Activation of erbB-mediated signaling may also represent one of the centrally originated, gonad-independent components that controls the initiation of puberty. Because erbB-1 ligands are able to activate estrogen-dependent events in the absence of estrogen, they could act in key hypothalamic regions to initiate such events before steroid production begins to increase at puberty and, thus, provide—in the absence of changes in gonadal output—one of the initial stimuli underlying the initiation of the pubertal process.

## V. PRESUMPTIVE UPSTREAM CONTROLLING GENES

Preceding, and likely controlling, the activation of the aforementioned cell–cell communication pathways, there appears to exist an “upstream” layer of specific regulators of gene transcription that, operating within specific cellular subsets of the hypothalamus, unleash the primary events leading to the pubertal increase in central drive. Two of these upstream regulators, known as Oct-2 and TTF-1/Nk-2.1, have been implicated in this process.

In the postnatal brain, the Oct-2 gene is expressed in several neuronal hypothalamic nuclei, but Oct-2 proteins are more abundant in cultured astrocytes than in neurons, indicating that Oct-2 gene products may play an important role in regulating the transcriptional activity of astroglial genes. One of the astroglial genes regulated by Oct-2 is TGF1. Its promoter contains a DNA sequence recognized by Oct-2 and is activated by Oct-2. Oct-2 appears to be important for the pubertal increase in hypothalamic TGF-α gene expression and for the initiation of puberty itself, because—like TGF-α—hypothalamic Oct-2 mRNA levels increase during juvenile development preceding the pubertal augmentation of steroid secretion. More importantly, blockade of Oct-2 synthesis results in reduced astrocytic TGF-α synthesis and delayed age at first ovulation, indicating that an intact Oct-2-initiated pathway involving TGF-α as a downstream regulated gene is required for the timely acquisition of female sexual maturity. Puberty-advancing lesions of the hypothalamus activate Oct-2 expression in astrocytes near the lesion site, further supporting this notion.

In addition to increasing Oct-2 gene expression in astrocytes, these lesion increased TTF-1 mRNA in neurons. Like Oct-2, the hypothalamic content of TTF-1 mRNA increases transiently before the initiation of normal puberty—also before the occurrence of any discernible change in gonadal steroid secretion. After birth, the hypothalamic expression of TTF-1 is limited to discrete cellular subsets, which include selected glial cells and neurons. The most prominent glial site of expression is in the median eminence of the hypothalamus, where it co-localizes

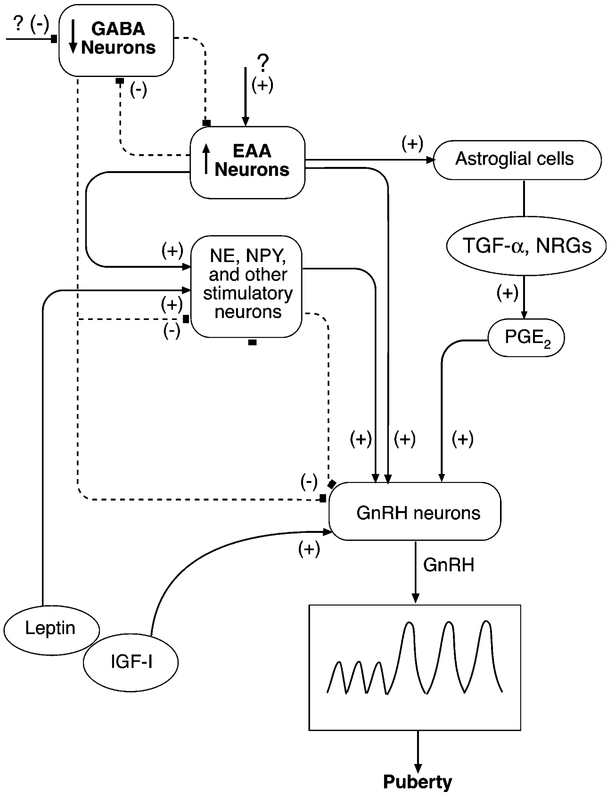

FIGURE 1 The central events underlying the initiation of puberty. An increase in episodic GnRH secretion is activated by an integrative mechanism involving both neuron-to neuron and glia-to-neuron communication processes. The neuron-to-neuron (transsynaptic) component involves the simultaneous activation of excitatory transsynaptic inputs and a reduction in inhibitory inputs to GnRH neurons. The main excitatory transsynaptic input affecting GnRH release is postulated to be provided by neurons that use excitatory amino acids (EAA) as neurotransmitters. Norepinephrine (NE) and Neuropeptide Y (NPY)-containing neurons provide a complementary facilitatory input to the GnRH neuronal network. The chief inhibitory neuronal input restraining GnRH release is provided by GABAergic neurons (GABA). The glia-to-neuron component of the process involves the production of the epidermal growth factor (EGF)-related peptides, TGF-α and neuregulins (NRGs), by astroglial cells, and the stimulatory effect of these peptides on the glial production of neuroactive substances, such as prostaglandin E2, which act directly on GnRH neurons to elicit GnRH secretion. Current evidence suggests that an increase in EAA neurotransmission (↑, EAA neurons) may be predominantly responsible for the pubertal activation of pulsatile GnRH secretion in the rat, whereas a decrease in an inhibitory GABAergic tone (↓, GABA neurons) may be a primary transsynaptic event underlying the process in the rhesus monkey. Evidence collected in rodents indicates that excitatory amino acids can act directly on GnRH neurons to enhance GnRH release (+), and on GABA neurons to inhibit (−) GABA neurotransmission. It is also possible, however, that a primary decrease in GABA release triggers the pubertal increase in EAA neurotransmission. EAA acting on astroglial cells appear to facilitate the glial production of TGF-α/NRGs. While the decrease in GABA transmission begins to relieve GnRH neurons from GABAergic inhibitory control, the increased production of EGF-related glial peptides stimulates the formation of prostaglandin E2, which acts directly on GnRH neurons to elicit GnRH release, amplifying the stimulatory effect of EAA and magnifying the loss of GABAergic inhibitory control. Upon the initiation of these changes, a further increase in EAA stimulation and a further decrease in GABAergic inhibitory tone lead to activation of NE and NPY neuronal systems. Both NE and NPY would then contribute to the progression of the pubertal process by stimulating GnRH secretion. There is also evidence that an inhibitory NPY influence on GnRH release is lifted at the end of juvenile development and, thus, contributes to the initiation of the pubertal process. The metabolic signals leptin and IGF-I further the process along by stimulating GnRH secretion either directly (IGF-I) or via functionally connected neuronal networks (leptin). The resulting increase in pulsatile GnRH secretion then results in the initiation of puberty ↓ = (−) = inhibition; ↑ = (+) = stimulation]. Modified with permission from Ojeda and Bilger (1999), *In* "Neuroendocrinology in Physiology and Medicine" (P. M. Conn and M. E. Freeman, eds.), pp. 197–224. Humana Press.

with erbB-2. The most salient site of neuronal expression is in a group of hypothalamic neurons that produce enkephalins, a family of inhibitory peptides. TTF-1 is also expressed to some extent in GnRH neurons. TTF-1 activates erbB-2 and GnRH gene expression, but it inhibits expression of the enkephalin gene, via binding to specific DNA sites present in the controlling (promoter) region of these genes. These findings have led to the hypothesis that TTF-1 is a controlling gene able to coordinate the (1) activation of hypothalamic cells required for the initiation of the pubertal process (such as GnRH neurons and erbB-2-containing glial cells of the median eminence) and (2) the repression of neuronal systems (such as enkephalinergic neurons) that may inhibit the initiation of puberty.

## VI. SUMMARY AND CONCLUSIONS

Clear evidence now exists indicating that the activation of GnRH secretion at puberty requires the concerted activation of both transsynaptic and astroglial regulatory systems (Fig. 1). The neuronal networks most critically involved in this process are those that utilize excitatory and inhibitory amino acids as neurotransmitters. Astroglial cells, on the other hand, contribute by producing cell–cell signaling molecules able to regulate neuronal function. Although in several brain regions the interaction of excitatory and inhibitory neuronal networks appear to follow the rules of a glutamate-to-GABA hierarchy, it is still unclear which of these two neurotransmitter systems initiates the transsynaptic cascade of events leading to the pubertal activation of GnRH secretion. Among the various cell–cell signaling molecules produced by astroglial cells, the TGF-α and EGF families of growth factors have been identified as central components of the communication pathway used by hypothalamic glial cells to facilitate GnRH release. Although IGF-I is also produced in astrocytes and facilitates the onset of puberty via stimulation of GnRH release, it does not appear to exert these effects upon release from hypothalamic astrocytes, but instead is produced by a peripheral source and reaches the hypothalamus via the bloodstream. In addition to the aforementioned glia-to-neuron signaling complexes, there is a neuron-to-glia communication pathway employed by neurons that use glutamate for neurotransmission and mediated by the coordinated activation of different glutamate receptors. Activation of these receptors on astrocytes sets in motion events that lead to activation of a different type of receptor, those that mediate the actions of TGF-α and neuregulins on glial cells. Finally, two genes have been implicated as members of the upstream regulatory hierarchy controlling some of the cellular events underlying the synchronized changes in neuronal/glial function that initiate puberty. The importance of neuronal and glial plasticity in the pubertal process remains to be established and should be the subject of extensive investigation in years to come.

## Acknowledgments

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## Glossary

**astrocytes** Nonneuronal, glial cells (from the Greek, meaning glue). They are required for the integration of cell–cell communication within the nervous system, regulation of synaptic transmission, facilitation of neuronal activity, generation of neurons, and production of growth factors needed for the integrity of neuronal function.

**gonadotropin-releasing hormone** The neuropeptide that controls pituitary secretion of gonadotropic hormones and that is produced by a specialized subset of neuroendocrine neurons of the hypothalamus. It is also known as luteinizing hormone-releasing hormone.

**transsynaptic** The mode of communication between neurons, which convey information to one another via chemical signals released into specialized points of contact known as synapses.

**upstream** Describes events that control and/or initiate subsequent events in a regulatory pathway.

## See Also the Following Articles

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# Gonadotropin-Releasing Hormone Neuron

Allan E. Herbison
*University of Otago, New Zealand*

I. TOPOGRAPHY AND MORPHOLOGY
II. ELECTROPHYSIOLOGICAL PROPERTIES
III. SYNAPTIC AND HORMONAL INPUTS
IV. SUMMARY

**The brain integrates multiple internal homeostatic and external factors to control reproductive functioning. The neural network responsible for this critical function ultimately uses a small group of 1000–2000 gonadotropin-releasing hormone (GnRH) neurons to control directly the secretion of gonadotropins from the pituitary gland. The release of the GnRH decapeptide from nerve terminals located in the median eminence into the pituitary portal system is episodic in nature and responsible for the pulsatile profile of the circulating gonadotropins. Furthermore, the changes in gonadotropin pulse amplitude and frequency that occur across the female ovarian cycle, or in response to various stressors, are thought to arise principally from alterations in the pattern of GnRH release. Thus, knowledge of the GnRH neuron and how it secretes GnRH is critical to our understanding of how the brain controls fertility. This article focuses on recent advances obtained in our understanding of the cellular properties of the GnRH neuron in mammalian species.**

## I. TOPOGRAPHY AND MORPHOLOGY

Remarkably, in all mammals examined to date, the gonadotropin-releasing hormone (GnRH) neurons responsible for regulating reproduction are not created in the brain but, instead, arise from the olfactory placode located within the nose. During midgestation, the GnRH neurons migrate along the axons of olfactory neurons to pass through the nose and enter the brain, whereupon they turn ventrally and proceed to the medial septum and hypothalamus. This pattern of migration is remarkable not only in the distance covered by the GnRH neurons but also in terms of the multiple different environments they must navigate to reach their final destination. Kallmann's syndrome, with the predominant features of infertility and anosmia, represents one such example of failed GnRH neuron migration in which these cells do not pass from the nose into the brain in humans.

One consequence of the extraordinary migratory development of the GnRH neurons is that the cell bodies of the GnRH neurons come to reside in a scattered manner along the entire midline migratory pathway within the postnatal brain (Fig. 1). Although this phenomenon is found in all species, differences the pulsatile infusion of the decapeptide can restore gonadotropin secretions in GnRH-deficient subjects, continuous administration of GnRH fails to sustain gonadotropin release. The pulsatile GnRH release pattern is thus considered a critical feature of the cascade of hormone secretions that constitute the reproductive axis. Those neurons and their processes that function to release the neuropeptide GnRH in this rhythmic manner are collectively referred to as the GnRH pulse generator.

At least one form of GnRH regulates reproduction in all vertebrate species. Thus far, at least nine different molecular forms of the decapeptide have been identified in lower vertebrates, and at least two different GnRH molecules, GnRH-I and GnRH-II, are expressed in many mammals. Two distinct genes encode the latter two GnRH molecules, and their expression patterns in the brains of primates and other animals are clearly dissimilar. With regard to function, much is known of the biology of GnRH-I, but little is known of the biological importance of GnRH-II. The hypophyseotropic functions of GnRH-I have been extensively studied in the years following its identification, and it is generally held that it serves as the predominant GnRH governing the reproductive axis in mammals. The neuroendocrine and behavioral functions of GnRH-II and other GnRH molecules are much less clear; although their distribution suggests a role in neuromodulation, this remains unproven. Thus, this article will focus largely on the anatomy, molecular and cellular biology, and physiology of GnRH-I neurons, and the appellation "GnRH" will henceforth be used synonymously with "GnRH-I," referring to the GnRH decapeptide: pyroGlu$^1$-His$^2$-Trp$^3$-Ser$^4$-Tyr$^5$-Gly$^6$-Leu$^7$-Arg$^8$-Pro$^9$-Gly$^{10}$amide.

## II. ANATOMY OF GnRH NEURONS

During embryogenesis, GnRH neurons originate from the olfactory epithelium and migrate along the nasal septum and terminal nerve to enter the basal forebrain. In most, if not all, animals the relatively small population of GnRH perikarya (e.g., 800 neurons in mice) assumes its final distribution along a medial septal, preoptic, and hypothalamic tissue continuum prior to birth. The number of GnRH neurons appears to remain constant throughout postnatal life, although the cytological appearance (e.g., smooth versus spiny contours) and the degree of glial ensheathment have been suggested to change during postnatal stages of development. The GnRH neuron in the mature animal is typically fusiform in shape and is relatively unremarkable at the ultrastructural level. Diminished or absent dendritic arborizations, as well as a minimum of synaptic contacts, are perhaps the most consistent features of GnRH neurons. This has been taken as presumptive evidence that only a limited number of afferent synaptic signals contribute to the integrative activity that occurs within GnRH neurons. Thus, nonsynaptic signals (neural or endocrine) and/or autoregulatory activity may predominate in the cell signal integration that controls spontaneous and regulated pulsatile GnRH secretion. A significant proportion of GnRH neurons co-express the structurally unrelated neuropeptide galanin, and this GnRH neuronal product has been suggested to play a role in the autoregulation of GnRH neurosecretion.

In rodents, the topography of GnRH is characterized by a scattered but stereotyped distribution pattern that typically forms an "inverted V" pattern at more rostral tissue levels, including the diagonal band of Broca, organum vasculosum of the lamina terminalis (OVLT), and preoptic nuclei. The distributions of GnRH neurons in monkeys, sheep, and guinea pigs additionally include subsets of GnRH perikarya that reside more caudally within the mediobasal hypothalamus. Although some GnRH neurons extend processes that make synaptic contacts with other neurons in the basal forebrain, it is clear that the majority of the GnRH neurons extend axons toward and within the lateral median eminence and form neurovascular junctions with fenestrated microcapillaries of the primary portal plexus. It is within this neurohemal organ that GnRH neurosecretion into the hypophyseal portal vasculature takes place and from which GnRH molecules are conveyed to a secondary plexus within the anterior pituitary gland.

A second GnRH axon terminal field is present within the OVLT, where GnRH neurovascular junctions are also evident. The functions of this ostensible GnRH neurosecretory site remain unknown. Axon terminals have also been found to extend through the ependymal lining of the ventral third ventricle, the septal lateral ventricle, the subfornical organ, and the cerebral aqueduct. The release of GnRH from these terminals into the cerebrospinal fluid has been inferred, and its functional importance in the regulation of pituitary function or behavior has been debated. Additional GnRH terminal fields of much smaller proportions have been characterized and include olfactory tissues, portions of pyriform cortex, medial, and cortical amygdalar nuclei, stria terminalis, ventral hippocampus, and subiculum. Several GnRH axons also course from the level of the medial septum and reach as far caudally as the midbrain central gray, where they have been implicated in the modulation of lordotic behavior in female rats.

## III. MOLECULAR BIOLOGY OF GnRH

The paucity and scattered topography of GnRH neurons hampered early molecular biological investigations of the neurohormone. These technical barriers were largely overcome following the development of immortalized hypothalamic GnRH neurons, including the GT1-1 and GT1-7 cells, as well as the GN10 and GN11 cell lines. Much information has since been obtained on the regulation of GnRH transcription, translation, processing, and secretion in a homogenous GnRH cell context *in vitro*.

The 5′-flanking sequences of the mouse, rat, and human GnRH gene contain a neuron-specific enhancer and dual promoters, each region being critical for transcription of the gene. The 300 bp enhancer is situated 1.8 kb upstream of the transcriptional start site and it confers a 50-fold activation of GnRH gene transcription. It contains sites that bind proteins important for neuron-specific expression of the gene, including POU-homeodomain (sharing a domain common to Pit-Oct-Unc families of transcription factors) and GATA (binding GATA nucleotide sequence) factors. In the human GnRH gene, the 5′-flanking region contains two transcriptional start sites at +1 and −579, each associated with respective promoter sequences. The proximal (or downstream) promoter for the proximal start site is active in hypothalamic GnRH neurons, whereas the distal promoter is fully active in cells derived from other reproductive tissues, including the placenta. The existence of dual promoters in the 5′-flanking region of the GnRH gene appears to be a conserved feature of GnRH genes across mammalian species.

Several neuroendocrine signals have been shown to regulate the transcriptional activity of the proximal GnRH gene promoter. Estrogen, progesterone, glucocorticoids, and retinoic acid are among the steroid hormones that have been shown to either directly or indirectly influence GnRH gene transcription, depending upon species and cell context. The negative and positive effects of estrogen and progesterone have received the greatest attention, with studies guided by the possibility that transcriptional regulation of the GnRH gene may mediate the negative and positive feedback actions of these steroids on GnRH release *in vivo*. Current evidence indicates that a negative estrogen-response element is localized in the proximal promoter region (−171 to −126), although the estrogen receptor does not bind to this region. An indirect regulatory mechanism has therefore been inferred. Progesterone has also been found to repress GnRH promoter activity through a region that encompasses the estrogen-response element. Three putative nonconsensus binding elements for the progesterone receptor (PR) are present within this region of the GnRH promoter. Glucocorticoids have also been found to suppress GnRH gene transcription. In the mouse GnRH gene, one response element is known to directly interact with the glucocorticoid receptor (GR) and a second binds the POU-homeodomain factor Oct-1, which in turn binds the GR. Both regions can confer negative regulation of the mouse GnRH gene promoter. GnRH promoters of various species have also been found to be regulated by signals within growth factor- and G-protein-coupled receptor-mediated pathways. Among these are signals conveyed via protein kinase C, insulin-like growth factor-I (IGF-I), bFGF, corticotropin-releasing hormone receptors, human chorionic gonadotropin/LH receptors, and GnRH receptors.

The coding region of the GnRH gene includes sequences distributed across four exons. Transcription and RNA processing yield a GnRH mRNA that encodes a 92-amino-acid prepro-GnRH protein, and the GnRH decapeptide and gonadotropin-releasing hormone-associated peptide are ultimately derived from the posttranslational proteolytic cleavage of this precursor molecule. The posttranslational processing of the molecule, including its C-terminal amidation, likely takes place within the nascent secretory granule and during axonal transport to the axon terminal. The GnRH secretory process involves a $Ca^{2+}$-dependent exocytotic mechanism, which is in turn directed by intermittent volleys of action potentials that invade the GnRH terminals and are coupled to both the entry of extracellular $Ca^{2+}$ into the cell and the mobilization of intracellular $Ca^{2+}$ stores.

A variety of neural and endocrine signals are known to regulate the rate and pattern of pulsatile GnRH release. In general, these inputs can be categorized as those that impact the amplitude of GnRH release or those that impact the frequency of GnRH pulse generation. Potential mechanisms for the regulation of GnRH amplitude include modulation of GnRH gene transcription, translation, posttranslational processing, intracellular transport, and stimulus-secretion coupling; the last process includes the enhancement or diminishment of cell signaling leading to the elevation of intracellular $Ca^{2+}$ and the exocytotic release of the GnRH decapeptide. Frequency modulation is likely dependent on inputs that alter the electrophysiological excitability of the GnRH neuronal membrane. Although the mechanisms that mediate such effects are not well known, the alteration of GnRH pulse frequency is most likely manifest as changes in the rate of a basic pulse-generating mechanism that is largely an intrinsic function of GnRH neuronal networks.

## IV. THE GnRH PULSE GENERATOR

The GnRH release process is almost invariably pulsatile, and in virtually all female and male mammals studied this pulsatile release pattern has been found to be critically important in sustaining gonadotropin secretions. The cellular mechanisms that govern the pulsatile GnRH release process, however, remain poorly understood. Electrophysiological correlates of pulse generator activity have been characterized using mediobasal hypothalamic, multiunit recordings in monkeys, sheep, goats, and rats. Although these studies have not allowed for microanatomical mapping of pulsing cells and/or circuitries, they have provided a functional definition of the GnRH pulse generator: a set of neurons that periodically fire a high-frequency volley of action potentials that eventuate in the neurosecretion of a GnRH pulse into the hypophyseal portal vessels. Beyond this definition, the electrophysiological studies have also underscored the existence of at least two important elements of the GnRH pulse-generating process—a pulse initiation mechanism and a mechanism for electrophysiological synchronization among GnRH neurosecretory cells.

The simplest model for pulsatility holds that pacemaking activity occurs within one or more GnRH neurons themselves and that the activities of "slave" GnRH neurons are entrained to the rhythm of the dominant pacemaker within the population. This idea is supported by the observations that GnRH release from immortalized GT1 cells in culture is pulsatile. Observations of pulsatile GnRH release from isolated guinea pig mediobasal hypothalamus and rat preoptic–mediobasal hypothalamic tissues *in vitro* have also been documented, and these findings, too, support the idea of intrinsic GnRH pulsatility, since GnRH perikarya in these species are contained within these respective tissues. Studies of cultured GnRH neurons, however, have not provided evidence that any specific GnRH neuron operates as a dominant pacemaker within a population of cells. Thus, a variation of the foregoing model of pulsatility (pseudo-pacemaking) holds that any neuron within the pulsing network can initiate a pulse and that the pulsatile rhythm follows from the continued repetition of (1) random activation of a GnRH neuron within the interconnected GnRH network; (2) stimulation of other neurons in the network by the excited cell; (3) a refractory period, during which no neurons fire; and (4) activation of another neuron in the network. This stochastic model has been strongly supported by studies of $Ca^{2+}$ elevations in cultured GnRH neurons.

Some mechanism must also operate to synchronize the pulsatile release activity among GnRH neurons. Synchronicity may be achieved through intercellular signaling via GnRH–GnRH synaptic contacts or gap junctions. Some GnRH immunopositive terminals have been demonstrated in apposition to GnRH perikarya, and recent evidence suggests that GnRH receptors are expressed in GnRH neurons. Other work suggests that a volume transmission mechanism, perhaps involving nitric oxide, could mediate synchronization among GnRH neurons.

## V. NEUROENDOCRINE REGULATION OF THE GnRH PULSE GENERATOR

Physiological regulation of the reproductive axis commonly occurs through GnRH pulse frequency modulation. Regulation of GnRH pulse frequency appears to be important in initiating prolonged changes in the activity of the reproductive axis, such as those occurring in puberty, during seasonal transitions in the reproductive state, and following gonadectomy. Frequency regulation also appears to mediate inhibitory responses of the reproductive axis to prolonged exercise and food restriction. The cellular mechanisms that mediate GnRH pulse frequency modulation are not currently understood. Based on the pseudo-pacemaking model of pulsatility, however, it may be assumed that any stimulus that tends to depolarize the membrane potential of a pulse-generating neuron would increase its likelihood of reaching the firing threshold in response to a stimulus. Conversely, any condition that tends to hyperpolarize the membrane would decrease the likelihood of a cell reaching the firing threshold in response to a stimulus. These expectations would apply regardless of the nature of the stimulus, viz. whether or not the cell is autoexcitatory or excited by another cell. Thus, if a significant portion of the entire pulsing neuronal network is rendered more or less excitable (more or less depolarized), the probability of any cell initiating a pulse at a given time following a preceding pulse would be increased or decreased, accordingly. It follows that any overall change in the probability of excitation in a pulsing network would produce a commensurate change in average interpulse interval and, hence, in GnRH pulse frequency.

How then is a given level of cell excitability manifest in an individual GnRH neuron, and how may it be altered? One likely mechanism depends upon the activity of ion channels, particularly those selective for $K^+$, which conduct hyperpolarizing currents that can prevent or retard depolarization toward firing threshold. Patch-clamp studies, using both whole-cell and perforated-patch configurations, have shown that immortalized GnRH neurons express several types of $K^+$ channels, including a $K^+$ inward rectifier ($K^+_{ir}$) and four outward $K^+$ channels: a delayed rectifier, a 4-AP-sensitive "A-type" channel, a 4-AP and TEA-insensitive channel, and a large-conductance $Ca^{2+}$-activated $K^+$ (BK) channel. A $K^+_{ir}$ channel has been shown to be active in embryonic GnRH neurons, as well as in GT1 cells; it has been postulated that it is these channels that may be regulated to produce changes in GnRH neuronal excitability.

## VI. NEUROENDOCRINE REGULATION OF GnRH RELEASE DURING THE OVULATORY CYCLE

During the ovulatory cycles of rats, monkeys, and other spontaneous ovulators, gonadotropin secretions maintained at low levels, principally through the negative feedback actions of the gonadal steroids, estrogen and progesterone, and protein hormones, such as inhibin. These homeostatic negative feedback actions are exerted via integrated actions on the GnRH pulse generator and pituitary gonadotropes (see Fig. 1). At midcycle, however, the sustained negative feedback regulation of GnRH and gonadotropin secretion is interrupted by the induction of the preovulatory gonadotropin surge. A rising tide of estrogen, secreted by the ripening follicle(s), evokes a primary surge of LH and FSH that in turn triggers ovulation. A major unresolved issue in the study of reproductive neuroendocrinology is the clarification of the mechanisms by which low levels of estrogen and progesterone may, on the one hand, exert homeostatic negative feedback actions on the GnRH pulse generator, while high levels of these steroids, on the other hand, exert positive feedback actions that culminate in the release of GnRH and LH surges.

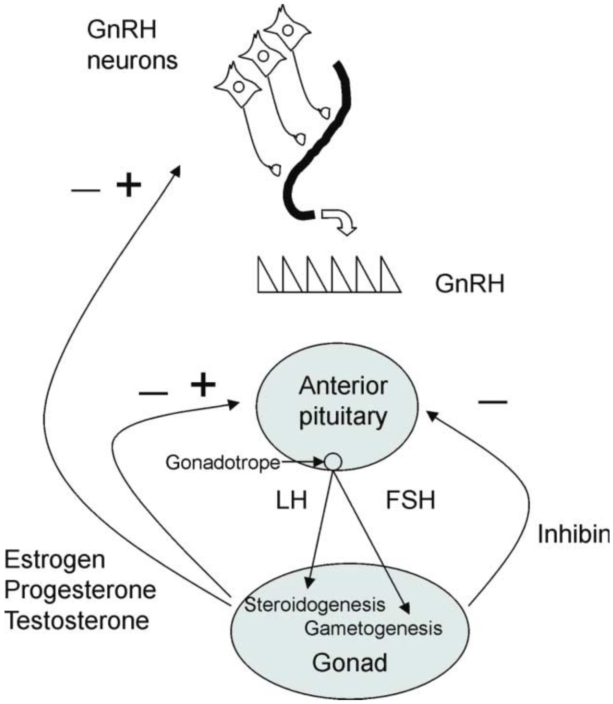

**FIGURE 1** Schematic diagram of hormonal signals in the hypothalamic–pituitary–gonadal axis. The "GnRH pulse generator," consisting of GnRH neurons and possibly other neuronal elements, governs the neurosecretion of pulsatile GnRH into the hypophyseal portal vessels. After delivery to the anterior pituitary gland, GnRH molecules bind GnRH receptors on the plasma membranes of gonadotropes and stimulate the synthesis and secretion of the gonadotropins, LH and FSH. At the gonads in both sexes, LH stimulates steroidogenesis and FSH supports gametogenesis. Steroids and protein hormones (e.g., inhibin) can exert homeostatic negative feedback actions within the hypothalamus to retard the activity of the GnRH pulse generator and at the anterior pituitary to suppress responsiveness to GnRH stimulation. In females, high levels of estrogen produced during the preovulatory period exert a positive feedback action, culminating in the release of preovulatory GnRH and gonadotropin surges.

## VII. PREOVULATORY GnRH SURGES

Preovulatory GnRH surges have been identified in rats, sheep, and monkeys and indirect measurements suggest that they may occur in women. In rats, where the necessity of this neurosecretory trigger in stimulating LH surges is unambiguous, a proestrous surge of GnRH in this species appears to be composed of a 2 to 4 h increase in the overall amount of GnRH released. Detailed analyses of GnRH release patterns during surges suggest that GnRH pulse amplitude, as well as a basal GnRH secretory component, may be acutely increased as a major portion of this surge release. An example of an estrogen-induced

GnRH surge, and its associated LH surge in the peripheral plasma, is depicted in Fig. 2. Mechanisms by which estrogen may exert these positive feedback actions include increased GnRH gene expression, activation of synaptic inputs to pulsing GnRH neurons, alterations in neurotransmitter receptor synthesis, and/or alterations in the expression of postreceptor signaling molecules. In some female mammals, notably many rodents, the release of GnRH surges is timed to occur during a restricted period on the afternoon preceding estrus. This mechanism has likely evolved to ensure that ovulation occurs in temporal association with the onset of behavioral estrus and that both processes occur during the nocturnal period of wakefulness for these species. Classical work showed that a daily neuronal signal, conveyed from the biological clock resident in the suprachiasmatic nucleus to GnRH surge-producing circuitries, directs the release of GnRH surges during the specified period on the afternoon of proestrus. Estrogen's actions appear to be important in coupling the daily neuronal signal to the GnRH surge release process. This coupling action is most evident in animals treated with prolonged, high levels of estrogen, in which daily afternoon GnRH and LH surges occur on successive days of treatment. Recent work suggests that estrogen couples the daily neural signal to the GnRH surge release mechanism via induction of transcription factors in estrogen-responsive neurons, which may thereafter be activated by neural signals for the surge. One such estrogen-induced transcriptional regulator may be the intracellular progesterone receptor. Evidence supporting this idea includes the observations that estrogen treatments fail to produce surges in progesterone receptor knockout mice.

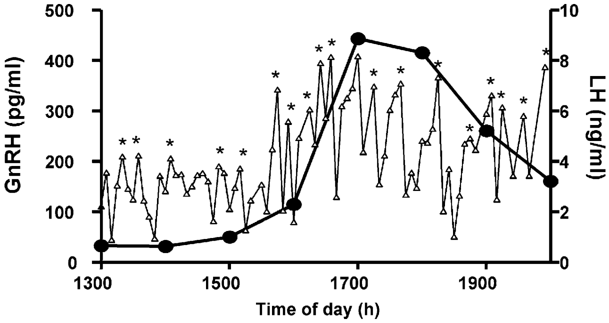

**FIGURE 2** Estrogen-induced GnRH and LH surge in an individual ovariectomized rat. A microdialysis procedure was used to monitor GnRH release in the median eminence of a rat that was acutely ovariectomized on the afternoon of diestrus and treated at the same time with 30 μg estradiol benzoate. Experiments were performed on the following day of presumptive proestrus. Radioimmunoassay of GnRH in microdialysates revealed an afternoon surge of GnRH that was accompanied by a surge of LH in the peripheral plasma. Open triangles indicate GnRH levels. Filled circles indicate LH levels. Asterisks represent significant pulses as determined by statistical analysis. Data are from Chappell *et al.* (2000). *Endocrinology* **141**, 1477–1485.

The cellular targets of estrogen's positive feedback actions on GnRH release have been extensively studied, and much remains to be clarified about this important signaling process. Three possible mechanisms of action have received the most attention in neuroendocrine investigation. One is a direct action of the steroid on GnRH neurons, whereby estrogen may directly alter gene transcription or cellular excitation. Early immunohistochemical analyses cast doubt on this scenario, as few if any GnRH neurons were found to express steroid receptors. More recent studies have resurrected this idea, however, as a small but significant portion of GnRH neurons have been found to express both the estrogen receptor-β (ER-β) mRNA and the ER-β protein. A second hypothesis holds that estrogen induces alterations in the glial ensheathment of GnRH neuronal projections and thereby affects changes in the rate and/or pattern of GnRH neurosecretion from neurovascular terminals in the lateral median eminence. Correlative immunohistochemical and ultrastructural microscopy studies have provided evidence supporting this idea; however, a causal link has yet to be established.

A third model for estrogen's positive feedback actions invokes the possibility that estrogen-receptive, non-GnRH interneurons mediate the positive feedback actions of estrogen on GnRH neurosecretion. Most investigators agree that the most important neural locus for the transduction of estrogen's positive feedback actions is a relatively discrete, sexually dimorphic region within the preoptic area, referred to as the anteroventral periventricular nucleus (AVPv). Although this area is replete with estrogen receptors, it contains a relatively small number of GnRH neurons. Lesions of this nucleus render female animals acyclic and unresponsive to estrogen's positive feedback actions. Thus, one prevailing model for the positive feedback actions of estrogen holds that non-GnRH, ER-expressing neurons in the AVPv transduce estrogen positive feedback signals and that these neurons thereafter convey first- or multi-order afferent signals to GnRH neurons for the initiation of GnRH surge release. Estrogen's actions in these transducer interneurons may include the induction of PRs, which may in turn be activated by neural signals emanating from the biological clock (via ligand-independent mechanisms) as well as by progesterone (via ligand-dependent mechanisms) secreted during the onset of the LH surge. The neurotransmitter phenotypes of AVPv neurons that may participate in this positive feedback circuitry include calcitonin gene-related peptide, dopamine, dynorphin, enkephalin, galanin, $\gamma$-aminobutyric acid (GABA), neurotensin, and substance P. Other estrogen-responsive cells situated outside of this area, such as noradrenergic or neuropeptide Y-producing neurons in the mediobasal hypothalamus or brainstem A2 cell group, may convey estrogen positive feedback signals via axo-axonic contacts within the median eminence.

## VIII. HOMEOSTATIC INHIBITION OF GnRH RELEASE

In the absence of the gonads, GnRH pulse generation in adult animals can occur at frequencies that approach one pulse per 10 min. In all normal physiological situations, however, the frequency and amplitude of GnRH pulses are continuously subject to feedback regulation by gonadal hormones. In male animals, the major feedback mechanism is mediated by testicular androgens. The GnRH pulses evoke LH pulses, which stimulate episodic testosterone secretion. Testosterone and its metabolites, estrogen and dihydrotestosterone, exert negative feedback influences at the hypothalamic level through the restraint of GnRH pulse generator activity. Inhibition is also maintained at the pituitary level through suppression of gonadotropin responses to GnRH pulses. In females, the negative feedback actions of estrogen and progesterone (as well as inhibin and other gonadal peptides) are also of primary importance in restraining GnRH pulse generation and in suppressing the responsiveness of the pituitary gland. Although the relative influence of the two ovarian steroids may vary among species and in different circumstances, it is likely that the appropriate homeostatic control over basal GnRH pulsatility requires the actions of both steroid hormones. There is some evidence to suggest that GnRH estrogen's negative feedback actions are principally manifest as a suppression of GnRH pulse amplitude and progesterone may primarily exert its effects on GnRH pulse frequency.

The sites and mechanisms of estrogen and progesterone negative feedback actions remain less well characterized than those mediating positive feedback. Some evidence suggests that the inhibitory effects of estrogen are exerted at neural loci that are at least partially dissociable from those that mediate positive feedback effects. That there are some conditions, e.g., in progesterone receptor deletion mutant mice, in which positive feedback is absent but negative feedback actions prevail suggests that the cellular mechanisms that mediate the two actions are distinct and separable. The involvement of multiple mechanisms in either or both feedback modes is also a consideration. The actions of estrogen, for example, may be transduced by both intracellular, ER-mediated genomic actions and nongenomic membrane effects of the steroid. The nongenomic effects of estrogen have been indicated by the observations that nanomolar concentrations of 17$\beta$-estradiol rapidly and directly hyperpolarized GnRH neurons in the guinea pig. The inhibitory actions of estrogen and progesterone may also be exerted through multiple intracellular routes; although some steroid effects may be exerted through direct actions on the GnRH neuron, there are other suggestions in the literature that negative feedback actions may be exerted indirectly, through the stimulation of inhibitory transmitter turnover or inhibition of stimulatory transmitter release. The strongest cases to be made in this regard are for GABAergic and endorphinergic neurons, which both express steroid receptors and release transmitters known to inhibit GnRH release.

## IX. ENVIRONMENTAL AND SOMATIC SIGNALS REGULATING GnRH NEUROSECRETION

Neurosecretion of GnRH is also regulated by numerous physiological signals from nongonadal sources. These include sensory stimuli, information retrieved from memory, and circulating hormonal and metabolic factors. Perhaps the best studied of these regulatory inputs are photoperiodic stimuli. In seasonally breeding animals, day length is registered through photic pathways that ultimately regulate the pattern of pineal melatonin secretions. Changes in the duration of pineal melatonin secretion, in turn, activate or depress GnRH pulse generation and thereby sustain or depress activity in the reproductive axis. In hamsters, long days are photostimulatory for the reproductive axis, and these responses are presumably mediated by activation and inhibition of the GnRH pulse generator, respectively. In sheep, exposure to short days leads to a stimulation of GnRH pulsatility, whereas long days are photoinhibitory; this mechanism dictates the birth of young in the spring, when the chances of survival are maximal.

There are numerous other examples of reproductively relevant sensory cues that prompt adaptive alterations in GnRH pulse amplitude or frequency. GnRH pulses are acutely stimulated in ewes following visual exposure to rams, coitus induces an ovulatory GnRH surge in rabbits through activation of the sensory pathways leading from the cervix to the hypothalamus, and pheromonal cues from male mice are critically important for the release of GnRH surges in female mice. In all of these circumstances, reflex pathways convey information regarding the presence of a sexual partner, and the GnRH pulse generator is activated to prepare the gonads for fertilization.

Chronic and acute stress can also alter the pattern of pulsatility, often in an inhibitory manner so as to favor the expenditure of metabolic energy on adaptive stress responses rather than on reproduction. Metabolic and hormonal cues, such as glucose, leptin, insulin, IGFs, thyroid hormones, or glucocorticoids, can also be important in mediating the adaptive physiological responses of the GnRH pulse generator. Food deprivation, for example, is a state that is not compatible with reproductive success; it is not surprising, then, that it is accompanied by suppression of the GnRH pulse generator in many species. Again, this regulatory mechanism may spare energy stores from being depleted during attempts to reproduce under adverse environmental conditions. In disease states, GnRH pulsatility may also be suppressed. Immune stress and inflammation responses may be accompanied by suppression of the GnRH pulse generator, possibly through the actions of cytokines that are known to be produced under these conditions.

## X. SUMMARY

The GnRH neuronal system functions as the final common pathway through which the brain regulates the activity of the reproductive axis. The physiological importance of the decapeptide can be easily appreciated by observing the drastic reproductive consequences of impaired GnRH secretion or action: near total reduction of LH secretion and reduced FSH secretion, greatly diminished steroidogenesis, anovulation in females, the absence of normal reproductive development, and a state of infertility in both sexes that prevails until GnRH stimulation of the pituitary gland is restored. The release of GnRH occurs in a pulsatile manner, providing an intermittent signal to pituitary gonadotropes that is necessary to sustain continued responsiveness to the decapeptide. The cellular mechanisms that function to produce this pulsatile GnRH release pattern remain ill-defined, but are proving amenable to study via homogenous tumor-derived cell lines and new transgenic animal models. Advanced genetic and molecular approaches are also opening up new pathways toward an understanding of the cellular and molecular pathways that mediate neural and endocrine regulatory inputs to the GnRH pulse generator, which remain a major focus of attention in the field of neuroendocrinology.

## Glossary

**GnRH pulse generator** The cellular and molecular mechanisms that produce and sustain pulsatile gonadotropin-releasing hormone neurosecretion; these mechanisms are incompletely understood.

**gonadotropin-releasing hormone** A decapeptide that is synthesized in neurons of the basal forebrain, released into the hypophyseal portal vasculature, and conveyed to the anterior pituitary gland, where it stimulates the synthesis and secretion of the gonadotropins luteinizing hormone and follicle-stimulating hormone; it is also referred to as luteinizing hormone-releasing hormone.

**hypothalamic–hypophyseal portal vessels** The specialized vasculature that forms a plexus in the median eminence of the hypothalamus and collects into larger vessels that extend into the hypophysis (pituitary gland); a major function of this vascular network is to convey substances that undergo neurosecretion from neurovascular junctions in the median eminence to the anterior pituitary gland, where these factors, such as gonadotropin-releasing hormone, can regulate hormone secretions.

**median eminence** The neural tissue at the base of the medial hypothalamus that contains neurovascular junctions, from which releasing factors, such as gonadotropin-releasing hormone, are secreted into the hypothalamic–hypophyseal portal vessels.

**pulsatile neurosecretion** Rhythmic releases of neurohormone pulses from neurovascular junctions in the median eminence into the hypothalamic–hypophyseal portal vessels.

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# Gonadotropin-Releasing Hormone and Puberty

Sergio R. Ojeda, Vincent Prevot, Sabine Heger, and Barbara Dziedzic

*Oregon National Primate Research Center*

I. INTRODUCTION
II. THE TRANSSYNAPTIC CONTROL OF GnRH NEURONS
III. THE GLIAL CONTROL OF GnRH NEURONS
IV. THE NEURONAL CONTROL OF NEUROENDOCRINE GLIAL FUNCTION
V. PRESUMPTIVE UPSTREAM CONTROLLING GENES
VI. SUMMARY AND CONCLUSIONS

**Gonadotropin-releasing hormone (GnRH), when it is released from the hypothalamus, acts on specialized hormone-producing cells in the pituitary, stimulating the secretion and synthesis of the luteinizing hormone and follicle-stimulating hormone. Once released into the bloodstream these hormones stimulate the gonads to secrete peptidergic and steroid hormones, which promote the growth of secondary sex organs and the development of traits of sexual dimorphism. The activation of GnRH secretion at puberty is not determined by a single mode of transsynaptic communication but instead requires the activation of both transsynaptic and astroglial regulatory systems.**

## I. INTRODUCTION

Puberty is initiated by events that take place within the central nervous system and that are set in motion even in the absence of the gonads. These events ultimately result in an increase in the release of gonadotropin-releasing hormone (GnRH) from a handful of specialized neurons located in a ventral region of the brain known as the hypothalamus. These neurons send their secretory axons to a region of the hypothalamus termed the median eminence, where they release GnRH into blood vessels that link the hypothalamus to the pituitary gland. Upon reaching the pituitary gland, GnRH acts on specialized hormone-producing cells known as gonadotrophs to stimulate the secretion and synthesis of gonadotropins. These hormones, known as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are released into the bloodstream and reach the gonads, where they stimulate the secretion of sex steroids and peptidergic hormones. In turn, steroid hormones promote the growth of the secondary sex organs and elicit the appearance of sexual dimorphism (such as fat distribution, muscle mass, breast development, and tone of voice).

It is now clear that the initial signals leading to the pubertal activation of GnRH secretion do not reside within the GnRH neuronal network itself but instead occur in neuronal and astroglial systems functionally connected to GnRH neurons. Some authors have postulated that the primary change in neuronal activity underlying the initiation of puberty is a loss of transsynaptic inhibitory inputs to GnRH neurons. According to this concept, a "central restraint" of puberty—imposed by neurons that utilize the amino acid $\gamma$-amino butyric acid (GABA) for neurotransmission—would operate at full capacity during juvenile development, preventing GnRH secretion from increasing prematurely. Other authors postulate that the pubertal activation of GnRH secretion is instead caused by an increase in excitatory inputs and that the main excitatory neurotransmitter system involved in this activation is composed of neurons that utilize the amino acid glutamate for transsynaptic communication. In addition to this dual neuronal occur in the locations along this pathway at which the majority of GnRH neurons stop their migration; in the rat, sheep, and mouse (Fig. 1), the great majority of GnRH neurons are found in the medial septum and rostral hypothalamus, whereas, in the primate, about half of the GnRH neurons end up in the mediobasal hypothalamus at the caudal extreme of the migratory pathway. The consequences of these neurons of being resident in one of several different areas of the medial septum and hypothalamus are not yet established, despite the observation that GnRH neurons located throughout the "GnRH neuron continuum" extend axons to the median eminence and are presumably all involved in the regulation of gonadotropin secretion.

In addition to the median eminence, a very substantial projection of GnRH fibers passes to the organum vasculosum of the lamina terminalis (Fig. 1), a brain region outside the blood–brain barrier. The role of this projection is completely unknown. Smaller numbers of GnRH fibers are encountered in other forebrain and brainstem regions where they may conceivably have roles in the coordination of gonadotropin hormone secretion with other facets of reproductive physiology. Whether individual GnRH neurons project to multiple locations within the brain has not yet been established. It is also unclear whether GnRH neurons located in different parts of the GnRH neuron continuum project to extrahypothalamic regions in a selective manner.

In many species, the cell body of the GnRH neuron exhibits a predominantly bipolar morphology with an orientation that reflects its embryonic migratory pathway. In other species, such as the sheep, the orientation and scattered distribution are maintained but the GnRH neurons display a variety of different morphologies. The significance of these different morphologies is unknown. Similarly, the functional relevance of bipolar GnRH neurons that exhibit either smooth or spiny contours, when assessed following GnRH immunocytochemistry, has not yet been established. Although GnRH perikarya are, for the most part, scattered along the GnRH neuron continuum, not all of the cells are completely isolated from one another, and clusters of adjacent GnRH neurons can be observed. A very small number of GnRH neurons are known to form

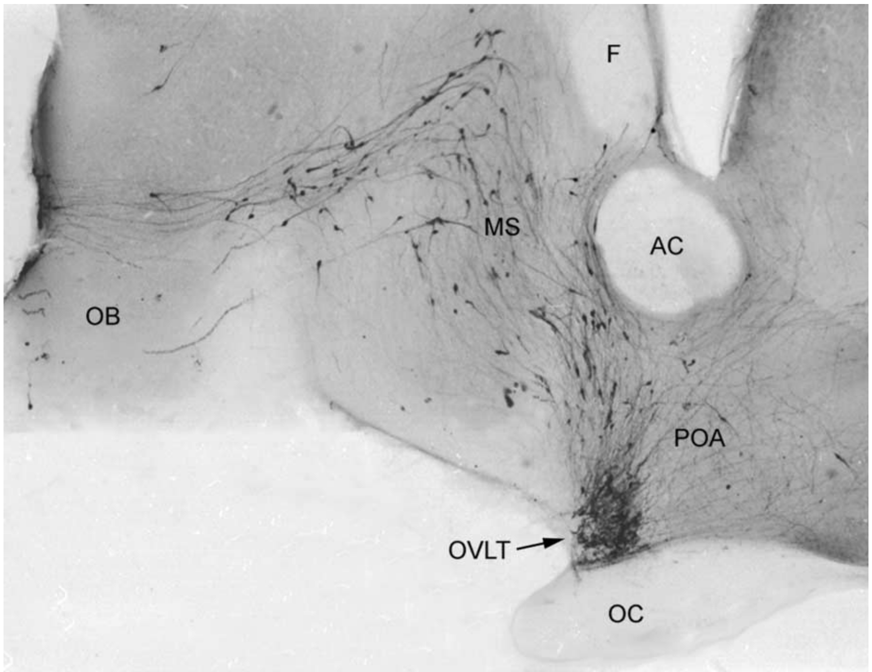

**FIGURE 1** Low-power photomicrograph of a midline parasagittal section of the adult mouse brain immunostained for GnRH (front of the brain to the left). Note that the cell bodies of the GnRH neurons are found scattered along their embryonic route of migration from the olfactory bulbs (OB) through the medial septum (MS) into the preoptic area (POA) and hypothalamus (right). The median eminence, where most GnRH neurons extend axons, is not shown and would be to the far right. The other major projection site for the GnRH neurons is the organum vasculosum of the lamina terminalis (OVLT). AC, anterior commissure; F, fornix; OC, optic chiasm.

cytoplasmic bridges with one another. Nevertheless, the overriding morphological and topographical features of the GnRH neuron are its relatively simple shape and its scattered distribution over a wide area within the basal forebrain.

## II. ELECTROPHYSIOLOGICAL PROPERTIES

The GnRH neurons secrete GnRH in a pulsatile manner to regulate gonadal function. As such, two of the most important features of the GnRH neuron that require explanation are (1) how they generate an episodic pattern of activity and (2) considering their dispersed topography, how this activity is synchronized among the GnRH neuronal population. Providing the answers to these questions has proven to be extremely difficult, and multiple different experimental strategies have been employed.

Because the GnRH neurons are not clustered together in a single brain region, it is currently impossible to identify them *in vivo* and undertake single-cell recordings. However, by targeting multiunit electrodes to the mediobasal hypothalamus, in the vicinity of the GnRH axons passing to the median eminence, a good correlation has been found between electrical activity in this region and pulsatile luteinizing hormone (LH) secretion *in vivo*. Although this has proven useful, the phenomenon is observed clearly only in ovariectomized animals, where GnRH pulsatility is at its most frequent. Furthermore, the identity and nature of this multiunit activity have not been defined and it is unclear whether this activity represents electrical activity in the GnRH neurons. Another strategy for examining electrical properties of the GnRH neuron has been that of making recordings from immortalized GnRH-secreting cell lines, such as the GT1 cells. Although these studies have demonstrated that GT1 cells have autonomous pulsatile activity and identified the presence of multiple neuron-specific channels and signaling pathways, the properties of an immortalized cell line *in vitro* are very unlikely to represent those of the native GnRH neuron within the brain. The use of organotypic explant cultures derived from the embryonic nose has provided a better model for the investigation of native GnRH neurons, and studies here have also suggested that GnRH neurons may have an inherent episodic nature. However, once again, it may be dangerous to interpret these results in terms of the GnRH neuron *in vivo*.

The most useful strategy for determining the electrical properties of the native GnRH neurons in the postnatal brain has come about through the acute brain slice approach. In this procedure, the electrical activity of native GnRH neurons can be recorded in their normal environment although the cells have usually had their axons, and possibly distal dendrites, cut off. The identification of the GnRH neuron in the brain slice preparation has remained troublesome due to their scattered topography but progress has been made through the "blind" recording of cells with a bipolar morphology and then determining whether they are GnRH neurons (or not) by postidentification GnRH immunocytochemistry or single-cell reverse transcriptase-polymerase chain reaction (RT-PCR). A further refinement to this technique has been to create transgenic rodents in which the GnRH promoter is used to direct the expression of molecules that can be visualized, such as green fluorescent protein, specifically to the GnRH neurons. This then enables GnRH neurons to be preidentified by examination of the brain slice under a fluorescence microscope. Although some uncertainties exist with this approach, it remains the most rapid method available for collecting electrophysiological data on GnRH neurons *in situ*.

Studies using the brain slice approach have now shown that, despite their unusual origins, the GnRH neurons exhibit conductances and general membrane properties similar to those of most other neurons in the brain. These include the identification of a variety of potassium ($I_A$, $I_{IR}$, and $I_K$) and calcium (high- and low-voltage-activated) channels as well as tetrodotoxin-sensitive sodium channels in spontaneously firing GnRH neurons. A little disappointingly, no clear electrophysiological "fingerprint" for the GnRH neuron has arisen as yet. However, one clear feature of the GnRH neurons that has emerged is the marked degree of heterogeneity present in their electrical behavior. Firing rates and patterns vary considerably and range from complete silence, through intermittent bursting, to continuous steady firing. Furthermore, the presence of different calcium and potassium channels is also variable within the GnRH neuronal population and, apparently, irrespective of their morphology or location within the GnRH neuron continuum. The same phenomenon of marked heterogeneity has been found in the expression of "activity-dependent" immediate-early genes by the GnRH neuronal population. Although some of the phenomena observed to date may be explained by technical considerations, it is apparent that the GnRH neurons do not exhibit nearly as homogenous an electrophysiological profile as other neuroendocrine populations involved in pulsatile pituitary hormone release, such as the magnocellular oxytocin or vasopressin neurons.

In terms of understanding the key issue of GnRH pulsatility and its origins, much has yet to be learned. Work involving the GT1 cells and organotypic embryonic GnRH neurons suggests that individual GnRH neurons may exhibit inherent pulsatility on an approximately 30 min periodicity. The electrical recordings made from GnRH neurons *in situ* have not yet confirmed or refuted this hypothesis. Although there is evidence that some GnRH neurons can exhibit bursting behavior, this is very variable in duration and timing, and its relationship to the pulsatile release of GnRH *in vivo* is unclear. Intriguingly, it appears that the electrical activity of the GnRH neuron is not altered markedly when evaluated before and after puberty. Clearly, this does not correlate with the marked increase in gonadotropin release that occurs at puberty in mammals and, interestingly, suggests that the absolute firing rate of individual GnRH neurons may not be strongly related to the release of GnRH from nerve terminals in the median eminence. Whether this results from a technical artifact, such as the cutting of critical inputs to GnRH neurons in the slice preparation, or provides us with a unique insight into the mechanisms underlying pulsatile GnRH secretion is unknown at this point. Although comprehensive data are currently lacking from GnRH neurons *in situ* within the mature brain, current data favor the likelihood that GnRH neurons do possess inherent pulsatile activity but that this needs to be entrained by external influences upon the cell.

In much the same way as the difficulty in obtaining good electrophysiological data on native GnRH neurons has prevented any definitive knowledge about GnRH neuron pulsatility, the nature of GnRH neuron synchronization remains obscure. It has not yet been established whether a pulse of LH arises from the activation of all GnRH neurons projecting to the median eminence or just a subpopulation or, even then, whether the same subpopulation of GnRH neurons is activated for each pulse. Nevertheless, it is reasonable to assume that a degree of synchronization must exist among some GnRH neurons. The potential mechanisms underlying synchronization are numerous. However, its seems unlikely that this occurs through direct electrical communication at the level of the cell body. Not only are nearly all GnRH cell bodies remote from one another, but no evidence of functional dye-coupling has been found in mouse GnRH neurons to support the presence of gap junctions in native GnRH neurons. Instead, the scattered distribution of these cells favors the existence of neurochemical intermediates that coordinate the GnRH neurons. This may exist in the form of diffusible molecules, such as nitric oxide, or more classically, as synaptic-released neurotransmitters from neurons within the GnRH neuronal network. The potential for GnRH neurons to communicate with one another through collateral innervation using GnRH itself as a neurotransmitter has long been suggested, but definitive proof for a role in pulsatility has not been forthcoming. Thus, although the need for synchronization among the GnRH neurons is likely, the underlying mechanisms remain unknown.

## III. SYNAPTIC AND HORMONAL INPUTS

The ability to regulate the pulsatile release of GnRH through the ovarian cycle and in response to factors such as stress and nutrition is critical for reproductive homeostasis. As such, it is important that the electrical activity of the GnRH neurons can be altered by the neural network responsible for regulating fertility. This may occur at the level of the GnRH cell bodies or at their nerve terminals located in the median eminence.

Currently, there is little evidence for the presence of synapses on GnRH nerve terminals. However, there is substantial *in vitro* evidence showing that a wide range of neurotransmitters and neuropeptides can influence GnRH secretion from the median eminence. This suggests that the nerve terminals are a site of regulation but that this may occur through nonsynaptic mechanisms. For example, "volume transmission" may exist whereby neurochemicals are released from nearby nerve terminals and diffuse a short distance through the extracellular matrix to influence the GnRH terminals. Although this would not provide specific targeting of any signal to the GnRH nerve terminals, the expression of specific receptors by GnRH nerve terminals may provide a degree of selectivity. The molecules capable of influencing GnRH secretion in this way may also arise from nonneuronal sources, such as glial and endothelial cells in the vicinity of the GnRH nerve terminals.

In contrast to the GnRH nerve terminal, there is good evidence for the presence of synapses on GnRH perikarya. However, a common observation has been that there are relatively few synapses on GnRH cell bodies and proximal dendrites compared with neighboring non-GnRH neurons. Although the innervation density of distal dendrites has not yet been examined, these data have suggested that the direct regulation of GnRH neurons at the level of the cell body may be undertaken by a relatively small number of afferent neurons. Importantly, the perikarya of GnRH neurons in the female rat and sheep have been shown to have twice as many synapses as GnRH neurons in males. Thus, sexually dimorphic afferent inputs to the GnRH neuron may underlie some of the marked sex differences that exist in the regulation of GnRH secretion in mammals.

An important goal has been to establish the identity of these primary afferents to the GnRH cell body, and electron microscopic experiments have identified the presence of a wide variety of different neurotransmitters and neuropeptides within nerve terminals synapsing on GnRH perikarya (Fig. 2). In association with *in vivo* data showing that these compounds alter LH secretion when infused into the brain, these findings have resulted in a perplexing situation whereby each of a large number of different neurotransmitters has been postulated to provide a small but direct input regulating the activity of the GnRH neuron. Furthermore, dual-labeling studies have provided ample evidence for the heterogeneous expression of various neurotransmitter receptors by GnRH neurons, once again adding the issue of heterogeneity to the problem of understanding the neural regulation of these cells.

Electrophysiological experiments in the mouse have now begun to provide a functional hierarchy for these multiple putative inputs to the GnRH cell body. Recent studies have shown that the great majority of postsynaptic currents in GnRH neurons originate from the actions of GABA or glutamate and that 100% of GnRH neurons respond to these two amino acids. The effects of GABA are mediated principally by the $\text{GABA}_A$ receptor, although $\text{GABA}_B$- may also have a role in other species, whereas both *N*-methyl-D-aspartate (NMDA) and AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) receptors are responsible for the excitatory effects of glutamate (Fig. 2). Experiments using single-cell RT-PCR have further been able to profile the $\text{GABA}_A$ receptor subunit messenger RNAs expressed by GnRH neurons in the mouse. These studies clearly indicate that, like most forebrain neurons, the amino acid neurotransmitters GABA and glutamate are the most important in terms of the ongoing regulation of GnRH neuron activity. The effects of the various putative neuropeptide regulators of GnRH neurons have yet to be examined in detail although GnRH neurons in the guinea pig have been

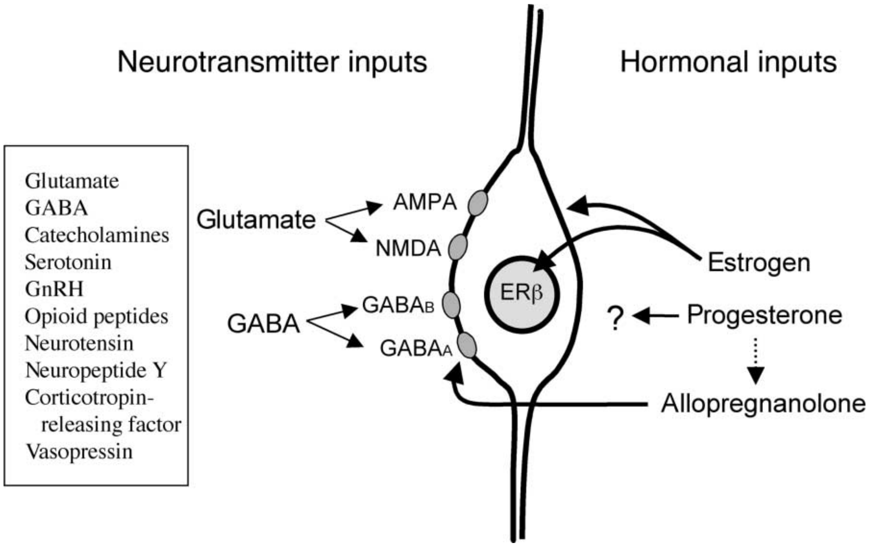

**FIGURE 2** Schematic diagram depicting established synaptic and hormonal inputs that impinge directly upon mammalian GnRH neurons. Neurotransmitters that have been identified in terminals synapsing on GnRH neurons by electron microscopy are indicated in the box (left). The amino acids glutamate and GABA have been shown by electrophysiology to mediate most ongoing postsynaptic activity in mouse GnRH neurons through AMPA and NMDA and through $\text{GABA}_A$ as well as $\text{GABA}_B$ receptors, respectively. Estrogen may exert both direct membrane and classical transcriptional influences (through estrogen receptor β) on GnRH neurons. Progesterone's ability to influence GnRH neurons directly is not clear but, following its rapid conversion to allopregnanolone in the brain, can enhance the inhibitory action of GABA through the $\text{GABA}_A$ receptor.

shown to express functional μ opiate receptors. Some of the neuropeptides may be found to subserve distinct functional roles in the regulation of GnRH neuron activity and secretion.

Whereas the synchronization and regulation of GnRH neurons are very likely to be dependent upon other neurons within the GnRH network, there is a possibility that one important influence upon the GnRH neurons occurs in a direct manner. Gonadal steroids act as important "feedback molecules" that relay information on the status of the ovaries and testes to the GnRH neuron. In the female, estrogen and progesterone can exert stimulatory (positive feedback) or inhibitory (negative feedback) actions upon GnRH biosynthesis and GnRH secretion. The initial site of positive feedback appears to occur remotely from the GnRH neurons in several species and must therefore require intermediary cells to transmit the information to the GnRH neurons, but some of the inhibitory effects may occur directly. Recent studies have demonstrated that GnRH neurons express estrogen receptor β (ER-β), thus providing a direct route through which estrogen can regulate gene expression in these cells (Fig. 2). The genes potentially regulated by estrogen in GnRH neurons are not firmly established but might conceivably include those regulating neurotransmitter and ion channel function.

Increasing evidence indicates that gonadal steroids may also exert rapid nonclassical, direct actions upon GnRH neurons. Studies in the guinea pig have shown that estrogen can exert rapid inhibitory effects on the membrane of GnRH neurons that involve the modulation of potassium channels. Although no evidence has yet been found for a direct effect of progesterone itself on GnRH neurons, electrophysiological studies in the mouse have shown that allopregnanolone, which is rapidly synthesized from progesterone in the brain, acts to allosterically enhance the activity of $GABA_A$ receptors expressed by GnRH neurons (Fig. 2). Thus, both estrogen and progesterone may contribute to negative feedback in a direct manner by direct actions at the GnRH cell membrane. The mechanisms through which androgens may influence GnRH neurons are not known. These studies indicate that the important regulatory influence of gonadal steroids on the GnRH neurons may occur through direct membrane actions or ERβ-mediated transcriptional events, as well as following the gonadal steroid modulation of glial or other neuronal elements within the GnRH network.

## IV. SUMMARY

The GnRH neurons are a critical element of the neural network controlling fertility. As a consequence of their unique embryonic origin within the olfactory placode, these neurons undertake an extraordinary migration through the nose and into the brain during embryogenesis. The resultant scattered distribution of the GnRH cell bodies within the septum and hypothalamus has greatly hindered the investigation of this phenotype but new approaches, particularly the use of GnRH promoter transgenics, are providing rewarding avenues for elucidating their properties. One recurring phenomenon encountered when investigating these cells is the marked degree of heterogeneity that exists at essentially all levels within the GnRH neuronal phenotype. The functional significance of this heterogeneity remains to be established. Although key issues such as the nature of pulsatility and synchronization within the GnRH population *in vivo* also remain unresolved, the amino acid neurotransmitters glutamate and GABA have now been identified as central molecules regulating the excitability of GnRH neurons. In addition, the important regulatory influence of gonadal steroids on the GnRH neuron appears likely to occur in part through both direct membrane and classical genomic mechanisms.

## Glossary

**gonadotropin-releasing hormone** A decapeptide released by a specific population of hypothalamic neurons to regulate the secretory activity of pituitary gonadotrophs. Also known as luteinizing hormone-releasing hormone.

**neural network** A group of interconnected neurons and associated glial cells that perform a specific brain function.

**organotypic culture** An *in vitro* experimental tissue preparation in which the organization of the (brain) cells resembles their *in vivo* organization.

**reverse transcriptase-polymerase chain reaction** A method through which RNA is transcribed into complementary DNA and then the amounts of a specific cDNA are amplified in an exponential manner to enable detection. This approach can be used to determine the mRNAs expressed by single neurons.

## See Also the Following Articles

**Gonadotropin-Releasing Hormone (GnRH)**
- **Gonadotropin-Releasing Hormone and Puberty**
- **Gonadotropin-Releasing Hormone Ontogeny**
- **Gonadotropin-Releasing Hormone Pharmacology**

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# Gonadotropin-Releasing Hormone Ontogeny

Oline K. Rønnekleiv[*] and
Robert B. Norgren, Jr.[†]

[*]*Oregon Health and Sciences University* • [†]*University of Nebraska Medical Center*

I. INTRODUCTION
II. DEVELOPMENT OF TERMINAL, VOMERONASAL, AND OLFACTORY NERVES AND GnRH NEURONS
III. DIFFERENTIATION OF GnRH NEURONS
IV. GnRH NEURONAL MIGRATION
V. FACTORS CONTROLLING GnRH NEURONAL MIGRATION
VI. SUMMARY

**Gonadotropin-releasing hormone (GnRH) neuronal cell bodies are diffusely located in the brain, primarily in the preoptic region and basal hypothalamus. Axons from this network of neurons project to the median eminence and secrete GnRH into the portal vessels of the anterior pituitary gland. GnRH acts on the pituitary gonadotropes to control the secretion of luteinizing hormone and follicle-stimulating hormone. GnRH neurons are unique in their development and also undergo critical functional changes related to two important events in human development: puberty and menopause. This article, however, will focus on early events in the development of GnRH neurons, specifically their differentiation and migration.**

## I. INTRODUCTION

Early in gestation, gonadotropin-releasing hormone (GnRH) neurons originate outside the brain in the nasal (olfactory) placode and migrate along olfactory, vomeronasal, or terminal nerves into the telencephalon. Once in the telencephalon, GnRH neurons migrate to their final destination, primarily the hypothalamus. The cellular substrates and molecular cues that guide GnRH neuronal migration have been extensively investigated because of specific interest in the development of this critical component of the reproductive system. In addition, this is an ideal model for the general study of neuronal migration due to easy identification of migrating GnRH neurons in some species of birds and mammals.

## II. DEVELOPMENT OF TERMINAL, VOMERONASAL, AND OLFACTORY NERVES AND GnRH NEURONS

The nasal placode (nasal epithelium) gives rise to the olfactory, vomeronasal, and terminal nerves. The olfactory sensory neurons located in the olfactory epithelium (OE) project to the olfactory bulb. This projection mediates the sensation of smell. Sensory neurons in the vomeronasal organ (VNO), located at the base of the nasal septum (Fig. 1), project to the accessory olfactory bulb in most vertebrates, with the exception of birds. The VNO is a chemoreceptive structure that is thought to mediate pheromone-stimulated reproductive behavior in mammals. In the human, this structure develops from the medial nasal mucosa at approximately gestational days 50–60, but almost completely degenerates by approximately day 80 of gestation (Fig. 1). It has long been known that neurons migrate out of the nasal placode and vomeronasal organ early in development and form neuronal clusters that connect the VNO to the brain. These clusters of neurons and their fiber projections are termed the terminal nerve. The cell

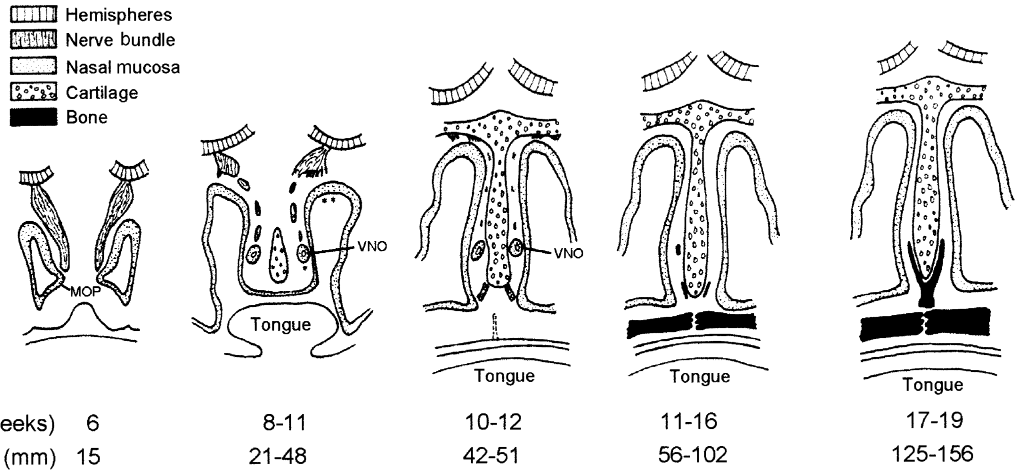

FIGURE 1 Stages of development of the human nasal cavity, evaluated on frontal sections of the nose from fetal ages 6–19 weeks. MOP, medial olfactory pit; VNO, vomeronasal organ; CR, crown rump. Adapted from Kjaer, I., and Fisher Hansen, B. (1996). The human vomeronasal organ: Prenatal developmental stages and distribution of luteinizing hormone-releasing hormone. *Eur. J. Oral Sci.* **104**, 34–40, with permission from Blackwell Publishing.

bodies of the terminal nerve, which are found at various locations along the VNO projection pathway, are referred to as the ganglia of the terminal nerve. The terminal nerve, which is distinct from the vomeronasal nerve, sends projections to a number of forebrain regions including the septum and preoptic area (POA).

The presence of GnRH-containing neurons within the peripheral and central parts of the terminal nerve was first described in mammals in the early 1980s. This led to the idea that the terminal nerve might be an additional source of GnRH besides the POA-basal hypothalamus (BH) population of neurons. However, in the late 1980s, based on studies of GnRH distribution in amphibians, it was proposed that GnRH neurons are derived embryologically from the terminal nerve, which originates in the nasal placode and VNO during development. Shortly thereafter, experimental evidence was obtained showing that GnRH neurons originating in the nasal (olfactory) placode migrate along vomeronasal and terminal nerves to reach the brain. This phenomenon has now been described in teleost fish, amphibians, birds, rodents, monkeys, and humans.

## III. DIFFERENTIATION OF GnRH NEURONS

The exact location of the source of GnRH neurons is controversial. Most investigators have suggested that these cells originate in the medial olfactory epithelium, which is where they are first observed during development. However, evidence has been put forth that they may arise in the respiratory epithelium or in the border zone between the olfactory epithelium and the respiratory epithelium. The olfactory and the respiratory epithelia are both derived from the nasal placodes, which are bilateral ectodermal thickenings on the ventrolateral sides of the head. In contrast to the respiratory epithelium, the composition of the developing OE has been studied extensively and a wealth of transcription factors that may be involved in the induction and differentiation of the various cell types are expressed in the OE during early development. Some of these factors, such as Olf-1, GATA-4, and nestin, are also transiently expressed in GnRH neurons. However, their specific roles in GnRH neuronal development are unknown. Experiments with null mutant mice suggest that the transcription factor activator protein 2 alpha, which is normally expressed by the respiratory epithelium, appears to prevent GnRH differentiation in this tissue. The origin of other cell types, such as those containing γ-aminobutyric acid (GABA), somatostatin, neuropeptide Y, tyrosine hydroxylase, and galanin, that migrate within the vomeronasal or terminal nerves remains to be determined.

## IV. GnRH NEURONAL MIGRATION

GnRH in some species, such as the rhesus monkey, the mouse, and the chicken, is expressed at the earliest stages of migration, i.e., as neurons leave the olfactory epithelium or VNO. Therefore, in these species, it is possible to describe the migration pathway using immunohistochemistry or *in situ* hybridization. Thus, it has been established that GnRH neurons migrate from the medial olfactory epithelium in close association with vomeronasal (mammals) or olfactory (chicken) nerves to the rostral telencephalon, where the future accessory olfactory and olfactory bulbs will form. During migration in the nasal cavity, GnRH cells form "tracks" that extend from olfactory areas to the forebrain (Fig. 2). As the fetus develops, the number of GnRH cells within the nasal area greatly decreases and the number within the brain increases. The period for development and migration is specific for each species that has been studied.

Thus, during development in mice starting at approximately embryonic day 11.5, GnRH cells migrate from the olfactory epithelium into the brain and then migrate caudally to reach their final destination by embryonic day 16.5 of a 20-day gestation period. In rhesus monkey, GnRH immunoreactive cells are detected within the medial olfactory epithelium and in nerve bundles of the nasal septum on days 32–36 of a 165-day gestation. During this period, GnRH cells are not found in the brain, but GnRH fibers extend from the nasal area into the brain and bilaterally along the ventral surface of the brain from the olfactory region rostrally to the basal hypothalamus caudally (Fig. 2). By day 38 of gestation, GnRH cells are also localized in the olfactory region of the brain. With increasing fetal age, there is a gradual caudal extension of GnRH cells, and the cells appear in the basal hypothalamus at approximately 47 days of gestation with an adult-like distribution by days 55–60 (Fig. 2). More limited information about GnRH neuronal migration is known in human, in which GnRH cells are present in the medial olfactory epithelium, nasal septum, and rostral forebrain at approximately day 42 of gestation.

GnRH cells migrate through the nasal septum and into the forebrain via the vomero-nasal and terminal nerves in mammals or via the medial part of the olfactory nerve in birds. After entering the brain, GnRH neurons appear to reach the septal–preoptic area and basal hypothalamus by routes that vary depending on the species. In mice the route appears to be associated with peripherin-containing axonal fiber pathways. In primates, a few "pioneer" GnRH neurons appear to lead the remainder "follower" GnRH neurons as they migrate out of the placode. Thus, GnRH neurons track along neurites of other GnRH neurons in what is termed neurophilic migration. The majority of GnRH neurons appear to follow the path laid by GnRH fibers through a

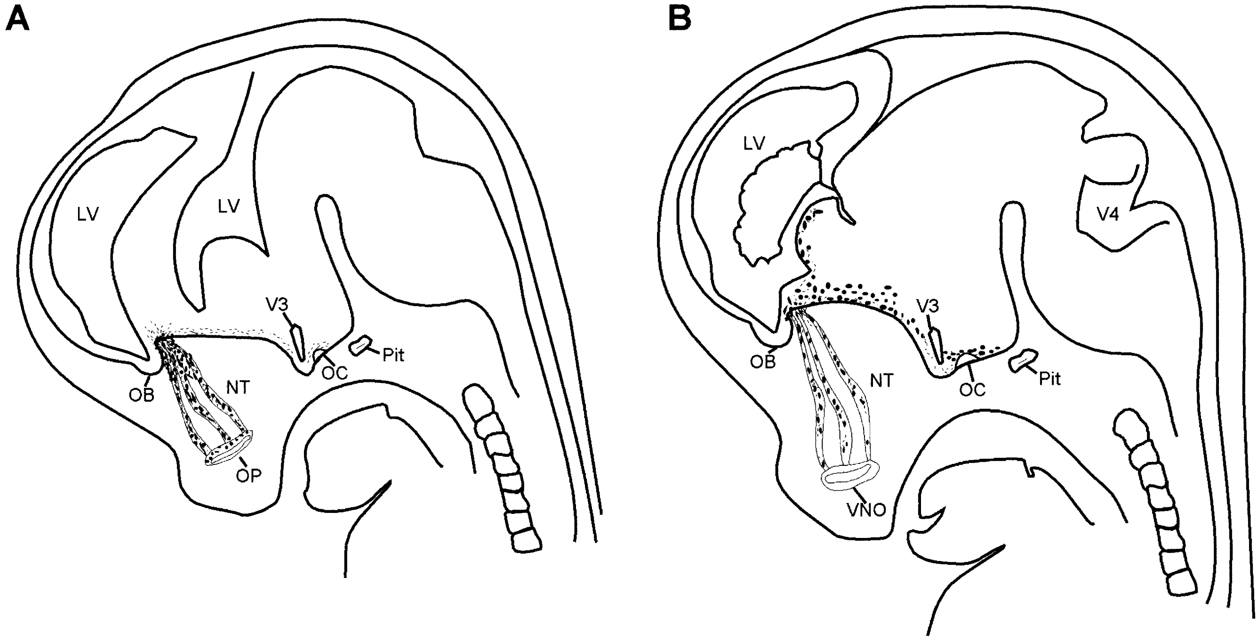

**FIGURE 2** Schematic drawings of the primate head at approximately 36 (A) and 50 (B) days of gestation, illustrating GnRH neuronal migration pathways. The large dots represent GnRH neurons, and the thin, stippled lines represent GnRH fibers. LV, lateral ventricle; NT, terminal nerve; OB, olfactory bulb; OC, optic chiasm; OP, olfactory placode; Pit, pituitary; V3, third ventricle; V4, fourth ventricle; VNO, vomeronasal organ.

ventral GnRH fiber path to the POA and basal hypothalamus. However, a portion of the migrating cells travel dorsally accompanied by GnRH fibers in the septal area, along periventricular regions of the lateral ventricles and interventricular foramen to hypothalamic regions. The guiding mechanism(s) of GnRH neuronal migration along a ventral and dorsal path is currently unknown.

There is widespread agreement that most neuroendocrine GnRH neurons originate in structures derived from the nasal placode. However, there is substantial evidence that there are other populations of GnRH neurons that do not appear to be derived from the nasal placode. These nonolfactory GnRH neurons are found in the striatum, thalamus, lateral septum, bed nucleus of the stria terminalis, and midbrain. Most likely, these neurons originate in the subventricular zone of the forebrain. They are substantial in number (at least 10,000 in primates), but their functions are unknown.

## V. FACTORS CONTROLLING GnRH NEURONAL MIGRATION

Several lines of evidence indicate that vomeronasal and terminal nerve axons are critical in guiding the GnRH neurons to the brain. First, in Kallmann's syndrome patients, who are anosmic and hypogonadal, olfactory, vomeronasal, and terminal nerves extend toward the brain but do not enter the telencephalon. GnRH neurons are produced and migrate within the nasal area but do not enter the brain. Instead, they remain outside the brain in the nasal cavity and within the dural layers of the meninges on the dorsal surface of the cribriform plate. Second, experimental studies in chicken embryos have demonstrated that GnRH neurons follow misdirected olfactory nerve axons to ectopic locations, including the pons, a region in which GnRH neurons are normally never found.

The dependence of GnRH neurons on nasal area nerves to gain entrance to the brain raises two questions: First, what are the molecular cues that guide olfactory, vomeronasal, and terminal nerve axons into the brain? And second, what is the molecular mechanism by which GnRH neurons maintain their close association with these nerve axons? The search for important guidance and adhesion molecules has led to many descriptive studies demonstrating that a variety of molecules are expressed along the GnRH neuronal migration pathway. Experimental evidence that these molecules are necessary for GnRH neuronal migration is much more limited.

The cloning of the KAL1 gene, which is mutated in Kallmann's syndrome, provides one obvious candidate for olfactory and vomeronasal nerve ingrowth to the brain. The KAL1 protein is predicted to be a secreted extracellular matrix protein with several fibronectin type III repeats, which occur in many neural cell adhesion molecules. Interestingly, studies in chick and humans are not in agreement regarding the expression profile of KAL1 mRNA in the developing olfactory bulb. However, in both species, KAL1 has been found not to be expressed in the nasal cavity along the path of migrating GnRH neurons. Detailed studies in chicken have demonstrated that olfactory neurons, GnRH neurons, and the mesenchymal tissue through which these neurons migrate do not express the KAL1 gene. Studies of human fetal brains, although more limited, similarly provide evidence that KAL1 is not expressed along the olfactory and GnRH migratory paths. In both species, the KAL1 gene is expressed in the olfactory bulb, which has led to the hypothesis that the genetic defect underlying Kallmann's syndrome is a disruption of the movement of nerve fibers from the nose to the brain. The failure to discover a mouse KAL1 homologue has prevented the production of null mutant experimental animals thus far. These problems have slowed the progress of developing models to explore the mechanism by which KAL1 facilitates GnRH migration.

A number of other molecules have been suggested to mediate GnRH neuronal migration in association with vomeronasal nerve axons. Neural cell adhesion molecule (NCAM) is strongly expressed in the olfactory and vomeronasal nerves during development; it is possibly expressed in migrating GnRH neurons, although this is controversial. Since NCAM is a well-studied homophilic adhesion molecule, it has been suggested that it may mediate GnRH neuronal migration. However, NCAM null mutant mice do not exhibit any deficits in either olfactory nerve ingrowth or GnRH neuronal migration. This suggests that NCAM is not necessary for GnRH neuronal migration, perhaps due to functional redundancy in that its role may be taken over by other cellular components. Also, a number of other factors may in certain circumstances be induced to direct migration of these neurons that are so critical for maintaining species survival.

The neurotransmitter GABA has been found to influence GnRH neuronal migration both *in vitro* and *in vivo* and multiple effects of GABA agonists and antagonists have been demonstrated. Within the nasal compartment, a subset of GnRH neurons express GABA, and treatment with $\text{GABA}_\text{A}$ agonists inhibits GnRH migration out of this area. Therefore, it is reasonable to suggest that GABA is involved in regulating the rate of GnRH neuronal migration from the nasal area to the brain. Interestingly, it has been shown in a number of species that migrating GnRH neurons normally "rest" for 1–3 days at the CNS border before entering the brain. The specific molecular mechanism, including a role for GABA in this delaying process, needs to be elucidated. The most striking result of GABA modulation is the disorganization of migrating GnRH neurons in the brain after the $\text{GABA}_\text{A}$ receptor is blocked with bicuculline. After such treatment, the GnRH neurons and the peripherin-containing fibers along which GnRH neurons are believed to migrate become disassociated. Thus, there is good evidence that GABA may be involved in GnRH migration; however, it is likely that additional molecules remain to be identified.

## VI. SUMMARY

Since the discovery in the late 1980s that GnRH neurons migrate into the brain in association with olfactory and vomeronasal nerves, significant progress has been made in understanding the development of these neurons. In normal intact animals, GnRH neurons are first observed in the olfactory placode. Therefore, the assumption has been that GnRH progenitors originate in the olfactory epithelium. However, various experimental manipulations have provided evidence that GnRH neurons may develop in the border zone between the respiratory epithelia and the chemosensory epithelia. Future studies are needed to solve this issue. GnRH neurons are dependent on vomeronasal or terminal nerves to reach the brain, in a process that may involve both NCAM and GABA, and most likely a number of currently unknown factors are also involved. The KAL1 protein is necessary for olfactory nerve and GnRH neuronal entry to the brain by an unknown mechanism. Once in the brain, GnRH neurons are no longer attached to large nerve bundles; they disperse and find their species-specific location within septal and hypothalamic areas. Not much is known about the mechanism of GnRH migration within the brain, although the expression of GABA receptors in GnRH neurons may be important for the accuracy of migration.

## Glossary

**activating enhancer binding protein 2 $\alpha$** A transcription factor that is important in embryogenesis and is expressed in the developing respiratory epithelium.

**fibronectin type III repeats** Repeat sequences found in the Kallmann gene; they were first detected in fibronectin, an extracellular matrix adhesion molecule, and also occur in many neural cell adhesion molecules. The function of these repeats is not well understood, but their presence seems to be important for axonal outgrowth.

**GATA-4** A transcription factor that is expressed in cells, including developing GnRH cells, in the olfactory epithelium.

**Kallmann's syndrome** A genetic disorder associated with an X chromosome deletion at Xp22.3, resulting in anosmia and hypogonadism due to failure of olfactory nerve ingrowth and GnRH neuronal migration during embryonic development

**nasal (olfactory) placode** Thickening of nasal ectoderm bilaterally; the precursor of the olfactory and vomeronasal epithelia.

**nestin** An intermediate neurofilament found in proliferating neural precursor cells. Nestin mRNA and protein are temporarily expressed in developing GnRH neurons.

**neural cell adhesion molecule** A large cell surface glycoprotein along which neurons may migrate.

**neurophilic neuronal migration** Movement of neurons that occurs via the preferential adherence of the neurons to the surface of apposing axons.

**Olf-1** A transcription factor that is expressed in cells, including developing GnRH cells, in the olfactory epithelium.

**terminal nerve** A ganglionated cranial nerve that is in part embedded within the olfactory nerves in the nasal area, enters the brain caudal to the olfactory bulbs, and has projections to the septal and preoptic areas of the brain.

**vomeronasal organ** Chemosensory organ that sends projections to the accessory olfactory bulbs and transmits pheromone-related stimuli to the brain.

## See Also the Following Articles

**Gonadotropin-Releasing Hormone (GnRH)**
**• Gonadotropin-Releasing Hormone and Puberty**
**• Gonadotropin-Releasing Hormone Neuron**
**• Gonadotropin-Releasing Hormone Pharmacology: Agonists and Antagonists • Sexual Differentiation, Molecular and Hormone Dependent Events in**

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# Gonadotropin-Releasing Hormone Pharmacology: Agonists and Antagonists

Jean Rivier* and P. Michael Conn†
*The Salk Institute, La Jolla • †Oregon National Primate Research Center

I. INTRODUCTION
II. PEPTIDE AGONISTS
III. PEPTIDE ANTAGONISTS
IV. NONPEPTIDE ANTAGONISTS

**The roles of endogenous peptides acting as regulatory molecules present potential strategies for diagnosis and pharmacologic intervention in a broad range of physiologic and pathophysiologic processes. Antagonists to these endogenous peptides are recognized as potential therapeutic agents in an ever-increasing number of disease states. In most cases, however, it is difficult to realize the therapeutic potential of a naturally occurring peptide because of short duration of action, multiple pharmacologic activities due to multiple sites of action, or low bioavailability within the bounds of convenient routes of administration. Peptide analogues are therefore designed to fulfill the needs that are either not satisfied or only partially satisfied by the parent compounds. Analogues with higher affinity for their receptor and higher resistance to biodegradation may be more potent and longer acting than the native peptides. This article examines the design of peptide gonadotropin-releasing hormone agonists and antagonists and nonpeptide antagonists, as well as their mechanisms of action and clinical significance.**

## I. INTRODUCTION

The structure of gonadotropin-releasing hormone (GnRH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$) was first disclosed at the Endocrine Society meeting on June 24, 1971, by Andrew V. Schally, preceded by the submission of three reports that were published a few weeks after this meeting. In the first report, Schally and co-workers described the isolation and properties of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH)-releasing hormone; in the second, the proposed structure of the porcine LH- and FSH-releasing hormone was given, and in the third, they confirmed the structure of porcine GnRH by conventional sequential analysis. This pioneering work in peptide isolation, sequencing, and synthesis was recognized in 1977 when Schally shared the Nobel Prize in Medicine with Roger Guillemin and Rosalyn Yalow. The structure of mammalian GnRH was confirmed by the total synthesis, isolation, and characterization of ovine GnRH. At present, 14 different members of the GnRH family have been fully characterized (Table 1). Except for 2 of these structures (guinea pig with a tyrosine at position 2 and lamprey I with a tyrosine at position 3), they all share six amino acids (four at the N-terminus including the posttranslationally modified pyroglutamic acid derived from glutamine and two at the C-terminus including the posttranslationally modified primary amide derived from a glycine residue followed by two basic residues in the precursor).

Competitive antagonists of several regulatory peptides (GnRH, vasopressin, parathyroid hormone, corticotropin-releasing factor, vasoactive intestinal peptide, and bombesin, among others) have been developed and shown to be useful for the elucidation of their mode(s) of action and ultimately for therapeutic applications. Development of orally active peptides is a high priority of pharmacologists, and preliminary results would indicate that both resistance to enzymatic degradation and small molecular size may play an important role for diffusion into the bloodstream after oral administration, and solubility in the physiological milieu may be important for controlled release after formulation in cases of parenteral administration.

This article reviews the design of peptide GnRH agonists and antagonists, nonpeptide antagonists, what is known of their mechanism of action, and some highlights of their clinical significance. Early structure–activity relationship (SAR) studies for both agonists and antagonists were described by Karten and Rivier in 1986. Patent and scientific literature of nonpeptide antagonists was reviewed recently.

## II. PEPTIDE AGONISTS

### A. Structure–Activity Relationships

The original premise for the design of GnRH agonists was both academic and pragmatic. Academically, it was important to understand the structural basis of GnRH agonists and this could be determined through SAR studies and physicochemical measurements such as NMR. Pragmatically, it was hypothesized that the LH-releasing and ovulation-inducing effects of GnRH observed in laboratory animals could be applied to the treatment of male and female infertility. However, the GnRH molecule is very flexible in solution and crystals could not be obtained, therefore jeopardizing efforts to generate structure-based models of the bioactive conformation of GnRH. Additionally, the half-life of GnRH is very short, and more potent and long-acting analogues were thought to be necessary for practical clinical utility.

**TABLE 1 Primary Sequence of Native GnRHs from Different Species**

| | Position[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Mammal (m) | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro | Gly-NH$_2$ |
| Guinea pig (gp) | — | Tyr | — | — | — | — | Val | — | — | — |
| Chicken I (C-I) | — | — | — | — | — | — | — | Gln | — | — |
| Rana (ra) | — | — | — | — | — | — | Tyr | — | — | — |
| Sea bream (sb) | — | — | — | — | — | — | — | Ser | — | — |
| Pejerrey (pj) | — | — | — | — | Phe | — | — | Ser | — | — |
| Herring (hr) | — | — | — | — | His | — | — | Ser | — | — |
| Catfish (cf) | — | — | — | — | His | — | — | — | — | — |
| Salmon (s) | — | — | — | — | — | — | Trp | Leu | — | — |
| Dogfish (df) | — | — | — | — | His | — | Trp | Leu | — | — |
| Chicken II (C-II) | — | — | — | — | His | — | Trp | Tyr | — | — |
| Lamprey III (l-III) | — | — | — | — | His | Asp | Trp | Lys | — | — |
| Lamprey I (l-I) | — | — | Tyr | — | Leu | Glu | Trp | Lys | — | — |
| Lamprey I (t-I) | — | — | — | — | Asp | Tyr | Phe | Lys | — | — |
| Tunicate II (t-II) | — | — | — | — | Leu | Cys | His | Ala | — | — |

[a] Only amino acids that are different from the sequence of mammalian GnRH are indicated.

Two major discoveries, the deletion of the C-terminal glycine amide and its substitution by an ethylamide and the introduction of a D residue at position 6, are the basis for the generation of long-acting, very potent agonists, also called superagonists. Ironically, these superagonists have antireproductive effects.

An abbreviated list of agonists used clinically in the past 25 years is shown in Table 2. Although the *in vitro* potency of these analogues varied considerably, their *in vivo* efficacy is comparable.

## B. Mode of Action

The mechanism of action of these agonists can be divided into two types. First, short-acting compounds, given in pulses or on a single occasion, can be used to stimulate the receptor or test functioning of the axis. Long-acting agents (given to maintain elevated levels of the peptide) result in a refractory status due to loss of receptor and loss of receptor–effector coupling. The result is diminished release of gonadotropins and, therefore, steroids. This is a valuable therapeutic approach to steroid-dependent cancers. This latter type is dependent on metabolically stable analogues useful to maintain serum levels.

## C. Indications

GnRH and long-acting GnRH agonists are currently being used for the management of the human disorders male and female infertility, precocious puberty, endometriosis, prostate cancer, uterine bleeding and leiomyoma, cryptorchidism, and others, and they are also used as protection during chemotherapy.

# III. PEPTIDE ANTAGONISTS

## A. Structure–Activity Relationships

The original premise for the design of GnRH antagonists was both academic and pragmatic. Academically, it was important to be able to block LH and FSH release in order to assess the physiological role of GnRH. Pragmatically, it was hypothesized that the LH-releasing and ovulation-inducing effects of GnRH observed in laboratory animals could be blocked with a competitive antagonist, thus opening new contraceptive avenues.

The design for potent GnRH peptide antagonists with adequate solubility, high affinity for the GnRH receptor, low potency in releasing histamine, and long duration of action was achieved following what is herein referred to as a “conventional approach” to the design of GnRH antagonists. Incremental increases in potency and duration of action were obtained mostly by intuition and trial and error. Several observations are now recognized as having played a critical role in the design of those antagonists that reached the clinic (see Table 3). Among these, substitution of Arg by ILys yielded analogues with considerably reduced histamine-releasing activity and substitution of the E-substituted lysines (L and D, respectively) at positions 5 and 6 by amino phenylalanines resulted in analogues with extended duration of action. Finally, optimization of the substituents on the ω-amino function of $Aph^5$ and $Aph^6$ yielded FE200486, currently under development.

With regard to the pharmacological properties of the analogues described in Table 3, ganirelix, abarelix, azaline B, cetrorelix, and FE200486 have very similar binding affinities and relative potencies. Additionally, some of them are more potent than others with regard to their ability to release histamine. They are listed in Table 3 in order of decreasing potency. For the safest analogues, the highest dose administered does not cause release of histamine to the full extent available in the assay.

What is most striking is that the duration of action after subcutaneous injection in mannitol (Table 3) is unpredictable on the basis of the primary structure although very reproducible from one assay to the next. The reason for that behavior of the peptides

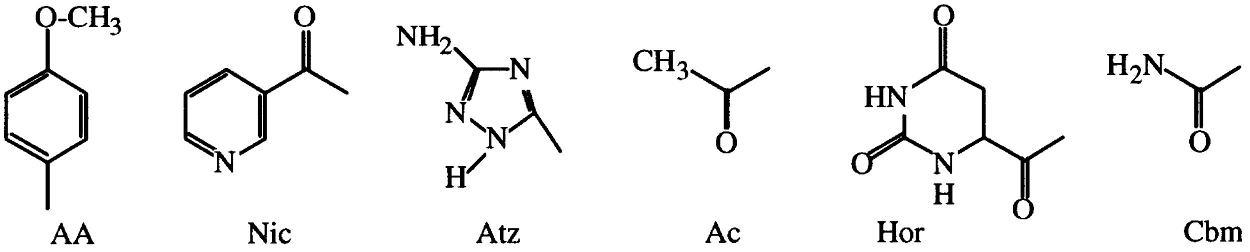
TABLE 2 GnRH Agonists in the Clinic

| Compound | Accepted designation | Company/sponsor |
|---|---|---|
| [DLeu$^6$,Pro$^9$-NHEt]GnRH | Leuprorelin (Leuprolide) | TAP Pharmaceuticals |
| [DSer(tBu)$^6$,Aza-Gly$^{10}$]GnRH | ICI 118630 | ICI Pharmaceuticals |
| [DSer(tBu)$^6$,Pro$^9$-NHEt]GnRH | Buserelin | Hoechst (Aventis) |
| [DHis(Bzl)$^6$,Pro$^9$-NHEt]GnRH | Histrelin | Ortho |
| [DTrp$^6$]GnRH | Tryptorelin | Debiopharm |
| [DTrp$^6$,Pro$^9$-NHEt]GnRH | Deslorelin | Salk Institute |
| [DTrp$^6$,N-MeLeu$^7$,Pro$^9$-NHEt]GnRH | Lutrelin | Wyeth |
| [D2Nal$^6$]GnRH | Nafarelin | Syntax Research |

**TABLE 3 Selected Structures of GnRH Antagonists in the Clinic**

| Structure | Name | Duration |
|---|---|---|
| Ac-$\Delta^3$Pro-DFpa-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-$NH_2$ | 4F antagonist | Short |
| Ac-DNal-DFpa-DTrp-Ser-Tyr-DArg-Leu-Arg-Pro-Gly-$NH_2$ | "Nal-Arg" antagonist | Short |
| Ac-DNal-DCpa-DPal-Ser-Arg-DGlu(AA)-Leu-Arg-Pro-DAla-$NH_2$ | "Nal-Glu" antagonist | Short |
| Ac-DNal-DCpa-DPal-Ser-Tyr-DCit-Leu-Arg-Pro-DAla-$NH_2$ | Cetrorelix | Long |
| Ac-DNal-DCpa-DPal-Ser-Tyr-DHArg($Et_2$)-Leu-HArg($Et_2$)-Pro-DAla-$NH_2$ | Ganirelix | Intermediate |
| Ac-DNal-DCpa-DPal-Ser-Tyr-DHci-Leu-ILys-Pro-DAla-$NH_2$ | Antarelix/Teverelix | |
| Ac-DNal-DCpa-DPal-Ser-*N*-MeTyr-DAsn-Leu-ILys-Pro-Dala-$NH_2$ | Abarelix | Very short |
| Ac-DNal-DCpa-DPal-Ser-Lys(Nic)-DLys(Nic)-Leu-ILys-Pro-DAla-$NH_2$ | Antide | |
| Ac-DNal-DCpa-DPal-Ser-Aph(Atz)-DAph(Atz)-Leu-ILys-Pro-Dala-$NH_2$ | Azaline B | Long |
| Ac-DNal-DCpa-DPal-Ser-Aph(Ac)-DAph(Ac)-Leu-ILys-Pro-DAla-$NH_2$ | Acycline | Long |
| Ac-DNal-DCpa-DPal-Ser-Aph(LHor)-DAph(Cbm)-Leu-ILys-Pro-DAla-$NH_2$ | FE200486 | Very long |

*Note.* Chemical structures of substituents shown in parentheses:



Definition of duration of action in castrated male rat assay (50 μg injection of antagonist is administered sc in 100 μl of mannitol): Very long = over 80% inhibition of LH release at 96 h; Long = over 80% at 72 h but not at 96 h; Intermediate = over 80% at 48 h but not at 72 h; Short = over 80% at 24 h but not 48 h; Very short = no inhibition at 24 h.

seems to lie in their ability to form gels at the site of administration.

## B. Mode of Action

Antagonists function by blocking the binding site of the GnRH receptor, thus preventing the (endogenous) agonist from occupying the site. At present, no alteration in the membrane receptor or activation of its effector coupling has been ascribed to antagonists. Much of the challenge of producing useful antagonists has been in minimizing histamine-releasing activity and, at the level of formulation, to achieve long duration of action.

## C. Indications

Abarelix is often a useful depot formulation that can be administered on days 1, 15, 29, and 57 and every 28 days thereafter. Because it is the first GnRH antagonist used for a major indication in which chronic administration is a must, the commercial success of this antagonist compared to that of the established superagonist preparations will be important in vindicating those of us who saw in those molecules an opportunity to avoid the undesirable "flare" and to obtain a more profound and prompt inhibition of gonadotropin than is observed with superagonists. This was demonstrated exactly 10 years ago when Pavlou and his colleagues showed that the combined administration of the GnRH antagonist (Nal-Glu) and testosterone replacement in men results in reversible azoospermia without loss of libido, a condition that cannot be attained with the administration of any dose of an agonist. In fact, Broqua *et al.* presented data that emphasized the difference in the efficacy of agonists versus that of antagonists on the growth of the androgen-dependent prostate tumor Dunning R-3327H.

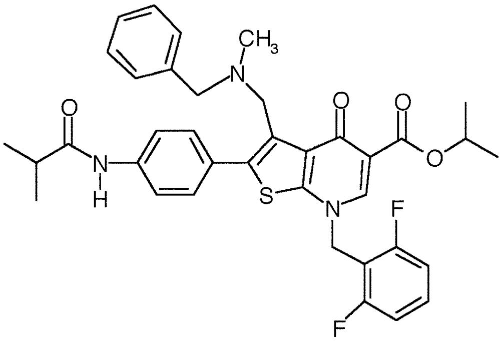


FIGURE 1 Structure of T98475.

The authors concluded that a rapid and complete suppression of plasma testosterone levels was necessary to stop the progression of the Dunning R-3327H rat prostatic tumor growth and therefore that the anti-tumoral activity of FE200486 makes it a promising candidate for prostate cancer treatment.

GnRH antagonists are currently being used for *in vitro* fertilization and are being considered for the same indications as those for which superagonists are promoted. It is believed that GnRH antagonists will replace the superagonists because of the absence of a stimulatory phase (i.e., immediate inhibition) and the observation that antagonists have a more profound effect on gonadotropin inhibition than the superagonists, an effect that is equal to castration in the male, suggesting additional uses such as contraception (suppression of ovulation and spermatogenesis).

## IV. NONPEPTIDE ANTAGONISTS

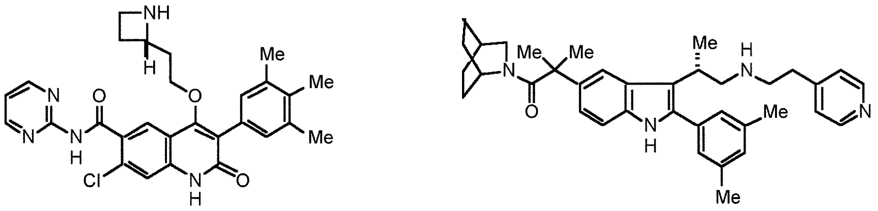
Development of orally active peptides is still in its infancy because of limitations due to gut adsorption, distribution, metabolism, and elimination (ADME).

With the availability of the cloned GnRH receptor, high-throughput screening programs for small nonpeptide molecules were initiated mainly by the pharmaceutical industry. Although there are several patents covering such molecules, only two groups have disclosed their most promising structures in the scientific literature. With an affinity of 0.2 nM on cloned human receptor-bearing cell lines, T98475 was described as having good oral bioavailability (see Fig. 1).

More recently, De Vita *et al.*, at Merck, initiated a program to develop nonpeptide GnRH antagonists by screening their compound collection for binding to the rat GnRH receptor. Leads were identified that ultimately produced the potent and selective GnRH antagonists represented by examples Q2 and In2 (Fig. 2).

The oral bioavailability (F) and pharmacokinetic properties of In2 were suitable for characterization of *in vivo* efficacy (rat $F = 8\%$, $t_{1/2} = 1.0$ h). In the castrated male rat, In2 reduced plasma LH when administered orally at doses of 5 and 10 mg/kg. The duration of effect in the 10 mg/kg experiment was approximately 12 h. This analogue was also reported to be active in the male rhesus macaque.

In short, inhibitory effects obtained *in vivo* with peptide GnRH antagonists are now also obtained with small nonpeptide molecules that bind to the GnRH receptor. Interestingly, these molecules are species specific in that they will bind with different affinities, for example, to rat, monkey, dog, or human GnRH receptors. This raises the question as to whether nonpeptide GnRH antagonists bind to the GnRH receptor at the same site(s) as do peptide antagonists. Additionally, this brings to light the fact that in order to carry out mandatory toxicology before administration to human, nonpeptide antagonists may have to have approximately the same affinity for both laboratory animal and human



**Q2**

$^{h}$GnRH binding $IC_{50} = 0.4$ nM

$^{h}$GnRH PI turnover $IC_{50} = 1.0$ nM

**In2**

$^{h}$GnRH binding $IC_{50} = 0.6$ nM

$^{h}$GnRH PI turnover $IC_{50} = 3.4$ nM

FIGURE 2 Merck nonpeptide GnRH antagonists. h, human.

receptors before the toxicology studies can be validated. This condition may limit the number of nonpeptide antagonist families that can be selected for development. However, nonpeptide antagonists are here to stay and, as with the GnRH peptide antagonists, further refinement of structures may be needed for improved ADME.

In conclusion, attractive physicochemical properties of new antagonists have been identified and new proprietary formulations of water-soluble ($>50$ mg/ml) and safe GnRH antagonists effective for about 1 month have been tested successfully in human. The question therefore is not whether a peptide antagonist will gain drug status but rather for which indication(s) it will be used. Also, nonpeptide GnRH antagonists may rapidly enter the market. It is unlikely that these will be used for the same indications as the current long-acting depot preparations that were developed to address prostate cancer (and the like); in comparison with peptide injectables, such compounds may be more versatile (allowing short- and long-term administration), better received (because of oral activity), and more economical as a result of improved ADME.

## Acknowledgments

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## Glossary

**agonist** A molecule that binds to a receptor and activates it.

**analogue** A molecule that is chemically similar to another.

**antagonist** A molecule that binds to a receptor and does not activate it but blocks agonists from binding.

**gonadotropin-releasing hormone** A hypothalamic peptide that is released into the portal system and stimulates the release of luteinizing hormone and follicle-stimulating hormone from the pituitary.

**indication** A disease state that is improved by a particular treatment.

**peptidomimetic** A nonpeptide structure designed to be recognized by a receptor and modeled by comparison to peptide structures.

## See Also the Following Articles

**Follicle-Stimulating Hormone (FSH) • Gonadotropin-Releasing Hormone (GnRH) • Gonadotropin-Releasing Hormone and Puberty • Gonadotropin-Releasing Hormone Neuron • Gonadotropin-Releasing Hormone Ontogeny • Luteinizing Hormone (LH)**

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different families of G-protein-coupled receptors were distinguished.

The amino acid sequences are similar among receptors within the same family, especially within the transmembrane domains. However, despite sharing a seven-transmembrane structure, the receptors in different families have no sequence similarity. The largest family by far is the Class A or rhodopsin-like receptor family, which contains several thousand members in mammals. Included in this family are receptors for light, calcium, odorants, small molecules (amino acids, neurotransmitters, peptides), and proteins (see Table 1). It should be noted that a given intercellular signaling chemical may interact with many types of receptors. For example, acetylcholine activates several rhodopsin-like G-protein-coupled receptors, originally identified pharmacologically as the muscarinic receptors. In addition, acetylcholine is the neurotransmitter that activates the nicotinic receptors, which are multisubunit receptor–channel complexes having no structural relationship to G-protein-coupled receptors.

Class B receptors include mainly receptors for peptides, such as secretin, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating peptide, glucagons, and corticotropin-releasing factor. Class C includes the G-protein-coupled receptors for the neurotransmitters glutamate and γ-aminobutyric acid (GABA). Several additional receptor families can be distinguished, including Class D receptors for pheromones, Class E receptors for adenosine 3′,5′-cyclic monophosphate (cAMP), and an additional class for the frizzled and smoothened receptors involved in the development of cellular polarity.

## III. GENERAL STRUCTURAL FEATURES

As shown in Fig. 1, all G-protein-coupled receptors have an extracellular amino-terminus, seven transmembrane domains that form the transmembrane core, three extracellular loops, three intracellular loops, and a carboxy-terminal segment. The membrane topology results in the segregation of secondary modifications to different receptor segments. For example, the amino-terminus can be modified by the attachment of polysaccharides (glycosylation), which may influence receptor stability. The carboxy-terminus segment may be modified by the attachment of palmitate to a cysteine residue (palmitoylation) that anchors this receptor domain to the membrane and results in a fourth cytoplasmic loop. Each of the seven-transmembrane domains is generally composed of 20–27 amino acids. On the other hand, amino-terminal segments (7–595 amino acids), loops

TABLE 1 G-Protein-Coupled Receptor Families

| Receptor family | Ligand type | Examples |
|---|---|---|
| Class A: Rhodopsin-like | Amine | Acetylcholine (muscarinic), adrenoceptors (α and β), dopamine, histamine, serotonin |
| | Peptide | Angiotensin, bombesin, bradykinin, chemokine, interleukin-8, endothelin, somatostatin, galanin-like |
| | Hormone protein | Follicle-stimulating hormone, luteinizing hormone/choriogonadotropic hormone, thyrotropin |
| | (Rhod)opsin | Rhodopsin |
| | Olfactory | Several types |
| | Prostanoid | Prostaglandin, prostacyclin, thromboxane |
| | Nucleotide-like | Adenosine, purinoceptors |
| | *Cannabis* | Cannabinoid receptors |
| | Platelet-activating factor | Platelet-activating factor receptor |
| | Gonadotropin-releasing hormone | Gonadotropin-releasing hormone receptor |
| Class B: Secretin-like | Calcitonin | Calcitonin receptor |
| | Corticotropin-releasing factor | Corticotropin-releasing factor receptor |
| | Glucagon | Glucagon receptor |
| | Parathyroid hormone | Parathyroid hormone receptor |
| Class C: Metabotropic glutamate | Glutamate | Metabotropic glutamate receptors |
| | γ-aminobutyric acid | $GABA_B$ receptors |
| Class D: Fungal pheromone | Fungal pheromone | Fungal pheromone STE2-like |
| Class E: cAMP receptors | cAMP | cAMP receptor in *Dictyostelium* |

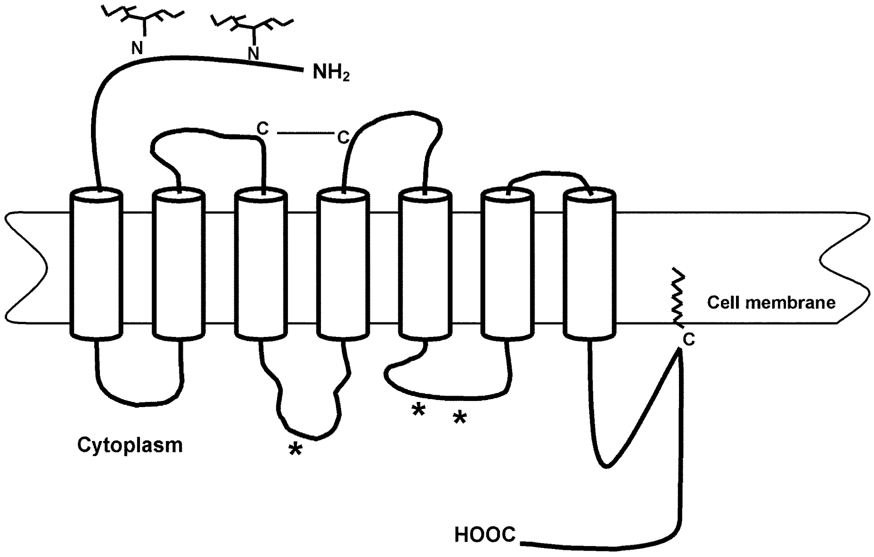

FIGURE 1 Two-dimensional model of a typical rhodopsin-like G-protein-coupled receptor showing some of the major structural features. The general structure of seven alpha-helical transmembrane domains is shown as cylinders connected by three extracellular and three intracellular loops. The amino-terminus usually contains asparagine (N)-linked glycosylation sites. Most receptors in this family have a cysteine (C) disulfide bridge between extracellular loops 2 and 3 and a conserved palmitoylation site in the carboxy-terminus, as shown. Potential regulatory phosphorylation sites are indicated by asterisks.

(5–230 amino acids), and carboxy-terminal segments (12–359 amino acids) vary considerably in size, an indication of their diverse structure and functions.

The seven transmembrane segments of rhodopsin are arranged as a closed loop in a clockwise direction from helix 1 to helix 7 when viewed from the intracellular surface. The orientation of the transmembrane segments imposes a stereo and geometric specificity on a ligand's entry into and binding to the transmembrane core. In this arrangement, the core is primarily composed of helices 1, 2, 3, 5, 6, and perhaps 7, whereas helices 1 and 4 are located peripherally (see Fig. 2).

The high-resolution three-dimensional structure of rhodopsin crystals provided the first detailed view of the three-dimensional structure of the transmembrane core. The structure of rhodopsin indicated that the transmembrane core does not have a vacant pocket, channel, or tunnel structure, as suggested earlier. Although the transmembrane core has hydrophilic, polar side chains, there are only a limited number (~10) of associated water molecules in the crystal structure. An extensive network of hydrogen bond interactions connects residues within the same transmembrane segments and between different segments.

Disulfide bonds play a major role in the structure and stability of G-protein-coupled receptors. Two conserved Cys residues in extracellular loops 1 and 2 are known to be linked by a disulfide bond. This bond appears to be required for proper assembly of most receptors, as its disruption interferes with either the expression or the function of various Class A receptors.

## A. Glycosylation

In common with most membrane proteins, G-protein-coupled receptors are usually modified by the attachment of oligosaccharides. Most Class A receptors contain at least one glycosylation site in the amino-terminal domain. However, some receptors, such as the $\alpha_{2B}$-adrenoceptor, do not have any sites for glycosylation. Oligosaccharides are linked to the side chain of asparagine residues (N-linked glycosylation) in a multistep process.

The contribution of glycosylation to receptor function differs for individual receptors. Glycosylation is important for the expression and stability of the gonadotropin-releasing hormone receptor and the vasopressin $V_{1A}$ receptor. Likewise, glycan chains are essential for correct folding and membrane insertion of the thyroid-stimulating hormone and follicle-stimulating hormone receptors. For some receptors, including receptors for somatostatin, $\beta_2$-adrenergic, thyroid-stimulating hormone, and

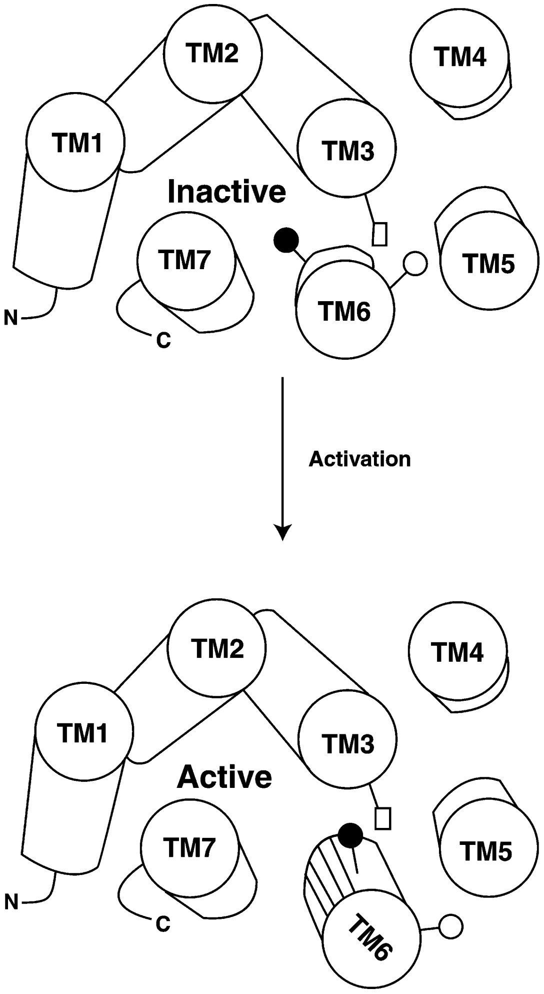

FIGURE 2 Three-dimensional schematic showing the arrangement of the transmembrane domains of an inactive and active Class A receptor as viewed from the inside of the cell. Selected side chains are shown to reveal the rotation and helix movements that occur during receptor activation.

gastrin-releasing peptide, glycosylation has been found to be important for proper receptor function, either ligand binding or receptor G-protein coupling. In other receptors, however, glycosylation has no known function. This latter group includes receptors for oxytocin, histamine $H_2$, muscarinic acetylcholine $M_2$, $NK_1$, bombesin $BB_1$, adenosine $A_{2A}$, and angiotensin. Thus, although N-linked glycosylation of receptors is an almost universal phenomenon, its effects on the properties of specific receptors may be variable.

For most G-protein-coupled receptors, glycosylation of at least one site contributes to normal expression or function.

## B. Palmitoylation

Covalent lipid modifications anchor numerous signaling proteins to the cytoplasmic face of the plasma membrane. These modifications mediate protein–membrane and protein–protein interactions and are often essential for receptor function. Protein fatty acylation occurs through either amide linkages (N-acylation) or thioester linkages (S-acylation). N-Acylation occurs on the amino-terminal glycine residue following removal of the initiator methionine by a methionyl-aminopeptidase. S-acylation occurs on cysteine residues through a thioester linkage in a wide variety of sequence contexts. Palmitate is the most commonly used S-linked fatty acid; thus, this posttranslational process is usually referred to as protein palmitoylation. However, other fatty acids can be incorporated into cellular proteins by a thioester linkage, including myristate, stearate, and arachidonate.

Palmitoylation is a posttranslational modification that is limited to a small subset of cellular proteins among which proteins involved in signal transduction are prevalent. This thioesterification of cysteine residues by palmitate distinguishes itself from other lipid modifications by its reversibility. The palmitoylation state of several proteins has been shown to be dynamically regulated. In particular, biological regulation of the palmitoylation state of the G-proteins and of their cognate receptors has been demonstrated.

Many G-protein-coupled receptors have been shown to be palmitoylated at cysteine residues in the intracellular carboxy-terminal tail, and these include the rhodopsin receptor, $\beta_2$- and $\alpha_2$-adrenoceptors, luteinizing hormone/chorionic gonadotropin receptors, endothelin $ET_A$ and $ET_B$ receptors, and the vasopressin $V_2$ receptor. The serotonin 5-$HT_{1A}$ and 5-$HT_{1B}$, dopamine $D_1$ and $D_2$, and metabotropic glutamate $mGlu_4$ receptors have also been reported to be palmitoylated.

Evidence suggests that palmitoylation serves to enhance the association of cytosolic proteins with the membrane. In this regard, palmitoylation of seven-transmembrane receptors anchor the carboxy-terminal tail to the plasma membrane, yielding a fourth intracellular loop. Biologically regulated changes in the palmitoylation state of either receptors or G-proteins may have important functional consequences and for some receptors, such as the endothelin receptor, have been shown to contribute to coupling to the mitogen-activated protein kinase cascade. The palmitoylation state of the receptor may also govern receptor internalization by regulating the accessibility of receptor to the arrestin-mediated internalization pathway.

### C. Phosphorylation

Regulatory processes collectively known as receptor desensitization usually attenuate the signal transduction pathways regulated by G-protein-coupled receptors. Thus, within milliseconds to minutes of agonist exposure, cells can diminish or virtually eliminate their agonist-evoked responses, via a process that involves phosphorylation of the receptors at one or more intracellular domains. After longer exposure to the agonist, receptor down-regulation also occurs, a process that includes receptor internalization (endocytosis) from the cell surface, protein degradation, and decreased receptor expression.

Two general types of phosphorylation-mediated receptor desensitization can be distinguished, homologous desensitization and heterologous desensitization. Homologous desensitization refers to the process in which the desensitization of the receptor results only from agonist-mediated activation of the same receptor. Heterologous desensitization refers to a process in which activation of one type of receptor can activate signal transduction leading to the desensitization of a different receptor.

The mechanisms underlying homologous and heterologous desensitization differ. Homologous desensitization is mediated by a unique class of serine/threonine protein kinases called G-protein-coupled receptor kinases (GRKs). Six different mammalian GRKs which differ in their tissue expression and receptor specificity, have been identified. Whereas GRKs usually phosphorylate only active-state, agonist-occupied receptors, second-messenger-activated kinases, such as protein kinase A or protein kinase C, may phosphorylate receptors that are not themselves being activated. Thus, heterologous desensitization may be caused by any cellular signaling that leads to activation of second-messenger-activated protein kinases that then phosphorylate sites on a particular receptor.

Phosphorylation of several receptors by GRKs leads to the receptor associating with a member of the arrestin family proteins. The association of arrestin with the receptor interferes with G-protein coupling and promotes internalization. Paradoxically, for some receptors, the arrestin interaction that attenuates G-protein coupling actually promotes the activation of mitogen-activated protein kinase pathways. Phosphorylation of receptors by signaling-activated protein kinases, such as protein kinase A, desensitizes the receptor because it causes a decreased efficiency in the activation of G-proteins.

## IV. BINDING SITE STRUCTURE

The various G-protein-coupled receptors manifest a remarkable diversity in the molecular mechanisms that they have developed for interacting with agonists. This evolutionary divergence on the shared seven-transmembrane template reflects the dual needs to maintain an efficient machinery for transmembrane signaling and to recognize specific activating signals as different as a photon of light and a 40 kDa protein.

### A. Photoreceptors: Rhodopsin

Photoreceptors have a retinal chromophore, which is attached through formation of a Schiff's base between the aldehyde moiety of retinal and the ϵ-amine of lysine in the seventh transmembrane helix of the receptor. The protonated Schiff base is paired with a conserved glutamate present at the boundary between the third transmembrane domain and the first extracellular loop. Light absorption causes isomerization of the retinal chromophore from 11-*cis* to all-*trans*, disrupting the salt bridge between lysine and glutamate and activating the receptor.

### B. Biogenic Amine Receptors

The biogenic amines acetylcholine, serotonin, adrenaline, noradrenaline, dopamine, and histamine activate their receptors by entering the transmembrane core. The binding site of these agonists lies approximately one-third of the way through the membrane. The amine nitrogen of these agonists forms an ionic interaction with an aspartate side chain in the third transmembrane helix of the receptor. This residue is a determinant for ligand binding but not for signal generation. The aromatic ring of most of these agonists interacts with a pocket in the fifth and sixth transmembrane domains to trigger receptor activation.

### C. Peptide Receptors

Peptide hormone receptors such as the formyl receptor, the thyrotropin-releasing hormone receptor, and the gonadotropin-releasing hormone receptor form a complex with the peptide through interactions involving both the extracellular domains and the transmembrane helix core.

### D. Protease-Activated Receptor

The ligands for these receptors are proteases, which activate their receptors by cleavage at specific amino-terminal domain sites. For example, the thrombin receptor has a specific site that is recognized by thrombin and cleaved. This results in the thrombin receptor having a new amino-terminus, which then functions as a tethered ligand that autoactivates the receptor.

### E. Glycoprotein Hormone Receptors

The glycoprotein hormones, which include luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, and thyroid-stimulating hormone, are the largest (30–40 kDa) and most complex G-protein-coupled receptor agonists. These hormones are heterodimers that consist of a common α-subunit and a hormone-specific β-subunit. Their receptors have a unique structure generated by fusing proteins from two gene families, the leucine-rich repeat glycoprotein family, which forms the amino-terminus, and the heptahelical Class A G-protein-coupled receptor family, which forms the transmembrane core. The high-affinity binding site of these receptors is contained within the large (350–400 residues) amino-terminal domain and the site of activation lies within the membrane-associated transmembrane core.

## V. ACTIVATION MECHANISM

When associated with an extracellular agonist, G-protein-coupled receptors undergo a conformational change that promotes the interaction and activation of the intracellular G-protein. The conserved amino acid side chains of the Class A receptors contribute to a network of interhelical interactions that subserve the shared requirement of these proteins to undergo conformational rearrangements during activation. Activation results from triggering a molecular switch within the receptor. An arginine located at the cytoplasmic side of the transmembrane segment 3 of all Class A G-protein-coupled receptors contributes to the conformational changes underlying receptor activation. Class A receptors activate their G-proteins via a rigid body movement and rotation of the helices, in which the transmembrane helices move as units (Fig. 2). The activation entails a displacement and rotation of helix 6 relative to helix 5. The movement of the helices then exposes the intracellular sites required for interaction with and activation of the G-proteins.

## VI. G-PROTEIN INTERACTIONS

G-proteins are heterotrimeric signaling proteins, consisting of an α-subunit, a β-subunit, and a γ-subunit. In the inactive state, the three subunits are associated and the α-subunit contains the nucleotide guanosine diphosphate in a nucleotide-binding site. Activation of a G-protein-coupled receptor causes a conformational change in the G-protein, which leads to guanosine triphosphate replacing guanosine diphosphate in the nucleotide site of the α-subunit and the dissociation of the α-subunit from the β-/γ-subunits. Both the α-subunit and the β-/γ-subunits can then interact with intracellular effector proteins to induce changes in signal transduction. The α-subunit has an enzymatic phosphatase activity for guanosine triphosphate. When the guanosine triphosphate is hydrolyzed to guanosine diphosphate, the α- and β-/γ-subunits reassociate, terminating the signaling.

The major specificity of G-proteins derives from the α-subunit. For example, an active $G_s$ α-subunit induces an increase in adenylate cyclase activity, which in turn generates the intracellular signaling molecule cAMP. An active $G_q$ subunit stimulates phospholipase C activity, ultimately promoting a release of calcium from intracellular stores and an activation of protein kinase C. The physiological effects induced by activation of a G-protein-coupled receptor are determined, in part, by the particular G-protein to which it couples. G-protein-coupled receptors preferentially activate a specific type of G-protein, such as $G_s$. However, many receptors have been found to be capable of activating more than one type of G-protein. The G-protein coupling preference displayed by an individual G-protein-coupled receptor is relative rather than absolute.

Given the structural conservation of G-protein-coupled receptors and heterotrimeric G-proteins, it is likely that the overall geometry of different receptor/G-protein complexes is generally conserved. Surprisingly, within a G-protein-coupled receptor family, the amino acids that are responsible for determining the G-protein coupling preferences show little relationship among receptors that display similar G-protein coupling profiles. Furthermore, receptors in different structural classes that have no sequence homology may couple to the same set of G-proteins. In the case of the agonist-binding site, amino acid similarities among receptors binding related agonists can be readily identified, such as an aspartate in the third transmembrane helix that interacts with biogenic amine agonists. The absence of evidence for a similar conservation pattern among the sites in different receptors involved in specifying the interaction with a particular G-protein, such as $G_s$, suggests that the structural basis for G-protein coupling specificity results from convergent evolution.

### A. Receptor Domains Involved in G-Protein Coupling

The selectivity of G-protein recognition is determined by multiple intracellular receptor regions. The most important regions are located in the second intracellular loop and in the amino-terminal and carboxy-terminal segments of the third intracellular loop. The intracellular domains act in a cooperative fashion to dictate proper G-protein recognition and efficient G-protein activation.

### B. Effects of Posttranslational Modifications on Receptor/G-Protein Selectivity

The selectivity of receptor/G-protein coupling may also be regulated by receptor phosphorylation. Activation of $\beta_2$-adrenergic receptors leads to a pronounced increase in intracellular cAMP levels via activation of $G_s$ (followed by activation of protein kinase A) and to the stimulation of mitogen-activated protein kinases through a pathway involving $G_i$-proteins. Studies with mutant $\beta_2$-adrenergic receptors lacking protein kinase A phosphorylation sites have shown that receptor-mediated activation of $G_i$-proteins is dependent on phosphorylation of the $\beta_2$-adrenergic receptor by protein kinase A. As protein kinase A phosphorylation decreases the efficiency of binding of $\beta_2$-adrenergic receptor to $G_s$, phosphorylation represents a switch mechanism for regulating the G-protein selectivity of the $\beta_2$-adrenergic receptor.

Many G-protein-coupled receptors have a conserved cysteine in their carboxy-terminus that may be modified by covalent attachment of palmitic acid. For some receptors, such as the endothelin receptor, this palmitoylation has been implicated in G-protein coupling selectivity.

## VII. MODIFYING PROTEINS

The function of specific G-protein-coupled receptors may be profoundly influenced by interactions between the receptor and specific proteins. A class of transmembrane proteins called receptor activity-modifying proteins (RAMPs) modulate the expression and/or function of several calcitonin-related G-protein-coupled receptors.

The calcitonin family of peptides comprises five known members: calcitonin (CT), amylin, two calcitonin gene-related peptides (CGRP1 and CGRP2), and adrenomedullin (ADM). CT, CGRP, ADM, and amylin have high-affinity receptors identified in tissue. The first RAMP, RAMP1, was discovered through attempts to clone the CGRP receptor. Human RAMP1 is a 148-amino-acid protein with a large extracellular amino-terminal domain, a single transmembrane-spanning domain, and a short cytoplasmic domain. RAMP1 is closely related to two other RAMPs. The RAMPs interact with specific receptors and have three potential effects: assisting in shuttling the receptor to the plasma membrane, altering receptor glycosylation, and altering the pharmacological specificity of the receptor. The calcitonin receptor-like receptor (CRLR) requires the presence of a RAMP to be transported to the cell surface plasma membrane. RAMP1 also alters the terminal glycosylation of CRLR. These effects of RAMP1 are direct, as the CRLR and RAMP1 are associated with each other.

## VIII. RECEPTOR DIMERIZATION

Many G-protein-coupled receptors function as dimers. Several G-protein-coupled receptors form heterodimers not only with closely related receptor subtypes but also with dissimilar G-protein-coupled receptors and even with members of other protein classes. Dimerization can affect receptor trafficking and G-protein coupling.

Receptor dimers may be present under basal conditions or may be generated following agonist stimulation. There are three patterns of dimerization: (1) dimers are detected under basal conditions and no change in their amount is observed upon agonist exposure; this pattern has been observed with the muscarinic $M_3$ receptor; (2) dimers are detected under basal conditions but agonists modulate the levels of the dimers, a pattern observed for the $\delta$ opioid receptor; and (3) dimerization requires the presence of agonist.

### A. Homodimerization

Many G-protein-coupled receptors have been shown to form homodimers. Indeed, the presence of receptor dimers may be the natural state of G-protein-coupled receptors. The presence of homodimers was originally suggested by the pattern observed with protein electrophoresis. Resonance energy transfer techniques have shown that receptor dimers are present in living cells.

### B. Heterodimerization

Heterodimerization provides a combinatorial mechanism that increases the population of distinct functional receptors. The first G-protein-coupled receptor recognized to form a heterodimer was the $GABA_B$ receptor. Each subunit of the dimer, the $GABA_{B(1)}$ and $GABA_{B(2)}$ proteins, is nonfunctional in the absence of a partner. Without $GABA_{B(2)}$, $GABA_{B(1)}$ is retained in the endoplasmic reticulum. $GABA_{B(2)}$ serves to chaperone $GABA_{B(1)}$ to the cell membrane and also contributes to coupling the complex to G-proteins and downstream effectors.

Heterodimerization of related receptors has also been found between $\kappa$ and $\delta$ opioid receptors and between $\mu$ and $\delta$ opioid receptors, which influences receptor internalization and pharmacology. Heterodimerization also occurs with serotonin 5-$HT_{1D}$ and 5-$HT_{1B}$ and with somatostatin $SST_5$ and $SST_1$ receptors.

In addition, heterodimerization occurs between more distantly related G-protein-coupled receptors. Somatostatin $SST_5$ and dopamine $D_2$ dopamine receptors can interact in the presence of agonist and function as a single signaling unit. Heterodimerization between dopamine $D_1$ and adenosine $A_1$ receptors and between angiotensin $AT_1$ and bradykinin $B_2$ receptors also occurs.

## IX. RECEPTOR MUTATIONS AND DISEASES

A large number of diseases result from mutations of G-protein-coupled receptors. The mechanisms by which receptor mutations lead to diseases include changes in the levels of receptor expression, in receptor signaling, in receptor desensitization, and in receptor specificity.

### A. Loss-of-Function Mutations

Mutations that lead to a loss of receptor function underlie a large number hereditary diseases. Loss-of-function mutations usually behave as recessive genetic traits. Representative examples are described.

Color blindness was the first disorder found to be caused by a defective G-protein-coupled receptor. The color-sensitive opsins, located in the retinal cone, are activated by light of a particular wavelength and couple to a specialized G-protein, cone transducin ($G_t$). A variety of mutations in cone opsin genes ranging from a single base change to large deletions have been correlated with loss of vision color. Mutations in the gene encoding the corresponding photoreceptor of retinal rod cells, rhodopsin, are responsible for autosomal dominant and recessive forms of retinitis pigmentosa, a degenerative disease of the retina.

### B. Constitutively Activated Mutations

Several naturally occurring mutations that cause constitutive activation of G-protein-coupled receptors have been identified. These mutations cause the receptor to be more likely to spontaneously assume the active state, even when no agonist is present, and thus to autonomously generate cellular signaling. The pathogenesis of these disorders results from the spontaneous signaling that occurs.

One type of retinitis pigmentosa is caused by mutations of the visual receptor rhodopsin that lead to a high level of spontaneous signaling. Interestingly, a mutation of rhodopsin that causes only a slight increase in spontaneous receptor signaling does not lead to degeneration of the retina but causes persistent desensitization of retinal cell signaling. This interferes with the ability of the retinal cells to adapt to low light levels and causes congenital night blindness. Activating mutations of the thyrotropin receptors have been identified in hyperfunctioning thyroid adenomas. A mutation of the luteinizing hormone receptor that causes increased spontaneous activity has been found to cause hereditary precocious puberty.

### C. Receptor Specificity

Diseases that result from a loss in receptor specificity have been identified. The thyrotropin-stimulating hormone receptor and luteinizing hormone/chorionic gonadotropin receptor are two Class A glycoprotein hormone receptors that have a high selectivity for their respective agonists. A hereditary mutation of the thyrotropin-stimulating hormone receptor has been identified that leads to a reduced ability to distinguish between thyrotropin-stimulating hormone and chorionic gonadotropin. During pregnancy, the elevated levels of chorionic gonadotropin levels cause an aberrant stimulation of the thyrotropin-stimulating hormone receptor. This mutation underlies the clinical disease familial gestational hyperthyroidism in which women become hyperthyroid only during pregnancy.

## X. SUMMARY

The remarkable heptahelical structure of the G-protein-coupled receptors has provided an adaptable template for the intercellular signaling of higher organisms. The signaling specificity of these receptors has been exploited both by evolution and by the pharmaceutical chemist. Receptor diversity is accomplished by the presence of a huge number of distinct receptor genes and is further augmented by combinatorial interactions of different receptors to form receptors with novel properties. The importance of the proper functioning of this signaling system for human health is indicated by the large number of diseases associated with function-altering receptor mutations. The G-protein-coupled receptor is a micromachine for facilitating the intercellular transfer of information required for homeostasis and survival that has proven versatile and efficient.

## Glossary

**α-helix** A basic structural element of proteins consisting of a continuous coil that contains 3.6 amino acids per turn.

**agonist** A chemical that specifically interacts with and activates a receptor by stabilizing the active conformation. The effects of agonists can be prevented by chemicals called antagonists or inverse agonists, which are usually synthetic compounds that interact with the receptor but oppose its activation.

**clone** A purified, specific DNA sequence, such as the sequence that provides the template for synthesis of a particular receptor.

**conformation** A particular structure or three-dimensional state that can be assumed by a chemical, such as a receptor protein. Receptor proteins can exist in several conformations and a given receptor molecule can transition between these conformations.

**dimerization** The association of two receptor molecules to form a bimolecular complex.

**G-protein** A signaling molecule that is activated by G-protein-coupled receptors. When active, it is associated with guanosine triphosphate.

**hormone** A chemical compound that is produced by an endocrine cell and that is delivered, usually via the circulatory system, to a distant target cell, where it interacts with a specific receptor protein to induce a particular biological response.

**mutation** A natural or laboratory-generated change in the DNA sequence of a particular gene. If the mutation alters the coding sequence of a receptor, it causes a corresponding change in the amino acid sequence for that receptor and may thereby alter the receptor's function. Naturally occurring receptor mutations may cause diseases by altering receptor function or level of expression. Site-directed receptor mutagenesis is a research tool in which alteration of specific amino acids is used to elucidate their role in receptor structure and function.

**neurotransmitter** A small chemical that provides signaling between neurons. It is released by a neuron and interacts with a receptor in an adjacent neuron to elicit a specific response.

**peptide** Two or more amino acids bound by covalent bonds. Many hormones are peptides.

**receptor** A cellular protein that interacts with a specific agonist and serves to initiate a molecular chain of events that converts the arrival of the agonist into a particular biological response in the cell.

**signal transduction** The process through which an extracellular signal, such as a hormone or neurotransmitter, is converted into a cellular response.

**transmembrane domain** The segment of a membrane protein that passes through the membrane. The transmembrane domain is often composed of an abundance of lipid-preferring (hydrophobic) amino acids that form an α-helix.

## See Also the Following Articles

**Calcitonin • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Heterotrimeric G-Proteins**

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When injected into mice or humans, G-CSF stimulates a dose-dependent increase in circulating levels of neutrophils and is the most potent cytokine to do so. The increase begins rapidly (within 4–5 h), reaches a plateau by 5–10 days, and is sustained until injections cease. With cessation of G-CSF administration, neutrophil levels return to normal within 1–2 days. Somewhat surprisingly, G-CSF also stimulates a dramatic increase in circulating levels of all hematopoietic progenitor cells and stem cells (several hundred-fold), peaking at day 5–6 then declining. These increases arise in part through mobilization of cells from the bone marrow into the blood but also through increased production of progenitor and mature cells.

G-CSF is also the major regulator of neutrophil production under steady-state conditions. Mice in which either the G-CSF gene or the G-CSF receptor gene had been deleted by homologous recombination in embryonic stem cells displayed only approximately 20% of the normal levels of circulating neutrophils and only approximately half the number of progenitor and maturing cells within this lineage in the bone marrow. These mice were also deficient in mobilizing stem cells from the bone marrow to the blood in response to some other stem cell-mobilizing factors, such as the chemokine interleukin-8 and the chemotherapeutic agent cyclophosphamide, but not to others, such as the Flt3 ligand. Although these mice were still able to respond to infections by near normal elevation of neutrophils (suggesting that G-CSF is not essential for this process), they nevertheless were less effective in clearing the infections, perhaps reflecting the other actions of G-CSF on neutrophil functions.

## III. PROTEIN STRUCTURE

Human G-CSF is synthesized as a 204-amino-acid precursor protein with the first 30 amino acids (the leader sequence) being cleaved during secretion from the producer cells (macrophages, endothelial cells, and fibroblasts). It is glycosylated with the attachment of sugar residues to a single threonine residue (Thr-133) (called O-glycosylation) and contains two disulfide bonds linking cysteines 36–42 and 64–74. The molecular weight of the mature protein is 18,627 (excluding the sugar chain). The sugar chain is not required for biological activity but increases the solubility and stability of the protein.

G-CSF folds into a three-dimensional conformation that is conserved among many different cytokines. It consists of a four α-helical bundle with relatively long helical lengths forming two antiparallel pairs (helices A with D and B with C). There is an additional small helix (E) in the loop between the A and the B helices (see Fig. 1). At least one major site of interaction of G-CSF with its receptor is the face presented by the A and C helices, with Glu-19 being particularly important. A second site of interaction has been postulated to involve residues on the E helix with the immunoglobulin-like domain of the receptor.

## IV. GENE STRUCTURE

The human G-CSF gene is on chromosome 17 and consists of five exons. The mouse G-CSF gene is on chromosome 11 and has an identical intron–exon structure. The major mRNA transcript in both cases is approximately 1500 nucleotides long and, in humans, there is a second minor transcript that contains an additional 9 nucleotides or 3 amino acids as a result of the use of an alternative splice donor sequence within intron 2. The alternative G-CSF thus encoded contains 3 additional amino acids (ValSerGlu) after amino acid 35 and has a reduced biological activity.

Control of G-CSF expression occurs at both the transcriptional and the posttranscriptional levels. Like the mRNA of many other cytokines, G-CSF mRNA contains an AU-rich 3′-untranslated region that confers instability on the mRNA. This sequence

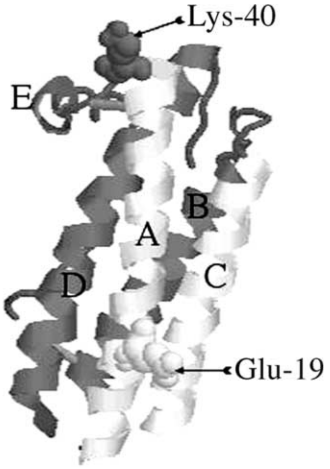

**FIGURE 1** The structure of human G-CSF as determined by X-ray crystallography. The main body of the molecule is made up of four antiparallel α-helices labeled as ribbons A–D in the figure (N-terminal) to (C-terminal). The short fifth helix is labeled E. The main receptor interaction site on the A–C helical face centered around Glu-19 is shown with CPK sphere models of the side chains, and the proposed interaction site with the immunoglobulin domain of the receptor, centered around Lys-40, is similarly shown. Disulfide bonds are shown as cylinders joining two helices.

is thought to bind specific proteins or ribonucleoproteins in a regulatable manner so that the mRNA can exist in a short or longer half-life form depending on extracellular signals. Inductive stimuli, such as bacterial lipopolysaccharides (LPSs), tumor necrosis factor (TNF), or interleukin-1 (IL-1), stabilize the G-CSF mRNA through this mechanism. The region 300 bp upstream of the transcriptional initiation site of the G-CSF gene is sufficient for both constitutive and inducible (in response to LPS, TNF, and IL-1) transcription of G-CSF in macrophages. Three *cis* regulatory elements (GPE1–3) have been identified involving nuclear factor κB (NF-κB)/NF-IL-6-binding sites, octamer-binding sites, and unidentified binding sites with the first two appearing to be the most important for LPS and TNF/IL-1-inducible transcription in macrophages, endothelial cells, and fibroblasts.

## V. G-CSF RECEPTOR

Specific receptors for G-CSF are expressed on all cells of the neutrophilic granulocyte series from blast cells to mature neutrophils. These cells generally display a few hundred receptors per cell but some monocytes also express lower numbers of receptors. Other hematopoietic cells do not express G-CSF receptors but some nonhematopoietic cells, including placental trophoblasts and endothelial cells, also express G-CSF receptors. The function of G-CSF receptors in these latter cells is currently unclear.

The G-CSF receptor was identified by expression cloning and shown to belong to the type I cytokine receptor family characterized by the presence in the extracellular domain of a paired fibronectin III module called the cytokine recognition domain (CRD). It also contains an N-terminal immunoglobulin (Ig) domain and three additional fibronectin III domains prior to the transmembrane domain.

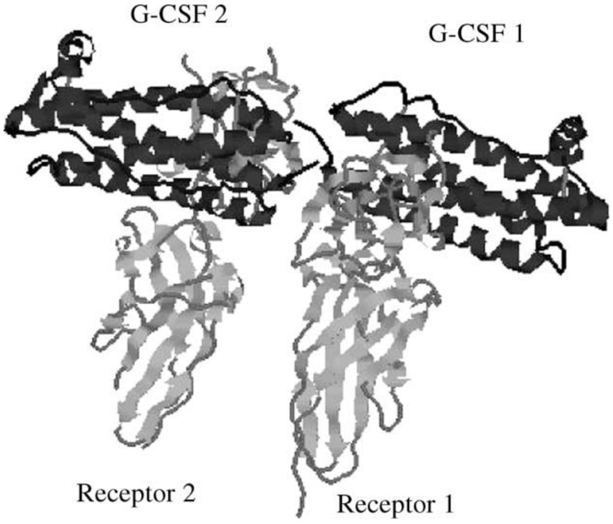

**FIGURE 2** The structure of the 2:2 G-CSF:G-CSF receptor complex as determined by X-ray crystallography. The two G-CSF molecules are shown as darker molecules with essentially the same structure as in Fig. 1, and the two receptor molecules are shown as the lighter colored molecules. The receptor fragments used in this study contained only the CRD domain and lacked the immunoglobulin domain. The β-strands of the two subdomains of each receptor are shown as arrows with each G-CSF molecule making contact with both receptor chains.

Somewhat surprisingly, the three-dimensional structure of G-CSF bound to a truncated G-CSF receptor containing only the CRD domain revealed a 2:2 complex rather than the 1:2 complex previously determined for the related growth hormone:growth hormone receptor complex (Fig. 2). Residues on the A and C helices of G-CSF (particularly Glu-19) interact in a hydrogen bond network with loops from both the N and the C subdomains of the CRD of one receptor chain (particularly Tyr-172 and Arg-287, respectively). The N-terminal residues of G-CSF (particularly Ser-7 and Gln-11) also interact with the C subdomain of the CRD of the other receptor chain (particularly Phe-259 and His-260, respectively), thus linking the two 1:1 complexes. This structure does not explain the importance of the Ig domains of the G-CSF receptor that has been noted in other studies but it is possible that these interact with other residues on G-CSF (previously called site I, which includes Lys-40 and Phe-144).

The cytoplasmic domain of the G-CSF receptor also contains conserved sequence elements typical of type I cytokine receptors including the box 1 and box 2 elements. Accordingly, activation of Janus tyrosine kinases (JAKs), tyrosine phosphorylation of the receptor, and activation of the signal transducers and activators of transcription (STATs) are thought to be critical for G-CSF-induced intracellular signaling. Other signaling pathways, including the Ras/MAP kinase and phosphatidyl inositol 3-kinase pathways, are also stimulated by G-CSF. Whereas only the membrane proximal regions of the receptor are required to signal cell proliferation, there is evidence that C-terminal regions are required for cellular differentiation in some cell lines and for feedback inhibition of G-CSF signaling. Interestingly, some patients with severe congenital neutropenias that develop into preleukemic myelodysplastic syndromes acquire mutations in the C-terminal part of the receptor cytoplasmic domain.

## VI. CLINICAL USES

The ability of G-CSF to elevate circulating neutrophils in mice and humans led to its application initially in patients receiving cytotoxic chemotherapy for malignancy. Neutropenia is a significant side effect of many chemotherapy regimens and may limit the benefits that patients accrue from treatment. G-CSF was tested in clinical trials for its ability to prevent or reduce severe neutropenia and its complications. Randomized trials demonstrated that, when commenced after chemotherapy, G-CSF accelerates the recovery of neutrophil numbers and reduces the duration of severe neutropenia when patients are at high risk of infections. As a direct consequence of this accelerated neutrophil recovery, G-CSF administration also reduced the incidence of inflammation of the oral mucosa, the need for antibacterial and antifungal antibiotics, and the duration of hospitalization. The benefits were shown to be more significant when more intensive chemotherapy was used and greatest after myeloablative chemo-radiotherapy and autologous bone marrow transplantation. G-CSF has been used to support chemotherapy in routine clinical practice for more than 10 years now, and specific indications for its use have been refined. Evidence-based guidelines for its cost-effective use have been published by the American Society of Clinical Oncology. G-CSF is used routinely to support chemotherapy of moderate intensity in patients with curable malignancies including lymphoma, leukemia, and breast cancer.

A second major clinical use for G-CSF is the mobilization of hematopoietic stem cells from the bone marrow into the blood. In early clinical studies, it was noted that the numbers of all types of CFCs and stem cells in the blood were elevated dramatically (about 100-fold) 4–7 days after the beginning of G-CSF treatment. These blood stem cells proved easier to collect in large numbers than bone marrow stem cells and they also were proved to reconstitute the hematopoietic system significantly faster than bone marrow when infused following myeloablative chemo-radiotherapy. In particular, platelet recovery was accelerated by 5–10 days. For patients undergoing autologous transplantation, blood stem cells are collected after mobilization either with G-CSF given alone or, more commonly, with G-CSF given during the recovery phase after chemotherapy when the number of stem cells in the blood may be increased 1000-fold over steady state. Patients undergoing allogeneic hematopoietic stem cell transplantation receive cells from normal donors. G-CSF administration to volunteer donors for patients undergoing allogeneic transplantation has been shown to be safe and effective. G-CSF-mobilized blood stem cells now have replaced bone marrow as the standard source of hematopoietic stem cells for both autologous and allogeneic bone marrow transplantation.

G-CSF has also found clinical utility in the treatment of patients with neutropenia unassociated with chemotherapy, such as severe congenital neutropenia, cyclic neutropenia, aplastic anemia, and some myelodysplastic syndromes. In particular, patients with severe congenital neutropenia have benefited substantially. Regular use of G-CSF in these settings increases the neutrophil counts toward normal, allowing previously refractory infections to resolve and preventing mouth ulceration, fevers, and recurrent infections.

The observation that serum levels of G-CSF are markedly increased during infection led to initial speculation that treatment with G-CSF may hasten the recovery of noncancer patients with serious bacterial or fungal infections. However, G-CSF administration does not accelerate the recovery of patients from pneumonia or septic shock, and G-CSF is not indicated in these conditions. Similar results were observed when this strategy was tested in patients with chemotherapy-induced neutropenia and indicate that the major benefit of G-CSF in chemotherapy patients is the previously mentioned reduction in the duration of severe neutropenia and consequent reduction in the risk of developing an infection. For neutropenic patients with life-threatening infections, transfusions of neutrophils may be beneficial. G-CSF is used to increase the number of neutrophils in the blood of donors by three- to fivefold, facilitating the efficient collection of these short-lived cells for immediate infusion into patients.

G-CSF is usually given by subcutaneous injection once per day to accelerate neutrophil recovery and either once or twice per day to mobilize stem cells into the blood. For patients requiring chronic treatment with G-CSF, administration once or twice a week is often sufficient. The side effects experienced by patients receiving G-CSF are generally mild and well tolerated. The most common are lethargy, headache, and bone pain. Bone pain is most common in patients who continue G-CSF after their neutrophil counts have recovered and in normal donors. It reflects an expansion of bone marrow tissue within the medullary cavity and a dramatic increase in local blood flow that accompanies this expansion. In volunteer donors, the only life-threatening complication is splenic rupture and this is a rare occurrence. In patients with acute myeloid leukemia, the presence of G-CSF receptors on leukemic cells raised the theoretical concern that G-CSF administration may accelerate the progression of the leukemia. This concern has not been borne out in clinical trials and short-term use of G-CSF is not thought to cause or promote leukemia. However, for patients with the preleukemic condition severe chronic neutropenia, prolonged G-CSF treatment is associated with an increased risk of the development of acute leukemia. This risk is offset by the marked improvement in the quality of life that these patients experience with G-CSF treatment. For the vast majority of people receiving G-CSF, it is a safe and highly effective therapeutic agent.

## Glossary

**bone marrow transplantation** The transplantation of a patient's own bone marrow (autologous) or bone marrow from a different donor (allogeneic) into a patient to provide a source of hematopoietic stem cells from which the entire hematopoietic system can be reconstituted after high-dose chemotherapy or radiotherapy.

**chemotherapy and radiotherapy** The use of toxic chemicals or radiation to attempt to kill (usually) cancer cells in patients. Side effects usually include the killing of normal hematopoietic cells (myelotoxicity), leading to infections, and the killing of intestinal and hair precursor cells, leading to vomiting and hair loss, respectively. Myeloablative chemotherapy is high-dose chemotherapy that permanently destroys the hematopoietic system and would prove fatal without bone marrow transplantation.

**colony-forming cell** The progenitor cell (blast cell) that can multiply and differentiate in semisolid cultures to form the differentiated cells that constitute a colony.

**colony-stimulating factor** A generic term for a family of proteins that stimulate the production of hematopoietic cell colonies from single-cell suspensions in semisolid medium such as agar or methylcellulose.

**differentiation** The process of morphological and functional maturation that occurs in cells as they progress from a blast cell stage to the mature cell (e.g., granulocyte).

**granulocyte** A white blood cell containing granules in the cytoplasm that is involved in attacking bacterial infections. There are three types, depending on the type of granules, called neutrophils, eosinophils, and basophils. The term granulocyte is sometimes used to mean only neutrophils, e.g., in the case of granulocyte colony-stimulating factor.

**granulocyte colony-stimulating factor (G-CSF) receptor** The protein displayed on the surface of cells that specifically recognizes, interacts with, and responds to G-CSF.

**neutropenia** A reduction in the normal circulating numbers of neutrophilic granulocytes from 4000–10,000/μl to 1000/μl or less. The reduction can be due to genetic diseases (congenital neutropenias) or to the myelotoxic effects of chemotherapeutic drugs.

**signal transduction** The series of molecular events occurring after G-CSF binds to its receptor, leading to the appropriate cellular responses. Signal transduction usually involves receptor modification, binding of intermediary signaling molecules, and activation of transcription factors, leading to the expression of specific genes.

## See Also the Following Articles

**Colony-Stimulating Factor-1 (CSF-1) • Crosstalk of Nuclear Receptors with STAT Factors • Erythropoietin, Biochemistry of • Flt3 Ligand • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Growth Hormone (GH) • Stem Cell Factor**

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eosinophil survival due to the production of GM-CSF by local lung epithelial cells. The priming of granulocytes for enhanced effector cell function is important to boost the innate immune response and facilitate phenomena central to inflammation such as adhesion to blood vessel wall, migration into tissues in response to chemotactic stimuli, and enhanced phagocytosis, degranulation, release of oxygen radicals, and cytotoxic activity. Although GM-CSF is an effective stimulus of all these functions, there is a hierarchy in terms of the concentrations of GM-CSF required. Cell survival is the function that requires the least amount of GM-CSF and hence receptor occupancy, highlighting the importance of this function and perhaps reflecting differences in signaling pathways according to the "strength" (level of receptor occupancy) of the signal.

GM-CSF also participates in the acquired immune response through its ability to up-regulate class II expression and stimulate dendritic cells. Interstitial myeloid dendritic cells can be generated *in vitro* by stimulating monocytes specifically with GM-CSF and interleukin-4. Similarly, both interstitial and Langerhans dendritic cells (found in the skin) can be uniquely generated *in vitro* by stimulating CD34$^+$ hematopoietic stem cells with GM-CSF and tumor necrosis factor α (TNFα). Such cells are dedicated to antigen uptake and processing and are unusually adept at stimulating naive T cells. The immunomodulatory properties of GM-CSF to enhance dendritic cell functions are currently being exploited in (1) vaccination strategies to prevent infection and (2) anti-cancer therapy.

In addition to its direct effect on hematopoietic and immune cells, GM-CSF exerts a synergistic effect in the presence of other cytokines. One example is with IL-4 in the production of dendritic cells. Another one is with stem cell factor or c-kit ligand, a cytokine that is a poor proliferative stimulus by itself but which, in combination with GM-CSF, has a strong proliferative effect on hematopoietic cells. Most of these *in vitro* effects of GM-CSF are recapitulated *in vivo* following the injection of GM-CSF in mice and humans. *In vivo* administration of GM-CSF results in increased numbers of circulating neutrophils, eosinophils, monocytes, and multipotent hematopoietic cells and increased numbers and activation of tissue macrophages, including alveolar macrophages. It also enhances dendritic cell maturation, proliferation, and migration and the proliferation and function of pulmonary type II alveolar epithelial cells. In the bone marrow, GM-CSF alters the kinetics of myeloid progenitor cells, causing rapid entry of cells into the cell cycle and decreasing cell cycle time by as much as 33%. There is also a synergistic response *in vivo* when GM-CSF is combined with other cytokines (such as erythropoietin and thrombopoietin) to stimulate multilineage hematopoietic recovery following chemotherapy or to mobilize stem cells (G-CSF) in the peripheral blood for collection pretransplant. To understand the true physiological role of GM-CSF, *in vivo* gene inactivation experiments have been performed in which the gene coding for GM-CSF has been disrupted. Surprisingly for a cytokine with such multilineage activities, no differences in the number of steady-state circulating granulocytes or macrophages were found; this has been explained by redundancy in the system and compensation by other hematological cytokines that act on myeloid cells, namely, G-CSF, macrophage colony-stimulating factor (M-CSF), and IL-3. However, these mice do display an inability to metabolize lung surfactant by lung-specific macrophages, resulting in an alveolar proteinosis-like syndrome. This syndrome can be corrected by administering GM-CSF to the lung or by bone marrow transplantation and is secondary to an inability of circulating monocytes to undergo further differentiation into alveolar macrophages in these mice. Humans deficient in GM-CSF via acquired antibodies or bone marrow genetic mutations in myeloid stem cells resulting in a nonfunctioning GM-CSF receptor also develop pulmonary alveolar proteinosis.

A role for GM-CSF *in vivo*, however, begins to be revealed under conditions of stress in which knockout mice are challenged under conditions that mimic disease situations. For example, GM-CSF −/− mice cannot engage a normal immune response against bacterial and fungal pathogens including *Listeria monocytogenes* and are predisposed to lung infection. Interestingly, GM-CSF −/− mice are refractory to experimentally induced arthritis and toxic shock syndrome (despite levels of TNFα being normal) and are protected against the development of a multiple sclerosis-like disease in mice. Thus, the physiological role of GM-CSF under steady-state conditions appears to be mainly the regulation of lung surfactant metabolism via alveolar macrophage differentiation with essentially no effect on leukocyte cell numbers. However, in response to infection, trauma, and inflammation, GM-CSF is a potent stimulator of both the hematopoietic system and the innate immune system with beneficial outcomes in certain cases but also with the potential to exacerbate chronic inflammation, leukemic cell load, and autoimmune responses.

## III. SITES OF PRODUCTION AND GENE REGULATION

Given its diverse and potentially harmful effects, it is not surprising that the production of GM-CSF in various cells throughout the body is under strict control. Copious amounts of GM-CSF are produced by activated T cells as part of either a type 1 or a type 2 helper T-cell response. Upon activation, T cells increase transcription of the GM-CSF gene via the effect of pro-inflammatory transcription factors such as nuclear factor κB (/Rel, activator protein-1, and NFAT. However, naive unstimulated T cells actively repress the GM-CSF gene. In this way, inflammatory responses can be directed in a localized and controlled fashion.

In addition to T cells, constitutive transcription of the GM-CSF gene at low levels has been detected in large variety of cells including monocytes, endothelial cells, fibroblasts, natural killer cells, mast cells, mesothelial cells, epithelial cells, and some tumor cells. In these cells, production of actual GM-CSF protein is regulated primarily by stabilization of the RNA message by an AU-rich element in the 3′-untranslated region. In the resting state, virtually no GM-CSF protein is secreted because of degradation of GM-CSF mRNA soon after it is transcribed. However, in response to inflammatory cytokines such as IL-1 or TNFα or the stimulation of Toll receptors, GM-CSF production can be dramatically and rapidly increased by inhibiting the GM-CSF mRNA degradation pathway. Removal of basal state inhibition of GM-CSF mRNA results in a lethal myeloproliferative disorder.

The GM-CSF gene may be co-regulated with other cytokines and pro-inflammatory genes on the same chromosome by formation of distinct chromatin domains of coiled DNA that are opened or repressed according to incoming nuclear signals. The human GM-CSF gene is localized to chromosome 5q21–q32 adjacent to the IL-3 gene and there is evidence that co-regulation of these genes occurs in some activated T cells.

## IV. RECEPTOR AND SIGNALING

The biological effects of GM-CSF are mediated via binding to specific receptors expressed on the surface of target cells. The GM-CSF receptor is a heterodimer consisting of two distinct subunits or polypeptide chains named α and $\beta_c$ ($\beta_c$). The α-subunit is the major binding component specifically binding GM-CSF with low affinity (2–5 nM). The $\beta_c$-subunit acts as an affinity converter, allowing the binding of GM-CSF to its heterodimeric receptor with high affinity (100 pM), a property that makes the binding of GM-CSF to its receptor essentially irreversible and ensures a biological outcome. Both subunits are necessary for receptor signal transduction with $\beta_c$ undergoing phosphorylation and being the major subunit associating with downstream signaling molecules. An important property of $\beta_c$ is that, unlike the α chain, it is not unique to the GM-CSF receptor but is shared with the IL-3 and IL-5 receptors. This predicts that in cells that co-express these receptors, the respective ligands will exert the same functions. Indeed, this is illustrated best on eosinophils, which show the same qualitative and quantitative biological responses to GM-CSF, IL-3, or IL-5.

The GM-CSF receptor has been detected by means of radiolabeled GM-CSF or antibodies to its receptor chains on the surface of many cells, in the hematopoietic as well as outside the hematopoietic system. These include neutrophils, eosinophils, basophils, monocytes, macrophages, undifferentiated bone marrow cells, dendritic cells, B cells, certain T lymphocytes, uterine cells, and astrocytes. In every case, both subunits of the GM-CSF receptor are present, and to date, there has been no unequivocal example of one subunit being expressed in the absence of the other, suggesting a coordinate control of gene expression and emphasizing the functional requirement for the heterodimer. Myeloid leukemias such as AML, CMML, CML, and JCMML and solid tumors such as prostate cancer, breast cancer, melanoma, and small-cell lung carcinoma can also express the GM-CSF receptor but the contribution that this expression makes to the malignant state remains unexplored.

The binding of GM-CSF to its receptor causes receptor dimerization and the formation of a high-order complex; however, the exact stoichiometry of this complex remains unknown (Fig. 1). Receptor dimerization is accompanied by phosphorylation of the receptor on $\beta_c$ and of several intracellular proteins. The phosphorylation on the receptor $\beta_c$ occurs on tyrosine as well as on serine and threonine residues and has the purpose of creating docking sites onto which signaling molecules can be recruited. Although $\beta_c$ lacks intrinsic kinase activity, GM-CSF has been shown to induce tyrosine phosphorylation of $\beta_c$ as well as of a diverse group of cytoplasmic proteins including (1) kinases, such as phosphatidylinositol 3-kinase and protein kinase B or AKT; (2) adapter proteins, such as growth factor receptor-binding protein 2, insulin receptor substrate-2, Cbl,

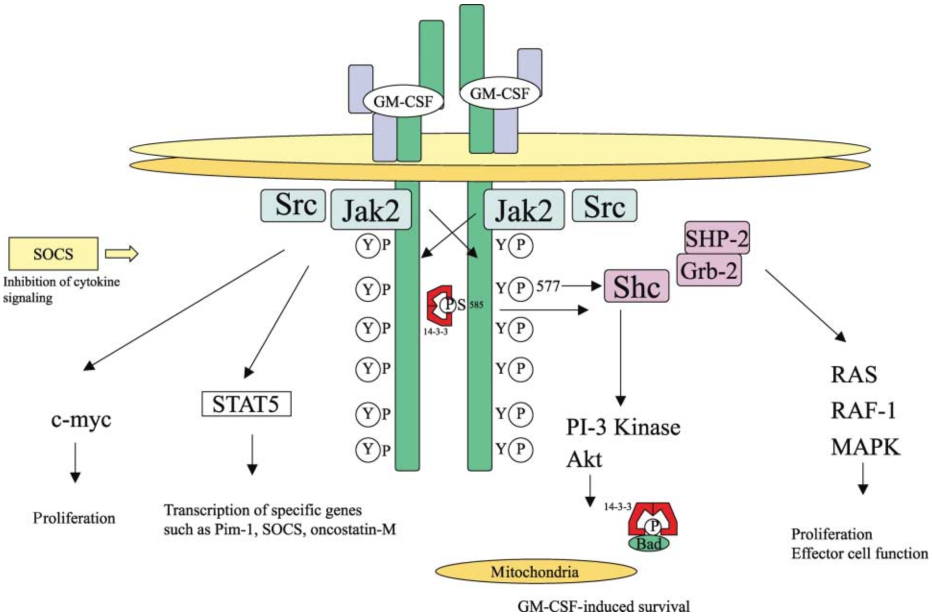

**FIGURE 1** Activated GM-CSF receptor signaling complex showing trans-phosphorylation of adjacent Jak2 tyrosine kinase molecules (crossed arrows) subsequent to receptor dimerization. α, GM-CSF receptor α-subunit; β, common β-subunit; GM, GM-CSF ligand. Src kinase is also involved in tyrosine phosphorylation of $\beta_C$ tyrosine residues and other adapter proteins. Some adapter proteins such as Shc, 14-3-3, and Grb2 are shown in close association with the cytoplasmic portion of $\beta_C$. Various downstream signaling pathways are shown with arrows indicating their putative means of activation and the biological outcomes.

and Src homology and collagen protein; (3) guanine exchange factors, such as Vav; (4) phosphatases, such as SH2-domain-containing protein tyrosine phosphatase-2 and SH2-containing inositol phosphatase, and (5) transcription factors, such as signal transducers and activators of transcription (STAT5). This tyrosine phosphorylation is mediated by the receptor-associated tyrosine kinase Janus kinase 2 (JAK2) and Src-family kinases. GM-CSF receptor signaling is initiated by trans-phosphorylation of the JAK2 kinase, which is associated with the proximal intracellular portion of $\beta_c$. The phosphorylation of $\beta_c$ on tyrosine residues by JAK2 in turn promotes the docking of SH2 domain-containing signaling molecules and the amplification of receptor signaling. Different tyrosine residues on $\beta_c$ couple the receptor to diverse signaling pathways. Although there is redundancy in terms of promoting the binding of STAT5 molecules (they can bind to any one of the six phosphorylated tyrosines in $\beta_c$), phosphorylation of Tyr-577 results in the specific recruitment of Shc, an adapter molecule involved in the activation of the ras pathway.

Phosphorylation of $\beta_c$ on certain serine residues also has been recently noted and shown to be important for GM-CSF receptor signaling. Phosphorylation of Ser-585 located immediately downstream from the Shc-binding site enables association of the 14-3-3 adapter protein and is required in signaling the cell survival response. Protein kinase A can phosphorylate Ser-585; however, it is not known how GM-CSF activates protein kinase A or indeed other potential serine/threonine kinases. Figure 1 summarizes the major known signaling pathways stimulated by GM-CSF.

## V. STRUCTURE

Human GM-CSF is a secreted glycoprotein containing 127 amino acids and two intramolecular disulfide bonds. The molecule is rich in N- and O-linked carbohydrates; however, unglycosylated GM-CSF (produced in bacteria) exhibits high-affinity binding, is fully functional, and in fact is approximately 10% more potent. The structure, as determined by X-ray crystallography, shows as its most important feature a four α-helix bundle with a double overhand topology. This fold is common to other cytokines such as growth hormone, IL-2, IL-4, and IL-5. This is particularly striking given the low degree of sequence similarity with these cytokines and suggests a common ancestral origin.

The human GM-CSF receptor α-subunit and $\beta_c$-subunit both contain conserved extracellular domains termed cytokine receptor modules (CRMs). Each CRM consists of two repeats of a fibronectin type III-like domain that contains four cysteines with conserved spacing and a WSXWS (where X is any amino acid, W is tryptophan, and S is serine) motif. Mutagenesis of the WSXWS motif has been shown to disrupt receptor activation and is important for correct folding of the extracellular domain of cytokine receptors. The B–C and F–G loops (where A to G are labels for the seven strands in each beta sheet of the CRM) in the domain containing the WSXWS motif are important for cytokine binding, with Tyr-421 in the F–G loop of $\beta_c$ being crucial for the high-affinity binding and signaling of GM-CSF as well as IL-3 and IL-5.

The exact manner in which GM-CSF interacts with its two receptor subunits is not known as the structure of the GM-CSF receptor complex has not yet been solved. Nevertheless, mutagenesis of GM-CSF and of its two subunits indicates binding through two different faces. One face of GM-CSF would be composed largely by its fourth α-helix binding to the receptor α chain through several weak molecular interactions that involve the surface residues Asp-112 and Glu-108. The second face of GM-CSF would comprise the first α-helix of GM-CSF and in particular Glu-21, which protrudes about halfway through the helix. This residue is essential for GM-CSF interacting with the receptor $\beta_c$-subunit and hence is crucial for GM-CSF high-affinity binding and biological activity.

These structure–function studies of GM-CSF and its receptor have facilitated the development of GM-CSF antagonists. The antagonist E21R was developed by substituting the key Glu-21 residue with amino acids of opposite charge, namely, Arg or Lys. These point mutation analogues of GM-CSF fully occupy the receptor α chain but they do not signal because they cannot engage $\beta_c$; hence, they behave as competitive antagonists of GM-CSF. E21R is currently undergoing phase II clinical trials for the treatment of certain leukemias. The antagonist BION-1 is an antibody directed to the cytokine-binding region of $\beta_c$. As expected, this antibody and its fragments antagonize the high-affinity binding and biological activities of GM-CSF as well as those of IL-3 and IL-5 but not of other cytokines. An antagonist with this specificity is postulated to be useful in pathological conditions in which all these three cytokines are involved. Since eosinophils respond to any of these three cytokines, antagonists such as BION-1 may be useful in diseases in which the eosinophil plays a major pathological role such as in asthma.

## VI. CLINICAL USES AND ROLE IN HUMAN DISEASE

The myeloid-stimulating properties of GM-CSF are currently utilized in the treatment of cancer in many centers. Myelosuppression following cytotoxic chemotherapy for the treatment of both hematological and solid cancers is an almost invariable side effect that, if severe, can result in life-threatening infections. Clinically described as febrile neutropenia, such infections usually occur between 6 and 14 days after exposure to chemotherapy when neutrophil counts (half-life 6–8 h) are at their lowest. Both G-CSF and GM-CSF have proven efficacious in hastening the time of neutrophil recovery, reducing the number of infections, and shortening hospital stays. Conventionally, GM-CSF is administered subcutaneously (250 $\mu g/m^2$ per day) 24–72 h after chemotherapy until neutrophil counts recover ($>10{,}000/\mu l$) but mild side effects such as flushing, flu-like symptoms, and myalgias have limited the use of GM-CSF compared with G-CSF.

Another common clinical use of GM-CSF is in peripheral blood stem cell transplantation. Hematopoietic stem cells normally circulate in the peripheral blood but at very low concentrations. Following the administration of certain hematopoietic growth factors such as GM-CSF or G-CSF, the concentration of hematopoietic progenitor cells in blood, as measured by colony-forming units or the expression of CD34 antigen, increases markedly. This has made it possible to harvest adequate numbers of stem cells from the peripheral blood for transplantation into a patient who has received myeloablative chemotherapy. Compared with autologous bone marrow, use of peripheral blood stem cells results in more rapid hematopoietic recovery, particularly with respect to platelet numbers. There are no major differences in the stem cell subsets mobilized with GM-CSF compared with G-CSF or engraftment outcomes but combination therapy appears to dramatically increase the progenitor cell number harvested.

The clinical indications for use of GM-CSF have expanded considerably since it first became available in the early 1990s as a stimulator of myelopoiesis for the treatment of cancer. Research in recent years has uncovered many other diverse biological effects, in addition to hematopoiesis, including responses to infection, inflammation, atherosclerosis, allergy, and pulmonary disease. Such immune-modulating and -enhancing properties of GM-CSF have sparked diverse clinical trials and approvals for its use in the treatment of human infections, vaccination strategies, and immunosuppression.

An example of the successful use of GM-CSF in stimulating the immune system has been in the treatment of fungal infections in severely immunocompromised patients. Substantial evidence that GM-CSF enhances the ability of neutrophils and macrophages to phagocytose and destroy bacteria and fungi has been obtained from *in vitro* and *in vivo* studies. Some of these effects are attributed to increased neutrophil survival, up-regulation of class II molecules to augment antigen presentation, and increased expression of mannose- and Toll-like receptors on both neutrophils and macrophages. Disseminated fungal infections in bone marrow transplant recipients or patients with acute myeloid leukemia are notoriously difficult to treat with standard antifungal drugs and result in mortality rates approaching 50%. Treatment with GM-CSF resulted in significantly reduced mortality from fungal infection (20 to ≈75%) in a phase III trial of 99 elderly patients undergoing chemotherapy for acute myeloid leukemia. Similarly, the pro-inflammatory properties of GM-CSF have been used in the treatment of chronic wounds that do not heal with conventional methods by subcutaneous injection of recombinant GM-CSF around the wound site.

Other emerging applications of GM-CSF utilize its immunomodulatory ability to up-regulate antigen presentation mainly by increasing the number and signal strength of the dendritic cell response. Vaccine strategies that result in the activation of the immune system specifically against proteins expressed by cancer cells have the potential to be effective, low-side-effect anti-cancer therapy. Success in treating metastatic cancer in mice and renal cell, melanoma, and prostate carcinoma in humans has been obtained by the insertion of the GM-CSF gene into cancer cells that are then used to immunize patients. These genetically modified tumor cells produce GM-CSF in the local environment of the tumor cells, specifically activating the patient's T cells and natural killer cells to destroy cancer cells.

Given its many pro-inflammatory properties, it is not surprising that GM-CSF has been detected at sites of acute and chronic inflammation in both infectious and autoimmune disease. Examples of the latter include rheumatoid arthritis, psoriasis, allergy, and graft-versus-host disease. Presumably, the role of GM-CSF at these sites is to recruit and stimulate mainly neutrophils and macrophages as well as to potentiate any autogeneic or allogeneic antigen presentation to T cells. Even atherosclerosis, which is now considered an inflammatory disease, involves GM-CSF secretion in localized tissues by endothelial cells, and macrophages can be stimulated to engulf lipids for atheroslerotic plaque formation in response to GM-CSF. Paradoxically, GM-CSF has also been shown to increase collateral blood flow formation in patients with coronary (atherosclerotic) artery disease.

## Glossary

**alveolar proteinosis** A rare human respiratory disorder characterized by the accumulation of periodic acid-Schiff-positive lipoproteinaceous material (derived from pulmonary surfactant) in the distal airspaces.

**cytokine** Secreted regulatory peptide or protein that controls certain functions in cells and includes growth factors, interleukins, lymphokines, and colony-stimulating factors.

**dimerization** The association of two identical molecules (monomers) to form a higher order complex.

**granulocyte** Any one of the three myeloid cell lineages that contain cytoplasmic granules, namely, neutrophils, eosinophils, and basophils.

**granulocyte colony-stimulating factor** A hematopoietic cytokine responsible for the regulation of neutrophil production.

**innate immune system** Ancient immune recognition system of host cells bearing germ-line-encoded pattern recognition receptors that directly recognize foreign antigens or pathogens and comprising natural killer cells, monocytes/macrophages, neutrophils, basophils, eosinophils, and dendritic cell precursors.

**JAK** Janus tyrosine kinase involved in hematopoietic cytokine receptor signal transduction.

**macrophage colony-stimulating factor** A hematopoietic cytokine responsible for regulation of monocyte/macrophage production and osteoclast function.

**SH2 domain** (Src homology-2) A structural domain present in many signal transduction protein molecules that can bind phosphorylated tyrosine residues in the context of a particular recognition sequence.

**stem cell transplantation** The transfer of stem cells, classically hematopoietic stem cells, from either another organism or the same organism to a recipient animal to reconstitute an entire multilineage cellular system.

## See Also the Following Articles

**Colony-Stimulating Factor-1 (CSF-1) • Erythropoietin, Biochemistry of • Flt3 Ligand • Granulocyte Colony-Stimulating Factor (G-CSF) • Growth Hormone (GH) • Stem Cell Factor**

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# Growth Hormone (GH)

Peter E. Lobie[*] and David J. Waxman[†]

[*]*National University of Singapore* • [†]*Boston University*

I. INTRODUCTION
II. GENE STRUCTURE AND EXPRESSION AND HORMONE SECRETION
III. GH RECEPTOR AND GH-BINDING PROTEIN
IV. PHYSIOLOGIC AND METABOLIC EFFECTS
V. CLINICAL SYNDROMES
VI. SIGNAL TRANSDUCTION
VII. SUMMARY

**Growth hormone (GH) is a member of a class of evolutionarily related hormones that includes prolactin, various placental lactogens, and proliferin. At the cellular level, GH action is mediated by a cell surface receptor, the GH receptor, which initiates multiple intracellular signaling pathways leading to alterations in cell biochemistry and physiology, in many cases by means of changes in gene expression. These cellular responses to GH translate into diverse physiological effects in different organ systems, including stimulation of long bone growth. This article provides a brief overview of the biology, function, and mechanism of action of GH.**

## I. INTRODUCTION

Many hormones, nutritional factors, and related cell regulators contribute to the regulation of mammalian growth and development; however, only growth hormone (GH) stimulates longitudinal bone growth in a specific and dose-dependent manner. Furthermore, only GH, when secreted at excessive concentrations prior to puberty, is able to induce excessive longitudinal bone growth. In 1909, Harvey Cushing described the relationship between pituitary hyperactivity and gigantism and between pituitary insufficiency and dwarfism. It was subsequently demonstrated that bovine anterior pituitary gland extracts are able to induce supranormal growth in intact rats and that hypophysectomy results in cessation of growth. GH was first isolated in 1944, and purified GH was demonstrated to restore growth in hypophysectomized rats.

## II. GENE STRUCTURE AND EXPRESSION AND HORMONE SECRETION

The human GH (hGH) gene is located within a gene cluster composed of five structurally and functionally related genes. Two hGH genes [hGH-N (normal) and hGH-V (variant)] and three placental lactogen (PL) genes constitute this gene cluster, which is located on the long arm of human chromosome 17 (Fig. 1).The GH–PL gene cluster is thought to have evolved from a single ancestral gene by a process involving gene duplications, insertion of gene regulatory elements, and at least one gene conversion event. The five genes in the cluster possess >92% nucleotide sequence identity in their coding and flanking regions. The molecular architecture of each gene is also identical; four small introns split the transcriptional units at identical positions and perfect codon co-linearity exists for the open reading frames of all five genes (Fig. 1, bottom).

Messenger RNAs for two isoforms of hGH-N (designated 22 and 20 kDa hGH) are generated in the somatotrope of the anterior pituitary by differential splicing of the primary transcript. The 22 kDa hGH-N is the predominant form, constituting 90% of pituitary gland GH mRNA. The 20 kDa GH form is identical to the 22 kDa form, except for deletion of amino acid residues 32–46 from exon 2 by an alternate splicing reaction. The hGH-V gene is transcribed and expressed in the placenta, where the protein product is secreted during pregnancy as a 22 kDa isoform.

Comparison of the nucleotide and amino acid sequences of GH from multiple species indicates that the sequence identity between GH and PL in humans (87%) is greater than in other species (65–73% sequence identity between GH and PL genes in porcine, bovine, ovine, equine, and murine). Despite this similarity, hPL has >1% of the growth-promoting potential of hGH. Homology between GH and prolactin family members is substantially lower (~27% sequence identity). Primate GHs, but not GH from other species, can bind to the human GH receptor. Under certain conditions, hGH will also bind and activate the prolactin receptor (e.g., in the rat).

GH is synthesized as a prohormone with a signal peptide at the amino-terminus that is removed during secretion from the cell. The mature 22 kDa hGH-N is a single chain, nonglycosylated 191-amino-acid polypeptide and is the most abundant isoform of hGH in plasma (~76% of total). The half-life of hGH in the human is ~25 min.

The three-dimensional structure of mammalian GH indicates the presence of four α-helices, each 21–30 amino acids in length. The helices are

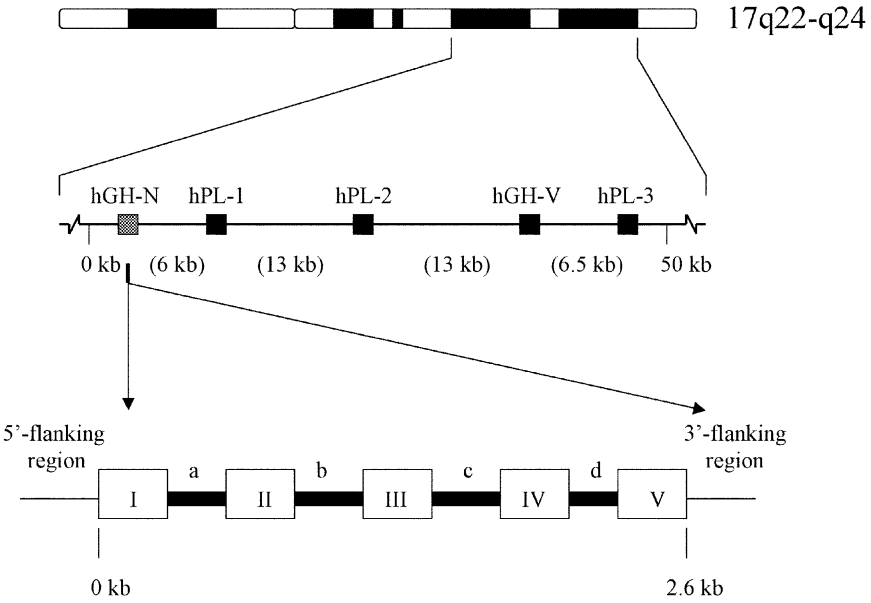

**FIGURE 1** The hGH–hPL gene cluster. The five genes shown comprise approximately 8 kb of structural sequences spread over 50 kb of DNA at chromosomal position 17q22–q24. Shown at the bottom are the five exons (I–V) and four introns (a–d) that characterize hGH-N, as well as the other four genes in the cluster.

arranged in a left-handed bundle orientation with an unusual up-up-down-down topology. Long connective loop structures link the two sets of parallel helices, and a shorter loop region connects helix 2 and helix 3. hGH contains disulfide bridges, at Cys-35–Cys-165 and Cys-182–Cys-189. These Cys residues are key components of the loop structures. The central core of the GH molecule is composed of ~20 hydrophobic amino acids. Smaller hydrophobic clusters of amino acids stabilize the four-helix bundle.

The 5′-proximal 0.5 kb promoter sequence of the hGH-N gene contains DNA regulatory elements responsible for the tissue-specific pattern of hGH-N transcription and gene expression. The proximal promoter of the hGH-N gene contains a typical TATA-box, as well as two binding sites for the pituitary transcription factor Pit-1. These sequences, together with more remote sequences as far as 15 kb upstream of the hGH-N transcription initiation site, are required for efficient expression of the hGH-N gene. The proximal hGH-N gene promoter also contains *cis*-acting elements that modulate transcription in response to hormonal signals.

Transcription of the hGH-N gene is stimulated by the hypothalamic GH-releasing hormone, GHRH. GHRH produced in the arcuate nucleus of the hypothalamus binds to and activates a specific GHRH receptor on the surface of the pituitary somatotrope. The GHRH receptor is a G-protein-coupled receptor whose activation by GHRH is linked to an increase in intracellular cyclic AMP (cAMP). The elevated cAMP in turn activates protein kinase A, leading to an increase in the concentration of Pit-1 in the somatotrope. Pit-1 is a member of the POU homeodomain family of transcription factors and binds to two Pit-1-binding sites in the proximal promoter of the hGH-N gene. Thus, GHRH-stimulated hGH-N gene transcription is Pit-1-dependent.

GH is stored in secretory granules of the somatotrope until a secretory signal leading to an increase in intracellular $Ca^{2+}$ or cAMP stimulates the secretion of GH. GH release from pituitary somatotrope cells occurs in a highly pulsatile fashion and is subject to neuroendocrine control. GH secretion is controlled by the opposing action of GHRH and the GH secretagogue GHrelin, both of which stimulate GH secretion, and somatostatin, which inhibits GH release and helps establish the timing and amplitude of the secretory pulse. Somatostatin, derived from the anterior periventricular nucleus in the hypothalamus, can override the stimulatory effects of GHRH. Somatostatin inhibits GH release from the somatotrope by binding to a $G_i$-protein-coupled cell surface receptor, leading to a decrease in intracellular cAMP levels. GHrelin is a 28-amino-acid polypeptide with a unique *n*-octanoly-serine modification; it is produced in the gastric mucosa and the hypothalamus. GHrelin binds to and activates the GH secretagogue receptor (GHSR) as do various synthetic GH secretagogues. GHSR is a seven-transmembrane G-protein-coupled receptor. Alternative splicing of the GHrelin gene (Gln14-GHrelin) yields a second ligand for GHSR that also promotes GH release. Increased somatrope $Ca^{2+}$ levels, induced by GHrelin, act in a synergistic manner with the increased production of cAMP in response to GHRH to stimulate pituitary secretion of GH. GH secretion can also be regulated by leptin through its effects on hypothalamic GHRH and somatostatin activity. Leptin is thought to be a major adipose tissue-derived signal responsible for the influence of nutritional status on GH secretion (e.g., increased amplitude of GH secretion in the fasted state).

Gonadal steroids play an important role in modulating pituitary GH secretory patterns, with estrogen acting at the level of the hypothalamus to induce more frequent pulses of GH secretion. This results in the occurrence of sex-dependent plasma GH profiles in several species, including humans. Sex differences in plasma GH profiles are most striking in rodents and lead to marked sex differences in GH-induced intracellular signaling and gene expression patterns in the liver. GH secretion in humans declines with age in both sexes, leading to an increase in body fat and a decrease in muscle mass associated with a decrease in GH-stimulated insulin-like growth factor-I (IGF-I) production.

GH is also expressed in various cell types and tissues outside of the pituitary gland, albeit at a much lower level. These include the central nervous system, the mammary gland, gonads from both sexes, and cells of the hematopoietic and immune systems. In some cells, GH expression is responsive to GHRH, and in others, notably lymphocytes, it is independent of Pit-1. GH therefore possesses autocrine/paracrine effects that are distinct from its endocrine effects.

## III. GH RECEPTOR AND GH-BINDING PROTEIN

The human GH receptor is encoded by a single-copy gene located in the proximal short arm of chromosome 5 in the region p13.1–p12 and spans ~87 kb (Fig. 2). The hGH receptor gene is characterized by at

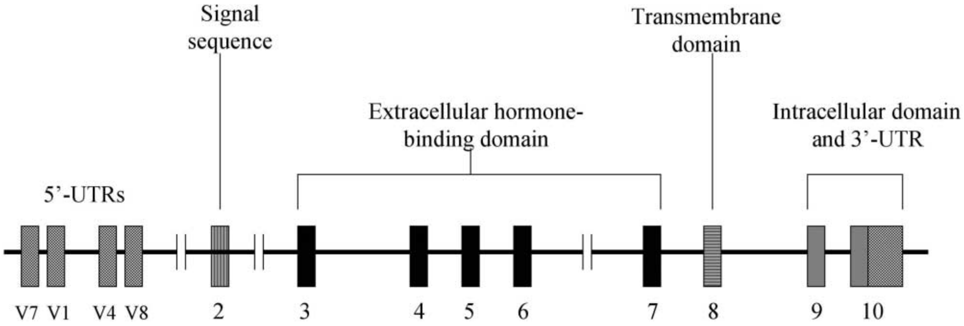
Signal sequence
Transmembrane domain
Extracellular hormone-binding domain
Intracellular domain and 3'-UTR
5'-UTRs
V7 V1 V4 V8 2 3 4 5 6 7 8 9 10

**FIGURE 2** Genomic structure of the hGH receptor gene.

least eight distinct, alternative 5′-untranslated region (5′-UTR) variants of exon 1. In liver, GH receptor exon 1 variant 1 (V1) is the most highly expressed form. The untranslated exon 1 is followed by nine protein-coding exons. Exon 2 encodes the last 11 bp of the 5′-UTR, an 18-amino-acid signal peptide, and the first 5 amino acids of GH receptor's extracellular domain. Exons 3–7 encode the majority of the receptor's extracellular domain. Exon 8 encodes the final 3 amino acids in the extracellular domain, a 24-amino-acid transmembrane domain, and the first 4 amino acids of the receptor's intracellular domain. Exons 9 and 10 encode the remaining 346 amino acids of the intracellular domain. Exon 10 also codes for a 2 kb 3′-UTR.

The GH receptor is an ~620-amino-acid single-pass transmembrane protein. The GH receptor contains two extracellular domains (amino acids 1–123 and 128–238) linked by a 4-residue spacer. Each extracellular domain contains seven β strands arranged to form a sandwich of two antiparallel β sheets. The GH receptor also contains three disulfide bonds, bridging Cys-38–Cys-48, Cys-83–Cys-94, and Cys-108–Cys-122. The overall extracellular domain is stabilized by these disulfides, in combination with hydrogen bonding between Arg-43 and Glu-169 and a salt bridge between Arg-39 and Asp-123. The molecular mass of the GH receptor predicted from its amino acid sequence is 70 kDa. However, the actual mass of GH receptor is 100–130 kDa, with the difference in size due to postranslational modifications such as glycosylation and ubiquitination. The GH receptor contains five N-linked glycosylation sites, each adding approximately 10 kDa in mass to the receptor. The GH receptor possesses 19 potential ubiquitination sites and can be polyubiquitinated on multiple lysine residues. Ubiquitination of the receptor is increased by ligand binding and may play a role in GH receptor internalization.

Human GH-binding protein (GHBP) is a proteolytic fragment of the GH receptor that circulates as a plasma-binding protein. It is generated by a metalloprotease-catalyzed cleavage of the plasma membrane-bound receptor form. Up to 60% of plasma GH is bound to GHBP, with the fraction bound decreasing at higher plasma GH concentrations. GHBP acts to increase the half-life of circulating GH by decreasing its rate of clearance and degradation. In rats, mice, and monkeys, GHBP is generated from the GH receptor at the RNA level, by alternative splicing of the GH receptor precursor RNA. Several short but membrane-anchored isoforms of GH receptor have also been described in humans.

GH receptor expression is highest in the liver, a major target organ of GH action. However, GH receptor expression has been observed in many organ systems, including the gastrointestinal tract, male and female reproductive systems, musculoskeletal system, cardiorespiratory system, hematopoietic and immune systems, central nervous system, the integument, renal and urinary systems, and endocrine system. Within each system, the GH receptor is expressed on both differentiated and undifferentiated cell types. The GH receptor is also expressed on cells derived from the ectoderm, mesoderm, and endoderm of the developing fetus.

The heterogeneity in the 5′-UTR exon 1 sequences of the GH receptor gene transcripts noted above indicates complex transcriptional control of the GH receptor locus. Indeed, the level of GH receptor mRNA can be regulated by both nutritional and hormonal factors, including GH itself. Experimental data derived from the GH receptor V1-like transcript of different mammalian species has revealed both positive- and negative-acting 5′-regulatory elements driving transcription. A proximal TATA-box is conserved across species, as is a positive-acting C/EBP and glucocorticoid-response element complex site and a negative-acting CCAAT site further upstream. The CCAAT site has been demonstrated to bind the transcription factors MSY-1 and NF-Y. Additional regulatory elements may exist further upstream.

## IV. PHYSIOLOGIC AND METABOLIC EFFECTS

GH exhibits diverse and pleiotropic effects on cell proliferation, differentiation, morphology, and metabolism. Some of these effects are a response to the direct action of GH in its target tissues, and other effects are indirect and are mediated by growth factors induced by GH, e.g., insulin-like growth factor (IGF-I), whose expression in liver and other tissues is strongly stimulated by GH. Both the direct and the indirect cellular effects of GH are subsequently translated into physiological effects in individual target organ systems.

A major mechanism by which GH affects cellular and, consequently, physiologic function is by regulating the rate of transcription of specific RNA species. Some of these GH-regulated RNAs code for tropic factors, such as IGF-I, which act in an intermediary role to execute cellular actions of GH. IGF-I is produced in many tissues and acts in an endocrine/paracrine manner through either the IGF-I receptor or the insulin receptor. GH also regulates other tropic factors, including hepatocyte growth factor in liver, epidermal growth factor in kidney, basic fibroblast growth factor in chondrocytes, interleukin-6 (IL-6) in osteoblasts, bone morphogenetic proteins 2 and 4 in fibroblasts, IL-1α, IL-1β, and thymulin in thymus, and preadipocyte factor-1 in adipocytes and islet beta cells.

The predominant phenotypic effect of GH is to promote longitudinal bone growth in the postnatal and pubertal growth periods. This is achieved by coordinated stimulation of both chondrocyte and osteoblast proliferation and effects on differentiated cell function. GH also exerts anabolic effects resulting in positive nitrogen balance and muscular hypertrophy consequent to increased protein synthesis.

GH exerts profound effects on body composition through a combination of anabolic, lipolytic, and anti-natriuretic actions. GH enhancement of the lipolytic activity of adipose tissue in combination with a reduction of triglyceride accumulation via inhibition of lipoprotein lipase activity appears to be the major mechanism by which GH acts to stimulate a reduction of total fat mass.

GH is important for cardiac function. hGH has been demonstrated to increase myocardial mass and improve myocardial energy metabolism and hemodynamics.

GH alters the size and morphology of the central nervous system during development and affects differentiated cell function with consequent modulation of cognitive function. Cognitive effects of GH include the enhancement of both long-term memory and delayed extinction responses, improved performance on operant discrimination learning, stimulation of maternal behavior, and an increase in rapid eye movement sleep with a concomitant decrease in slow-wave sleep.

GH increases the renal glomerular filtration rate and also increases renal absorption of sodium, phosphate, and water. GH also increases the activity of 25-hydroxy-1-α-hydroxylase (thereby increasing calcitriol production), as well as renin and erythropoietin synthesis.

GH is required for sexual differentiation and pubertal maturation and it participates in gonadal steroidogenesis, gametogenesis, and ovulation. GH is also required for fetal nutrition and growth during pregnancy and for mammary development and lactation.

GH stimulates hypertrophy throughout the gastrointestinal tract especially by stimulation of the epithelial component. GH also stimulates increased water and mineral transport and stimulates the absorption of specific vitamins such as vitamin B12. Studies in rodents show that GH regulates the expression of a large number of liver metabolic genes and other genes in a sexually dimorphic manner. This sexual dimorphism can be directly attributed to the differential stimulation of gene expression by the male versus female pattern of pituitary GH secretion.

GH has several immunomodulatory functions including the proliferation of B cells and T cells, stimulation of cytokine and immunoglobulin production, and regulation of the activity of neutrophils, macrophages, and natural killer cells.

Postnatal body weight and composition differ between males and females due to sexual dimorphism of the somatotropic axis and its impact on GH secretory patterns, as noted above. Such sex differences predominantly originate at the hypothalamic–pituitary level but are also due to altered responsiveness of target tissues to somatotropic-axis hormones.

## V. CLINICAL SYNDROMES

The absence or decrease of pituitary GH production is referred to as isolated GH deficiency (IGHD)

(1 in 500 human births). A small proportion (5–30%) of known clinical cases are hereditary. Four IGHD types can be differentiated according to their pattern of inheritance, severity, and response of the affected individuals to recombinant hGH treatment. Among them, type 1A is an autosomal recessive disorder in which endogenous GH is absent. The loss of GH production is due mainly to homozygous deletion of the hGH-N gene. IGHD type 1A subjects often exhibit short stature and hypoglycemia at birth and by 6 months of age display readily obvious signs of dwarfism.

Acromegaly, a condition due to excessive secretion of GH, is associated with an increased morbidity and mortality. This is largely due to cardiovascular, cerebrovascular, respiratory, and metabolic diseases.

Laron syndrome is a recessively inherited disease characterized by GH resistance. Multiple primary defects that cause the disease include deletion, nonsense, frameshift, missense, and splice site mutations, most often in the extracellular domain of the GH receptor. Mutations in the receptor's intracellular domain, as well as postreceptor signaling defects leading to a typical Laron phenotype, are also known. These mutations result in truncation, abnormal processing, loss of ligand binding, lack of receptor dimerization, and interference with signal transduction of the GH receptor. Humans with Laron syndrome exhibit short stature, trunkal obesity, and low serum IGF-I, but elevated serum hGH concentration and low serum GHBP. Deletion of the GH receptor gene in mice produces a phenotype similar to Laron dwarfism.

## VI. SIGNAL TRANSDUCTION

The GH receptor was the first identified member of the cytokine receptor superfamily. Other members of the superfamily include prolactin, erythropoietin, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, ciliary neurotropic factor, thrombopoietin, leptin, interleukins 2–7, IL-9, IL-11, and IL-12 ('class 1' family members). The superfamily also includes receptor subunits that interact with more than one cytokine receptor. Such receptor subunits include gp130 [shared by leukemia inhibitory factor (LIF), oncostatin M, IL-6, IL-11, and IL-12], the β-subunit of the LIF receptor, the γ-chain of the IL-2 receptor (shared by IL-2, IL-4, IL-7, IL-9, and IL-15) and the IL-3 receptor common β chain.

Common features and characteristics of the class 1 cytokine receptor superfamily members include the following (see Fig. 3): (1) possession of a single transmembrane domain; (2) limited amino acid homology (14–44% sequence identity) in a region spanning ~210 amino acids in the extracellular domain (corresponding to two fibronectin III domains); (3) conserved pairs of cysteine residues in the extracellular domain and a conserved tryptophan residue adjacent to the second cysteine in the N-terminal fibronectin domain; (4) a WSXWS (Trp-Ser-X-Trp-Ser)-like motif in the C-terminal fibronectin domain (YXXFS in the mammalian GH receptor); (5) the absence of a canonical tyrosine kinase consensus sequence; (6) two short proline-rich sequences in the intracellular domain; and (7) multiple intracellular domain tyrosine residues, several of which become phosphorylated upon activation of the receptor by ligand binding. In addition, members of the cytokine receptor superfamily utilize similar mechanisms of signal transduction, which is directed by the receptor's C-terminal cytoplasmic tail. Two conserved regions within

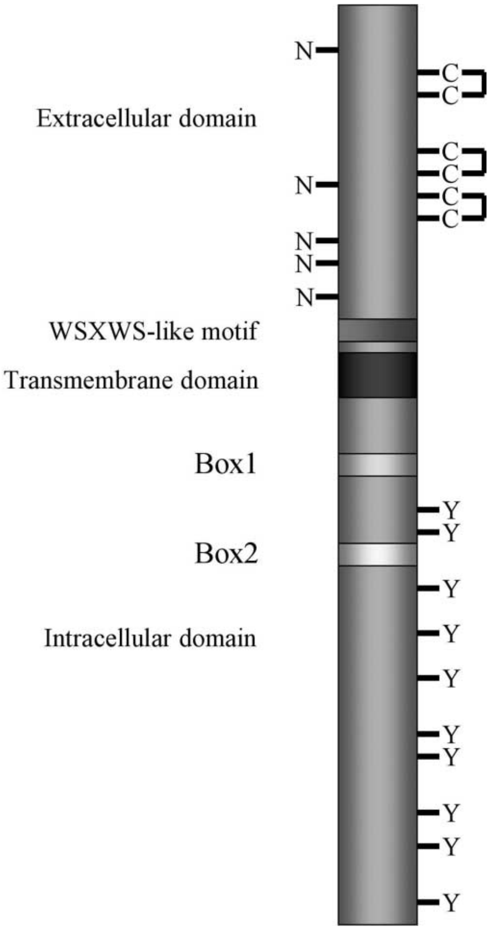

**FIGURE 3** Schematic structure of the hGH receptor.

the cytoplasmic tail of the GH receptor and other cytokine receptor family members are designated Box 1 and Box 2. Box 1 is an important site of association of Janus kinase 2 (JAK2) with the GH receptor. Box 1 is located within 20 residues of the transmembrane domain and displays an alΨPXalPXP or ΨXXXalPXP consensus sequence, where al is any aliphatic residue, Ψ is any hydrophobic residue, and P is proline. In mammalian GH receptors, the Box 1 sequence is ILPPVPVP. Box 2 is less well defined and comprises approximately 15 amino acids situated 30 residues C-terminal to Box 1. It consists of a cluster of hydrophobic and acidic residues ending with 1 or 2 basic residues.

GH activates the GH receptor by a sequential receptor dimerization reaction. This dimerization initiates the signal that generates GH's multiple downstream signaling events and biological responses. Each GH molecule contains two receptor-binding sites, a high-affinity binding site, designated site 1, and a lower affinity site, site 2. GH initially binds to one molecule of the GH receptor, via GH site 1. The resultant GH site 1–GH receptor complex then binds to a second receptor molecule via GH site 2. Essentially the same ligand-binding site is used by each GH receptor molecule in forming the GH–(GH receptor)$_2$ complex. In the final, active trimeric complex, one GH molecule bridges two receptor molecules, i.e., (GH receptor)–GH–(GH receptor). GH derivatives with mutations at site 2 form inactive, monomeric GH site 1–GH receptor complexes and provide a basis for the development of effective GH receptor antagonists.

The GH receptor lacks intrinsic tyrosine kinase activity, as do all other members of the cytokine receptor superfamily. The GH receptor therefore recruits the nonreceptor Janus family tyrosine kinase JAK2 to initiate signal transduction in response to GH binding. Unique structural features of JAK2 include the absence of SH2 or SH3 domains and the presence of seven conserved JH regions (JH1–JH7), of which JH1 comprises the functional tyrosine kinase catalytic domain and JH2 is a catalytically inactive pseudo-kinase domain. The pseudo-kinase domain of JAK2 interacts with the kinase domain to negatively regulate JAK2's tyrosine kinase activity. This interaction is required to maintain JAK2 in the inactive state in the absence of a hormonal stimulus.

Upon GH receptor dimerization, the JAK2 protein molecule associated with the box 1 region of each GH receptor chain is spatially positioned and/or conformationally modified to enable the pair of JAKs to carry out a trans-phosphorylation reaction. This reaction results in the phosphorylation of JAK2 on two C-terminal tyrosine residues of each JAK molecule, leading to the activation of JAK2's tyrosine kinase activity. The activated JAK2 then phosphorylates the intracellular domain of the GH receptor on multiple cytoplasmic domain tyrosine residues. The tyrosine phosphorylated GH receptor–JAK2 complex in turn provides docking sites for a variety of signaling molecules that contain Src-homology 2 (SH2) or other phosphotyrosine-binding motifs.

Four major signaling cascades are subsequently initiated by the JAK2-activated GH receptor (Fig. 4). (1) GH-stimulated tyrosine phosphorylation of STAT proteins (signal transducers and activators of transcription) that dock to the GH receptor–JAK2 complex leads to a direct stimulation of target gene transcription (see below); (2) GH activation of the Ras–Raf–MEK pathway results in the activation of mitogen-activated protein kinase (MAP kinase), a dual-specificity kinase whose activity is linked to intranuclear phosphorylation, c-fos transcription, and cell proliferation. GH-induced MAP kinase activation thus links multiple GH-activated signaling pathways; (3) GH activation of phosphatidylinositol 3-kinase (PI 3-kinase), at least in part by tyrosine phosphorylation of insulin receptor substrates (IRS-1 and IRS-2), is thought to contribute to the metabolic and proliferative activities of GH; and (4) GH activation of protein kinase C may impact on the metabolic, differentiative, and proliferative actions of GH. Significant cross talk between these and other GH signaling pathways may also occur.

GH can activate four of the seven known mammalian STAT forms, STATs 1, 3, 5a, and 5b. Each STAT protein resides in the cytoplasm in an inactive form until it is converted by tyrosine phosphorylation to an active dimeric DNA-binding form, which translocates into the nucleus where it activates certain GH target genes. STAT5b appears to be the most important STAT in mediating GH's effects on pubertal growth in male mice. STAT5b is also a key regulator of the sex-dependent transcriptional effects of GH in the liver. Consistent with this role, the pulsatile pattern of plasma GH stimulation that characterizes adult male rodents induces a repeated, pulsatile translocation of liver STAT5b from the cytoplasm to the nucleus. This enables the activated STAT5b to bind to STAT5 DNA-response elements found upstream of male-expressed liver GH target genes. STAT1 and STAT3 may play a role in GH's induction of c-fos, an early GH-induced transcriptional response. STAT5a plays a major role in the effects of prolactin on mammary gland differentiation

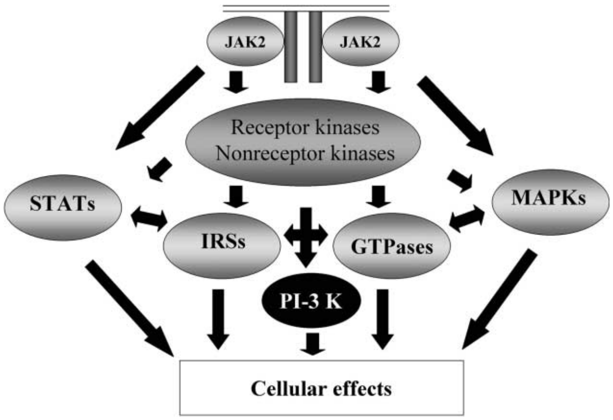

FIGURE 4 GH signal transduction. Shown is a simplified diagram of the major intracellular pathways of GH signal transduction. GH binding to its cell surface receptor induces dimerization of the receptor with subsequent association and activation of the tyrosine kinase JAK2 in association with the recently described protein signaling protein SH2b-β (not shown). JAK2 then induces the subsequent activation of several major groups of signaling molecules. These include (1) STAT family members, including STATs 1, 3, 5a, and 5b, which constitute a major mechanism for transcriptional regulation by GH; (2) small Ras-like GTPases and their downstream target members of the MAP kinase family, including p44/42 MAP kinase, p38 MAP kinase, and JNK/SAPK and their respective downstream signaling pathways; (3) members of the IRS group, including IRS-1, IRS-2, and IRS-3, which serve as docking proteins for further activation of signaling molecules including phosphatidylinositol 3-kinase; and (4) protein kinase C. For further details, see text. GH can also activate other receptor (EGF receptor) and nonreceptor (c-Src, c-Fyn, and FAK) kinases not shown in the figure.

but may also contribute to GH responses in other tissues.

Modulation and attenuation of GH signaling is thought to occur by multiple mechanisms. Two major mechanisms involve (1) the action of specific phosphotyrosine phosphatases, which dephosphorylate and thereby deactivate several key tyrosine phosphorylated GH signaling molecules. These include GH receptor itself, the tyrosine kinase JAK2, and the GH-activated STATs, which are recycled from the nucleus back to the cytoplasm in their inactive, monomeric forms upon dephosphorylation; and (2) the STAT-dependent activation of several immediate-early response genes belonging to the SOCS/CIS (suppressor of cytokine signaling) family. The induced SOCS and CIS proteins feedback inhibit the overall signaling pathway by binding to the tyrosine phosphorylated GH receptor–JAK2 complex via the SOCS protein's SH2 domain. This recruitment of a SOCS/CIS protein blocks further receptor signaling by one of several mechanisms: inhibition of JAK2 activity; competition between SOCS and STAT for GH receptor–phosphotyrosine-binding sites; and stimulation of GH receptor degradation by a proteasome-dependent mechanism. The precise relationship of this latter signal termination mechanism to the established pathways of GH receptor ubiquitination and internalization remains to be clarified.

## VII. SUMMARY

GH is the major hormonal regulator of postnatal somatic growth. It is produced predominantly by the pituitary gland as a 191-amino-acid single-polypeptide chain. The cellular actions of GH are initiated by binding to its specific plasma membrane bound receptor in a 1:2 stoichiometry. Signal transduction by GH is predominantly mediated by Janus kinase 2, which tyrosine phosphorylates a variety of intracellular substrates, including the GH receptor and itself. GH also regulates the development and function of the different organ systems, often by stimulation of intermediary tropic factors, such as the insulin-like growth factor IGF-I. The major phenotypic effect of GH is promotion of longitudinal bone growth.

## Acknowledgments

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## Glossary

**growth hormone-binding protein** A soluble, circulating form of the growth hormone receptor that is composed of the receptor's extracellular ligand-binding domain and can be formed in different species by proteolytic cleavage of the membrane-bound form or by alternative splicing of the growth hormone receptor primary transcript.

**growth hormone secretagogue** A synthetic or naturally occurring peptide that interacts with the growth hormone secretagogue receptor to stimulate secretion of pituitary growth hormone.

**Janus kinase 2** A nonreceptor tyrosine kinase that associates with the growth hormone receptor and is activated by growth hormone, leading to the initiation of multiple pathways of intracellular signal transduction.

**signal transducers and activators of transcription** A family of latent cytoplasmic transcription factors whose members contain an SH2 domain, are activated by phosphorylation at a single carboxy-terminal region tyrosine residue in response to cytokine or growth factor receptor stimulation, and when activated, dimerize and translocate to the nucleus where they induce transcription of specific target genes.

**somatotrope** A secretory cell type in the anterior hypophysis that is the major site for synthesis and secretion of growth hormone.

## See Also the Following Articles

**Crosstalk of Nuclear Receptors with STAT Factors • Erythropoietin • Granulocyte Colony-Stimulating Factor (G-CSF) • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Growth Hormone-Releasing Hormone and the GHRH Receptor • Growth Regulation: Clinical Aspects of GHRH • Leptin • Prolactin and Growth Hormone Receptors**

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# Growth Hormone Inhibiting Factor

See *Somatostatin*

# Growth Hormone-Releasing Hormone (GHRH) and the GHRH Receptor

SHANE R. CUNHA AND KELLY E. MAYO
*Northwestern University*

I. INTRODUCTION
II. GHRH
III. REGULATION OF GHRH
IV. GHRH RECEPTOR
V. GHRH RECEPTOR SIGNALING
VI. REGULATION OF THE GHRH RECEPTOR
VII. GHRH AND THE GHRH RECEPTOR IN DISEASE

**Growth hormone-releasing hormone (GHRH) is the preeminent neuroendocrine factor positively regulating growth hormone (GH) synthesis and secretion. Together with the negative regulator somatostatin, it forms a regulatory network that maintains an appropriate pulsatile pattern of GH release in the adult vertebrate organism. As such, GHRH is itself subject to regulation by a wide variety of neurotransmitters, hormones, and metabolic cues, leading to an appropriate integration of sensory information and a corresponding activation of the GH axis. GHRH exerts its actions through a specific G-protein-coupled receptor expressed on the somatotroph cells of the anterior pituitary. The predominant signaling pathway downstream of the GHRH receptor, leading to increased GH synthesis and secretion, is a cyclic AMP-mediated pathway. In addition, recent studies suggest that activation of other signaling pathways, such as the mitogen-activated protein kinase cascade, play an important role in mediating the early developmental effects of GHRH on somatotroph cell proliferation and differentiation. Consistent with the key role that GHRH and its receptor play in the regulation of GH secretion, the hormone and receptor have been implicated in several diseases or disorders affecting growth in**

**human. Future study of the receptor and its ligand-binding properties, leading to the design of more effective agonists and antagonists, therefore holds substantial therapeutic promise. Finally, the finding that GHRH and its receptor are expressed in numerous tissues in a regulated fashion suggests additional roles of the hormone that are not well understood and represent a significant challenge for the future.**

## I. INTRODUCTION

A variety of organs must work together to ensure the proper development and homeostasis of an organism. The endocrine system facilitates communication between these organ systems through the actions of secreted steroid and peptide hormones. Key to the functions of this system is the hypothalamus, which integrates sensory information and regulates hormone secretion from the pituitary gland, producing an appropriate endocrine response. With respect to the growth hormone (GH) axis, the hypothalamus regulates GH secretion from somatotroph cells of the anterior pituitary through the opposing actions of two hormones: growth hormone-releasing hormone (GHRH) and somatostatin (SRIF). GHRH stimulates GH synthesis and secretion, and SRIF suppresses GH secretion (see Fig. 1). GH has important systemic, anabolic functions, many of which are mediated by insulin-like growth factors (IGFs). Many tissues including the liver secrete IGF-I following GH stimulation. Some IGF-mediated functions include stimulation of amino acid uptake by muscles for protein synthesis, activation of cartilage formation and calcification in bones, and inhibition of glucose uptake in adipose tissue, thereby reducing fat deposition.

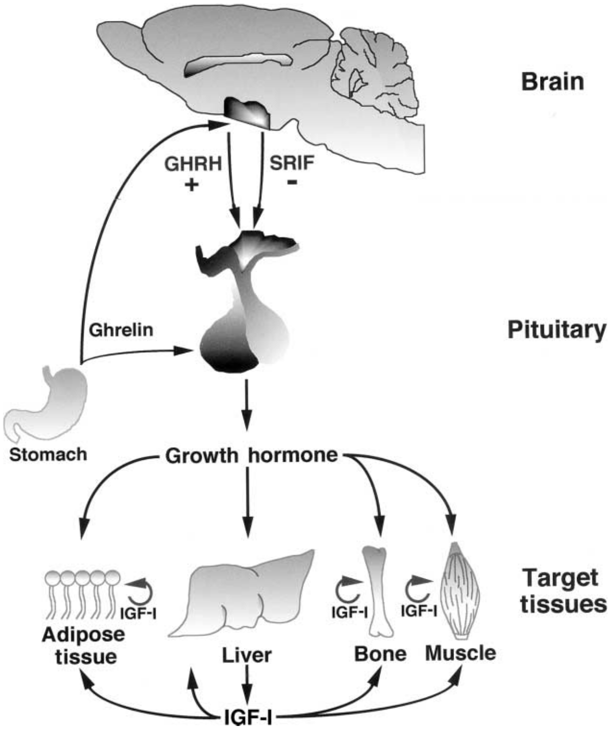
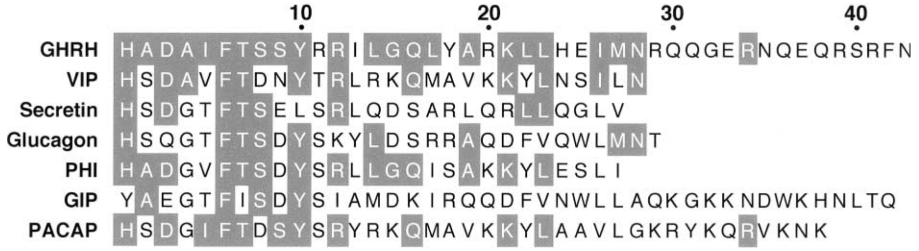


FIGURE 1 Overview of the growth hormone axis. GHRH and somatostatin (SRIF) are the predominant stimulatory and inhibitory factors released from the hypothalamus to modulate GH synthesis and secretion in the pituitary. Ghrelin is a stimulatory factor released from the stomach. GH acts directly on the liver, fat, bones, and muscle. Additionally, some of the effects of GH are mediated by insulin-like growth factor I (IGF-I), which is secreted from the liver and other tissues. GH and IGF-I modulate proliferation, differentiation, and metabolism in the target tissues.

## II. GHRH

### A. Characterization

GHRH was initially isolated from human pancreatic tumors that caused acromegaly, an endocrine disorder resulting from excessive GH secretion in the adult. Three isoforms were identified: GHRH(1–37)-OH, (1–40)-OH, and (1–44)-$NH_2$. All three isoforms stimulate GH secretion, but the shorter isoforms are less potent and probably arise from differential processing of the GHRH precursor protein or proteolytic degradation of the full-length GHRH product. The first 29 amino acids of GHRH are remarkably conserved across species and contain the necessary amino acids to confer biological activity. The amino-terminus forms an α-helical structure that is important for receptor binding. This domain has served as the template for subsequent generations of GHRH receptor agonists and antagonists. The carboxyl-terminus increases peptide stability, and amidation of the carboxyl-terminus of GHRH(1–44) further enhances this stability. Unlike human GHRH and GHRH from domestic animals, the mouse and rat GHRH peptides have 42 and 43 amino acids, respectively, and are not C-terminally amidated. Human GHRH is inactivated in serum by cleavage of the first 2 amino acids by dipeptidylaminopeptidase, producing a more stable metabolite, GHRH(3–44)-$NH_2$, which is 1000-fold less effective at eliciting GH secretion. Subsequent to its isolation from pancreatic tumors, GHRH was isolated from the human and rat hypothalamus. The neurons in the arcuate nucleus of the hypothalamus synthesize GHRH and secrete it into the median eminence,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GHRH HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQRSRFN
VIP HSDAVFTDNYTRLRKQMAVKKYLNSILN
Secretin HSDGTFTSELSRLQDSARLQRLLQGLV
Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
PHI HADGVFTSDYSRLLGQISAKKYLESLI
GIP YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKNDWKHNLTQ
PACAP HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK

**FIGURE 2** Amino acid sequence identity between GHRH and other brain–gut peptides in the rat. Conserved residues are shaded. VIP, vasoactive intestinal peptide; PHI, peptide hormone isoleucine; GIP, gastric inhibitory peptide; PACAP, pituitary adenylate cyclase-activating polypeptide.

where it is released into the portal system, which terminates on the pituitary gland. GHRH or GHRH-like peptides have been characterized in mammals, birds, amphibians, and fish. Based on amino acid sequence homology, GHRH belongs to the family of brain–gut peptides that includes secretin, pituitary adenylate cyclase-activating polypeptide (PACAP), glucagon, glucagon-like peptide-1, vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP), and peptide hormone isoleucine/peptide hormone methionine (see Fig. 2).

## B. Biosynthesis and Tissue Expression

In mammals, GHRH is proteolytically processed from a precursor protein that consists of a secretatory signal sequence, the mature GHRH peptide, and a C-terminal peptide that is distantly related to GHRH (see Fig. 3). This related peptide is reported to stimulate expression of stem cell factor, which may be involved in reproduction and is necessary for hematopoiesis. The precursor proteins for mouse and rat GHRH are 103 and 104 amino acids long, respectively. In the human, there are two precursor proteins of 107 and 108 amino acids, with the latter isoform containing an additional serine in the carboxyl-terminus as a result of alternative RNA splicing. In nonmammalian vertebrates, a single precursor protein encodes GHRH and the related peptide PACAP.

The gene for GHRH is found on chromosome 20 in human and on chromosome 2 in mouse. In human, rat, and mouse, the gene has four exons encoding the precursor protein, with an alternative first exon encoding unique 5′-untranslated sequences for hypothalamic, placental, or testicular tissue (see Fig. 3). The unique first exon allows for tissue-specific regulation, whereas the remaining four exons, utilized in all tissues, encode the same full-length GHRH precursor protein. Within the gene, the unique testis-specific exon is located upstream of the placental and hypothalamic exons. Alternative splicing of the GHRH gene in the testes results in three transcripts, two of which also include the unique placental exon.

In addition to its expression in the hypothalamus, placenta, and testis, GHRH mRNA has been detected in lymphocytes, pancreas, gastrointestinal tract, and ovary. In the ovary, GHRH modulates the actions of follicle-stimulating hormone on granulosa cells and may promote follicular maturation. Testicular GHRH may act as an intragonadal regulatory factor by modulating gonadotropin-induced steroidogenesis, and placental GHRH probably acts to regulate secretion of fetal growth hormone and maternal placental lactogen. In lymphocytes, GHRH might

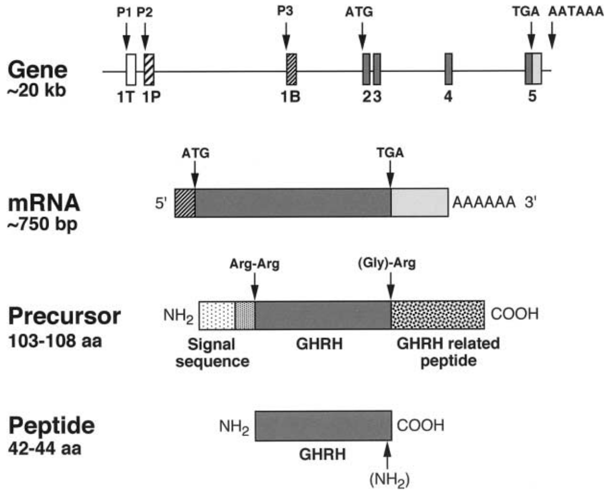
Gene
~20 kb
P1 P2
P3
ATG
TGA AATAAA
1T 1P
1B
23
4
5
mRNA
~750 bp
ATG
TGA
5'
AAAAAA 3'
Precursor
103-108 aa
Arg-Arg
(Gly)-Arg
$NH_2$
COOH
Signal sequence
GHRH
GHRH related peptide
Peptide
42-44 aa
$NH_2$
COOH
GHRH
$(NH_2)$

**FIGURE 3** Summary of GHRH biosynthesis in mammals. The structure of the GHRH gene, mRNA, precursor protein, and mature peptide are schematically presented. P1, P2, and P3 represent distinct promoters upstream of the first exons used in testis (1T), placenta (1P), or brain (1B) mRNAs. Exons 2–5 are conserved. Arg-Arg and (Gly)-Arg, sites of proteolytic processing; $(NH_2)$, amidation of carboxyl-terminus in GHRH, which occurs in all species except in rodents.

function to modulate immunity, whereas in the pancreas and gastrointestinal tract, GHRH may be involved in hormone secretion.

## III. REGULATION OF GHRH

### A. Neuroendocrine Regulation of GHRH Secretion

Regulation of GHRH secretion by a variety of neurotransmitters produces a pulsatile secretion pattern. In humans, GHRH pulses are secreted throughout the day with a large bolus of GHRH released at the onset of sleep. Exercise, nutrition, and stress can alter this secretion pattern. This pulsatility in GHRH secretion is partly the result of inhibitory actions by SRIF on GHRH neurons. SRIF receptors are expressed on GHRH neurons and receptor activation inhibits GHRH secretion. Similar to GHRH, SRIF is released in a pulsatile manner and SRIF neurons express GHRH receptors, by which GHRH stimulates SRIF release. Based on findings from rodent studies, a model of alternating patterns of SRIF and GHRH secretion has been proposed. In this model, peaks in SRIF secretion correspond to troughs in GHRH secretion and vice versa. These alternating secretion patterns for GHRH and SRIF could account for the pulsatility in GH secretion.

Additional neurotransmitters regulate GHRH secretion by stimulating GHRH release and/or inhibiting SRIF release (see Fig. 4). Three neurotransmitters that stimulate GHRH secretion by acting directly on GHRH neurons are γ-aminobutyric acid, epinephrine, and norepinephrine. In contrast, galanin inhibits SRIF release and stimulates GHRH release. Interestingly, GHRH neurons co-secrete galanin with GHRH, and galanin receptors are expressed on SRIF neurons, suggesting that galanin may provide an additional mechanism through which GHRH neurons can regulate SRIF release. Opiates increase GHRH secretion by inhibiting SRIF release. The effects of dopamine and serotonin on GHRH secretion vary depending on the species studied. In rodents, dopamine increases SRIF release and inhibits GHRH release. Mice that have elevated levels of dopamine as a result of genetic disruption of the dopamine transporter also exhibit dwarf phenotypes with reduced GHRH mRNA content. In contrast to rodents, humans demonstrate reduced levels of SRIF following dopamine treatment. The effect of serotonin on GHRH secretion is also species-specific. Serotonin stimulates the release of GHRH in rats but inhibits it in sheep.

| | Hypothalamus | |
|---|---|---|
| | GHRH secretion | SRIF secretion |
| **SRIF** | ↓ | |
| **GHRH** | | ↑ |
| **Epinephrine** | ↑ | |
| **Norepinephrine** | ↑ | |
| **Dopamine** | ↓ | ↑ |
| **Serotonin** | ↑ | |
| **Galanin** | ↑ | ↓ |
| **GABA** | ↑ | |
| **Opioids** | | ↓ |

FIGURE 4 Neuroendocrine regulation of GHRH and SRIF secretion in rodents. Arrows indicate changes in GHRH and/or SRIF secretion in response to a variety of hormones or neurotransmitters.

### B. Hormonal Feedback Regulation

Hormones of the GH axis, including GH and IGF-I, act on the hypothalamus and pituitary to attenuate the GH response to GHRH (see Fig. 5). GH inhibits GHRH synthesis and secretion in the hypothalamus. In transgenic mice overexpressing human GH, GHRH mRNA synthesis is decreased. In contrast, GHRH mRNA synthesis is increased in the absence of GH following hypophysectomy. These inhibitory effects of GH on GHRH synthesis and secretion in the hypothalamus may be mediated by SRIF input, which is released from SRIF neurons following GH stimulation. In the pituitary, GH acts on somatotroph cells to inhibit GH synthesis, thereby establishing a negative regulatory loop. Similarly to GH, IGF-I reduces GHRH mRNA content in the hypothalamus. IGF-I also inhibits GH synthesis and GHRH-induced GH secretion from the pituitary.

### C. Metabolic Cues

Nutritional status and metabolism affect GHRH synthesis and secretion through the actions of thyroid hormones, leptin, ghrelin, and glucocorticoids (see Fig. 5). Thyroid hormones decrease GHRH synthesis and secretion. The ability of thyroid hormones to stimulate GH synthesis may mediate this effect. In hypothyroidism, the reduction in GH synthesis would result in a compensatory increase in GHRH synthesis and secretion. In contrast, GHRH synthesis

| | Hypothalamus | | Pituitary | |
|---|---|---|---|---|
| | GHRH synthesis | GHRH secretion | GHRH-R mRNA expression | GHRH-induced GH secretion |
| **GH** | ↓ | ↓ | ↓ | |
| **IGF-I** | ↓ | ↓ | ↓ | ↓ |
| **Ghrelin** | | ↑ | | ↑ |
| **Leptin** | ↑ | ↑ | | ↑ |
| **Thyroid hormone** | ↓ | ↓ | ↑ | |
| **Glucocorticoids** | ↓ | ↓ | ↑ | |
| **Testosterone** | ↑ | ↑ | | ↑ |
| **Estrogen** | ↓ | ↓ | ↓ | ↓ |

FIGURE 5 Hormonal regulation of the GH axis in rodents. Arrows indicate changes in hypothalamic synthesis and secretion of GHRH and pituitary responsiveness to GHRH in response to a variety of peptide and steroid hormones.

and secretion would be reduced in hyperthyroidism as a result of the elevated GH levels.

Leptin is a hormone released from white adipose tissue that signals satiety to the hypothalamus. In addition to down-regulating mRNA for Neuropeptide Y, a potent stimulator of food intake, leptin increases GHRH synthesis and decreases SRIF synthesis in the hypothalamus. Leptin also enhances GHRH secretion from the hypothalamus and GHRH-induced GH release from somatotroph cells of the pituitary. By increasing GHRH synthesis and GH secretion, leptin may reduce glucose uptake by adipose tissue through the actions of GH and, thereby, inhibit fat deposition.

Ghrelin is a hormone that is released from the stomach and that stimulates food intake, adipogenesis, and GH release from the pituitary. GH secretion is partly stimulated by GHRH released from the hypothalamus following ghrelin stimulation. In addition, ghrelin acts directly on somatotroph cells of the anterior pituitary to stimulate GH secretion. Interestingly, somatotroph cells release GH synergistically following GHRH and ghrelin stimulation.

Glucocorticoids are important regulators of the GH axis. They have a suppressive effect on growth, but they have variable effects on individual hormones in the GH pathway. Glucocorticoids reduce GHRH synthesis and content in the arcuate nucleus of the hypothalamus, suggesting that glucocorticoids negatively regulate hypothalamic expression and content of GHRH.

### D. Sex Steroids

Regulation of GHRH by sex steroids may be responsible for the distinct sexual dimorphism in GH secretion. Males exhibit GH secretion profiles characterized by low levels of basal GH release punctuated with regular large-amplitude pulses. In contrast, females have high levels of basal GH secretion with irregular small-amplitude pulses. Studies in rodents have shown that the unique male pattern of GH secretion is the result of neonatal imprinting by androgens. Sex steroids influence mRNA expression, hypothalamic content, and secretion of both GHRH and SRIF (see Fig. 5). Males demonstrate higher levels of mRNA synthesis and content of GHRH and SRIF than females.

Correspondingly, there is a greater release of GHRH from the hypothalamus of males than females. Testosterone treatment increases GHRH synthesis and secretion in the hypothalamus. Furthermore, neonatal testosterone treatment increases the population of hypothalamic GHRH neurons. GHRH synthesis is reduced following castration, an effect that is reversed with testosterone replacement. Contrary to the effects of testosterone treatment, estrogen treatment reduces the hypothalamic content of GHRH in male and female rats. Following ovariectomy, female rats demonstrate elevated levels of GHRH mRNA expression, an effect that is reversed with estrogen treatment.

## IV. GHRH RECEPTOR

### A. Characterization

Two different approaches were used to isolate the GHRH receptor: (1) polymerase chain reaction amplification of pituitary cDNAs using degenerate oligonucleotides designed using the sequences of the receptors for secretin, parathyroid hormone, and calcitonin and (2) low-stringency cDNA library hybridization using probes for these receptors. The receptor has now been isolated from human, rat, mouse, pig, sheep, goat, cow, and goldfish. It is a G-protein-coupled receptor (GPCR) that binds a stimulatory heterotrimeric G-protein and activates adenylyl cyclase to increase production of the cellular second messenger cyclic AMP (cAMP). Based on amino acid sequence identity, the GHRH receptor belongs to the B-III family of GPCRs, which includes the receptors for brain–gut peptides. Some of these receptors are closely related to the GHRH receptor, but other receptors are more distantly related, including the receptors for calcitonin, corticotropin-releasing factor, and parathyroid hormone (see Fig. 6). The hallmark characteristics of the B-III family

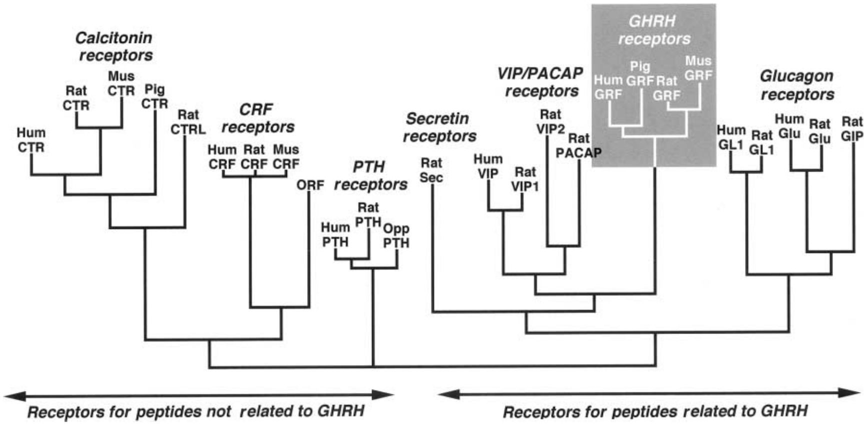

**FIGURE 6** Phylogenetic tree of the B-III family of GPCR receptors that includes the GHRH receptor. Relatedness of the GHRH receptor to the receptors for secretin, VIP, PACAP, glucagon, parathyroid hormone (PTH), corticotropin-releasing factor (CRF), and calcitonin is illustrated. This figure was adapted from the GPCR database (http://swift.embl-heidelberg.de/7tm). ORF, open reading frame.

include complex gene structures and six conserved cysteine residues in the extracellular amino-terminus.

Although the GHRH receptor has a high degree of amino acid sequence identity among species, the length of the receptor is quite variable. In human, rat, mouse, and cow, the GHRH receptor is 423 amino acids long. The pig receptor is 451 amino acids long and the sheep and goat receptors are 407 amino acids long. Interestingly, the last 16 amino acids in the human GHRH receptor, missing in sheep and goat, is reported to inhibit receptor signaling by attenuating cAMP production.

## B. Gene Structure and Tissue Expression

The gene for the GHRH receptor is located on mouse chromosome 6 and human chromosome 7p14–15. In the rat, the genomic structure of the GHRH receptor consists of 14 exons spanning 15 kb with exons ranging in size from 42 to 456 bp, interrupted by introns ranging in size from 111 bp to more than 2 kb. A longer isoform of rat GHRH receptor is generated by alternative splicing of exon 11 so that 41 amino acids are inserted into the third intracellular loop (see Fig. 7). The short and the long receptor isoforms bind GHRH with similar affinity, but only the short isoform stimulates cAMP production. The long receptor isoform has yet to be associated with a cellular second-messenger pathway, but other receptors in the brain–gut peptide family have alternative isoforms that signal through different second-messenger pathways. For example, two isoforms of the PACAP receptor act distinctly through $G_{\alpha s}$ or $G_{\alpha q}$ to increase cAMP production or to stimulate phospholipase C hydrolysis, respectively. Additional GHRH receptor isoforms have been identified in mouse, pig, and human. The functional significance of these additional isoforms has yet to be clarified within the context of normal physiology. Interestingly, an alternative GHRH receptor isoform, found in human pituitary adenomas, is truncated in the third intracellular loop. It is reported that this receptor isoform is unable to stimulate cAMP production but inhibits cAMP production by the full-length GHRH receptor, suggesting that this alternative isoform may act as a dominant negative protein.

Using sensitive reverse transcriptase-polymerase chain reaction techniques, GHRH receptor mRNA has been detected in the pituitary, gonads, placenta, kidney, hypothalamus, and other brain regions. Of these regions, GHRH receptor (GHRH-R) mRNA is expressed predominantly in the anterior pituitary. This is likely to be due to the dependence of GHRH-R gene transcription on the pituitary-specific transcription factor Pit-1. Pit-1 is a POU domain-containing transcription factor that regulates GH transcription and terminal differentiation of somatotroph, lactotroph, and thyrotroph cells. Consistent with this finding, expression of GH and the GHRH receptor on day 18 during rat embryonic development follows Pit-1 expression on day 13.5. Potential binding sites for Pit-1 have been identified in the promoter regions of both the rat and the human GHRH receptor genes. These sites confer Pit-1-dependent expression of the GHRH receptor promoter in pituitary cell lines that express endogenous Pit-1 or in heterologous cell lines transfected with Pit-1.

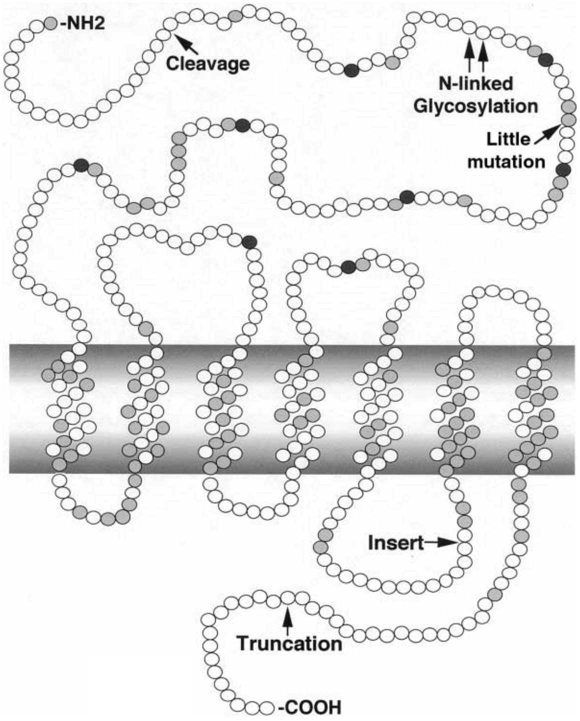

**FIGURE 7** Schematic representation of the rat GHRH receptor. The seven membrane-spanning domains are shown crossing the lipid bilayer. The gray circles represent conserved amino acids found in the related receptors for secretin, VIP, GIP, glucagon, PACAP, parathyroid hormone, and calcitonin. The black circles represent the conserved cysteine residues; those in extracellular loops 1 and 2 are found in most GPCRs, whereas those in the N-terminal domain are unique to the GPCRs in the B-III family. The arrowheads indicate the putative signal-peptide cleavage site, the consensus sites for N-linked glycosylation, the conserved aspartic acid residue at position 60, which is mutated in the *little* mouse, the insertion site for the long receptor isoform, and the truncation of the last 16 amino acids in the sheep and goat receptors.

### C. Receptor Domains That Interact with GHRH

Hormone binding by the GHRH receptor and related family B-III GPCRs has been investigated through the analysis of mutant and chimeric receptors. Although discrete ligand-binding domains have not been identified, these studies suggest that determinants in both the amino-terminal extracellular domain of the receptor and the three extracellular loops contribute to specific and high-affinity hormone binding. Receptor truncations reveal that although the amino-terminal extracellular domain does not appear to be sufficient for GHRH binding, it is necessary. The importance of the amino-terminus of the receptor for GHRH binding is further supported by studies of the *little* mouse. The *little* mouse exhibits a dwarf phenotype and GH deficiency as a result of a naturally occurring missense mutation that converts amino acid 60 in the amino-terminus of the GHRH receptor from an aspartic acid to a glycine residue (see Fig. 7). This missense mutation disrupts GHRH binding and receptor signaling. A direct interaction between GHRH and the amino-terminus of the GHRH receptor has been demonstrated in mammalian cells using protein cross-linking approaches and in yeast cells using the two-hybrid protein interaction system.

## V. GHRH RECEPTOR SIGNALING

### A. Protein Kinase A Activation of GH Secretion and Synthesis

GHRH acts through protein kinase A (PKA) to catalyze membrane depolarization and calcium influx, which is required for GH release (see Fig. 8). Specifically, nonselective cation currents and voltage-gated calcium currents are increased by PKA to induce membrane depolarization and to facilitate GH secretion, respectively. Although GHRH stimulates $Ca^{2+}$ influx in pituitary cells, it has no effect on the release of calcium from intracellular stores. Consistent with this finding, some studies have reported that the GHRH receptor does not activate the phospholipase C second-messenger pathway, which mediates protein kinase C activation and intracellular calcium mobilization. In contrast, a recent study of the pig anterior pituitary found two subpopulations of somatotroph cells, distinguishable by the ability of GHRH to activate either adenylyl cyclase or phospholipase C.

As addressed earlier, GH gene expression requires the transcription factor Pit-1 (see Fig. 8). Signaling through cAMP increases Pit-1 transcription and expression. This effect is mediated by the cAMP-responsive element-binding protein (CREB), which binds two cAMP-responsive elements in the Pit-1 promoter. Another mechanism through which cAMP may regulate Pit-1 function includes direct interaction between the CREB-binding protein (CBP) and Pit-1. Activation of Pit-1 by cAMP depends on the amino-terminal domain of CBP. Interestingly, PKA phosphorylation may regulate the interaction between CBP and Pit-1.

### B. Somatotroph Cell Proliferation and the Mitogen-Activated Protein Kinase Pathway

In addition to stimulating GH synthesis and secretion, GHRH induces somatotroph cell proliferation. The initial observation that GHRH stimulates

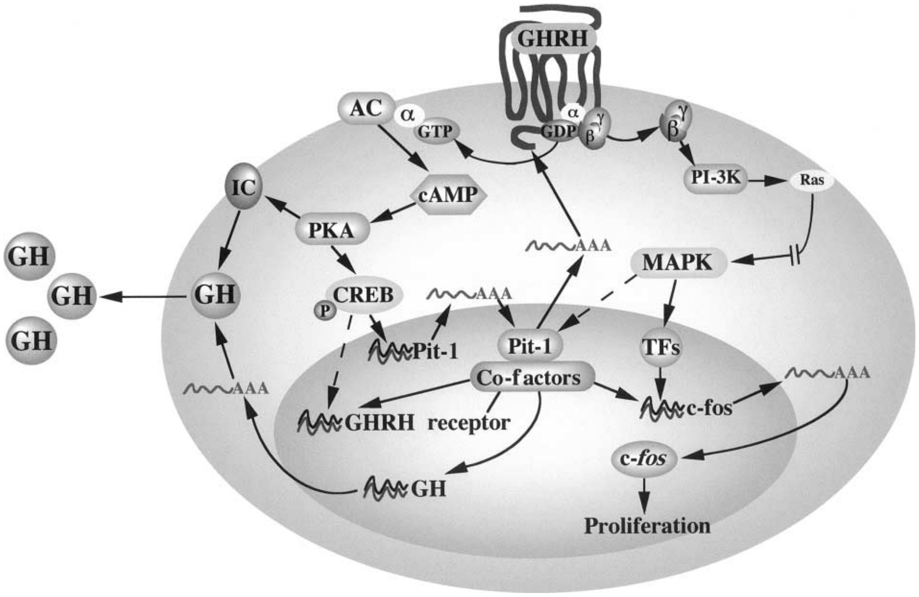

**FIGURE 8** The GHRH signaling pathway in the pituitary somatotroph cell. Binding of GHRH to its receptor causes the dissociation of the G-protein into the β γ-subunits and the GTP-bound α-subunit. The α-subunit activates adenylyl cyclase to increase the production of cAMP, which in turn releases the catalytic subunits of protein kinase A (PKA) from its regulatory subunits. PKA acts on ion channels (IC) to facilitate GH release. Additionally, PKA phosphorylates and thus activates cAMP-response element-binding protein (CREB). Activated CREB increases transcription of the pituitary-specific transcription factor Pit-1. CREB may also increase transcription of the GHRH receptor gene. Pit-1 interacts with co-factors to increase the transcription of the GH and GHRH receptor genes. In addition to stimulating GH synthesis and secretion, GHRH activates the mitogen-activated protein kinase (MAPK) pathway that may underlie the ability of GHRH to stimulate somatotroph cell proliferation. MAPK activation by GHRH may occur in part through the actions of the β γ-subunits, phosphoinositol 3-kinase (PI-3K), and Ras. Transcription factors (TFs) activated by MAPK increase transcription of a variety of genes involved in cell proliferation, including c-*fos*. Transcription of c-*fos* may also be stimulated by Pit-1.

somatotroph cell proliferation was described in human case studies that reported somatotroph cell hyperplasia resulting from ectopic secretion of GHRH from endocrine tumors. Further validation of this observation comes from a variety of genetic studies in mice. Overexpression of human GHRH in a transgenic mouse model results in excessive proliferation of somatotroph cells. In contrast, the number of somatotroph cells is significantly reduced in the anterior pituitary of the *little* mouse, which has disrupted GHRH receptor signaling, as previously discussed. Genetic models have also addressed somatotroph cell proliferation in the context of disrupting intermediaries in the GHRH signaling pathway. For example, constitutive activation of the α-subunit of the stimulatory heterotrimeric G-protein following selective targeting of a cholera toxin transgene results in hyperproliferation of somatotroph cells. In contrast, there is a hypoproliferation of somatotroph cells in the anterior pituitary of transgenic mice in which a dominant negative CREB protein was targeted to somatotroph cells. GHRH induces expression of c-*fos*, a transcription factor associated with proliferation, and somatotroph cell proliferation in cultured pituitary cells.

Detailed studies in both pituitary and nonpituitary cell lines have shown that GHRH receptor activation stimulates the mitogen-activated protein kinase (MAPK) pathway. It has been reported that this activation is partly mediated through the actions of the β γ-subunits of the heterotrimeric G-protein, phosphatidylinositol 3-kinase, and $p21^{ras}$ (see Fig. 8). Pituitary cells proliferate following activation of the MAPK pathway by GHRH, an effect that is blocked by co-treatment of the cells with MAPK inhibitors or SRIF.

The transcription factor Pit-1 is also required for somatotroph cell proliferation. Mutations in Pit-1 that disrupt its ability to bind DNA result in hypoplastic anterior pituitaries lacking somatotroph, lactotroph, and thyrotroph cell types. This finding from Snell and Jackson dwarf mice suggests that

Pit-1 is important in the expansion of pituitary cell types that normally express Pit-1. As previously addressed, Pit-1 expression can be regulated by CREB. Inhibition of somatotroph cell proliferation by the dominant negative CREB transgene mentioned above may therefore be the result of reduced Pit-1 expression. Consistent with this notion, inhibition of Pit-1 expression by antisense oligonucleotides significantly reduces proliferation in pituitary cell lines. Pit-1 may regulate somatotroph cell proliferation in part by binding the serum-response element in the c-*fos* promoter and activating transcription.

## VI. REGULATION OF THE GHRH RECEPTOR

### A. Hormonal Feedback Regulation by the GH Axis

GH and IGF-I inhibit GHRH receptor mRNA expression (see Fig. 5). When GH signaling is disrupted, as demonstrated in two rodent models with GH receptor deficiencies, there is an increase in GHRH receptor mRNA expression. These findings suggest that GH negatively regulates GHRH receptor mRNA expression. When pituitary cells are treated with IGF-I, GHRH binding and GHRH-stimulated GH secretion are significantly reduced, indicating that IGF-I also negatively regulates the GHRH receptor.

GHRH-binding activity in the pituitary is significantly reduced following prolonged exposure to GHRH. This reduction in binding activity may arise from receptor desensitization and internalization. Receptor desensitization, or the process of attenuating the ability of GPCRs to activate heterotrimeric G-proteins, is often followed by receptor internalization. Currently, no studies with the cloned receptor have addressed GHRH receptor desensitization and internalization, but studies on the related receptors for secretin and glucagon have described receptor inactivation by these processes.

Whether homologous down-regulation of GHRH receptor mRNA expression occurs following prolonged ligand exposure is controversial. Whereas one study concluded that GHRH up-regulates receptor mRNA expression, another study found that GHRH down-regulates receptor expression. In yet another study, the inhibitory actions of GHRH on receptor expression were made evident only when SRIF was immunoneutralized, suggesting that homologous down-regulation of the GHRH receptor is obscured by the inhibitory effects of SRIF on receptor expression. Interestingly, GHRH has been localized to secretatory granules of somatotroph cells, suggesting a possible autocrine role for GHRH in perpetuating GH secretion and expediting receptor down-regulation.

### B. Metabolic Cues

Nutritional status and metabolism affect the expression of the GHRH receptor through the actions of thyroid hormones and glucocorticoids (see Fig. 5). Pituitary cells treated with thyroid hormones demonstrate an increase in GHRH receptor mRNA expression. Correspondingly, the loss of thyroid hormones after thyroidectomy significantly reduces GHRH receptor mRNA expression, but normal levels can be restored with thyroid hormone replacement. Similar to thyroid hormones, glucocorticoids positively regulate GHRH receptor mRNA expression. The loss of endogenous glucocorticoids after adrenalectomy reduces the number of GHRH-binding sites, and these sites are restored by glucocorticoid replacement. Interestingly, glucocorticoids and thyroid hormones act synergistically to up-regulate GHRH receptor mRNA expression. Glucocorticoids are also important differentiating factors for somatotroph cells. Glucocorticoid treatment of rat fetuses advances expression of GH and GHRH receptor mRNAs, both hallmark transcripts of the somatotroph cell phenotype. In contrast, inhibition of glucocorticoid synthesis delays expression of GH and GHRH receptor mRNAs in the developing rat fetus.

### C. Sex Steroids

Sex steroids can influence pituitary responsiveness to GHRH (see Fig. 5). Specifically, estrogen inhibits GHRH-induced GH release and GHRH receptor expression in the pituitary. Consistent with this finding, the promoter regions of both the rat and the human GHRH receptors contain estrogen-response elements. In some studies, a sexual dimorphism in GHRH-R expression has been reported, with males expressing elevated levels of the mRNA compared to females.

## VII. GHRH AND THE GHRH RECEPTOR IN DISEASE

As mentioned earlier, acromegaly is an endocrine disorder resulting from adult-onset excessive GH secretion. Symptoms include broadening of the brow and chin, exaggerated growth of hands and feet, hypertrophy of soft tissue, headaches, fatigue, visual abnormalities, and sleep apnea. Most cases of acromegaly are the result of GH-secreting pituitary adenomas. Many of these pituitary adenomas arise from a mutation in the $G_{\alpha s}$ protein (*gsp*) so that the stimulatory α-subunit is constitutively activated and chronically stimulates adenylyl cyclase to produce cAMP in somatotroph cells. Other cases of acromegaly are the result of excessive GHRH secretion from hypothalamic gangliocytomas or ectopic GHRH-secreting tumors. Hypothalamic gangliocytomas secrete GHRH into the hypophyseal portal vessels and promote somatotroph cell proliferation and adenoma formation. Ectopic GHRH-secreting tumors have been found in pancreatic, pulmonary, and intestinal tissues. Few of these tumors secrete the full-length GHRH(1–44)-$NH_2$ product, but rather secrete the truncated products GHRH(1–37)-OH and (1–40)-OH. Excision of ectopic GHRH-secreting tumors often leads to full regression of acromegalic symptoms. Conditions of GHRH excess can also be created in a transgenic mouse model in which human GHRH is overexpressed. In this model, mRNA for the transgene is expressed in all tissues, but only tissues that normally express GHRH such as the pituitary, pancreas, and brain produce the mature GHRH(1–44)-$NH_2$ and (1–40)-OH forms. The remaining tissues produce the inactive GHRH metabolite, GHRH(3–44)-OH. The phenotype of this transgenic mouse model includes gigantism, somatotroph hyperplasia, and pituitary adenoma formation in the older mice. In contrast, pituitary adenoma formation is not found in cases of acromegaly resulting from ectopic GHRH-secreting tumors.

Isolated GH deficiency (IGHD) is an endocrine disorder with symptoms that include dwarfism and low levels of circulating GH, IGF-I and IGF-II. An excellent animal model of IGHD is the *little* mouse. In addition to the above-mentioned symptoms, the *little* mouse exhibits a hypoplastic anterior pituitary that is deficient in somatotroph cells. As discussed in a previous section, this is the result of an autosomal recessive mutation that converts an aspartic acid residue at position 60 in the amino-terminus of the GHRH receptor to a glycine residue. This mutation renders the GHRH receptor incapable of binding GHRH and activating downstream signaling pathways. Introduction of a GH transgene in the *little* mouse produces normal or excessive growth. Although not responsive to GHRH treatment, somatotroph cells cultured from *little* mice secrete GH in response to cAMP analogues, suggesting that the *little* mouse has an intact cAMP signaling pathway capable of eliciting GH secretion. More recently, human studies have reported cases of IGHD arising from mutations in the GHRH receptor. Three kindreds from India, Sri Lanka, and Pakistan have an identical point mutation in the GHRH receptor, which truncates the receptor before the first transmembrane domain. A kindred in Brazil has a donor splice mutation in the receptor that also produces a truncated receptor. All of these patients have short stature, anterior pituitary hypoplasia, and undetectable serum GH concentrations. These patients are unresponsive to GHRH treatment, but they show an increase in growth following GH therapy. These studies have described IGHD in the context of inactivating mutations of the GHRH receptor. Currently, mutations that render the GHRH receptor constitutively active have not been reported, although several studies have looked for such mutations in human GH-secreting tumors.

GHRH has been implicated in promoting tumor proliferation. GHRH acts in an endocrine manner to stimulate tumor proliferation by elevating the circulating levels of GH and subsequently IGF-I, and it may also act in an autocrine manner by directly stimulating tumor tissue. A variety of tumor cell lines locally produce GHRH and express GHRH-binding sites. GHRH treatment of these cell lines usually results in an increase in cAMP production and proliferation. Additionally, some cell lines increase the synthesis and secretion of IGF-I and/or IGF-II, which may then act in an autocrine regulatory manner. Interestingly, GHRH receptor antagonists are effective inhibitors of proliferation in most of these tumor cell lines, including those from ovarian, mammary, prostatic, and pancreatic cancers. These findings suggest that GHRH antagonists may provide a novel approach to the treatment of endocrine cancers.

## Glossary

**alternative splicing** Rearranging the same pre-mRNA in multiple ways to yield multiple different mRNAs that produce distinct proteins.

**autocrine** Mechanism of regulation in which a released hormone acts on its cell of origin.

**dominant negative protein** A defective protein that interferes with the function of the normal protein in the same cell.

**hypophysectomy** Surgical removal of the pituitary gland.

**somatotroph cells** One of the six distinct hormone-producing cell types present in the anterior pituitary. These cells synthesize and secrete growth hormone.

**synergism** The action of two or more hormones to achieve a response that each hormone is incapable of achieving individually.

## See Also the Following Articles

**Growth Hormone (GH) • Growth Regulation: Clinical Aspects of GHRH • Insulin-like Growth Factor (Igf) Signaling • Prolactin and Growth Hormone Receptors • Somatostatin**

## Further Reading

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# Growth Regulation: Clinical Aspects of GHRH

A. V. B. Castro and S. Melmed
*Cedars Sinai Medical Center, Los Angeles*

I. INTRODUCTION: GROWTH AND GH REGULATION
II. GROWTH HORMONE-RELEASING HORMONE
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**Linear growth results from the interaction of genetic and environmental factors. The key hormone involved in the growth process during these phases is insulin-like growth factor, controlled directly or indirectly by peripheral and central influences. Growth hormone is the principal insulin-like growth factor regulator during the postnatal period.**

## I. INTRODUCTION: GROWTH AND GH REGULATION

Appropriate growth hormone (GH) pulsatile secretory patterns are essential for physiologic GH effects and occur as a result of the interplay between growth hormone-releasing hormone (GHRH) and somatotropin release-inhibiting factor (SRIF; somatostatin) on pituitary somatotrophs, as well as additional peripheral and central regulators. Physiologic and clinical roles of GHRH and its receptor on growth have been elucidated in sporadic and transgenic models in which they may be overexpressed or mutated. Synthetic GHRH has been used for diagnostic and therapeutic purposes in growth disorders.

The GH pulsatile pattern is important for exerting an effect on target tissues, and the mechanisms involved in GH pulse generation are complex. GH pulses depend on interactions between hypothalamic GHRH and SRIF and the on-modulatory role of ghrelin, an endogenous GH secretagogue. Neurotransmitters, including NO, neuropeptides, hormones (hypothalamic, pituitary, or peripheral), cytokines, and metabolic signals, influence GHRH and SRIF. In addition, long, short, and ultrashort insulin-like growth factor-I (IGF-I) feedback loops also regulate GH secretion. GH pulses (10 to 20 pulses/24 h) occur concomitantly with GHRH pulses and are assumed to be 180° out of phase with hypothalamic secretory SRIF pulses. Intermittent SRIF withdrawal and/or the release of GH cosecretagogues drive GH pulse timing, and the pulse amplitude is generated primarily by GHRH. GH secretion maintains a nocturnal rhythm in which the bulk (70%) of daily GH secretion occurs during the slow-wave of sleep stages 2 and 3.

GH pulse amplitudes vary throughout life; they are high in the fetus and at birth, decline gradually during infancy, increase significantly at puberty, return to prepubertal levels during early adulthood, and decline progressively beyond middle age. Mechanisms involved in GH pulse variability involve variations in somatotroph sensitivity to GHRH or somatostatin, which is modified by other hormones, including GH secretagogues, sex steroids, and neurotransmitters. In the elderly, the GH secretory capability of the somatotroph is preserved given appropriate external stimuli, such as administration of GHRH, somatostatin inhibitors, or GH secretagogues (GHSs). These hypothalamic influences determine age-related GH secretion patterns.

Sexual dimorphism also characterizes GH secretion patterns. Men and premenopausal women have similar GH pulse frequencies, but premenopausal women exhibit higher baseline GH levels and GH pulse amplitudes. Women are also less prone to the negative impact of GH secretion modifiers, such as age, obesity, and physical fitness. These sexual differences in GH secretion pattern also influence hepatocyte GH signaling. GH signaling is mediated by signal transducer and activator of transcription proteins (Stat3 and Stat5) and is terminated by expression of the suppressor of cytokine signaling (SOCS) protein. A male pulsatile GH pattern elicits increased intracellular Stat levels and an intermittent increase in SOCS levels, which leads to transient hepatocyte unresponsiveness to GH. The more steady female GH secretion pattern elicits more prolonged GH unresponsiveness due to continuous induction of intrahepatic SOCS levels. Other influences that increase GH secretory patterns are exercise, stress, and hypoglycemia (Fig. 1). Conversely, hyperglycemia suppresses GH secretion, as do obesity and hypothyroidism.

## II. GROWTH HORMONE-RELEASING HORMONE

GHRH is encoded by a 10-kb gene that contains five exons. Exon 3 segregates the 1–31 sequence responsible for the functional domain of the molecule. GHRH is produced predominantly by neurons localized in hypothalamic arcuate and ventromedial nuclei and is also modestly expressed in normal leukocytes and normal (pituitary, gonadal, adrenal, placental, gut, pancreatic) or tumoral (pituitary, breast, endometrial, and gastrointestinal) tissues.

### A. Structure

The size of the active isoform varies from 42 to 44 amino acids among species, differing mainly in the extension and composition of the carboxy-terminal (COOH) region. Conversely, the amino terminus ($NH_2$), specifically the highly conserved (1–29) residues, plays a key role for the full GH-evoking activity of the peptide, as also does the GHRH tertiary structure (Fig. 2).

Human GHRH is derived from cleavage of a precursor molecule, preproGHRH (containing 107–108 amino acids), yielding a proGHRH molecule that is cleaved by endoproteases into human (h) hGHRH (1–40)-$NH_2$, hGHRH(1–44)-OH, and a carboxyl-terminal peptide designated GHRH-related peptide (GHRH-RP). After release into the circulation, GHRH is rapidly (within minutes) metabolized to inactive peptide by circulating dipeptidylpeptidase IV. The three sequenced GHRH isoforms, GHRH(1–44)-$NH_2$, GHRH(1–40)-OH, and GHRH(1–37)-OH, differ in their C-terminal extension.

### B. Function

GHRH signaling through the GHRH receptor directly controls somatotroph proliferation and function. The potent GHRH proliferative effect on somatotrophs is corroborated by the observation of pituitary enlargement within days after GHRH

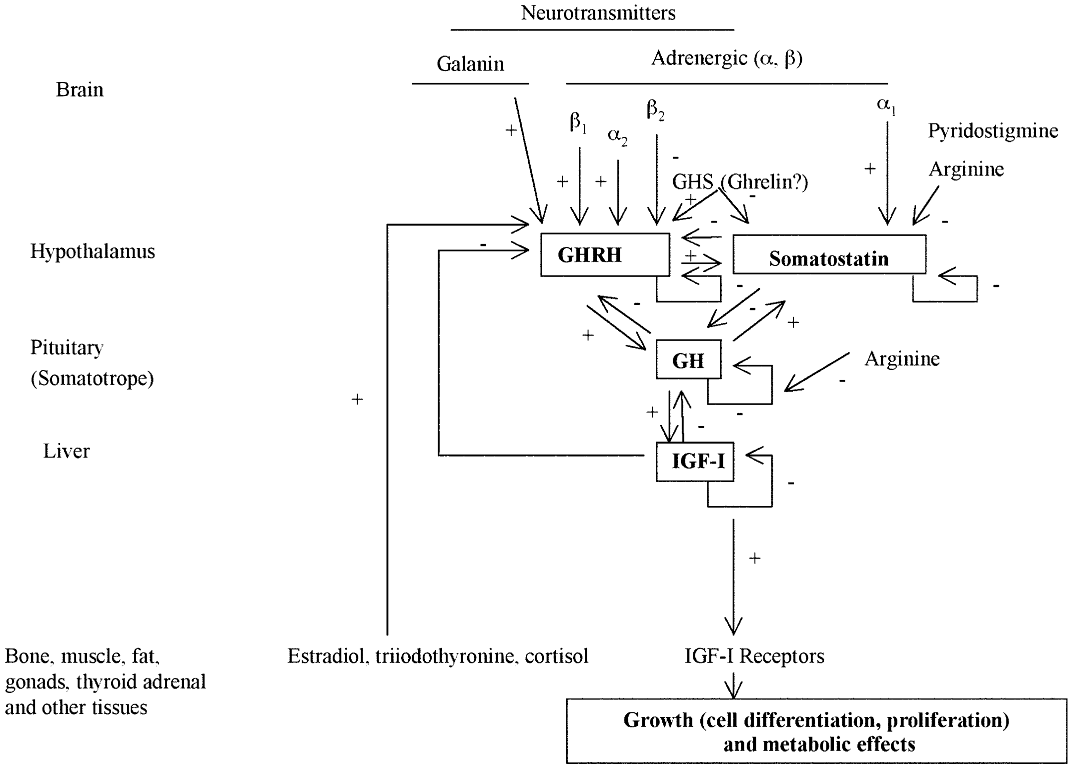
Neurotransmitters
Galanin
Adrenergic (α, β)
Brain
β1
α2
β2
α1
Pyridostigmine
Arginine
GHS (Ghrelin?)
Hypothalamus
GHRH
Somatostatin
Pituitary
(Somatotrope)
GH
Arginine
Liver
IGF-I
Bone, muscle, fat, gonads, thyroid adrenal and other tissues
Estradiol, triiodothyronine, cortisol
IGF-I Receptors
Growth (cell differentiation, proliferation) and metabolic effects

FIGURE 1 Central and peripheral influences on the growth hormone axis, showing the systemic involvement in growth control. Positive (+) and negative (−) neuroendocrine feedbacks contribute to the ultimate secretion of the main components of the growth axis (boxed). Also shown are the arginine and pyridostigmine sites of action. Adapted from Cunha *et al.* (2000) and Giustina and Veldhuis (1998).

administration to normal rats. Furthermore, the direct effect of GHRH in pituitary proliferation is reinforced by the observation of somatotroph hyperplasia and adenomatous transformation in GHRH transgenic mice lacking the GH receptor (MT-hGHRH/GH-R − /−). A paracrine/autocrine role of GHRH is also suggested by a direct correlation between neoplastic progression of somatotroph adenomas and pituitary GHRH content. GHRH also controls GHRH-R, GHS-R, and ghrelin gene expressions, and placental GHRH may play a role in fetal growth.

### C. Regulation

Glucocorticoids (acutely administrated), sex steroids, triiodothyronine, and GH-releasing peptide may amplify GHRH-stimulated GH secretion. *In vivo*, glucocorticoid administration decreases hypothalamic GHRH expression. GHRH expression is also down-regulated by GHRH, somatostatin, and GH/IGF-I.

## III. GHRH RECEPTOR

The GHRH-R belongs to the G-protein-coupled secretin subfamily B; it is mainly expressed in the pituitary, but also in the placenta, gonads, kidney, and hypothalamus.

### A. Structure

The human GHRH-R gene, mapped to the short arm of chromosome 7p14, comprises more than 10 exons spanning 15 kb of DNA. The cDNA encodes a 47-kDa, 423-amino-acid protein containing seven putative transmembrane domains, characteristic of secretin family receptors. The N-terminal extracellular domain plays an important role in GHRH binding. Single mutations or deletions at position 60 of this domain, for example, render the receptor nonfunctional. Other domains are also important for ligand selectivity and binding. Two variants of GHRH-R mRNA have been described in a human bronchial tumor; the truncated nonfunctional isoform was related to the less aggressive behavior of neuroendocrine tumors.

## B. Signaling

At the somatotroph, GHRH binding to its receptor activates the G ($G_{\alpha}$) stimulatory protein, which sequentially stimulates adenylyl cyclase and cellular cyclic adenosine monophosphate (cAMP). Cyclic AMP binds to cAMP response element binding (CREB) protein, mediating expression of Pit-1 and GH gene transcription. Activation of the mitogen-activated protein kinase (MAPK) pathway by GHRH-R-stimulated βγ subunits accounts for the proliferative actions of GHRH. Other pathways involved in GHRH signaling include inositol phosphate, calcium influx and intracellular mobilization, and calmodulin (Fig. 2).

## C. Function

At early stages of pituitary development, several homeodomain transcription factors are active. Among them, factors named prophet of pituitary transcriptional factor-1 (Prop-1) and POU1F1 are critical for specialization of cell lineages, including thyrotrophs, gonadotrophs, somatotrophs, and mammosomatotrophs. Full differentiation of these cells is achieved by the permissive effect of other transcriptional and nontranscriptional factors. Somatotrophs, for example, require the presence of GHRH-R for their proliferation. GHRH stimulates expression of POU1F1, which plays an important role in GHRH-R expression.

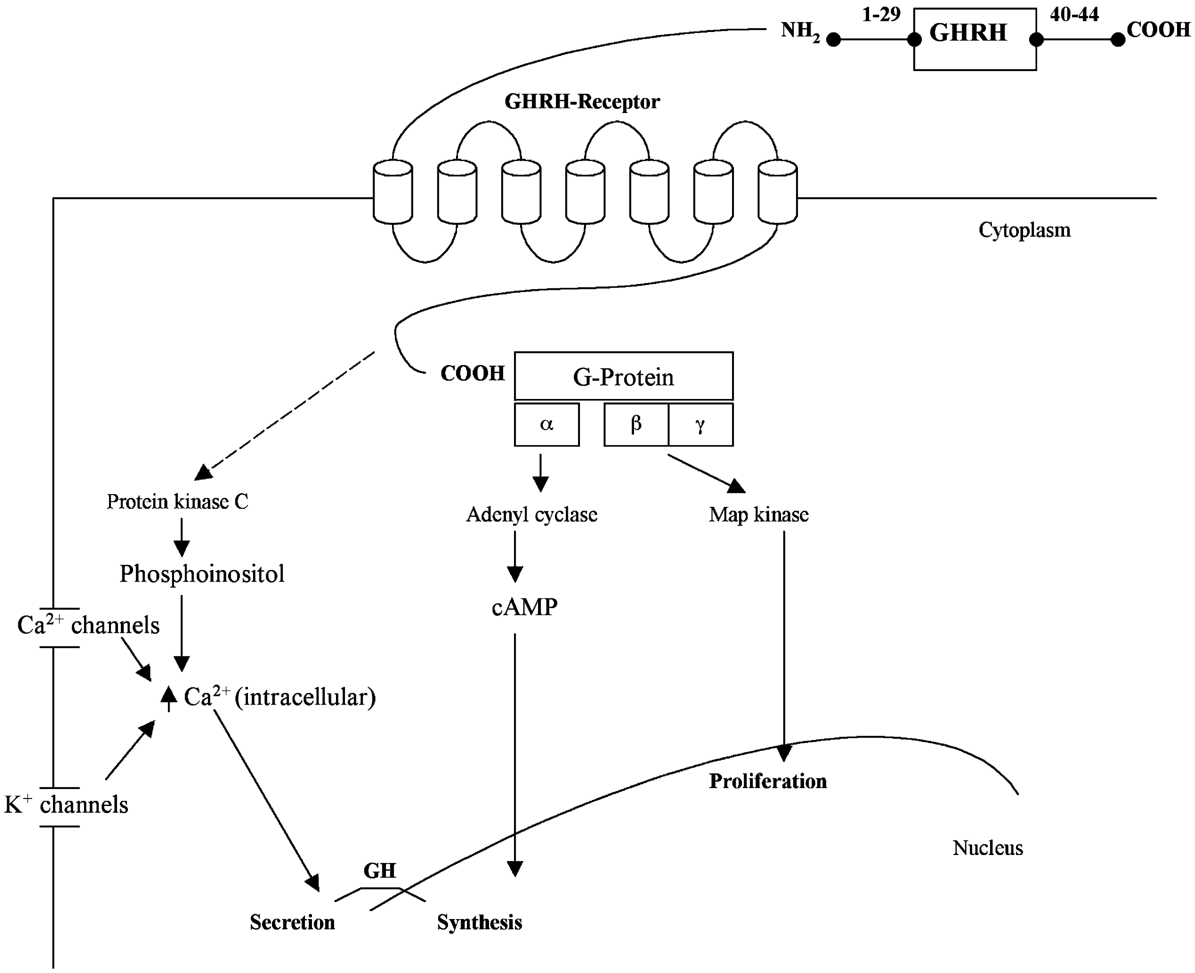
$NH_2$
1-29
GHRH
40-44
COOH
GHRH-Receptor
Cytoplasm
COOH
G-Protein
α
β
γ
Protein kinase C
Phosphoinositol
Adenyl cyclase
cAMP
Map kinase
$Ca^{2+}$ channels
$Ca^{2+}$ (intracellular)
$K^+$ channels
Proliferation
Nucleus
GH
Secretion
Synthesis

FIGURE 2 Growth hormone-releasing hormone (GHRH) receptor and GHRH signaling in the somatotroph, illustrating the seven-transmembrane domain of the GHRH receptor; its amino-terminal ($NH_2$) domain, which binds to GHRH (at Asp-60), and the carboxyl-terminal (COOH) domain (linked to G-protein). Binding of GHRH to its receptor elicits activation of the α and βγ G-protein subunits, which activate the cAMP and mitogen-activated protein (MAP) kinase cascades, leading to GH synthesis and somatotroph proliferation, respectively. GHRH also activates protein kinase C and opens ion channels, resulting in an increase in intracellular calcium ($Ca^{2+}$) and GH secretion.

## D. Age-Related GHRH and GHRH-R

Human fetal GHRH neurons appear between 18 and 29 weeks of gestation. In the mouse and rat, GHRH neurons and the GHRH-R develop on embryonic days 16.5 and 18, respectively, reaching adult levels by postnatal day 30. GHRH gene expression and content reach a nadir at 12 days, increase at puberty onset, and subsequently decline in the aging rat. GHRH-R mRNA is highly expressed in neonatal rats and is decreased in aged rats (18 months), which may contribute to the diminished GH response to GHRH observed with aging; the same decrease is also seen in elderly human subjects. However, GHRH sensitivity can be restored by treatment with GHRH.

## E. Regulation

In both rats and humans, GHRH-R expression is positively regulated by transcription factors, glucocorticoids, thyroid hormones, and GHRH; GHRH-R expression is down-regulated, however, by estrogen, which may explain the sexual dimorphism observed in GHRH regulation and GH secretion. POU1F1 is also a key determinant of somatotroph differentiation. Hypothalamic GHRH and GHRH-R mRNA levels are higher in male than in female rats, as are the responses of GH to GHRH in *ex vivo* and *in vitro* models. Testosterone enhances GHRH activity in rats, whereas estrogen down-regulates GHRH-R mRNA expression.

# IV. DISORDERS OF GROWTH REGULATION

Genetic, nutritional, physical, and psychological conditions interact to determine final physical stature. Growth retardation or overgrowth may occur due to disorders at all hormonal levels of the somatotropic axis, as well as to defective pituitary development because of mutations in pituitary transcriptional factors. Some of these disorders have been described in spontaneous and/or animal transgenic models.

# V. GHRH AND GHRH RECEPTOR: CLINICAL ASPECTS

## A. Excessive GHRH Secretion

Overexpression of the mouse metallothionein promoter-driven human GHRH (MT-hGHRH) results in increased growth rate, increased body mass, and increased pituitary, liver, kidney, spleen, testis, and vesical weights (Table 1). Massive pituitary enlargement as a result of somatotroph hyperplasia occurs as early as 8 months; adenomatous transformation is observed by 10–12 months in addition to lactotroph and somatomammotroph hyperplasia. Immunoreactive GH, prolactin, $G_{\alpha s}$, and, less frequently, thyroid-stimulating hormone β (TSHβ) are observed in pituitary adenomas in the hyperplastic pituitary of the transgenic mice.

GH secretion and consequently IGF-I levels are markedly increased, with decreased GHRH mRNA levels and increased somatostatin receptor subtype 2 and subtype 5 mRNA expression. Pituitary GHRH-R and GHS mRNA levels are unchanged in relation to wild-type mice. Other alterations that have been described in these mice include increased abdominal fat, hyperinsulinemia, hyperleptinemia, and elevation of prolactin, corticosterone, and galanin.

## B. Human GHRH-Secreting Tumors

Gigantism or acromegaly secondary to eutopic (hypothalamic hamartomas, gliomas, and astrocytomas) or ectopic (pancreatic, lung, thymus, and gastrointestinal neoplasias) GHRH secretion occurs

TABLE 1 Genetic and Transgenic Animal Models of Growth Retardation or Overgrowth Related to GHRH

| Model | Defect | Phenotype |
|---|---|---|
| *Little* (*lit*) mouse | GHRH receptor missense mutation (autosomal recessive) | Moderate to severe dwarfism, low levels of GH/IGF-I, GHRH-specific absence of response of somatotroph cells, anterior pituitary hypoplasia |
| Zucker rat | GHRH decrease (unknown mechanism) | Growth retardation, obesity, hyperinsulinemia, and hyperlipidemia; decreased sensitivity to GHRH *in vitro* decreased GHRH and GHRH mRNA levels |
| Methallothionein human GHRH transgenic mouse (hGHRH) | Overexpression of GHRH gene | Excessive linear growth; extremely high levels of GH/IGF-I, prolactin, and cortiscosterone; organomeglay, dramatic somatotroph hyperplasia (eventually adenoma) |

rarely and is frequently associated with multiple endocrine neoplasia. The clinical presentation is related to GH/IGF-I and GHRH excess as well as to syndromes related to primary tumor mass localization and function. Patients also present with pituitary hyperplasia and/or adenomatous transformation, as observed in genetically altered mice (Table 2). Somatotroph adenomas overexpressing intratumoral GHRH also show more aggressive tumor behavior.

## C. Decreased GHRH

Obese Zucker rats exhibit growth retardation, pituitary hypoplasia, and decreased pituitary GH content and secretion. Some investigators have suggested a hypothalamic abnormality based on the decreased hypothalamic GHRH and GHRH mRNA levels and the unchanged somatostatin levels or secretion present in this model.

## D. Decreased GHRH-R

The *little* mouse harbors an inherited autosomal missense GHRH-R mutation that results in inability of the receptor to bind to its ligand. Consequently, a 70% reduction in somatotroph number occurs and these animals exhibit moderate to severe growth retardation, decreased pituitary GH and GH mRNA, extremely low GH plasma levels, and absence of GH responses to GHRH. *In vitro* studies demonstrate that lack of somatotroph GH response is restricted to GHRH in these mice. GHRH postreceptor events are intact, as demonstrated by somatotroph GH response to cholera toxin, to forskolin (a direct stimulator of the α-subunit of $G_s$), and to dibutyryl cAMP.

In humans, the disturbance of isolated GH deficiency corresponding to that observed in the *lit/lit* mouse has been described in kindreds from India, Pakistan, Sri Lanka, and Brazil. In familial dwarfism with an autosomal recessive pattern of inheritance, both nonsense and missense mutations lead to disruption of GHRH-R function. These patients have proportional dwarfism, delayed bone age and puberty, pituitary hypoplasia, and impaired GH response to GHRH. GH pulsatility is preserved in these patients, but pulse amplitudes are low, corroborating the amplifier role for GHRH in generation of GH pulses. The response to the GH secretagogues is also decreased either because of somatotroph hypoplasia or because of the key role of the intact GHRH response in the integral somatotroph response to GHSs. The *lit/lit* mouse and patients with GHRH-R mutations are usually fertile, suggesting that GHRH does not play a key role in gonadal function, although GHRH and GHRH-R are expressed in testis and ovary.

TABLE 2 **Clinical Features of GHRH Receptor Abnormalities and GHRH-Secreting Neoplasias in Humans**

**GHRH-R-related dwarfism**
- Autosomal recessive, nonsense or missense mutation
- Proportional dwarfism
- Delayed bone age and puberty
- Pituitary hypoplasia

**GHRH excess related**
- Somatotrope hyperplasia and/or adenomatous transformation
- Excessive GH secretion
  - Excessive IGF-I levels
    - Skeletal and other tissue overgrowth; arthropathy; compressive neuropathy; cardiovascular disease (systemic arterial hypertension, atherosclerosis, myocardial hypertrophy, or cardiomegaly); metabolic disorders (diabetes mellitus or hypercalcemia); respiratory dysfunction (sleep apnea)
- Tumor-related (type, localization, cosecreted substances)
  - Benign (carcinoids): facial flushing, diarrhea, peptic ulcers, renal lithiasis, hypoglycemia
  - Malignant: cachexia

## VI. GHRH AS A DIAGNOSTIC TOOL

## A. GH Deficiency

GHRH has been used diagnostically and therapeutically in GH deficiency. Differential diagnosis of short stature may be complex, and GH deficiency is only one cause. The first goal is to determine which child is to be investigated (Table 3). Once criteria have been met for investigation, and systemic diseases (diabetes, chronic renal failure, and hypothyroidism) have been excluded or controlled, the next step is to investigate pathologies intrinsic to the somatotropic axis. Pitfalls related to the accuracy of functional diagnostic tools commonly lead to false positive or negative results; care must be taken to assess sensitivity, reproducibility, and arbitrary cutoff levels of hormone measurements, as well as age- and sex-related variations in the patients. Standard tests (insulin-induced hypoglycemia; glucagon, arginine, and clonidine) act through hypothalamic signals to elicit pituitary GH. Thus, an altered GH response

TABLE 3 Short Stature: Clinical Criteria for Investigation[a]

| |
|---|
| Severe short stature—height ≥ 3 SD below mean for chronological age |
| Moderate short stature—height between 2 and 3 SD below mean for chronological age and growth |
| Deceleration (height velocity below 25%) |
| Growth deceleration |
| Predisposing condition to growth hormone deficiency (hypothalamic injury, irradiation, etc.) and growth deceleration |
| Neonatal signs of pituitary deficiencies (hypoglycemia, microphallus, midline craniofacial defects) |

[a] Adapted from Rosenfield and Cutler (2001).

does not necessarily distinguish hypothalamic or pituitary disturbance, which potentially might have therapeutic implications.

GHRH is a powerful GH secretagogue in children and adults and may distinguish a hypothalamic or pituitary etiology for GH deficiency. Normal GH responses confirm pituitary integrity. An abnormal GH response to GHRH strongly points to pituitary abnormalities in the etiology of GH deficiency. Some studies have shown that most patients with severe GH deficiency and without demonstrable anatomical hypothalamic–pituitary alterations are in fact GHRH deficient. However, an intact hypothalamic–pituitary connection is required for the GHRH test to be useful.

Currently, the synthetic analogue GHRH(1–29)-$NH_2$ is commercially available in the United States and Europe for diagnostic and therapeutic purposes in GH deficiency. This compound is equipotent to the natural analogues GHRH(1–40)-OH and GHRH(1–44)-OH in evoking GH secretion.

## B. GHRH Testing

GH response to GHRH is dose related; a maximally stimulating dose ~1.0 μg/kg, detectable in 5 min, peaks between 15 and 45 min and returns to baseline after 90–120 min. After an overnight fast, intravenous GHRH(1–29)-$NH_2$ (1.0 μg/kg) is administered as a bolus and blood is sampled 15 min before and each 15 min thereafter for 60 min. The test may provoke mild and transient side effects such as warmth, face flushing, local inflammation, nausea, vomiting, and chest tightness. GHRH administered continuously or as repeated intravenous boluses provokes GH release and maintains GH pulsatility, although a tendency to subsequent decreased pulsatile frequency has been observed.

### *1. GH Response to GHRH*

There is poor reproducibility and some inter- and within-subject variability when identifying GH status using the GHRH test alone, which limits its diagnostic value.

### *2. Factors That Interfere with GH Responses to GHRH*

**a. Age.** GH response to GHRH varies throughout life, partially due to variation of somatostatinergic and/or cosecretagogue tones and sensitivity to these molecules. In the immediate postnatal period, there is GH hyperresponsiveness to GHRH. In the prepubertal period, during different stages of puberty and in young adults, the GH response to GHRH remains constant. After the third and fourth decades in men and during menopause in women, GH responsiveness decreases. Increased somatostatin tone is the most likely mechanism for this age-related decline in GH response.

**b. Sex.** Although both sexes exhibit similar responses, slightly higher GH levels are achieved in women. Menstrual cycle and sex steroid priming do not alter GH responses, suggesting that the related steroids act mainly at the hypothalamic level.

**c. Obesity.** Obesity, particularly abdominal obesity, suppresses the GH response to GHRH. This negative influence on GH responsiveness may be partially overcome by a combination of GHRH with arginine or GHSs. Blood sugar, free fatty acids, and GH also decrease GH responses to GHRH.

### *3. Indication*

Currently, the main role for GHRH testing is to select children who are candidates for GHRH treatment.

### *4. GHRH Test Combinations*

Combination of GHRH with somatostatinergic inhibitors (such as arginine) and GHSs (such as GHRP-6) may overcome some limitations of GHRH testing.

**a. Arginine.** Simultaneous intravenous infusion of arginine (0.5 g/kg; administered over a 30-min period) and GHRH is performed and blood is sampled before and at 30-min intervals for 120–180 min. In normal children and adolescents, the ranges in GH response evoked by the combination of arginine/GHRH (22.4–108 μg/liter) are similar and are not influenced by pubertal stage, gender, body mass index, and IGF-I levels. In adults, combined

GHRH/arginine usually overcomes the declining GH response to GHRH with aging, and does not vary with gender. The combination of GHRH and arginine has been proposed as the best alternative to the insulin tolerance test (ITT) in the diagnosis of GH deficiency, due to reproducible efficacy and safety. The combination of GHRH and arginine usually leads to GH peaks greater than the threshold for GH deficiency in adults (3 μg/liter). The proposed diagnostic cutoff for GH deficiency and GH insufficiency in adults was 9 (first percentile) and 16.5 μg/liter (third percentile), respectively.

**b. GHS–GHRP-6 (hexarelin).** GHRP-6 is synergistic with GHRH in evoking GH secretion. Combined intravenous administration of a bolus of hexarelin (1 μg/kg; blood sampled before and at 15-min intervals for 30–120 min), simultaneously with, before, or after GHRH administration, is safe, well tolerated, and potently evokes GH secretion; this protocol has also been proposed to replace the ITT. Another advantage of this combination is that although anatomic disruption of the hypothalamic–pituitary unit invalidates the GHRH test alone, this may be reversed by addition of a GHS. The proposed threshold of normality has been 15 μg/liter; however, diagnostic cutoff values ≥20 μg/liter for normality and ≤10 μg/liter for GH deficiency have yielded 100% sensitivity and specificity.

### C. Excessive Growth

One of the few indications for the measurement of GHRH levels is to distinguish between central and peripheral (elevated ectopic GHRH syndromes) causes of gigantism/acromegaly.

## VII. GHRH: THERAPEUTIC ROLE

The mainstay therapy for GH deficiency, related to both pituitary or hypothalamic causes, and GH insufficiency (GHI) syndrome in childhood has been GH. GHRH treatment is potentially a therapeutic choice for patients who are GHRH deficient (so-called idiopathic GH deficiency) but who have intact somatotroph responses to GH secretagogues. Moreover, GHRH treatment preserves the pulsatile pattern of GH secretion as well as endogenous GH and IGF-I feedbacks. GH is administered daily as subcutaneous or intramuscular injections (25–50 μg/kg/day; usually 30 μg/kg/day for children and a starting dose of 200 μg/day, for adults).

### A. Indication

The United States Food and Drug Administration (FDA) has approved GHRH(1–29)-$NH_2$ for use in GH-deficient children according to the criteria and dosage delineated in Table 4.

### B. Response to GHRH Therapy

Either GHRH(1–29) or GHRH(1–44)-$NH_2$ may be administrated and yield similar dose–response patterns. Studies are still inconclusive regarding effects on final growth stature, although improvement in growth velocity has been observed. Similar to what is observed with GH treatment, children who have a higher height standard deviation and slower growth rates for their chronologic age respond well to GHRH treatment. In contrast, GH response levels in GHRH testing have not proved to be a good predictor of response to GHRH treatment in these children.

Administration of GHRH, either in pulses or once or twice daily, to GH-insufficient children promotes linear growth. Continuous nocturnal GHRH administration augments nocturnal GH release, which suggests that production of a long-lasting GHRH release product can be useful. A combination of GHRH and GHRP elicits a more pronounced growth velocity response than does the use of either compound alone.

#### 1. *Adverse Effects*

Local injection site inflammation, urticaria, headache, flushing, dizziness, dysphagia, and hyperactivity have been reported. Hypothyroidism (5%) and the need to increase thyroid hormone doses have been observed in children. This effect is also described with GH treatment, perhaps due to an enhanced somatostatinergic tone induced by GH, which subsequently suppresses TSH secretion. Formation of GHRH antibodies has frequently been described, but does not have a negative effect on growth or GH responses to GHRH.

**TABLE 4 Current Criteria and Dosage for GHRH Treatment in Growth-Retarded Children**[a]

Growth hormone-deficient children with an intact hypothalamic–pituitary axis
Adequate growth hormone response (>2 μg/liter) to GHRH
Prepubertal children (bone age <7.5 years for girls and <8 years for boys)
Dosage: 30 μg/kg/day, once a day, subcutaneously, at bedtime

[a] Adapted from Rosenfield and Cutler (2001).

## Glossary

**acromegaly** Clinical syndrome characterized by overgrowth of extremities and organs and by metabolic disturbances secondary to growth hormone excess, usually due to a growth hormone-secreting pituitary adenoma.

**ghrelin** Polypeptide growth hormone secretagogue predominantly produced by the stomach; exerts a direct or indirect stimulatory effect on growth hormone secretion by somatotrophs.

**growth hormone** Polypeptide produced and secreted by the pituitary gland; responsible for inducing growth.

**growth hormone-releasing hormone** Peptide predominantly produced by the hypothalamus; responsible for inducing growth hormone synthesis and secretion.

**growth hormone secretagogues** Peptide and nonpeptide compounds produced exogenously (e.g., hexarelin) or endogenously (e.g., ghrelin); stimulate growth hormone secretion directly or synergistically with growth hormone-releasing hormone.

**insulin-like growth factor** Peptide produced mainly by the liver in response to growth hormone stimulation.

**POU-homeodomain-1** Transcription factor-1, previously known as pituitary transcription factor-1; responsible for development of pituitary cell lines (somatotroph, lactotrophs, and thyrotrophs).

**somatotroph** Pituitary cell responsible for growth hormone production and secretion.

**somatotropin release-inhibiting factor** Somatostatin; peptide ubiquitously produced in the central nervous system and other tissues. In the pituitary, it exerts an inhibitory effect on growth hormone secretion by somatotrophs.

## See Also the Following Articles

**Growth Hormone (GH) • Growth Hormone-Releasing Hormone (GHRH) and the GHRH Receptor • Insulin-like Growth Factor (Igf) Signaling • Prolactin and Growth Hormone Receptors • Somatostatin**

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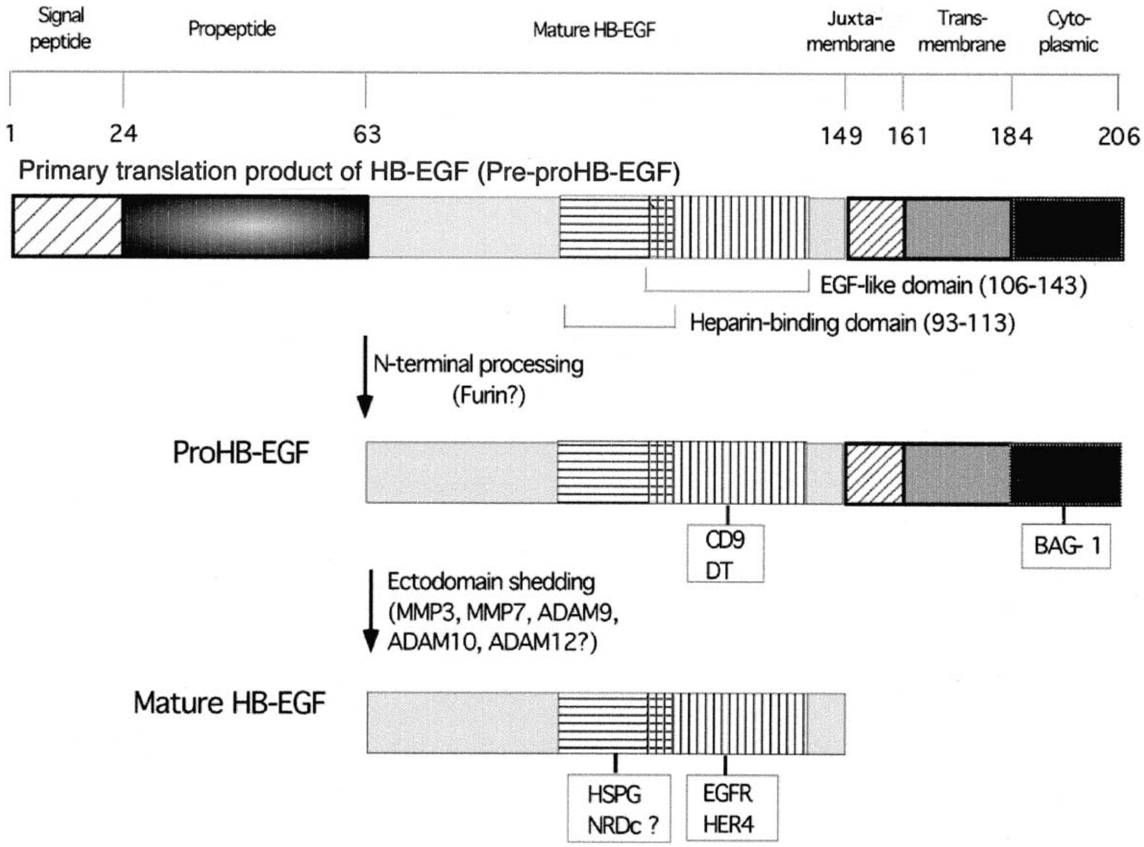
Signal peptide
Propeptide
Mature HB-EGF
Juxta-membrane
Trans-membrane
Cyto-plasmic
1
24
63
149
161
184
206
Primary translation product of HB-EGF (Pre-proHB-EGF)
EGF-like domain (106-143)
Heparin-binding domain (93-113)
N-terminal processing (Furin?)
ProHB-EGF
CD9
DT
BAG-1
Ectodomain shedding (MMP3, MMP7, ADAM9, ADAM10, ADAM12?)
Mature HB-EGF
HSPG
NRDc ?
EGFR
HER4

**FIGURE 1** HB-EGF domains and binding to various receptors. (Top) The open reading frame of 206 amino acids. The numbers correspond to the amino acids that delineate the various HB-EGF domains. (Middle) PreproHB-EGF is cleaved to proHB-EGF. Binding sites for CD9, diphtheria toxin (DT), and BAG-1 are depicted. (Bottom) Mature HB-EGF is generated by ectodomain shedding induced by a variety of enzymes. HB-EGF receptors are depicted.

contributes to the strong binding of HB-EGF to heparin and HSPG.

## B. Gene Structure and Its Regulation

The HB-EGF gene locus has been mapped to chromosome 5 in humans and to chromosome 18 in mice. Cytogenetic localization of human HB-EGF is at 5q23, which is consistent with the finding that diphtheria toxin sensitivity is localized to 5q23. The human gene encoding HB-EGF contains six exons and five introns spanning 14 kb of DNA. So far, only one splice variant of human HB-EGF cDNA has been reported. It encodes a short form of HB-EGF containing the signal peptide, the propeptide, the heparin-binding domain, the first two disulfide loops of the EGF domain, and an additional nine C-terminal amino acids. The biological activity, if any, of this short form of HB-EGF has not been determined.

The gene expression of HB-EGF is highly regulated and a number of regulatory factors have been described. Positive regulators for HB-EGF gene expression include inflammatory cytokines (e.g., tumor necrosis factor-α and interleukin-1), growth factors (e.g., EGF family members, basic fibroblast growth factor, and platelet-derived growth factor), agonists of G-protein-coupled receptors (GPCRs) (e.g., angiotensin II, thrombin, and platelet-activating factor), and atherogenic lipids (e.g., oxidized low-density lipoproteins and lysophosphatidylcholine). Physical stresses, such as sheer stress, stretch, and hyperosmolarity, also up-regulate HB-EGF gene expression. HB-EGF mRNA induction is typically rapid and transient and is not inhibited by blocking protein synthesis, characteristic of immediate-early genes. The HB-EGF promoter has been described and transcription factors such as MyoD and PDX-1 have been shown to be involved in HB-EGF gene regulation.

## III. TRANSMEMBRANE HB-EGF

Transmembrane HB-EGF has a number of activities that are summarized in Fig. 2. The details are presented below.

### A. Juxtacrine Activity

The mode of cell-to-cell signaling that is mediated by interaction of a transmembrane growth factor with its receptor on a neighboring cell has been termed "juxtacrine." Juxtacrine growth factor/receptor interactions were first demonstrated for the membrane-anchored forms of TGF-α and tumor necrosis factor α (TNFα). The juxtacrine activity of proHB-EGF was demonstrated using a coculture system of cells expressing proHB-EGF and cells expressing EGFR. Mouse L cells transfected with proHB-EGF stimulated DNA synthesis in 32D cells expressing EGFR. In some cases, however, juxtacrine interactions have induced growth inhibition. ProHB-EGF has also been demonstrated to be a survival factor for renal epithelial and hepatoma cells. Those cells transfected with proHB-EGF show significant resistance to apoptosis induced by $H_2O_2$, etoposide, or serum starvation. This survival effect appears to be unique for proHB-EGF in that mature HB-EGF does not promote cell survival readily.

### B. Diphtheria Toxin Receptor

A unique property of transmembrane HB-EGF is that it is the specific receptor for DT. Expression cloning using a Vero cell cDNA library that was intended to identify DT receptor (DTR) revealed the DTR to be identical to proHB-EGF. Human and monkey proHB-EGFs are functional DTRs whereas mouse and rat proHB-EGFs are not. DT interacts with the EGF-like domain of human proHB-EGF, in which three amino acid residues, Phe-115, Leu-127, and Glu-141, seem to be critical for DT binding. Some of these amino acids are altered in mouse/rat proHB-EGF. The steps in the cytotoxic action of DT are internalization of the DT/proHB-EGF complex, translocation of the A fragment of DT from acidified vesicles to the cytosol, and subsequent inhibition of protein synthesis by ADP-ribosylation of elongation factor 2.

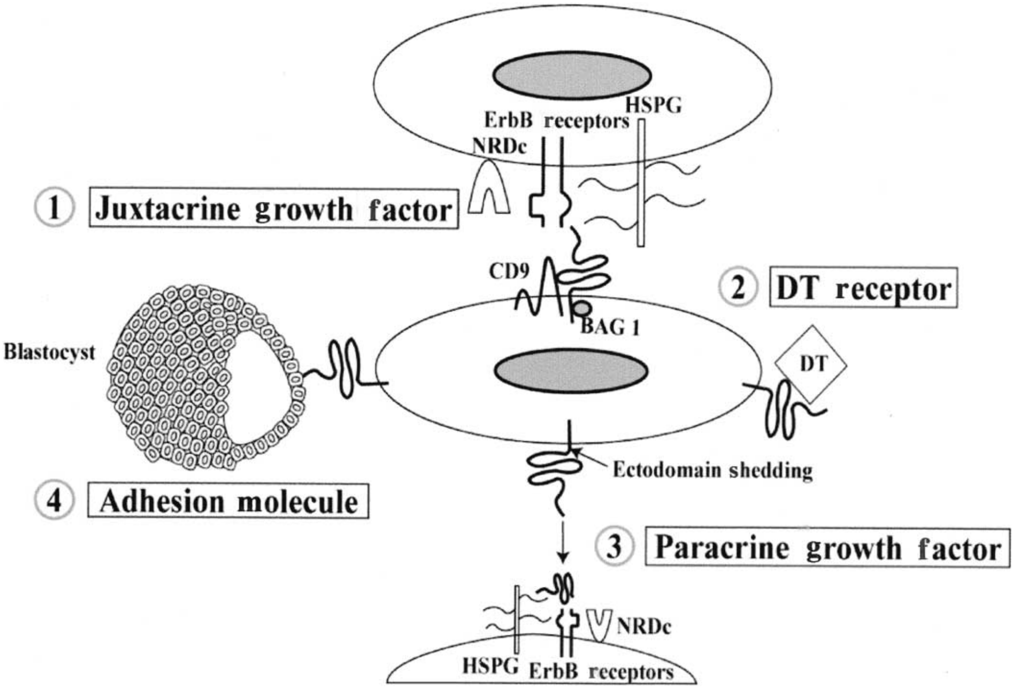

FIGURE 2 Biological properties of HB-EGF. ProHB-EGF is depicted as a juxtacrine growth factor (1), as the receptor for diphtheria toxin (DT) (2), as a target for ectodomain shedding that generates mature paracrine HB-EGF (3), and as an adhesion molecule for the trophoectodermal cells of the blastocyst (4).

Although the internalization rate for the DT is much slower than is typical of classical endocytic receptors, successful toxin effects occur because a single internalized enzymatically active toxin molecule is sufficient to inhibit protein synthesis in the cell. The cytoplasmic domain of proHB-EGF is not required for the internalization step.

## C. ProHB-EGF Complexes

Several membrane proteins form complexes with proHB-EGF. CD9/DRAP27 was first identified as the antigen for a monoclonal antibody that inhibited the binding of DT to Vero cells. The antigen was identified as the monkey homologue of human CD9. CD9 is a tetramembrane-spanning protein that physically interacts with the proHB-EGF ectodomain and up-regulates the number of functional DT receptors and enhances sensitivity to DT. CD9 does not affect the expression level of proHB-EGF or the binding affinity of DT. CD9 also potentiates the juxtacrine activities of proHB-EGF. Anti-CD9 antibody does not inhibit the mitogenic activity of mature HB-EGF, but suppresses the juxtacrine activity of proHB-EGF in mouse L cells expressing both CD9 and proHB-EGF, suggesting complex formation. CD9 also interacts with the transmembrane forms of amphiregulin and TGF-α and modulates their bioactivities. On the other hand, other tetramembrane-spanning molecules, CD63, CD81 and CD82, associate with proHB-EGF, but do not affect proHB-EGF activity. Integrin $\alpha_3\beta_1$ forms a complex with proHB-EGF at cell–cell contact sites in epithelial cells. A yeast two-hybrid screen identified BAG-1 as a protein that binds to the cytoplasmic domain of proHB-EGF. BAG-1 has been demonstrated to bind to Bcl-2 and several other signaling molecules and is capable of suppressing apoptosis. Expression of BAG-1 along with proHB-EGF suppresses etoposide-induced apoptosis and increases mature HB-EGF secretion induced by etoposide.

# IV. MATURE HB-EGF

Mature HB-EGF is a potent stimulator of cell proliferation and migration. Target cells include epithelial cells, fibroblasts, and SMCs, but not ECs. Target cell specificity may correlate with the expression of HB-EGF receptors. Mature HB-EGF is not only a paracrine factor but also an autocrine factor for some cell types, including keratinocytes, urothelial cells, and prostate stromal cells. Autocrine activity has been demonstrated by the inhibition of cell growth *in vitro* by HB-EGF blocking antibody and CRM197.

Mature HB-EGF binds strongly to heparin–sepharose and is eluted with approximately 1 *M* sodium chloride. Site-directed mutagenesis and synthetic peptide studies indicate that the interaction of mature HB-EGF with heparin is primarily mediated by a stretch of amino acids extending from Lys-93 to Lys-113 of the protein, within which there are three separate basic amino acid stretches. The heparin-binding domain is mostly N-terminal to the EGF-like domain, but one of the three basic amino acid stretches is between the first and the second cysteine of the EGF-like domain.

HB-EGF binding to cell surface HSPG enhances its binding to EGFR and HB-EGF bioactivity in some cell types. Chinese hamster ovary cells mutant in HSPG production and transfected with EGFR do not bind significant levels of HB-EGF unless heparin is present in the medium. Treatment of bovine aortic SMCs with heparitinase or chlorate, an inhibitor of HSPG sulfation, significantly inhibits HB-EGF-stimulated cell migration. A similar inhibition occurs when SMCs are treated with a synthetic peptide corresponding to the heparin-binding domain of HB-EGF. Cell surface HSPG enhances DT sensitivity and DT binding to proHB-EGF/DTR.

# V. ECTODOMAIN SHEDDING OF HB-EGF

## A. Protease-Mediated Shedding

Ectodomain shedding of proHB-EGF is induced by various stimuli, including phorbol esters, calcium ionophore, and lysophosphatidic acid (LPA). Multiple signaling cascades, such as the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways, appear to regulate HB-EGF shedding. Metalloproteinases have been implicated as sheddases of proHB-EGF because various metalloproteinase inhibitors efficiently inhibit the ectodomain shedding of HB-EGF. Matrix metalloproteinase 3 (MMP3), MMP7, a disintegrin and metalloproteinase 9 (ADAM9), ADAM10, ADAM12, and ADAM17 have been implicated as responsible proteases. The specific proteases involved in the shedding process of HB-EGF depend on cell type and biological environment. For example, MMP7 appears to be involved in the shedding process in epithelial cells, especially on the apical surface of cells. On the other hand, ADAM12 seems to be responsible for HB-EGF shedding in cardiomyocytes.

Pro148Val149 and Glu151Asn152 have been suggested as the shedding sites of proHB-EGF. Highly specific HB-EGF shedding inhibitors have been found by a screening of hydroxamic acid-based compounds. They inhibit cutaneous wound healing and cardiac hypertrophy in mice, and submandibular gland development in organ culture.

### B. G-Protein-Coupled Receptor Ligand-Induced EGF Receptor Transactivation

The transactivation of EGFR by G-protein-coupled receptors (GPCRs) is critical for the mitogenic activity of ligands such as LPA, endothelin, and thrombin. This transactivation is dramatically attenuated by specific HB-EGF inhibitors such as CRM197 and anti-HB-EGF neutralizing antibody. Metalloproteinase inhibitors specific for inhibiting HB-EGF shedding also inhibit EGFR transactivation by GPCRs, indicating that HB-EGF shedding is associated with EGFR transactivation induced by GPCR ligands (Fig. 3). EGFR transactivations induced, for example, by insulin-like growth factor-I and *Helicobacter pylori* also appear to be dependent on HB-EGF shedding.

## VI. HB-EGF RECEPTOR

EGFR (HER1) was originally identified as the receptor for HB-EGF. HB-EGF binds to EGFR and induces tyrosine phosphorylation of the receptor. HB-EGF also binds to cells with HER4 receptors and induces chemotaxis but not proliferation in those cells, whereas HB-EGF induces both chemotaxis and proliferation via EGFR. HB-EGF-induced chemotaxis of HER4-expressing cells is inhibited by wortmannin, indicating that phosphoinositol 3-kinase (PI3K) activity is involved in the HER4-mediated chemotaxis. More recently, NRDc has been identified as a highly specific 140-kDa non-tyrosine kinase receptor for HB-EGF, unlike EGFR, which also binds EGF, TGF-α, amphiregulin, betacellulin, and epiregulin. NRDc was initially identified as a metalloendopeptidase with an inverted consensus zinc binding site (HXXEH), thereby placing it in the inverzincin/M16 family of metalloendopeptidases. NRDc expression enhances HB-EGF-induced cell migration via EGFR. Although NRDc is a metalloendopeptidase, the enzyme does not cleave HB-EGF and its enzymatic activity is not required for HB-EGF binding or enhancement of cell motility.

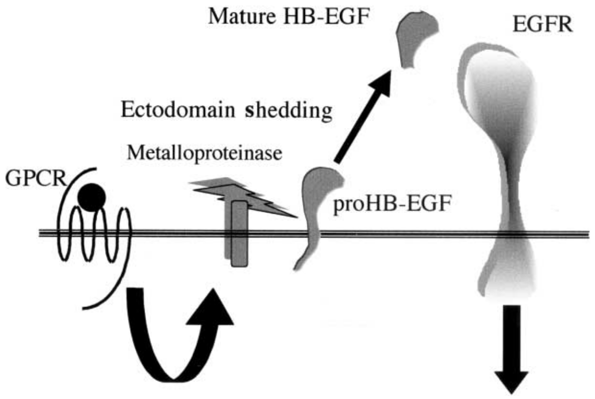

**FIGURE 3** Model of transactivation of epidermal growth factor receptor (EGFR) by G-protein-coupled receptor (GPCR) agonists. Agonist binding and activation of the GPCR results in an intracellular signal that induces the extracellular activity of a transmembrane metalloproteinase. Activated metalloproteinase induces the ectodomain shedding of proHB-EGF, resulting in the release of mature HB-EGF. Mature HB-EGF binds to the ectodomain of EGFR and induces an intracellular signal.

## VII. PHYSIOLOGICAL ACTIVITIES OF HB-EGF

HB-EGF has been implicated in a number of physiological and pathophysiological processes.

### A. HB-EGF in Tumors

HB-EGF expression is enhanced in hepatocellular, pancreatic, gastric, and breast cancer tumors, when compared to normal tissue. Up-regulation of HB-EGF is also detected in a model rat of spontaneous hepatocarcinogenesis. Anti-HB-EGF blocking antibody diminishes the growth of some tumor cells, e.g., glioblastoma and multiple myeloma, suggesting an autocrine loop. Furthermore, transfection of HB-EGF into normal rat kidney cells or chicken embryo fibroblasts induces oncogenic transformation and anchorage-independent growth, indicating an important role of HB-EGF in tumorigenesis. HB-EGF is a direct transcriptional target of oncogenic Raf kinases and the tumor suppressor p53. Inhibiting HB-EGF function with anti-HB-EGF neutralizing antibody inhibits Raf-induced Jun N-terminal kinase (JNK) activation and p53-induced MAPK activation, respectively.

### B. Wound Healing

Successful wound healing involves a number of processes, including inflammation, cell proliferation, cell migration, vascular permeability, and angiogenesis, as well as matrix deposition and tissue remodeling. In cutaneous wound healing, keratinocytes play a central role, not only as a key structural cell type in the repair of skin but also as the source of a number of growth factors, among which the EGF family are prominent. Keratinocyte proliferation and migration are in part mediated in an autocrine manner by EGFR–ligand interactions. Wound stimuli induce keratinocyte shedding of EGFR ligands *in vitro*, particularly HB-EGF. The released mature HB-EGF stimulates transient activation of EGFR, which is essential for keratinocyte migration. HB-EGF, like EGF and TGF-α, is a potent stimulator of keratinocyte proliferation. Metalloproteinase inhibitors such as OSU8-1 and KB-R7785, which are relatively specific for proHB-EGF, block HB-EGF shedding and abrogate the wound-induced activation of EGFR, causing suppression of keratinocyte migration *in vitro*. The application of OSU8-1 to wound sites in mice greatly retards reepithelialization as the result of a failure in keratinocyte migration. This effect can be overcome by including recombinant soluble HB-EGF along with OSU8-1. Based on these findings, the shedding of HB-EGF represents a critical event in keratinocyte migration leading to wound healing.

## C. SMC Hyperplasia/Cardiac Hypertrophy

The process of atherogenesis in the arterial wall is characterized by the formation of fibrous lesions and the proliferation of neointimal smooth muscle cells. HB-EGF is a potent chemoattractant and mitogen for vascular SMCs. In human atherosclerotic plaques, marked production of HB-EGF mRNA and protein is detected in SMCs and macrophages in the plaques. Balloon catheter injury of rat carotid arteries induces migration and proliferation of SMCs, with subsequent neointimal formation concomitant with production of HB-EGF mRNA and protein in SMCs. Other stimulants such as osmotic pressure, tension, and other growth factors and cytokines also up-regulate HB-EGF production in SMCs.

Cardiac hypertrophy is a primary and common adaptive response of the heart to a variety of cardiovascular diseases. Prolonged cardiac hypertrophy eventually culminates in chronic heart failure or sudden cardiac death. A variety of endogenous vasoactive reagents that act via GPCRs, such as phenylephrine, norepinephrine, angiotensin II, and endothelin-1, are associated with cardiac hypertrophy. Ectodomain shedding of HB-EGF by metalloproteinases is a key event in GPCR agonist-induced cell signaling via EGFR transactivation. When cardiomyocytes are stimulated by GPCR agonists, shedding of HB-EGF via metalloproteinase ADAM12 activation and subsequent transactivation of EGFR result in cardiac hypertrophy. An inhibitor of HB-EGF shedding, KB-R7785, blocked this GPCR-mediated signaling. In mice with cardiac hypertrophy, KB-R7785 inhibited the shedding of HB-EGF and attenuated hypertrophic changes. These data suggest that shedding of HB-EGF by ADAM12 plays an important role in cardiac hypertrophy, and that inhibition of HB-EGF shedding could be a potent therapeutic strategy for cardiac hypertrophy.

## D. HB-EGF in Blastocyst Implantation

Uterine luminal epithelial cells express HB-EGF mRNA at the site of blastocyst implantation 6–7 h before attachment. Immunohistochemical analysis shows that proHB-EGF is expressed in a spatial and temporal manner similar to that of HB-EGF mRNA. Cells expressing proHB-EGF, but not cells expressing mature HB-EGF, adhere to day 4 mouse blastocysts, suggesting that proHB-EGF is an adhesion factor for blastocysts. Blastocysts isolated from egfr − /− mice still interact with blastocysts, indicating that the attachment is mediated by HB-EGF receptors other than EGFR, possibly erbB-4/HER4, NRDc, or HSPG. Mature HB-EGF promotes blastocyst development and hatching in mice, rats, and humans. Local application of HB-EGF-coated beads to the uterine lumen mimics many of the same discrete local responses elicited by the blastocyst, including increased localized vascular permeability, decidualization, and expression of bone morphogenetic protein-2 (BMP-2) at the sites of the beads. Other EGF family ligands, including EGF, TGF-α, and epiregulin, have no such effects. Together, these results suggest an important role for HB-EGF in blastocyst adhesion and blastocyst development.

# VIII. SUMMARY

HB-EGF is found in two forms, the precursor proHB-EGF and its cleaved product, mature HB-EGF. ProHB-EGF plays a vital role in a number of processes such as juxtacrine activation, diphtheria toxin binding, and blastocyst implantation. Mature HB-EGF is a potent chemotactic and mitogenic factor. The transition of proHB-EGF to mature HB-EGF is tightly regulated by specific metalloproteinases.

This shedding process plays a central role in GPCR-mediated EGFR transactivation. Antagonizing these metalloproteinases can inhibit transactivation of the EGFR and may be of therapeutic value, for example, in cardiac hypertrophy. Future investigation with transgenic mice (for example, HB-EGF-deficient mice, mice expressing only mature HB-EGF, and mice expressing only a noncleavable mutant of HB-EGF) will yield clues that clarify the biological roles of HB-EGF.

## Glossary

**HB-EGF ectodomain shedding** Irreversible posttranslational modification that converts membrane-anchored HB-EGF into its soluble mature form.

**heparin binding** Ability of a protein to bind tightly to heparin or heparan sulfate proteoglycan.

**juxtacrine** Mode of cell-to-cell signaling mediated by interaction of a transmembrane growth factor on one cell with its receptor on another cell.

## See Also the Following Articles

**Cancer Cells and Progrowth/Prosurvival Signaling • Epidermal Growth Factor (EGF) Family • GPCR (G-Protein-Coupled Receptor) Structure • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor B (VEGF-B)**

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# Hepatocyte Growth Factor

**See *HGF***

# Heptahelical Receptor Superfamily

Karin A. Eidne

*Western Australian Institute for Medical Research,*
*The University of Western Australia*

I. INTRODUCTION
II. CLASSIFICATION OF HEPTAHELICAL RECEPTORS
III. HEPTAHELICAL STRUCTURE
IV. SIGNAL TRANSDUCTION PATHWAYS OF GPCRS
V. ATTENUATION OF GPCR SIGNALING
VI. RECEPTOR–PROTEIN INTERACTIONS
VII. GPCR OLIGOMERIZATION
VIII. GPCR MUTATIONS AND DRUG TARGETS
IX. SUMMARY

**G-protein-coupled receptors constitute extraordinarily large and diverse gene families in fungi, plants, and animals and form the central component of one of the primary mechanisms used by eukaryotic cells to receive and interpret a signal and then respond to it.**

## I. INTRODUCTION

G-protein-coupled receptors (GPCRs) account for approximately 1% of the human genome. Mutations in GPCR genes can result in altered function and have been associated with a number of hereditary or somatic disorders ranging from infertility to cancer. Members of the superfamily of GPCRs mediate the cellular responses to an enormous range of physiological processes such as the perception of light, taste, smell, and pain, synaptic neurotransmission, and chemotaxis. GPCRs also mediate the effects of numerous therapeutic agents such as the analgesic effects of opiates, the hormonal control of many physiological processes, the stimulation and regulation of mitotic events, and even the entry of viruses into cells.

GPCRs are functionally linked to G-proteins, the means by which they broadcast an external ligand message to the internal compartment of the cell and subsequently activate a cascade of second-messenger events to convert the extracellular signal into an intracellular response. Members of this receptor family have conserved protein sequences consisting of a single polypeptide chain that snakes back and forth across the lipid bilayer seven times, hence the term seven-transmembrane domain or ‘heptahelical’ receptors.

## II. CLASSIFICATION OF HEPTAHELICAL RECEPTORS

When the first gene encoding a GPCR, the β2-adrenergic receptor, was cloned in the 1980s, it was revealed that receptors involved in hormonal signaling shared their characteristic heptahelical structure with that of the visual receptor, rhodopsin. However, there is little conservation of amino acid sequence across the entire superfamily and this variability is thought to accommodate the diverse range of ligands that bind to these molecules. Only approximately 25% of sequence homology is shared within each family, occurring mainly within the transmembrane regions with very little homology beyond the predicted transmembrane core domain. GPCRs have been identified in all species thus far examined and represent the most evolutionarily ancient of signaling devices (more than 800 million years old), as they are also present in the earliest metazoa and protozoa. The recent sequencing of the *Caenorhabditis elegans* genome has revealed that GPCRs constitute the most abundant family of genes in this organism, with more than 1000 GPCRs encoded by 5% of the genome. In addition to the seven transmembrane domains, characteristic structural features are the extracellular N-terminal domain and loops and the intracellular C-terminal tail domain and loops. Key sequence motifs can be found within phylogenetically related subclasses of receptors and these have been useful for the classification of receptors into six families (Families A to F; Table 1). In addition to each of these classes, many GPCRs exist as subtypes of a particular receptor. These subtypes allow for further discrimination of cellular responses based on tissue distribution or expression levels and in some cases provide for negative feedback regulation or inhibition. The majority of GPCRs are grouped into Families A, B, and C (Fig. 1, Table 2).

### A. Family A

Family A constitutes the largest family that has key residues conserved, such as the DRY (Asp-Arg-Tyr)

TABLE 1 **Classification of Hepthelical GPCR Families**

| Family | Classified GPCR |
|---|---|
| A | Rhodopsin-like |
| B | Secretin-like |
| C | Metabotropic glutamate/pheromone |
| D | Fungal pheromone |
| E | Class E cAMP receptors (*Dictyostelium*) |
| F | Frizzled/Smoothened family |
| Family | Classified putative GPCR |
| | Ocular albinism proteins |
| | *Drosophila* odorant receptors |
| | Plant Mlo receptors |
| | Nematode chemoreceptors |
| Family | Unclassified putative GPCR |
| | Orphans |

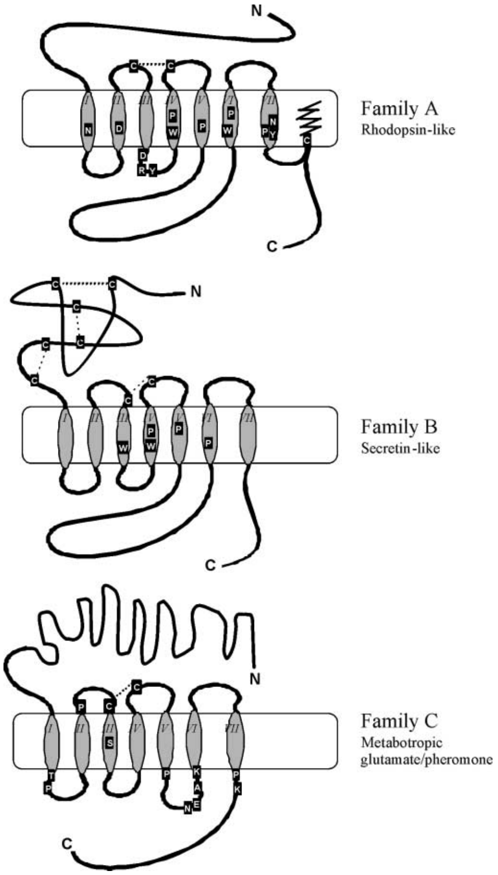


**FIGURE 1** Major heptahelical receptor subfamilies. Of the receptors that have been classified into the main family groups, the majority have characteristic features and key residues. Members of Family A, the largest family, have an Asp-Arg-Tyr (DRY) motif in transmembrane 3 (TM3), an Asn-Pro-Tyr (NPY) motif in TM7, conserved Pro residues in TMs 4, 5, 6, and 7, Trp residues in TMs 4 and 6, an extracellular disulfide bridge, and a palmitoylation group in the C-terminal tail. Family B is characterized by a distinctively large extracellular N-terminus that contains several Cys residues thought to form a network of disulfide bridges. Pro residues in TMs 4, 5, and 6 and Trp residues in TMs 3 and 4 (different than those found in Family A) are conserved. Family C members have a very long N-terminus (approximately 600 amino acids), which is thought to contain the ligand-binding domain. In contrast to Families A and B, the third intracellular loop is extremely short and highly conserved. A putative extracellular disulfide bridge is shown.

motif in the third transmembrane domain (TM3) (Fig. 2). Among this family of receptors are the amine receptors (e.g., adenosine, adrenergic, dopamine, histamine, muscarinic, and serotonin receptors), the peptide receptors (e.g., angiotensin, bradykinin, chemokines, endothelin, and opioid receptors), and the hormone receptors (e.g., follicle-stimulating hormone, luteinizing hormone, and thyrotropin-stimulating hormone receptors). The sensory receptors, including rhodopsin (detects photons in the rod photoreceptor cell) and the olfactory receptors (detects smells), also belong to this family. Olfactory receptors make up the majority of this family, although to date it has approximately 200 nonolfactory members that have been functionally characterized.

## B. Family B

This family has none of the distinctive motifs apparent in Family A (i.e., the DRY motif is missing and different prolines are conserved). Common to all

**TABLE 2** Three Major GPCR Subfamilies

| Family A | Family B | Family C |
|---|---|---|
| Adenosine | Calcitonin | Calcium-sensing |
| Adrenergic | CGRP | Metabotropic GABA |
| Angiotensin | CRF | Metabotropic glutamate |
| Bombesin | Gastrin | Taste |
| Bradykinin | GHRH | Vomeronasal |
| Cannabinoid | Glucagon | |
| Chemokine | Glucagon-like | |
| Dopamine | Latrotoxin | |
| Eicosanoid | PACAP | |
| Endothelin | PTH | |
| FSH | PTHrP | |
| Galanin | Secretin | |
| GnRH | VIP | |
| Histamine | | |
| LH | | |
| Melatonin | | |
| Muscarinic | | |
| Neuropeptide Y | | |
| Neurotensin | | |
| Olfactory | | |
| Opioid | | |
| Oxytocin | | |
| Protease-activated | | |
| Rhodopsin | | |
| Somatostatin | | |
| Serotonin | | |
| TRH | | |
| TSH | | |
| Vasopressin | | |

*Note.* CGRP, calcitonin gene-related peptide; CRF, cortiotropin-releasing factor; FSH, follicle-stimulating hormone; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; VIP, vasoactive intestinal peptide.

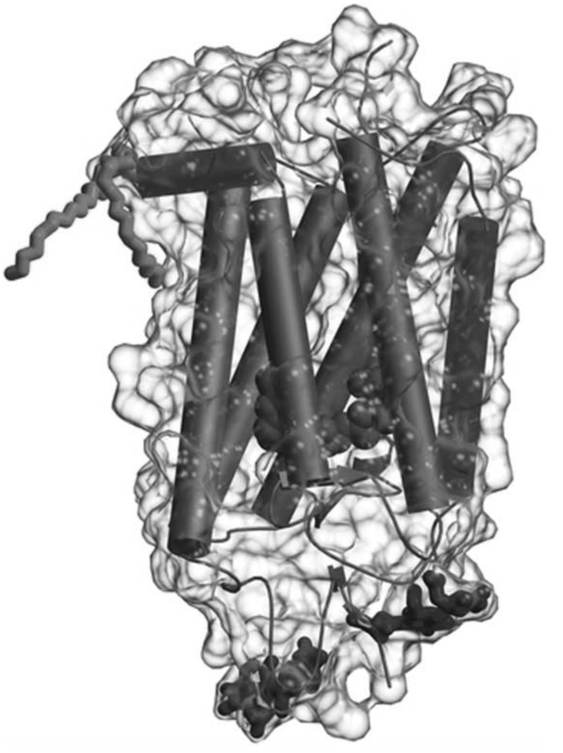


**FIGURE 2** Three-dimensional structure of bovine rhodopsin. Helical portions of the protein, including the seven transmembrane helices with the 11-*cis*-retinal (RET) chromophore bound, are shown as rods, and the transparent envelope around the protein represents the molecular surface. The ball-and-stick groups at the bottom of the figure denote carbohydrate groups attached to the protein and two palmitoyl groups covalently attached to the protein are shown at the top left. Figure drawn by Craig Behnke. Modified with permission from Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., Stenkamp, R. E., (2001). Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein coupled receptors (GPCRs). *Biochemistry* **40**, 7761–7772. Copyright 2001 American Chemical Society.

Family B receptors is a network of disulfide bridges connecting the first and second extracellular loops (ECLs), consequently forming a large extracellular amino-terminus consisting of a number of cysteine residues. This family comprises approximately 25 members that include calcitonin, gastrin, glucagon, vasoactive intestinal peptide, growth hormone-releasing hormone, parathyroid hormone, and secretin receptors. Most of the members of this family have receptors that couple to the guanosine triphosphate (GTP)-binding protein, $G_s$, and signal via adenylyl cyclase.

## C. Family C

Receptors belonging to this family are distinguishable by a remarkably long amino-terminus (500–600 amino acids). As in Families A and B, in Family C, disulfide-forming cysteines in ECL1 and ECL2 are present and it is believed that the amino-terminus of Family C receptors contains the ligand-binding site. A relatively small number of receptors belong to this family, which consists of the metabotropic glutamate and γ-aminobutyric acid (GABA) receptors, the calcium receptors, the vomeronasal mammalian pheromone receptors, and the recently identified putative taste receptors.

## D. Family D

This family encompasses the yeast pheromone receptors, STE2 receptors and STE3 receptors.

## E. Family E

Family E is a minor but unique family that is composed of four different cyclic AMP-linked receptors from *Dictyostelium discoideum*.

## F. Family F

Frizzled (FZD) and smoothened (SMO) are integral membrane proteins with characteristic G-protein-coupled receptor features of seven membrane-spanning domains and extracellular N-terminal and hydrophilic C-terminal domains. FZD and SMO are segment polarity genes required for the transmission of polarity information during tissue morphogenesis. Secreted Wnt glycoproteins, important in developmental control, are ligands for FZD receptors, whereas the smoothened receptor mediates hedgehog signaling. FZD receptors are mostly coupled to the β-catenin canonical signaling pathway, which results in activation of disheveled proteins, inhibition of glycogen synthase kinase-3, nuclear accumulation of β-catenin, and activation of Wnt target genes.

## G. Classified Atypical GPCRs

Additionally, there are atypical (or putative) GPCR families: These include ocular albinism proteins, *Drosophila* odorant receptors, plant Mlo receptors, nematode chemoreceptors, and vomeronasal receptors (V1R and V3R). All the different GPCR families share the same seven-membrane-spanning domain topology; however, the evolutionary relationships among the different families are uncertain since there is no significant degree of sequence similarity among them. It is likely that these families evolved independently and convergently adopted the G-protein signal transduction pathway.

### H. Orphan GPCRs

A number of structurally similar yet novel receptors for which the endogenous ligands are not yet known have also been isolated. These receptors, known as "orphan" GPCRs, show homology to known GPCRs at levels that are too low to classify them into any known receptor subfamily with any degree of confidence. It is possible that these receptors represent novel subfamilies of GPCRs with functions and physiology that are distinct from those of identified GPCRs. Orphan GPCRs represent a valuable resource for novel drug targets and more than 200 orphans have been identified following the completion of the Human Genome Project. The challenge lies in being able to characterize these novel genes and to understand their physiological and pathophysiological roles. Identification of the activating ligand of a receptor with unknown function has proven to be extremely difficult and time-consuming. The overall strategy for characterizing orphan GPCRs involves a "reverse pharmacology" approach in which many ligands are used to screen for the functional activation of a particular orphan GPCR. But despite a great deal of effort by many groups working in this area, the ligands for only a very few orphan GPCRs have been identified.

## III. HEPTAHELICAL STRUCTURE

The characteristic membrane-spanning heptahelical regions of GPCRs are generally composed of 20–27 amino acids, whereas the other segments can vary in size: N-terminal segments are 7–595 amino acids, C-terminal segments are 12–359 amino acids, and extracellular and intracellular loops are 5–230 amino acids. Only recently has the structure of the first GPCR, rhodopsin, been solved, and to date, this is the only GPCR that has yielded a crystal structure. To obtain crystals, bovine rhodopsin was purified from rod outer segment membranes and the crystal structure was determined to 2.8 Å resolution. A diagram of the rhodopsin molecule with its bound 11-*cis*-retinal chromophore intact is shown in Fig. 2 and confirms the presence of an anti-clockwise bundle of seven transmembrane α-helices that are connected by extracellular and intradiscal loops of varying size. The helices are irregular, differing in length, and tilting at various angles with respect to the membrane surface. Although the rhodopsin structure can be useful as a template for other GPCRs, it is not ideal. Although rhodopsin shares similarities with other GPCRs, it also possesses many specialized features, such as visual pigments.

Ligands can bind to the TM, extracellular loops, and amino-terminus. Evidence suggests that smaller ligands bind primarily to the TM core and larger peptides can bind to the amino-terminus, extracellular loops, and TMs. Binding of the ligand is thought to induce a change in the position of the seven TMs, leading to a conformational change in the receptor. This results in increased coupling of the receptor to heterotrimeric G-proteins and consequently G-protein activation; however, this theory is still under intense investigation.

An intriguing question is why are there seven TMs? Possible reasons for this might be that the receptor must be of sufficient size in order to provide the versatility required for the ligand specificities, regulatory mechanisms, and G-protein contact sites as well as other signaling molecules. Perhaps five TMs would not be large enough to form a stable TM core with sufficient flexibility, but nine TMs would be too many. Also, an uneven number of TMs allows the terminal segments to be positioned at opposite membrane surfaces, thus permitting glycosylation and ligand binding at the N-terminal segment and phosphorylation and palmitoylation at the C-terminal segment (important for desensitization and internalization).

## IV. SIGNAL TRANSDUCTION PATHWAYS OF GPCRS

### A. G-Proteins

The generally accepted dogma for the action of a GPCR is that upon activation by ligand, the receptor binds to and activates a G-protein. G-proteins belong to a superfamily of highly conserved GTPases that play a role in many aspects of cell regulation. They are heterotrimeric, consisting of three different subunits: α, β, and γ. The α-subunit has a site for binding GDP or GTP and a catalytic center for hydrolyzing GTP to GDP. G-protein subunits have lipid anchors that permit lateral diffusion, protein–lipid interactions, and protein–protein interactions. The α-subunit is usually modified by a fatty acyl lipid

TABLE 3 **Classes of G-Protein α-Subunits**

| G-protein | Types | Function | Receptor |
|---|---|---|---|
| $G_s$ | $G_{\alpha s}$, $G_{\alpha olf}$ | Activates cAMP pathway | ACTH, adenosine A2, β-adrenergic, CCK, dopamine D1, FSH, histamine H2, LH, MSH, olfactory, PGE1, PGE2, PGE12, TSH, taste |
| $G_i$ | $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o1}$, $G_{\alpha o2}$, $G_{\alpha t}$, $G_{\alpha gus}$, $G_{\alpha z}$ | Inhibits cAMP pathway and regulates ion channel function through release of $G_{\beta\gamma}$ | Adenosine A1, adrenergic A2, dopamine D2, GABA$_B$, histamine H3, muscarinic M2, opioid (μ, κ, δ), prostaglandin, 5HT-1a |
| $G_q$ | $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 16}$ | Activates inositol phosphate pathway | Adrenergic, bradykinin, CCK, histamine H1, GnRH, muscarinic M1, PAF, thromboxane, TRH, substance K, substance P, vasopressin V1, VIP, 5H2C |
| $G_{12}$ | $G_{\alpha 12}$, $G_{\alpha 13}$ | Activates Rho-GEF, which modulates the cytoskeleton | Angiotensin AT1, lysophosphatidic acid, muscarinic, thrombin |

*Note.* ACTH, adrenocorticotropic hormone; CCK, cholecystokinin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MSH, melanocyte-stimulating hormone; PAF, platelet activating factor, PGE1, 2, and 12, prostaglandin E1, 2, and 12; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone, VIP, vasoactive intestinal peptide; 5HT-1a, 5-hydroxytryptamine 1A; 5H2C, 5 hydroxytryptamine 2C.

anchor (myristoyl or palmitoyl), and the γ-subunit has an isoprenoid lipid anchor (farnesyl).

There are four main classes of G-proteins that are categorized according to their α-subunit (Table 3). The $G_{s\alpha}$ and $G_{i\alpha}$ subunits both interact with adenylyl cyclase isoforms. Their actions, however, are opposite: $G_s$ stimulates and $G_i$ inhibits the synthesis of cyclic AMP from ATP. Members of the $G_{i\alpha}$ family are also defined by their ability to activate $K^+$ channels. The actions of these two α-subunits may be differentiated in the laboratory by the bacterial toxins cholera toxin and pertussis toxin (Table 4). The $G_{q\alpha}$ family members are not substrates for cholera or pertussis toxin but stimulate the β class of phospholipase C (PLC-β) enzymes. Members of the fourth class of G-proteins are also insensitive to cholera and pertussis toxins and include $G_{12\alpha}$ and $G_{13\alpha}$, which have been implicated in the regulation of small GTP-binding proteins.

## B. G-Protein GTPase Cycle

Heterotrimeric G-proteins differentially control the kinetics and amplitude of signal transduction via a regulated G-protein/GTP cycle of binding and hydrolysis (Fig. 3A). In the "inactive" or "basal" state, the G-protein α-subunit is bound to the βγ-subunit (forming the heterotrimer) with GDP bound to the guanine nucleotide-binding site of the $G_\alpha$ subunit. Upon activation of the receptor by its ligand, the subunits dissociate, GDP is released from the Gα subunit, and GTP binds to and activates $G_\alpha$. Thus, the activated receptor acts as a guanine nucleotide exchange factor. Both $G_\alpha$ (with GTP bound) and the $G_{\beta\gamma}$ subunits regulate effector molecules until GTP is hydrolyzed, and the heterotrimer reassociates, returning the system back to the beginning of the cycle. The rate at which $G_\alpha$ is able to hydrolyze receptor-bound GTP determines the duration of the active state. GTPase-activating proteins (GAPs) accelerate the rate of GTP hydrolysis. Examples of these include regulators of G-protein signaling (RGS) (see below), $G_\alpha$-interacting proteins, and phospholipase C-β.

## C. Regulators of G-Protein Signaling

RGS proteins function to regulate the hydrolysis of GTP to GDP, thereby promoting conversion of the $G_\alpha$

TABLE 4 **Bacterial Endotoxins and Their Effect on Signal Transduction**[a]

| Bacterium | Toxin | Cellular target/residue |
|---|---|---|
| *Bordetella pertussis* | Pertussis | $G_{i\alpha}$/Cys |
| *Vibrio cholerae* | Cholera | $G_{s\alpha}$/Arg |

[a] Pertussis toxin and cholera toxin have ADP-ribosyltransferase activity and their intracellular substrates are $G_{i\alpha}$ and $G_{s\alpha}$ subunits, respectively. Pertussis toxin targets a Cys residue of $G_i$, which then becomes ADP-ribosylated, thus locking it in the inactive state. Cholera toxin targets an Arg residue of $G_s$, which then becomes ADP-ribosylated; GTPase activity is abolished, and $G_s$ is "locked" in the active monomeric state.

subunit from an active to an inactive state (Fig. 3B). These proteins provide another mechanism for shutting off G-protein signaling; however, this process differs from that of arrestins and G-protein receptor kinases (GRKs) by acting at the level of the G-protein instead of the receptor. RGS proteins constitute a family of 20 proteins. They were first discovered when discrepancies were noted between the rate at which certain physiological responses were terminated (approximately milliseconds) and the rate at which isolated $G_\alpha$ subunits hydrolyzed GTP (approximately seconds) in systems such as photoreception. The duration of GPCR signaling is subject to the rate of $G_\alpha$ GTP hydrolysis, and signal deactivation of the GTP-bound G-protein can occur only by means of the intrinsic guanosine triphosphatase activity of the $G_\alpha$ subunit. Clues to this timing inconsistency came from the discovery that certain effectors can also act as GAPs by enhancing the rate of GTP hydrolysis by $G_\alpha$ subunits (Fig. 3B).

In addition to their role as effectors for G-proteins, RGS proteins can also act as positive regulators of $G_s$-coupled receptors, or by increasing the concentration of free $G_{\beta\gamma}$ subunits. Specific RGS proteins inhibit different $G_\alpha$ subfamilies. For example, RGS4 is thought to inhibit signaling from some $G_{q\alpha}$-coupled receptors. GRKs also contain RGS domains, which can provide an explanation for the direct effect that GRK2 has on $G_{q\alpha}$ activity.

## D. Receptor–G-Protein Interactions

GPCRs interact with an ever-increasing number of G-proteins (Table 3), giving rise to the question of how a GPCR dictates the recruitment of a particular G-protein. Numerous studies using either synthetic peptides or minigenes encoding GPCR cytoplasmic domains, receptor chimeras, and site-directed mutagenesis have provided strong evidence that the important receptor contact sites for G-proteins are located in the third intracellular loop of a variety of GPCRs. Specificity for a particular G-protein may be due to different contact sites within the same domain or multiple domains within the receptor.

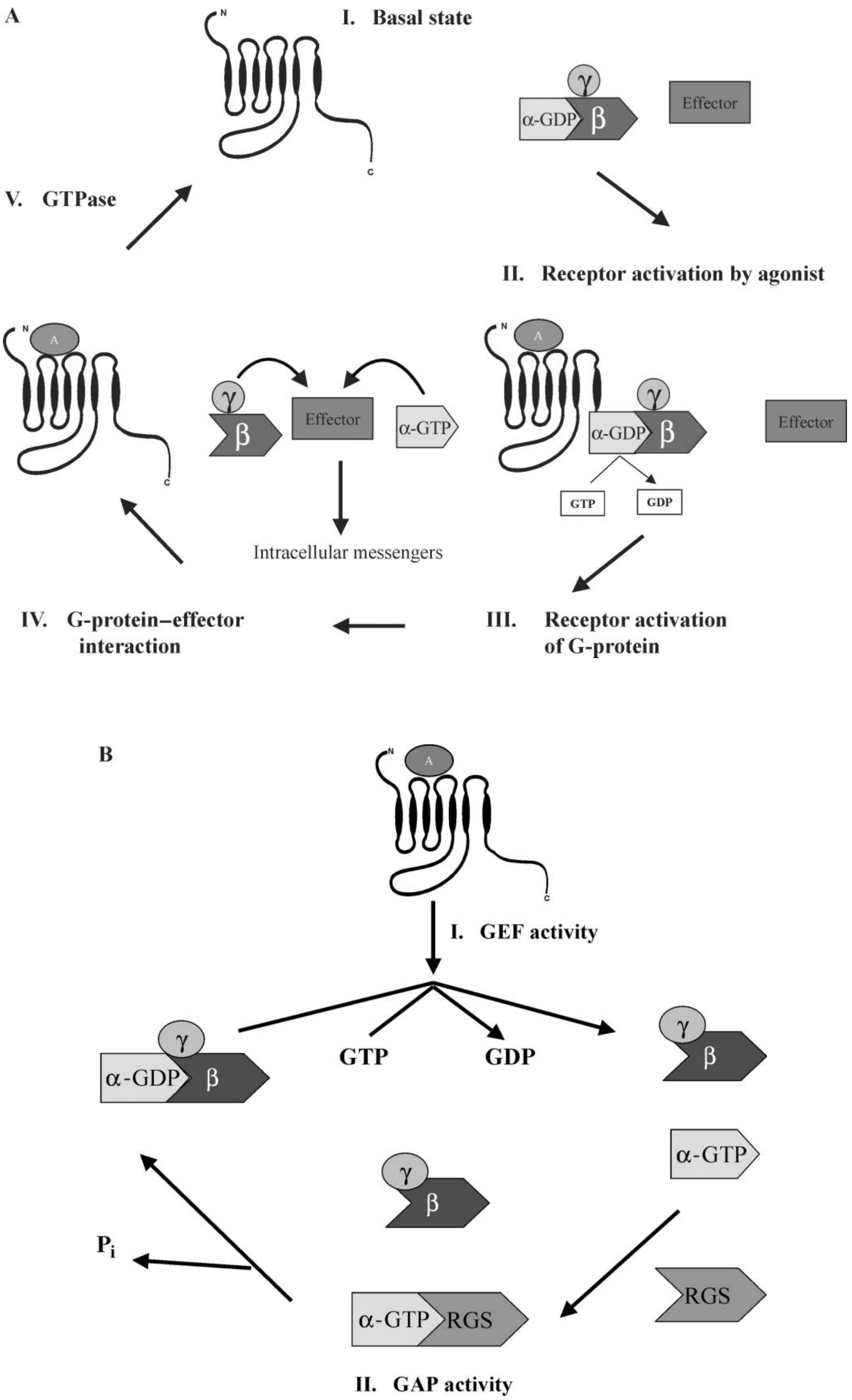
Receptor–G-protein interactions do not use only the $G_\alpha$ subunit, as $G_{\beta\gamma}$-binding sites have also been identified in some GPCRs. With regard to $G_{\beta\gamma}$, current evidence suggests that these tightly associated subunits can also regulate effector molecules including adenylate cyclase, PLC-β, and phospholipase A2, regulate the activation of mitogenic-activated protein kinase (MAPK) pathways, and, in a more recently proposed role, function as a docking protein to provide an interface for an array of signaling pathways.

## E. GPCR Signaling

GPCRs translate an extracellular signal into an intracellular response, regulating events such as proliferation, apoptosis, secretion, and differentiation. This signal transduction event involves a complex network of interwoven signaling cascades in which the signal becomes amplified and directed in a specific manner. The propagation and amplification of the primary signal involve a wide array of enzymes with very specialized functions. Many of these signaling enzymes propagate the signal by posttranslationally modifying other cellular proteins involved in the signaling cascade. Enzymes such as protein phosphatases and kinases play an important role with respect to the phosphorylation state of cellular proteins. Altering the phosphorylation state of proteins determines whether or not a signal is transduced.

Following ligand binding by GPCRs, the receptor undergoes a conformational change that is thought to involve helical rearrangements within the structure of the receptor. The activation signal is then transmitted via the cognate G-protein located on the cytoplasmic surface of the membrane, to the interior of the cell. Activation of the receptor causes initiation of a number of downstream effectors, such as second-messenger-generating adenylyl cyclases, phospholipases, and ion channels. There is also a concomitant increase in GTPase activity of the $G_\alpha$ subunit.

Classical examples of GPCR signaling are shown in Fig. 4. Binding of GPCRs to $G_{s\alpha}$ activates adenylyl cyclase to produce cAMP, which acts as a second messenger to activate protein kinase A (PKA). PKA is a serine/threonine kinase that is able to phosphorylate a plethora of substrates leading to a cellular response. $G_{i\alpha}$ has an inhibitory effect on adenylyl cyclase activity and regulates ion channel function through release of the βγ-subunits. Binding of GPCRs to the $G_{q\alpha}$ family of proteins activates the plasma membrane-associated PLC-β, causing hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$). $IP_3$ activates $IP_3$ receptors located on intracellular calcium stores, causing a rise in intracellular $Ca^{2+}$. DAG can also activate calcium channels. Both DAG and $Ca^{2+}$ activate protein kinase C (PKC), which in turn can initiate signaling of the MAPK cascade pathways. MAP kinases


A
I. Basal state
N
C
γ
α-GDP
β
Effector
II. Receptor activation by agonist
N
A
C
γ
α-GDP
β
Effector
GTP
GDP
III. Receptor activation of G-protein
IV. G-protein–effector interaction
γ
β
Effector
α-GTP
Intracellular messengers
N
A
C
V. GTPase
B
N
A
C
I. GEF activity
γ
α-GDP
β
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RGS
$P_i$
II. GAP activity

play a central role in mitogenic signaling and growth-promoting pathways. They complete the signal transduction pathway by translocating themselves into the nucleus and initiating the transcription process. This process ultimately regulates the expression of genes essential for cellular processes such as proliferation and development.

## V. ATTENUATION OF GPCR SIGNALING

GPCRs provide a rapid response to a variety of ligands and are often rapidly attenuated in their signaling despite the continued presence of the stimuli. Failure to terminate GPCR signaling resulting in prolonged stimuli can have dramatic consequences for the cell. The balance between receptor signaling, desensitization, and resensitization is very important in maintaining cell function and GPCR responsiveness. From a clinical perspective, receptors undergoing rapid desensitization can limit the therapeutic effects of many receptor agonists. GPCR systems have a number of mechanisms for attenuating the agonist-stimulated signal. These include ligand removal, receptor desensitization, internalization, and down-regulation. Following internalization, receptors are either degraded via the lysosomal pathway or recycled back to the cell surface, contributing to the process of resensitization. The cell's ability to either maintain responsiveness or shut off response to repeated signals represents a coordinated balance between the molecular mechanisms governing receptor signaling, desensitization, internalization, and recycling.

### A. Removal of Ligand

Certain receptors utilize a mechanism whereby the signaling molecule is removed from the extracellular fluid. Neurotransmitters such as acetylcholine have a specific degrading enzyme (acetylcholinesterase) that effectively can accomplish ligand removal. Inhibition of acetylcholinesterase results in prolonged signaling at the neuromuscular junction and can have detrimental effects in humans such as leading to uncontrollable spasms. On the other hand, acetylcholinesterase inhibitors can be used as a form of therapy for patients with Alzheimer's disease. These patients have fewer cholinergic neurons and inhibitors of acetylcholinesterase can compensate for the loss in cholinergic stimuli. Transporters for ligands such as dopamine, serotonin, and GABA are able to remove these neurotransmitters from the synapse, thus terminating signaling. This is in contrast to the degrading enzyme, as reuptake conserves neurotransmitter so that it can then be recycled back to the receptor. Drugs such as cocaine act by inhibiting reuptake of dopamine or adrenaline, thus preventing termination of signaling.

### B. Desensitization

Desensitization refers to the phenomenon in which the prolonged stimulation of a response induced by a receptor begins to wane. Desensitization consists of three processes: receptor phosphorylation, which occurs in seconds to minutes; internalization, which occurs in minutes to hours; and down-regulation, which continues for hours to days. There are two types of desensitization: homologous desensitization, which is defined as the agonist-induced reduction in cellular response that occurs when the receptor is stimulated only by this particular agonist, and heterologous desensitization, which is defined as the reduction in response of a particular receptor following activation of other receptors in the cell by their respective agonists. These processes are reversible following removal of the agonist, after which a process known as resensitization occurs and GPCRs gradually regain their ability to respond to ligands.

### C. Receptor Phosphorylation

Following exposure to ligand, receptors undergo rapid phosphorylation events and this modification

FIGURE 3 (A) G-protein GTPase cycle. In the basal state (I), the G-protein is a heterotrimer with GDP bound tightly to the $G_\alpha$ subunit. Agonist-bound receptor (II) acts with the G-protein and catalyzes the release of bound GDP, allowing GTP to bind (III). This exchange results in the dissociation of the $G_\alpha$ from the $G_{\beta\gamma}$ subunit, which in turns interacts with an effector protein to produce an intracellular signal (IV). Effector regulation by G-proteins is transient and terminated by an intrinsic GTPase activity of $G_\alpha$, resulting in the hydrolysis of bound GTP to GDP and a return to the basal state where the $G_\alpha$ has high affinity for $G_{\beta\gamma}$, thus reassociating to form the heterotrimer. (B) Regulators of G-protein signaling (RGS proteins). Following agonist-induced stimulation, RGS proteins accelerate the rate of GTP hydrolysis as a mechanism of reducing receptor signaling. (I) Agonist binding to receptors enhances guanine nucleotide exchange (GEF) activity, resulting in the association of GTP with the $G_\alpha$ subunit. (II) RGS proteins act as GTPase-activating proteins (GAPs) for heterotrimeric G-proteins by accelerating the rate of GTP hydrolysis by $G_\alpha$, leading to reassociation of $G_\alpha$ with $\beta\gamma$-subunits.

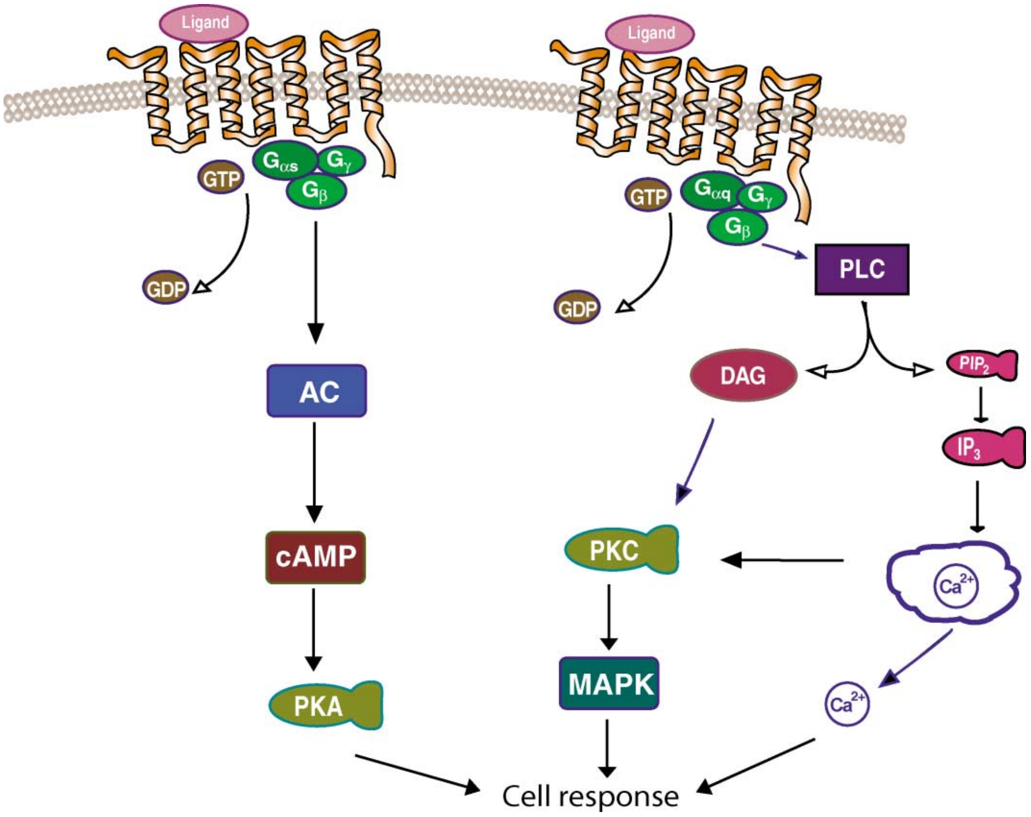

**FIGURE 4** G-protein receptor signal transduction pathways. Simplified signaling pathways highlighting cyclic nucleotide and phospholipase cascades triggered by ligand-activated GPCRs. Ligand binding to a $G_s$-coupled GPCR results in the activation of adenylate cyclase (AC), causing an accumulation of cyclic AMP (cAMP). Cyclic AMP binds to protein kinase A (PKA), which then phosphorylates targets in the cytoplasm and nucleus, resulting in a cellular response. Ligand binding to $G_q/G_{11}$-linked GPCR results in activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphonate ($PIP_2$) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$). The $IP_3$ interacts with a calcium channel in the endoplasmic reticulum, raising intracellular $Ca^{2+}$ levels. The increase in $Ca^{2+}$ levels activates protein kinase C (PKC), which then initiates signaling via the mitogen-activated protein kinase (MAPK) cascade pathways, resulting in a downstream cellular response.

is thought to be responsible for G-protein uncoupling. Phosphorylation consensus sites are found in the intracellular domain of the GPCR, particularly within the third intracellular loop and the C-terminal tail. Different kinases have been implicated in GPCR phosphorylation and these include the second-messenger kinases (PKA, PKC), the family of GRKs (GRK1–GRK7), and casein kinases (1a and II). Of these, the GRKs have received the most attention for their roles in GPCR desensitization and internalization. There are currently seven members of the GRK family, all of which share significant sequence homology, but differ in their tissue distribution and regulatory properties. GRK1 is confined to the retina and pineal gland; GRK4 is localized to the testis and GRK7 to the retina. GRK2, GRK3, GRK5, and GRK6 are ubiquitously expressed. Distinct consensus sites for GRK phosphorylation are largely unknown although Ser and Thr residues that are GRK substrate sites have been characterized for some GPCRs. Interestingly, cross-talk between PKC and GRKs exists (e.g., PKC can phosphorylate GRK2, which enhances its kinase activity).

## D. Model of GPCR Internalization

The molecular mechanisms surrounding internalization of numerous GPCRs have been studied, and the following model, which implicates key players regulating this event, has been proposed (Fig. 5). Briefly, the first step in this process is the binding of agonist to receptor. This then triggers GPCR

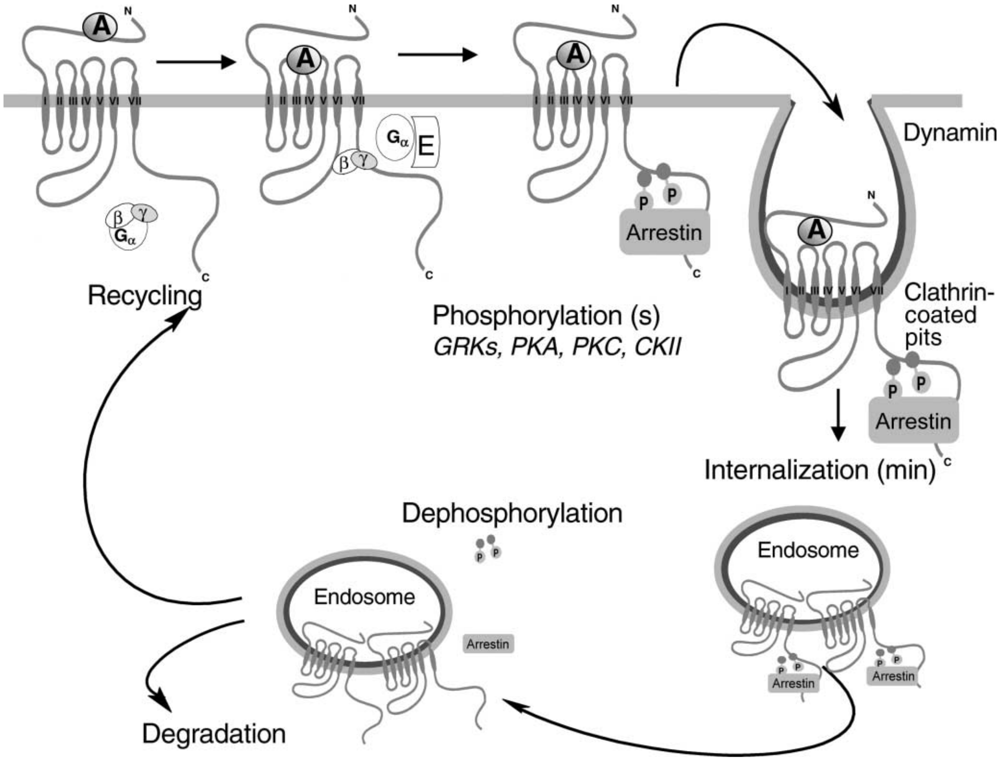

**FIGURE 5** Heptahelical receptor trafficking. Agonist occupancy of the receptors leads to activation of G-proteins, resulting in the dissociation of α- and βγ-subunits. G-protein receptor kinases are recruited to the receptor by the free βγ-subunits and phosphorylation of intracellular domains of the receptor then occurs. Agonist-induced receptor phosphorylation occurs within seconds, causing the receptor to become desensitized to further stimuli. Subsequently, β-arrestins facilitate recruitment of the receptors to clathrin-coated pits and they undergo internalization into the interior of the cell. Dynamin plays a role in pinching off vesicles from the cell membrane. Following dephosphorylation, receptors either are recycled back to the cell surface ready for reactivation by ligand or enter the lysosomal pathway and undergo degradation.

phosphorylation by phosphorylating enzymes (i.e., GRKs), which then results in the creation of high-affinity binding sites for the β-arrestins. β-Arrestins then bind to the receptor, forming a complex with the adapter protein, activator protein-2 (AP-2) as well as the heavy chain of clathrin. AP-2 is thought to be responsible for targeting the receptor–β-arrestin complex to clathrin-coated pits. Thereafter, the vesicles containing the receptors begin to bud from membranes, a step that requires the GTPase activity of dynamin. β-Arrestin interacts with another protein, c-Src, a tyrosine kinase that regulates the phosphorylation state of dynamin. Vesicles are then targeted to the endocytic compartment of the cell via the endosomes. The fate of the receptor is dependent on whether it is retained within the endosomes before being recycled back to the cell surface or whether it is destined for the lysosomal pathway to be degraded. Specific molecular determinants that determine receptor fate are contained within the GPCR intracellular domain and clusters of serine residues in the carboxy tail have been implicated in this regard.

## E. Arrestins

Binding of arrestins to agonist-activated GPCRs results in the deactivation of the signaling pathway. Arrestins are cytoplasmic adapter proteins that rapidly translocate to the cell surface to bind to GPCRs upon their phosphorylation. The binding of arrestins to phosphorylated receptors has been implicated in G-protein uncoupling, desensitization, and internalization. To date, the arrestin gene family consists of at least four members: the visual arrestins (rod and cone) and β-arrestins 1 and 2. β-Arrestin 2 is also referred to as arrestin-3, although an additional two arrestin isoforms, D- and E-arrestins, may also exist. D- and E-arrestins are partial clones that are expressed in a broad range of tissues and have not yet been properly characterized. Visual arrestins are found mainly in the retina and less so in the pineal gland, cerebellum, and primary blood leukocytes. Cone arrestins are found mostly in cone photoreceptors but are also localized to the pineal gland and pituitary. β-Arrestins 1 and 2 are ubiquitously expressed. The phenomenon of cytosolic arrestins being recruited to the cell surface where they colocalize with receptors has been demonstrated using confocal microscopy with β-arrestins fused to green fluorescent protein.

## VI. RECEPTOR–PROTEIN INTERACTIONS

GPCRs appear to be capable of interacting with a bewildering array of adapter/scaffolding proteins, some of which play a role in pulling proteins together. Some of these protein–protein interactions are thought to lead to other signaling pathways for GPCRs, beyond the classical G-protein-dependent pathways, represented in Fig. 4. In addition to facilitating GPCR–effector interactions, these proteins can play a role in determining the specificity of the activation of downstream signaling cascades as well as ensuring that these signaling complexes are directed to the appropriate compartments within the cell.

Thus far, a number of novel binding partners for GPCRs that can activate nonclassical G-protein-mediated signaling have been discovered. Of particular note is the large family of PDZ-domain-containing proteins (named after the first three proteins in which they were discovered, i.e., the postsynaptic density protein PSD-95, the *Drosophila* tumor suppressor protein discs-large, and the tight junction protein ZO-1) that interact with the C-terminal domains of GPCRs (e.g., $\beta_2$-adrenergic receptor, rhodopsin, dopamine D2 receptor, and somatostatin receptor 2). In the case of the $\beta_2$-adrenergic receptor, the interaction of the PDZ protein NHERF allows receptor regulation of a $Na^+/H^+$ exchange, whereas for the rhodopsin receptor, InaD acts as a multisignaling scaffold that can interact with downstream effectors such as PLC, PKC, and ion channels. Since many more GPCR/PDZ interactions are being reported, this has led to the suggestion that signaling via these proteins may be a common feature among GPCRs. Other non-G-proteins involved in GPCR signaling bind to proline-rich domains in the receptor, e.g., the interaction between Homer 1 and metabotropic glutamate receptor, which facilitates the interaction with $IP_3$ receptors, which in turn regulate intracellular calcium release. In addition, dopamine D4 receptors interact via their SH3 domain with the adapter proteins Grb2 and Nck, providing a mechanism for GPCR activation of tyrosine kinase signaling cascades.

Transactivation of growth factor receptors by GPCRs represents another non-G-protein-mediated mechanism for GPCR activation of the signaling pathways (MAPK pathways) for receptor tyrosine kinase. GPCRs are known to transactivate the growth factor receptors, such as epidermal growth factor receptor, by a currently unknown mechanism. Recent studies have shown that the $\beta_2$-adrenergic receptor and insulin-like growth factor receptor exist as complexes, suggesting that these two receptors may physically interact or dimerize to achieve this transactivation.

There are several examples of adapter/scaffolding proteins that link GPCRs to effectors within the classical G-protein-linked pathways. For instance, the A-kinase anchoring proteins link GPCRs with $G_{\alpha 12}$ and PKA. In some cases, the mechanism of linking proteins together in this manner may facilitate the rapid signaling necessary for certain functions such as fly vision.

Another receptor–protein interaction that has been shown to occur is one between spinophilin and the third intracellular loop of the dopamine D2 receptor. Spinophilin, also known as neurabin-2, contains several protein-binding domains including a PDZ domain and an F-actin-binding domain. It is also known to bind the third intracellular loop of the α2-adrenoceptor, suggesting that this protein might serve to localize or coordinate signaling for a range of GPCRs, although the precise functional role of this interaction is not fully understood.

Certain GPCRs such as the calcitonin receptor-like receptor (CRLR) have been shown to form associations with receptor activity-modifying proteins (RAMPs). This association was shown to be important both for targeting the receptor to the cell surface and for altering its pharmacological properties. RAMP1 converted CRLR into a receptor with properties resembling calcitonin-gene-related-peptide receptor, and RAMP2-associated receptors displayed properties similar to that of an adrenomedullin receptor. It is not yet clear whether RAMPs form associations with other GPCRs and if they do, what roles they may play in GPCR function.

## VII. GPCR OLIGOMERIZATION

The recent literature suggests that GPCRs might exist as oligomers, undergoing homo- and/or hetero-oligomerization. For Class A receptors, hydrophobic interactions between transmembrane domains are thought to be important in receptor–protein interactions. In addition, there is evidence that extracellular cysteine residues might be involved in covalently cross-linking receptors, and another possibility is that the intracellular carboxy tails might provide a mechanism for holding the receptor dimers together.

### A. Approaches for Demonstrating Receptor Oligomerization

Early biochemical evidence was mainly provided by co-immunoprecipitation studies that demonstrated both homo- and hetero-oligomerization of GPCRs. However, more definitive evidence of oligomers forming a functional unit resulted from studies using 'split receptors.' Upon co-expression of each 'half' of the receptor, full receptor function could be restored even when the halves of two different receptors were co-expressed as chimeras, suggesting the existence of heterodimers. Similarly, transcomplementation experiments also provide evidence for the existence of a functionally active receptor complex. Here, individual receptors are inactivated by mutations in distinct receptor domains and have restored function upon co-expression of each mutant receptor. Resonance energy transfer techniques such as fluorescence resonance energy transfer and bioluminescence resonance energy transfer have been useful for detecting oligomerization in live cells for a number of GPCRs, such as the β2-adrenergic receptor, thyrotropin-releasing hormone receptor, gonadotropin-releasing hormone receptor, and somatostatin and opioid receptor subtypes.

### B. Functional Significance of GPCR Oligomerization

In some cases, the occurrence of homo- versus hetero-oligomerization has been proposed to play an important role in determining the functional characteristics of receptors, thus generating the possibility of pharmacological diversity. The importance of hetero-oligomer formation between GPCRs is highlighted by an example provided by the $\mathrm{GABA_B}$-R1 receptor, which forms a heterodimer with the $\mathrm{GABA_B}$-R2 receptor. The R1 receptor is incapable of forming a functional receptor by itself as it becomes trapped in the *trans*-Golgi network and is unable to traffic to the cell surface. Only when it forms a hetero-oligomer with the $\mathrm{GABA_B}$-R2 receptor can it be delivered to the cell surface. Interestingly, the $\mathrm{GABA_B}$-R2 receptor expressed as a homo-oligomer can form a functional receptor; however, there have been reports that the homo-oligomer receptor displays different signaling properties than the hetero-oligomer.

Localization of GPCRs to their site of action within a cell is critical for the correct functioning of these receptors. This involves both the targeting of the receptor to its proper location in the membrane and the retention of the receptor after it reaches its destination. Receptor oligomerization may occur either at biosynthesis or after the receptors have reached the cell surface, the latter opening the possibility for ligand-induced effects. The majority of GPCRs studied thus far appear to undergo oligomerization constitutively, although for some receptors, the ligand may also have a role in regulating oligomerization.

The fact that this process has been reported for such a variety of receptors across different subclasses suggests that dimer and/or oligomer formation is a universal process among this superfamily of receptors. If so, the current level of understanding is just at the initial stage of deciphering how and why this occurs for different receptor systems.

## VIII. GPCR MUTATIONS AND DRUG TARGETS

Many agonists and antagonists of GPCRs are used for therapeutic purposes, as GPCRs are the target for more than half of all known medicines. For instance, the dopamine agonist L-DOPA is the agent of choice in the treatment of Parkinson's disease, and a dopamine antagonist is used to treat schizophrenia as well as Huntington's disease in its early stages. Agonists and antagonists of adrenoceptors are used for the treatment of hypertension and other cardiovascular disorders as well as anxiety and asthma. Muscarinic agonists are used in the treatment of glaucoma and tachycardia, serotonin antagonists are effective tools to treat migraine, and histamine H1 antagonists are used against allergic and anaphylactic reactions, hay fever, itching, and motion sickness.

Mutations in GPCRs can result in altered function and have been associated with a number of hereditary or somatic disorders ranging from infertility to cancer. Examples of these disease-causing mutations include retinitis pigmentosa, nephrogenic diabetes insipidus, and asthma. Defects of some GPCRs result in a gain-of-function phenotype, the consequences of which is constitutive bioactivity. This is in contrast to loss-of-function phenotypes that result in deficient bioactivity. Table 5 gives a brief summary of some receptors and their associated disease states, effects, and treatments.

The process of heterodimerization occurring between GPCRs has also been implicated in certain disease states and an example is a recent observation that heterodimers formed between the $AT_1$ receptor for the vasopressor angiotensin II and the $B_2$ receptor for the vasodepressor bradykinin are involved in preeclampsia. As hetero-oligomer formation can result in altered pharmacology, it provides a novel target for drug discovery. Therefore, by using combined treatments that selectively target the hetero-oligomer, it could be envisaged that some of the unwanted side effects encountered with some GPCR agonists could be overcome.

**TABLE 5 Disorders and Treatments Related to GPCR Dysfunction**

| Receptors | Related diseases, effects, and treatments |
|---|---|
| Adrenoreceptors | Asthma, hypertension, anxiety |
| Angiotensin II/bradykinin B2 | Preeclampsia |
| Bradykinin | Inflammation, pain, shock |
| Calcitonin | Paget's bone disease |
| CCK | Anxiety |
| Dopamine | Parkinson's disease, schizophrenia, Huntington's disease |
| FSH | Infertility |
| $GABA_B$ | Analgesics |
| GnRH | Prostate cancer, precocious puberty |
| Histamine 1 | Hay fever, itching, motion sickness |
| Metabotropic | Tinitus |
| Muscarinic | Glaucoma, tachycardia |
| Opioid | Pain |
| Opsins | Color blindness |
| Oxytocin | Induces labor and promotes lactation |
| PAF | Inflammation, asthma |
| PTH | Chondrodysplasia |
| Rhodopsin | Retinitis pigmentosa |
| Serotonin | Depression, migraine, postoperative vomiting |
| Somastostatin | Tumors, glucagonoma |
| TSH | Hyperthyroid |
| Vasopressin | Nephrogenic diabetes insipidus |

*Note.* CCK, cholecystokinin; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; PAF, platelet activating factor; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone.

## IX. SUMMARY

GPCRs are elegantly engineered switches designed to turn on (and off) an enormous range of physiological responses. These include sensory perception (pain, taste, smell, and light), hormonal control of most physiological functions, synaptic neurotransmission, regulation of chemotaxis, stimulation of mitosis, and entry of viruses into cells. In addition to responding to such a range of natural ligands and stimuli, GPCRs form the targets for more than half of all prescription drugs currently on the market. However, despite their pivotal role in all these functions, how the heptahelical receptors actually operate as ligand-activated control systems is still not fully understood. Although the structure of rhodopsin has been solved, inherent difficulties have been experienced in obtaining the crystal structure of any other GPCR, GPCR–ligand, or GPCR–G-protein complex. GPCRs have been found to form either homo-oligomers or hetero-oligomers with a structurally different GPCR. Additionally, GPCRs are able to interact with a range of "partner proteins" such as β-arrestins and RAMPs. This ability to form interactions with adapter proteins and scaffolding proteins has resulted in a paradigm shift away from the more classical linear signaling pathways, instead focusing on more complex signaling networks in which common components orchestrate the responses of multiple interlinking pathways. The identification of the natural ligands for the many orphan GPCRs is still awaited and it is anticipated that this information will greatly aid in the intervention and treatment of a variety of diseases that involve the GPCR family.

## Glossary

**arrestins** Proteins that arrest receptor function by uncoupling the receptor from its cognate G-proteins to target the receptor to the intracellular compartment of the cell by means of endocytosis. The arrestin family consists of visual arrestins (rod and cone arrestins), which have limited tissue distribution, and β-arrestin 1 and β-arrestin 2, which have a more widespread distribution.

**desensitization** Loss of functional response; can be short-term (seconds or minutes) or long-term (hours).

**down-regulation** Loss of total receptor number (not just on the cell surface) due to agonist-induced endocytosis and subsequent degradation.

**endocytosis** Movement of receptor–ligand from the plasma membrane to an internal compartment.

**G-protein-coupled receptors** A pharmacologically important protein superfamily with more than 400 genes identified to date. Pathways involving these receptors are the targets of hundreds of drugs, including antihistamines, neuroleptics, antidepressants, and antihypertensives.

**G-proteins** Guanine nucleotide-binding proteins are heterotrimeric and composed of α-, β-, and γ-subunits. A number of these subunits have been identified (to date, 20 α-, 5 β-, and 12 γ-subunits) and make up at least four general classes of G-proteins: $G_s$, $G_i$, $G_{q/11}$, and $G_{12}$. G-proteins are responsible for transducing signals from G-protein-coupled receptors to effectors, including adenylyl cyclase and phospholipases.

**heptahelical receptors (G-protein-coupled receptors)** Relates to the characteristic structure in which stretches of hydrophobic amino acids span the cell's membrane seven times.

**internalization** Also termed sequestration; the loss of surface receptor number determined by a combination of the effects of endocytosis and recycling. A commonly used route for G-protein-coupled receptor internalization is via clathrin-coated pits.

**receptor** A protein molecule that binds very specifically to its cognate hormone to generate a receptor–hormone complex. This complex activates a cellular signal transduction pathway, resulting in the appearance of a biological response.

**receptor–ligand binding** The binding of a ligand to a receptor can be described mathematically by a dissociation constant ($K_d$), whose unit is concentration. This equilibrium constant provides information on the amount of ligand needed to elicit a biological effect. The lower the magnitude of $K_d$, the greater the affinity between ligand and receptor.

**receptor recycling** Movement of a receptor from an internal compartment to the cell surface.

**RGS proteins (regulators of G-protein signaling proteins)** Effectors that act as guanosine triphosphatase-activating proteins, enhancing the rate of GTP hydrolysis by $G_\alpha$ subunits.

## See Also the Following Articles

**GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G Proteins • Multiple G-Protein Coupling Systems • Receptor–Receptor Interactions • RGS Protein Superfamily**

## Further Reading

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## Useful GPCR Web Sites and Public Domain Databases

Entrez is a retrieval system for searching several linked databases provided by the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/Entrez/

GPCRDB (Information system for G-protein-coupled receptors): http://www.gpcr.org/7tm/

Kyoto Encyclopedia of Genes and Genomes (KEGG) information pathways of interacting molecules or genes: http://www.genome.ad.jp/kegg/ortholog/tab04030.html

GRAP and tGRAP (Databases of mutants of Family A G-Protein-Coupled Receptors covering ≥7000 mutants): http://tinygrap.uit.no/GRAP/homepage.html

List of G-protein-coupled receptors expressed in different cell lines: http://www.tumor-gene.org/GPCR/gpcr.html

Database of olfactory receptor and chemoreceptor sequences: http://senselab.med.yale.edu/senselab/

Compendium of GPCR fingerprints (group of conserved motifs used to characterize a protein family): http://www.bioinf.man.ac.uk/dbbrowser/gpcrPRINTS/

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# Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors

Gerald Litwack

*Thomas Jefferson University, Philadelphia*

I. INTRODUCTION
II. GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS
III. HETERODIMERIZATION AND EFFECTS

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**Glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) are proteins with ligand-binding sites for glucocorticoids, such as cortisol, and for aldosterone, respectively. If both GRs and MRs are present in the same cell and the ligands for both receptors are also present, heterodimerization as well as homodimerization of the receptor molecules may take place. The relative amounts of the homodimers GR–GR and MR–MR and of the heterodimer GR–MR that are present will depend on the number of molecules of each receptor type as well as the amounts of the ligands in the cell. The heterodimer produces effects that differ from those produced by the homodimeric species.**

## I. INTRODUCTION

Glucocorticoids, such as cortisol, are generated in the adrenal cortex (most cortisol comes from the middle layer of cells called the zona fasciculata) in response to a stress signal. This stress signal emanates from the brain through the hippocampus, the anterior pituitary, and finally the adrenal cortex, with specific hormones being released from the hippocampus and pituitary, each signaling the next organ in the sequence and ultimately causing cortisol to be synthesized and secreted into the bloodstream. Elevated levels of cortisol in the bloodstream, in turn, feed back negatively on the brain, the hippocampus, and the anterior pituitary to shut off the original signals. This is accomplished by the binding of cortisol to intracellular (cytoplasmic) glucocorticoid receptors (GRs) whose actions lead to a reduction of the forward-feeding hormone that culminated in the release of cortisol. Thus, the system is poised to receive another set of signals when a new stress arises. However, stress resulting in the secretion of cortisol from the adrenal cortex is not the only mechanism whereby this hormone is released. The adrenal cortex is also under the control of cells in the brain that release serotonin, and the release of serotonin occurs by an endogenous mechanism that may be independent of the stress mechanism. However, once serotonin is released, the same mechanism that is seen during stress is activated except that the secretion of serotonin is elevated in the early morning, declines for the rest of the day, and rises again in the early morning of the next day; thus, this cycle can be viewed as a biorhythmic mechanism. Superimposed on the serotonin mechanism are stress events that can take place at any time during the day.

On the other hand, aldosterone, the ligand for the mineralocorticoid receptor (MR), is released from the outer layer of the adrenal cortex (zona glomerulosa) essentially only as the result of a stress stimulus. Although both of these receptors are located in the cellular cytoplasm before the ligand enters the cell, their actions take place in the cellular nucleus after the cognate ligand binds to its receptor. First, the receptor complex in the cytoplasm is “activated” or “transformed” and carried into the nucleus by a complex mechanism. Here, the receptor binds to a hormone-responsive element on the promoter of specific genes, causing RNA to be synthesized from the specific gene, and the RNA is transported into the cytoplasm where it is translated into proteins. These may be catalytic proteins (enzymes) that alter the cellular metabolism and account for the cellular response to the hormone. Overall, the summation of all the cellular responses (that may be different from different target cells) lead to the systemic (bodily) response. Glucocorticoids affect a large number of genes in different cells. This is exemplified by inhibition of inducible cyclooxygenase II, an inflammation-causing enzyme by virtue of its catalytic role in the synthesis of certain prostaglandins. Inhibition of the enzyme activity by reduction of its synthesis produces an anti-inflammatory response. Over a prolonged period of stress, the production of antibodies may be reduced, making it more difficult to fight off infections, and there may be a breakdown of proteins, causing muscle wasting after surgical stress, for example.

Mineralocorticoids, such as aldosterone, affect a smaller set of genes; they function primarily to increase the uptake of sodium ions in the kidney, distal colon, and elsewhere by stimulating the components of the sodium conductance channel in the apical cells lining lumens. Mineralocorticoids also increase the levels of sodium–potassium ATPase on the basolateral cell membrane (blood side), where both effects increase the uptake and transport of sodium ions across the cell and result in the accumulation of sodium ions in the bloodstream. If the uptake of sodium ions into the blood is sufficient, the effect on the posterior pituitary, which senses the increased sodium ion concentration, is such that water is reabsorbed by the kidney tubule, increasing the partial pressure in the blood vessels. Recently, several genes have been discovered to be under the control of aldosterone, and the studies on such genes are ongoing.

In some cell types, for example, cells found in the kidney and the distal colon, both GRs and MRs are produced by the same cell. Also, both cortisol and aldosterone can be taken up by these cells. Cortisol will usually be available at least through the serotonergic mechanism in the absence of stress, and both aldosterone and more cortisol will be released during a stress response. Thus, in a stress situation, both ligands will be available to the target cells and presumably both receptors could be activated and generate transcriptional responses (new RNA molecules) in the nucleus. When solely cortisol is taken up by the cell, its effects would be manifested. The liganded cortisol (glucocorticoid) receptor will homodimerize (two receptor molecules bound to cortisol form a complex by binding head to head) and in that form can bind to the hormone-response element on DNA and generate effects associated with glucocorticoid actions, some of which are mentioned above. If the concentration of cortisol is high enough, both GRs and MRs could be activated to generate homodimers and heterodimers, although little is known about the *in vivo* situation. However, after a stress event, aldosterone will also become available and will bind preferentially to the MR in that same cell, and the two liganded receptors can interact by a process called heterodimerization. In this case, the actions of both receptors are expected to be different at the level of DNA so that the individual responses to cortisol and to aldosterone are different when heterodimers are formed compared to only homodimers.

## II. GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS

Glucocorticoid and mineralocorticoid receptors are unique among members of the steroid receptor gene family because they are located exclusively in the cytoplasm of the target cell in the absence of the hormonal ligand (cortisol for the GR and aldosterone for the MR), whereas the other well-known receptors in this family are located exclusively in the nucleus or partitioned between the nucleus and the cytoplasm. Each receptor structure can be divided into domains, from the N-terminus to the C-terminus: the N-terminal domain, the DNA-binding domain, containing a "hinge" domain, and the steroid-binding domain at the C-terminal end. There are great similarities in the DNA-binding and steroid-binding domains, such that both receptors can bind to the same DNA sequence (hormone-responsive element) and the steroid-binding domains can bind many of the same compounds. Importantly, both receptors bind cortisol with approximately equal affinity so that expression of the receptor type in a given cell and the availability of cortisol and/or aldosterone to the cell become important to the biological effects. The N-terminus of each receptor is quite different, however, so that in this region there is only approximately 15% similarity between the two receptors. It may be that differences in receptor functions are reflected in the different sequences in the N-termini. Additionally, there are two classes of nuclear receptors: those that are associated with heat-shock proteins (Class I) and those that are not (Class II). Both the GR and the MR are Class I molecules, as are the progesterone receptor, the androgen receptor, and the estrogen receptor. On the other hand, Class II includes those receptors in this supergene family that appear to be completely compartmentalized in the nucleus in the absence of ligand; these receptors include the thyroid hormone receptor, the retinoic acid receptor, the vitamin D receptor, and peroxisome proliferator-activated receptor. Thus, the association of the GR and MR in the cytoplasm with heat-shock proteins and other proteins constitutes a "repressed" state until the specific steroidal ligand becomes available to the cell, after which cytoplasmic activation (or transformation) occurs to develop the active form, which probably dimerizes in the cytoplasm and which is able to translocate to the nucleus. Once in the nucleus, either receptor will bind to DNA (in dimerized form) and will associate with co-activator molecules and the specific gene promoter to trigger the transcriptional process. Pure nuclear forms of receptors in this gene family (Class II receptors) will be expected to reside in the nucleus in the absence of ligand and associate with co-repressors, and in the presence of ligand they would dissociate from the co-repressor proteins and associate with co-activators to enable the transcriptional process. GR and MR would not be expected to associate with co-repressors in the nucleus since their association with heat-shock proteins in the cytoplasm would constitute the repressed state.

## III. HETERODIMERIZATION AND EFFECTS

Either GR or MR can be associated with specific gene promoters in the homodimerized state. Thus, two molecules of the GR would be associated in head-to-head fashion, allowing the DNA-binding domains of each to participate in binding the major groove in DNA. The DNA-binding domain consists of two zinc-fingers in each molecule; the upstream (toward the N-terminus) zinc-finger of each binds in the major groove of DNA at the hormone-responsive element, which is the same sequence for both glucocorticoid and mineralocorticoid receptors (AGAACAnnnTGTTCT). The second, more downstream zinc-fingers of each receptor, in addition to recognizing the spatial distance between the half-sites, interact, stabilizing the dimeric complex. In the case where both the GR and the MR are present in the same cell and ligands for both receptors are present, heterodimerization may take place between each type of receptor in addition to homodimerization. The relative amounts of GR–GR, MR–MR, and GR–MR (the heterodimer) should be a consequence of the number of molecules of each receptor type as well as the amounts and types of the ligands. Interestingly, the presence of the heterodimer produces effects that are different from those produced by the homodimeric species. Thus, in hemopoietic cell systems, there may be down-regulation of the effects of either the GR or the MR. In other systems, the heterodimer results in greater activity than the homodimer or generates different outcomes altogether.

In this new area of research, there are a few specific examples to explain the effects of GR–MR heterodimers compared to GR–GR or MR–MR homodimers. It is of significance to clinical therapeutics when a receptor heterodimer targets genes differently than the homodimer. Thus, it has been shown recently in rats that a single dose of dexamethasone (specific for the GR) will generate apoptotic loss of cells that contain both GR and MR in the dentate gyrus of the hippocampus. High doses of corticosterone (equivalent to cortisol in the human), which will bind to both GR and MR, did not lead to apoptotic cell death. Furthermore, in rats pretreated with corticosterone before dexamethasone was administered, there was no cell death, suggesting that corticosterone, which activated both the GR and the MR to form heterodimers, regulates genes mediating cell survival differently from the effect of dexamethasone, which activates the GR–GR alone. Thus, clinicians may want to consider using a glucocorticoid such as prednisolone for an anti-inflammatory effect that activates GR–MR rather than a drug such as dexamethasone that activates GR–GR alone.

Another observation concerns repression of the 5-HT1A receptor promoter. This is a neuronal serotonin receptor whose repression by glucocorticoids may lead to clinical depression. Using different cells in culture, it was determined that transcriptional inhibition of the 5-HT1A receptor was greater in the presence of GR–MR heterodimers than with either GR–GR or MR–MR alone. This work suggested that GR and MR form a novel head-to-tail heterodimeric complex with a negative GRE (glucocorticoid-response element). The negative GRE contains two half-sites separated by 6 bases compared to the normal palindrome in which the two half-sites are separated by 3 bases. Apparently, the head-to-tail MR–GR accommodates binding to the negative GRE with the 6-base spacing. In the negative GRE, elimination of 3 bp between the two half-sites reverts the GRE to a positive element.

Finally, interesting studies have been carried out on the formation of GR–MR heterodimers in solution in the absence of DNA. In the formation of the GR–GR homodimer, the hinge region is sufficient to promote this dimerization in solution; peptides that mimic this region have been synthesized, and these peptides can associate with the GR to form a dimer. In the case of formation of the MR–GR heterodimer in solution, some grouping in the MR structure binds to the ligand-binding domain (in the C-terminal region) of the GR. It appears likely that the dimerization process occurs in the cytoplasm and that the dimer is transported into the nucleus in this or in a more complex form. There may be factors involved in the formation of GR–MR heterodimers in cells that are, as yet, unidentified. Figure 1 presents a concept of the dimerization of GR–GR, MR–MR, and GR–MR and how these receptor forms could interact with gene targets in the cell.

## Glossary

**glucocorticoid receptor** A protein with a ligand-binding site for glucocorticoids, the major one of which in the human is cortisol. In the human, this receptor is located in all tissues except hepatobiliary cells and pars intermedia-like cells in the pituitary.

**mineralocorticoid receptor** A protein with a ligand-binding site for specific ligands, in this case, those related to aldosterone, which is the primary ligand in the human. The receptor is not ubiquitously distributed throughout the body, like the glucocorticoid receptor, but is found mainly in epithelial cells that line lumens (tubes).

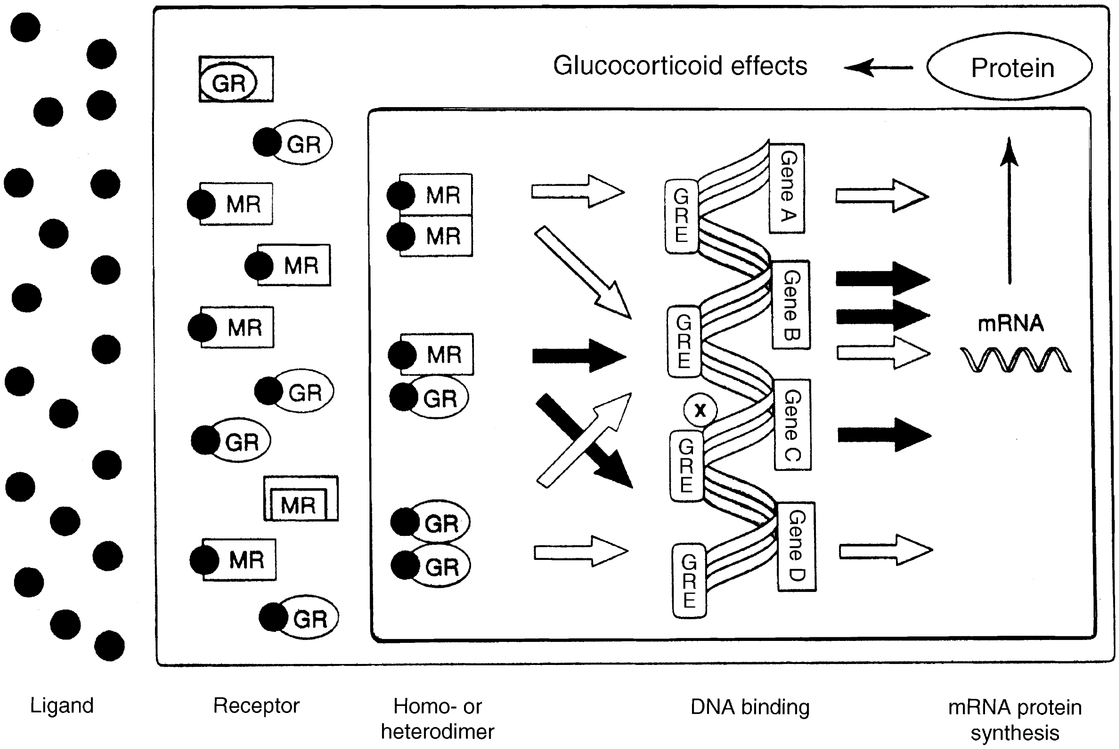

FIGURE 1 Model of corticosteroid-activated gene expression. Steroids enter the target cell by passive diffusion, where they bind to the cognate receptor (MR). This binding leads to a conformational change in the receptor and its dissociation from the inhibitory hetero-oligomeric structure. After intranuclear translocation, the MR and GR homodimers or the corticosteroid heterodimer is formed, depending on the relative concentrations of both receptors. The different corticosteroid receptor dimers bind to GREs in the flanking regions of target genes with particular DNA-binding kinetics. The binding of the dimer to the GRE leads to mRNA production and protein synthesis. The unique transcriptional activities and DNA-binding kinetics of the corticosteroid receptor are indicated by black and white arrows. The use of different genes in this illustration indicates that different corticosteroid dimers may regulate an overlapping set of genes and activate the expression of dimer-specific candidate genes. The regulation of certain genes by a specific corticosteroid receptor dimer may predominantly depend on dimer-specific protein–protein interactions with other transcription factors that are repressed in this model by a DNA-binding factor X. Reprinted from Trapp and Holsboer (1996), with permission from Elsevier.

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## Further Reading

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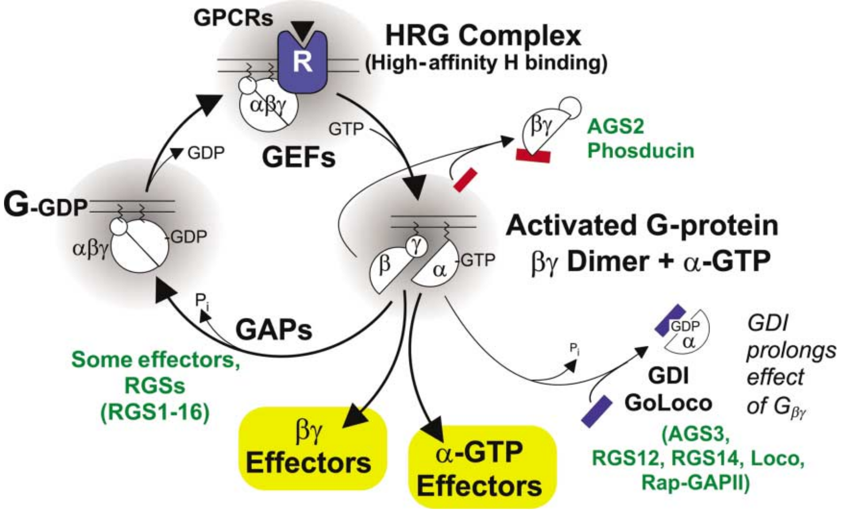

**FIGURE 1** Hormone receptor complexes act as guanine nucleotide exchange factors (GEFs) to catalyze the activation of the trimeric G-protein with formation of α–GTP plus βγ, both of which modulate effector functions. Spontaneous decay of α–GTP to α–GDP can be modulated by GTPase-activating proteins (GAPs), such as RGSs and some effectors, and by activators of G-protein signaling (AGSs) that tilt the balance between effects resulting from α–GTP and those resulting from βγ.

GTP to GDP—and reassociation of the separated members in preparation for reactivation by a new round of GDP/GTP exchange promoted by the activating hormone receptor (HR) complex (bottom panel of Fig. 1). As shown in Fig. 1, the basic cycle is affected by regulators of G-protein signaling, the RGSs, which increase the rate at which α–GTP deactivates, and the AGS2- and AGS3-type modulators, which sequester either α–GDP or $G_{\beta\gamma}$ and thereby bias signaling through α–GTP versus signaling through $G_{\beta\gamma}$ (see below).

## II. *Ras,* THE PROTOTYPIC REGULATORY GTPase

As the name indicates, regulatory GTPases are proteins that bind and hydrolyze GTP. Their regulatory power lies in the fact that their conformation differs when occupied by GTP or GDP. They are also referred to as molecular switches. Due to their intrinsic GTPase activity, they carry a built-in inactivating timer that prevents the GTP state from being long-lived. The crystal structure of the 180 aa regulatory GTPase *ras*, in its GTP- and GDP-liganded forms, shows two principal regions that differ in the GTP state compared to the GDP state, referred to as Switch I and Switch II. Switch I, amino acids 32 through 40, is a large loop connecting α-helix 1 (α1) to β-strand 2 (β2). Mutations in this region interfere with activation of several of the downstream effectors of *ras*. Switch II, amino acids 60 through 75, changes conformation even more drastically than Switch I, to the extent that upon binding of GTP, aa 66 through 74 rearrange into a well-ordered α-helix (α2). Switches I and II are required for activation of a downstream effector(s) by *ras*-GTP, the activated form of *ras*. *Ras*-GDP appears to be neutral. Mutations of Gln-61 (at the start of Switch II after β3) and of Gly-12 (at the base of α1) reduce the intrinsic GTPase activity of *ras,* prolonging the lifespan of the activated GTP state. As is the case for most, if not all, regulatory GTPases, the *ras* GTPase is under the regulation of two types of proteins, a guanine nucleotide exchange factor (GEF), responsible for promoting the transition of *ras*-GDP to *Ras*-GTP, and several GTPase-activating proteins (GAPs), which, as their name indicates, shorten the lifespan of *ras*-GTP by increasing the catalytic efficacy of its intrinsic GTPase. Sos (*son of sevenless*) and *ras*-GAP1 are the prototypes of *ras*-GEF and *ras*-GAP, respectively. Mutation of Ser-17 to Asn-17, roughly in the middle of α2, interferes with the nucleotide exchange reaction and locks *ras*-GDP in an inactive conformation in which it may still bind to proteins regulated by *ras*-GTP but without affecting their activity. By reason of occupying the site to which *ras*-GTP should bind to activate effectors, Asn-17 *ras* interferes with signaling by wild-type *ras*-GTP and is referred to as a dominant negative form of *ras*. Figure 2 depicts *ras* in its GTP and GDP conformations and highlights the locations of Gly-12 (G12), Ser-17 (S17), Gln-61 (Q61), Switch I, and Switch II. The upper left panel of Fig. 2 presents this basic GTPase cycle in schematic form with GEF and GAP regulating the lifetimes of the GTP and GDP states. The upper right panel of Fig. 2 is a schematic two-dimensional representation of the main three-dimensional features of this regulatory GTPase.

## III. HETEROTRIMERIC G-PROTEINS

Trimeric G-proteins are responsible for transducing the effects of the seven-transmembrane superfamiliy of receptors and are regulatory GTPases, which, though more complex than *ras*, nevertheless preserve the basic features of the *ras*-type regulatory GTPases. G-proteins activated by seven-transmembrane receptors are αβγ trimers, of which the α-subunit is the GTPase-bearing subunit. The β- and γ-subunits form a dimer that exists either free or in association with α–GDP. $G_\beta$ and $G_\gamma$ have never been found isolated as individual proteins.

### A. Subunit Structure

α-Subunits were crystallized in the laboratories of Paul Siegler at Yale University and of Steven Sprang at the University of Texas at Dallas, in collaboration with Heidi Hamm then at the University of Illinois (Chicago) and Alfred Gilman at the University of Texas at Dallas. From a structural viewpoint, α-subunits of heterotrimeric G-proteins are made up of a *ras*-like GTPase domain and a helical domain with six α-helices (αA through αF) inserted in the center of what would be *ras*'s Switch I (Figs. 3 and 4). In its GDP-liganded form, $G_\alpha$s have a disorganized Switch II region and exhibit high affinity for $G_{\beta\gamma}$. $G_\alpha$–GDPs are therefore found associated with $G_{\beta\gamma}$s as heterotrimers. $G_{\beta\gamma}$ locks GDP into its binding

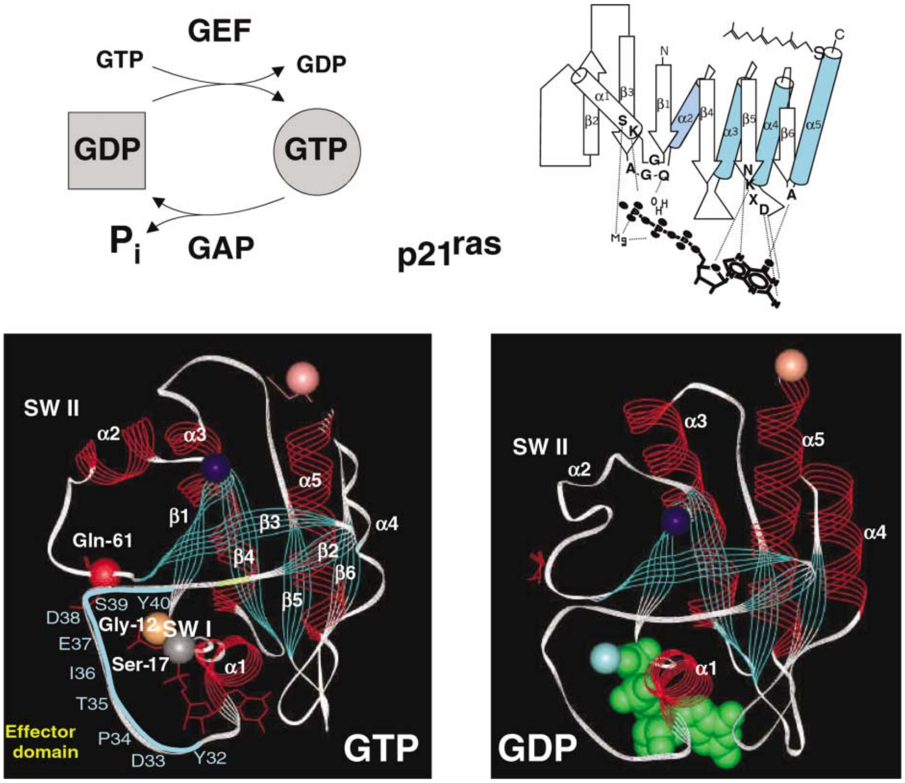

FIGURE 2 Model of the crystal structure of *ras* in its GTP and GDP states (PDB Accession Nos. 521P and 1Q21), highlighting secondary structures (α-helices and β-strands) as well as Switch 1 (effector domain) and Switch 2 regions. Top left illustrates the basic GTPase regulatory cycle and its two principal modulators: GEFs, responsible for activation, and GAPs, responsible for deactivation of the regulatory GTPases. The top right diagram is a two-dimensional representation of the three-dimensional features of the basic GTPase fold found in all regulatory GTPases.

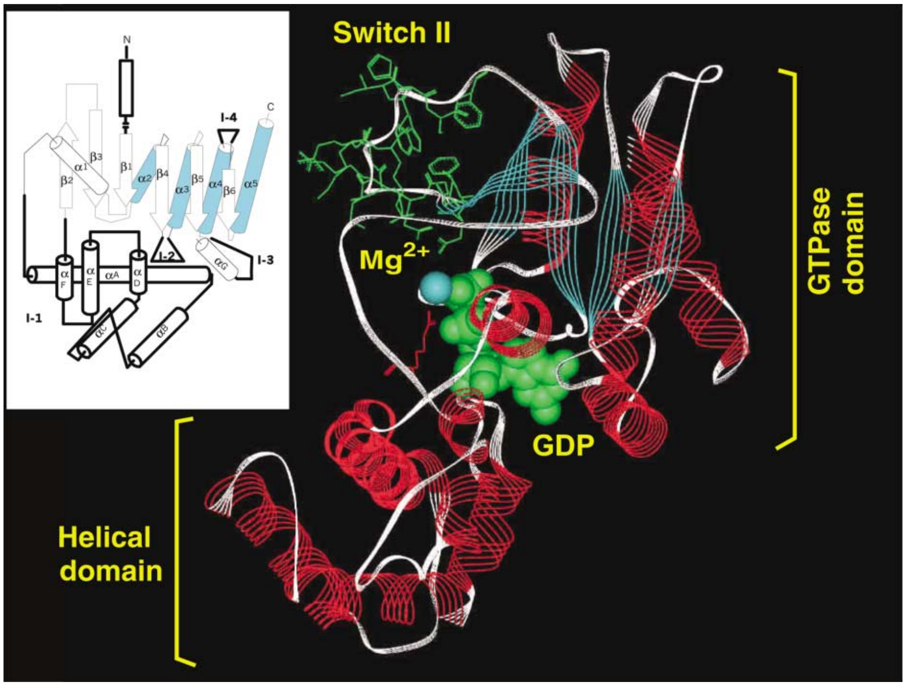

**FIGURE 3** Crystal structure of an α-subunit of a heterotrimeric G-protein in its GDP state (PDB Accession No. 1TAG). Its GTPase domain is oriented in the same way as *ras* in Fig. 2. Atoms of amino acid side chains of Switch II are displayed as sticks; space-filling CPK atoms were used to represent GDP. Mg is represented by a ball. (Inset) Two-dimensional diagram of the three-dimensional features of heterotrimeric G-protein (α-subunit). Additions to the *ras* structure are shown as boldface lines. GTPase and helical domains are highlighted.

site on $G_\alpha$, causing its dissociation rate to drop by a factor of 10. At the same time, $G_{\beta\gamma}$ also shields the Switch II region from interacting with possible effectors (Fig. 4). Consequently, activation of a heterotrimeric G-protein requires two events: (1) the exchange of GTP for GDP and (2) the dissociation of $G_{\beta\gamma}$ from $G_\alpha$. Subunit dissociation has not been measured in intact cells in an unequivocal way but it is readily seen *in vitro*. Dissociation exposes the regulating surface of $G_\alpha$–GTP and allows for effector regulation with direct involvement of Switch II. The converse also applies. That is, $G_{\beta\gamma}$ is a signaling molecule able to regulate effector functions and its association with $G_\alpha$–GDP results in occlusion of its ($G_{\beta\gamma}$'s) signaling surface.

The structure of $G_{\beta\gamma}$ deserves separate comment (Figs. 4 and 5). $G_\beta$ is a seven-bladed propeller, each blade of which is made up of four anti-parallel β-strands running from the center to the periphery. The innermost β-strand runs parallel to the axis of rotation of the propeller. The next two strands change pitch to approach the orientation of the outermost strand, which runs along the periphery of the propeller and is co-planar with the circle described by the rotating propeller. Blade VII is made up of three β-strands contributed by the very C-terminus of $G_\beta$ and a fourth (outermost) "zipping" β-strand recruited from the sequence immediately preceding those that create blade I. Preceding the zipping β-strand is an extended N-terminus with a long α-helix that interacts with $G_\gamma$ by forming a coiled coil. $G_{\beta\gamma}$ dimers have thus far been crystallized only in association with $G_\alpha$ subunits or with a retinal regulatory protein, phosducin, but not in isolation. Conformational changes in $G_\beta$ that occur upon dissociating from $G_\alpha$, if they occur, have not been observed as yet.

## B. Lipid Modifications

$G_\alpha$ and $G_{\beta\gamma}$ subunits engaged in signal transduction are membrane bound by virtue of lipid modifications. $G_{\beta\gamma}$ dimers are anchored to membranes through a C-15 or C-20 polyisoprene attached to the Cys of a C-terminal CAAX motif. Posttranslational processing not only attaches the polyisoprene but also removes the last three amino acids and methylates the new C-terminus. Prenylation is not necessary for association of $G_\gamma$ with $G_\beta$, but is required for association

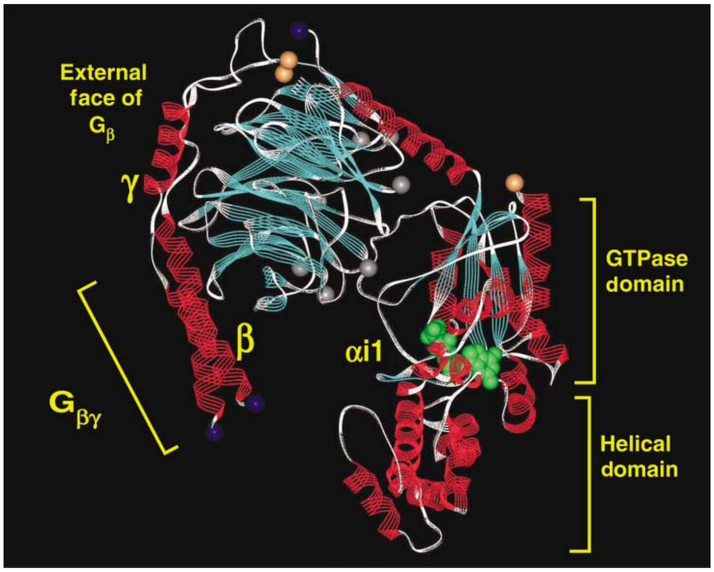

**FIGURE 4** Model of $G_{i1}$ heterotrimer with bound GDP deduced from crystal X-ray diffraction studies (PDB Accession No. 1GG2). Note that the internal face of $G_\beta$ shields the $G_\alpha$ subunit's Switch II from being accessible to effectors. N-terminal α-carbons, C-terminal α-carbons, and α-carbons of conserved aspartic acids are shown.

of $G_{\beta\gamma}$ to $G_\alpha$–GDP and for regulation of effector, e.g., adenylyl cyclase. Furthermore, prenylation contributes to the association of the $G_\gamma$ dimer to membranes. Most $G_\alpha$ subunits engaged in signal transduction are palmitoylated near their N-terminus. Palmitoylation facilitates their anchoring to the plasma membrane. $G_\alpha$ subunits of the $G_i/G_o$ family are also myristoylated at N-terminal glycines (Gly-2 of the primary transcript). $G_\alpha$ myristoylation increases affinity for $G_{\beta\gamma}$ dimers. Removal of myristic acid by Gly-2 to Ala-2 mutation renders $G_i\alpha$ subunits inactive as inhibitors of adenylyl cyclase. Some but not all nonpalmitoylated $G_\alpha$ subunits fail to localize to membranes and are found in the cytosol. Lipid modification of $G_\alpha$ and $G_\gamma$ subunits is therefore essential for their normal biological activity.

## C. Molecular Diversity of G-Proteins

Each of the subunits that make up a heterotrimeric G-protein is encoded by a family of structurally homologous genes. There are 16 $G_\alpha$ (one with two splice variants), 5 $G_\beta$, and 11 $G_\gamma$ genes, leading to the theoretical possibility of close to 1000 distinct heterotrimers. $G_\alpha$ subunits are the longest of the three subunits, ranging from 350 to 390 amino acids. Their sequence similarities vary from almost identical when $G_{i1\alpha}$ is compared to $G_{i3\alpha}$ (86% identical) to only 40% identical when $G_{s\alpha}$ is compared to $G_{16\alpha}$. Amino acid sequence alignments show $G_\beta$s to be a structurally very closely related family, with β1–4 all being 350 aa long and differing by no more than 17% in their amino acid sequences. $G_{\beta5}$, with 395 aa, exhibits the same degree of similarity, differing primarily by a 45 aa N-terminal extension. $G_\gamma$s are the shortest (68–75 aa) and are the most diverse, differing in amino acid sequence between 40 and 65%. GGL ($G_\gamma$-like) domains of RGS 6, 7, 9, and 11 (for RGS proteins, see below) constitute an additional group of $G_\gamma$ subunits that interact with $G_{\beta5}$'s atypical N-terminal extension.

The actual number of G-protein isoforms in any given cell is much lower than 1000, for two reasons: (1) there is no cell known to express all G-protein subunit genes and (2) there are structural limitations that do not allow all βγ dimers to form; e.g., whereas β1 interacts with γ1, γ2 and γ3, β3 does not interact with γ1 or γ2 but instead partners with γ4. Vice versa, γ1 partners with β1 but not with either β2 or β3, and γ2 partners with β1 and β2 but fails to do so with β3. The complete spectrum of permissible interactions among the 5 β- and 11 γ-subunits still needs to be worked out. One $G_\beta$, $G_{\beta5}$, interacts preferentially with the GGL domain of γ-subunits 6, 7, 9, and 11. It is quite possible that even for biochemically permissible interactions, there may be βγ dimers that never form because they are not coexpressed in the same cell.

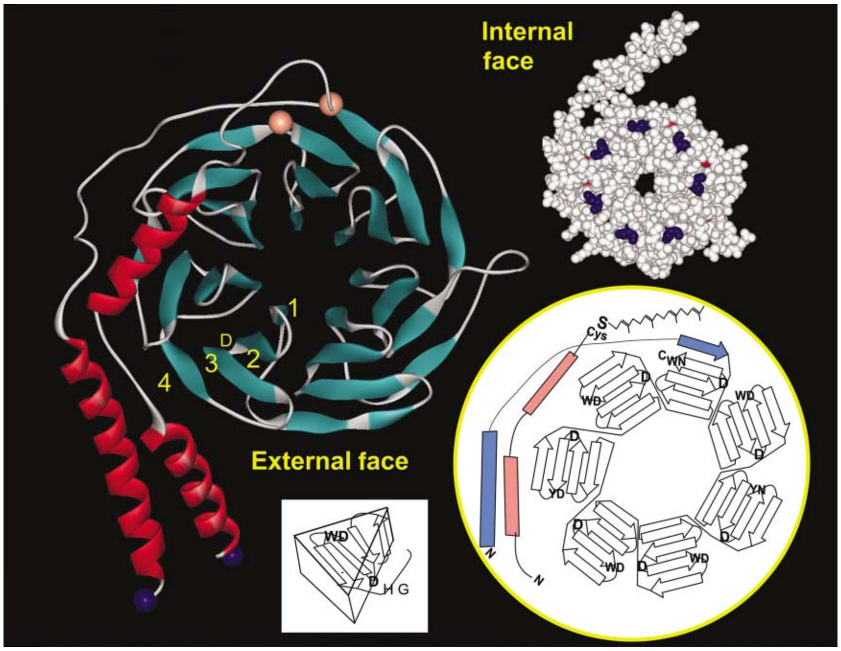

**FIGURE 5** View of a seven-bladed $G_\beta$ propeller from the "external" side determined by its orientation when complexed with α–GDP. (Top right) CPK representation of the same propeller viewed from the side facing α–GDP in its trimeric form. Conserved aspartic acids (D) are present in each propeller blade. (Bottom right) Two-dimensional diagram of the three-dimensional features of a $G_\beta$ propeller highlighting the "zipping," outermost β-strand of blade VI and location of conserved D residues shown in the space-filling representation of the model. Note the γ coiled-coil interaction of the γ-subunit with the N-terminus of the $G_\beta$ subunit.

G-proteins are named after their α-subunit. This nomenclature originated from the fact that for first two G-proteins discovered, $G_s$ and transducin ($G_t$), the major (then sole) signaling function resided in their α-subunits. $G_{\alpha s}$ subunits activate adenylyl cyclases (ACs), and $G_{\alpha t}$ subunits activate visual phosphodiesterase (PDE, a tetramer of one α, one β, and two inhibitory γ subunits). Activation of visual PDE results from the association of αt–GTPs with the PDEγs, thereby suppressing their inhibitory effects on PDEαβ. A phylogenetic tree of G-protein α-subunits (Fig. 6) clusters their sequences into four subfamilies: $G_s$, $G_q$, $G_{12/13}$, and the pertussis toxin (PTX)-sensitive $G_\alpha$ subunit subfamily. The latter includes three α-subunits that play roles in light and taste perception, plus the α-subunits of the $G_i/G_o$ family. PTX-sensitive α-subunits not only show higher sequence similarity to one another than to the remaining α-subunits, but as their name indicates, they are substrates for the ADP-ribosyltransferase activity of the S1 subunit of pertussis toxin. The ADP-ribosylated amino acid is a Cys at position −4 from their C-termini. PTX uncouples this group of G-proteins from activation by receptors by virtue of creating of a steric hindrance to the G-protein–receptor interaction. Included in this structural group is the α-subunit of $G_z$ (αz), which lacks a Cys at −4 from the C-terminus and is PTX-insensitive. It is functionally a $G_i$, its closest structural homologue.

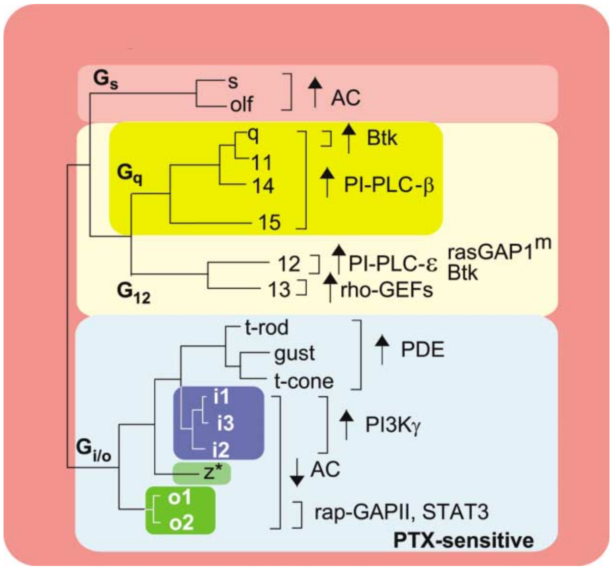

**FIGURE 6** Phylogenetic analysis of G-protein α-subunits reveals structural subdivisions that have functional correlates.

As illustrated in Fig. 6 and in more detail in Fig. 7, α-subunits modulate the activity of a large and diverse group of enzymes, including ACs, phospholipase C-βs (PLC-βs), phosphatidylinositol 3-OH kinases (PI3Ks), type 6 visual and type 3 gustatory PDEs, and a Rho-GEF for regulation of cytoskeletal remodeling.

Both $G_{\alpha}$ subunits and $G_{\beta\gamma}$ dimers modulate, positively or negatively, an extraordinarily diverse set of cellular functions. In some instances, the effects of $G_{\beta\gamma}$ dimers are in concert with those of α-subunits; in other instances, regulation of the effector by $G_{\beta\gamma}$ is unrelated to regulation by a $G_{\alpha}$. It is not clear at this time whether effectors distinguish between $G_{\beta\gamma}$ isoforms. As illustrated in Fig. 8, the gamut of $G_{\beta\gamma}$ effectors is as complex and as diverse as that of the α-subunits. Among the regulated functions worth mentioning are inhibition of type 1 AC, but co-stimulation with $G_{s\alpha}$ of type 2 and type 4 ACs; stimulation of type 3 PLC-β independent of co-existing stimulation by the $G_{q/11}$ group of α-subunits; co-stimulation with $G_{\alpha i2}$ (possibly also $G_{\alpha i1}$ and $G_{\alpha i3}$) of PI3Kγ; and co-stimulation of PI3Kβ with tyrosine-phosphorylated p85, the regulatory subunit of PI3K. *In vitro* reconstitution experiments in which PI3Kβ was incubated with $G_{\beta\gamma}$ and a tyrosine-phosphorylated peptide corresponding to the tyrosine-phosphorylated sequence of p85 showed that stimulation by each was 3- to 5-fold but became 100-fold when the tyrosine-phosphorylated peptide and the $G_{\beta\gamma}$ were added together. This type of cross-dependence on dual inputs is highlighted in Figs. 7 and 8. $G_{\beta\gamma}$ also modulates, sometimes positively and other times negatively, a variety of ion channels and thereby provides a nexus between regulation of second-messenger formation by enzymes and regulation of cell excitability by voltage, which it can augment (activation of potassium channels) or dampen (inhibition of presynaptic $Ca^{2+}$ channels) (Fig. 8).

In conclusion, signal transduction by G-proteins is the result of structurally similar receptors activating structurally very similar G-proteins, which then regulate positively or negatively the activity of a diverse gamut of structurally unrelated cellular functions that affect intracellular levels of cAMP, inositol 1,4,5-triphosphate, $Ca^{2+}$, diacylglycerol (DAG), and phosphatidylinositol 3 phosphates, as well as ion channel activity, formation of lamellipodia and filopodia, and the attendant cytoskeletal changes. Moreover, since second messengers such as cAMP,

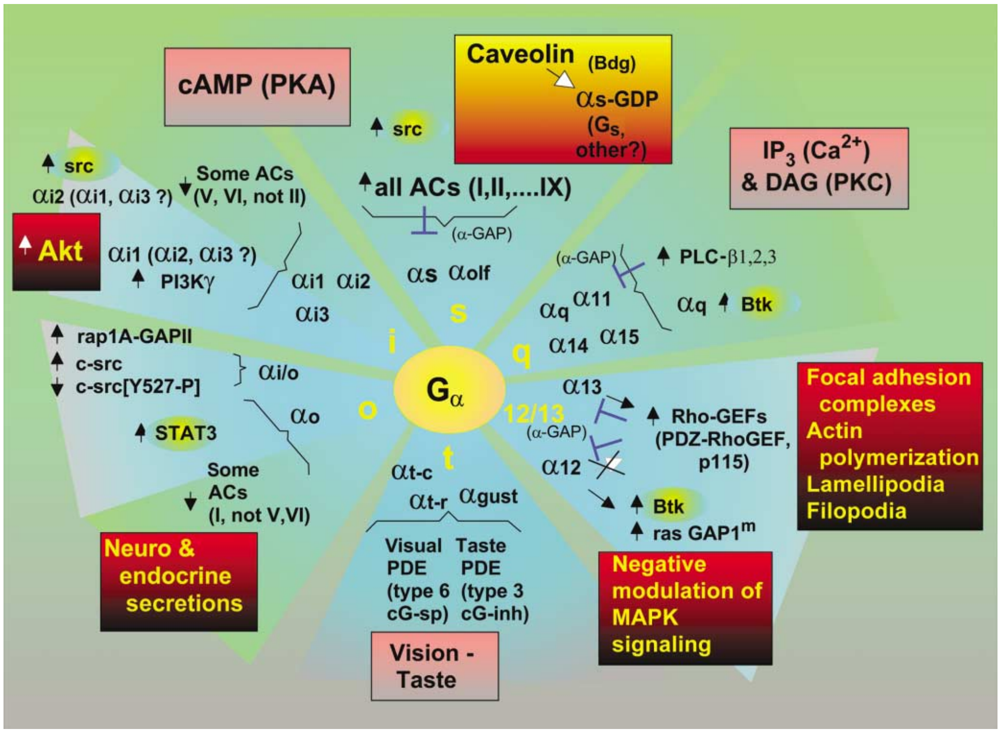
cAMP (PKA)
Caveolin (Bdg)
αs-GDP (Gs, other?)
↑ src
IP₃ (Ca²⁺) & DAG (PKC)
↑ src
αi2 (αi1, αi3 ?)
↓ Some ACs (V, VI, not II)
↑ all ACs (I,II,....IX)
(α-GAP)
↑ Akt
αi1 (αi2, αi3 ?)
↑ PI3Kγ
αi1 αi2 αi3
αs αolf
(α-GAP)
↑ PLC-β1,2,3
αq α11
αq ↑ Btk
α14 α15
↑ rap1A-GAPII
↑ c-src
↓ c-src[Y527-P]
αi/o
αo
s
i
q
o
Gα
12/13
t
↑ STAT3
α13
(α-GAP)
↑ Rho-GEFs (PDZ-RhoGEF, p115)
α12
Focal adhesion complexes
Actin polymerization
Lamellipodia
Filopodia
Some ACs
↓ (I, not V,VI)
αt-c
αt-r αgust
↑ Btk
↑ ras GAP1m
Visual PDE (type 6 cG-sp)
Taste PDE (type 3 cG-inh)
Neuro & endocrine secretions
Vision - Taste
Negative modulation of MAPK signaling

FIGURE 7 Effectors of activated α-subunits. The figure shows the different signaling systems affected by α-subunits at the periphery, with increasing detail toward the center. ⊣, inhibition.

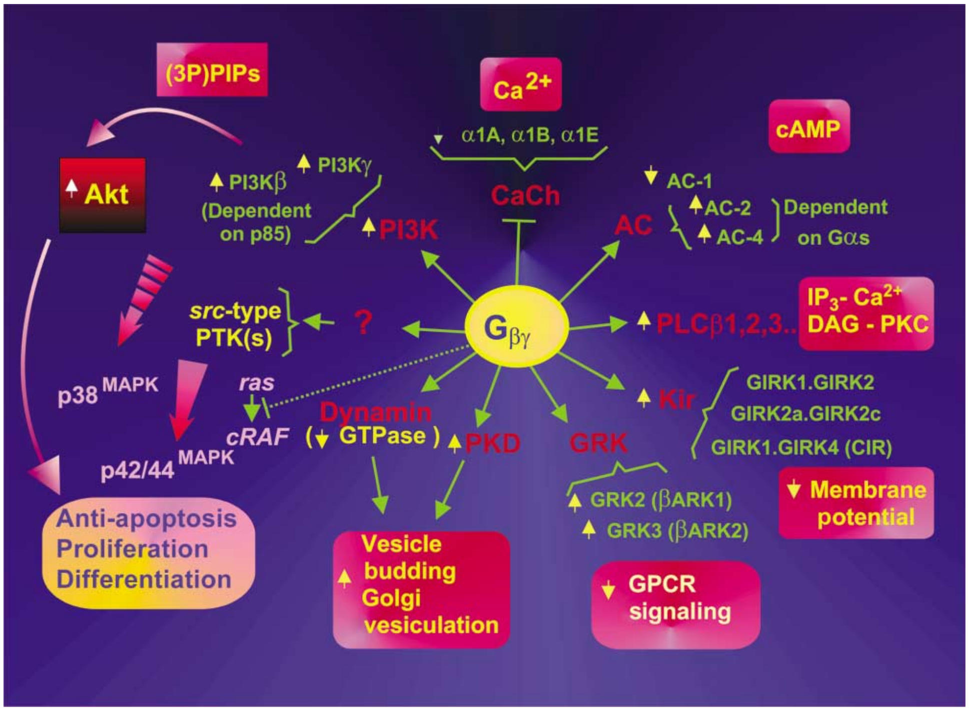
(3P)PIPs
Ca2+
α1A, α1B, α1E
cAMP
Akt
PI3Kγ
PI3Kβ
(Dependent on p85)
PI3K
CaCh
AC
AC-1
AC-2
AC-4
Dependent on Gαs
src-type PTK(s)
?
Gβγ
PLCβ1,2,3..
IP3- Ca2+ DAG - PKC
p38 MAPK
ras
cRAF
Dynamin
GTPase
PKD
GRK
Kir
GIRK1.GIRK2
GIRK2a.GIRK2c
GIRK1.GIRK4 (CIR)
p42/44 MAPK
GRK2 (βARK1)
GRK3 (βARK2)
Membrane potential
Anti-apoptosis Proliferation Differentiation
Vesicle budding Golgi vesiculation
GPCR signaling

FIGURE 8 Effectors of βγ dimers. The different intracellular signaling systems affected by $G_{\beta\gamma}$ dimers at the periphery are shown, with increasing detail toward the center. → , stimulation; —|, inhibition; ·····|, stimulation or inhibition.

DAG, and $Ca^{2+}$ affect protein kinases, it is unlikely that there is a cellular function that in one way or another is not under the controlling or modulatory influence that emanates from activation of heterotrimeric G-proteins. Indeed, as indicated in Figs. 7 and 8, cellular responses included are the PI3K–PDK–Akt–nuclear factor κB anti-apoptotic response of cells to extracellular signals and the effects of $G_{\beta\gamma}$ on dynamin, Golgi vesiculation, and vesicle budding.

## IV. MECHANISM OF G-PROTEIN ACTIVATION BY RECEPTORS AND MODULATION OF ACTIVITY

Receptors acting through heterotrimeric G-proteins are referred to as G-protein-coupled receptors (GPCRs). They play the role of GEFs in the regulatory GTPase cycle. Ligand binding to GPCRs, all of which belong to the rhodopsin superfamily of seven-transmembrane receptors, has as its final effect the GDP/GTP exchange with attendant subunit dissociation into $G_\alpha$–GTP plus $G_{\beta\gamma}$ (Fig. 1). The final effect of a hormone acting through a GPCR on any given cell depends on the type of G-protein activated by the receptor and the repertoire of effectors, i.e., regulatable enzymes, ion channels, and other affected molecules in the target cell.

### A. Mechanism of Activation of a G-Protein by a Receptor

At the molecular level, activation of a G-protein by a rhodopsin-like receptor is still poorly understood. This is because of lack of knowledge of which amino acids of the receptor make contact with which amino acids of the G-protein. In contrast, the regions of each molecule important for productive interaction are well known, as are some of the changes in the kinetic and molecular states that occur when a receptor under the influence of an activating ligand, i.e., agonist, interacts with and activates a G-protein. Thus, binding of an agonist to a GPCR in the absence of guanine nucleotide (GTP or GDP), as can be done *in vitro* with purified membranes, has two consequences: (1) a shift of the equilibrium between two states of the receptor, from being mostly in state I (inactive), characterized by having low affinity for agonist as well as for the G-protein(s), to being mostly in state II (active), characterized by having higher affinity for the activating ligand; and (2) the stable association of the agonist–receptor complex with the G-protein. The latter causes the G-protein to reduce its affinity for GDP. Bound GDP, or prebound [$^3$H]GDP, will thus dissociate under these conditions. $Mg^{2+}$ ion must be present if the receptor is to cause GDP dissociation. Addition at this point of GTP or a GTP analogue, such as GTPγS or GMP-P(NH)P, leads to its binding in place of GDP and to the activation of the G-protein as seen by stimulation of the activity of an effector, such as adenylyl cyclase or visual phosphodiesterase. For most of the cases in which this has been studied, a high concentration of $Mg^{2+}$ ion, ca. 50 m*M*, mimics the action of the agonist-activated receptor. With a purified G-protein, incubation with $Mg^{2+}$ ion and GTPγS or GMP-P(NH)P leads not only to accumulation of $G_\alpha$-bound guanine nucleotide, but also to subunit dissociation, i.e., formation of $G_\alpha$–GTPγS plus free $G_{\beta\gamma}$, Dissociation is evident in several ways, the easiest to determine being a shift in sedimentation velocity from that corresponding to a protein with a $M_r$ of approximately 100,000 ($G_{\alpha\beta\gamma}$) to that of two co-sedimenting proteins of $M_r$ ca. 50,000 ($G_\alpha$–GTPγS + $G_{\beta\gamma}$). $M_r$s of α-subunits are in the range 40,000–50,000 and those of $G_{\beta\gamma}$ complexes are also approximately 50,000.

In intact membranes, where activation of a G-protein of the αβγ type by agonist occupancy of a receptor can be measured in terms of stimulation of the activity of an effector, e.g., adenylyl cyclase, phospholipase C, visual phosphodiesterase, or an inwardly rectifying potassium channel, the net effect of receptor activity is thus facilitation of the activation of the G-proteins by $Mg^{2+}$ ion. This comes about as a consequence of a receptor-induced shift in the apparent $K_m$ for $Mg^{2+}$ ion from high millimolar to low micromolar concentrations. In other words, a receptor appears to act by reducing the concentration of $Mg^{2+}$ required for activation of the G-protein by GTP from being above physiologic to being below physiologic concentrations. Free cytosolic $Mg^{2+}$ is on the order of 0.5 m*M*. The effect of glucagon (receptor) shifting the concentration of $Mg^{2+}$ ion required for activation of liver $G_s$ (the stimulatory regulatory component of adenylyl cyclase) by GTPγS, is illustrated in Fig. 9.

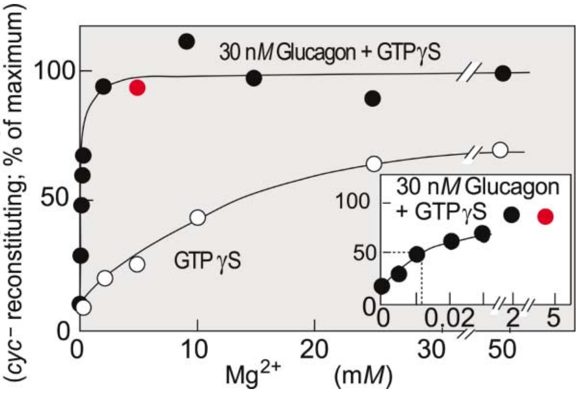

**FIGURE 9** GPCRs reduce the concentration of $Mg^{2+}$ ion required for G-protein activation by GTP. The results of a three-step reaction are shown. In the first step (not shown), the adenylyl cyclase enzyme of liver membranes was inactivated by treatment with *N*-ethyl maleimide, leaving an intact receptor–G-protein system in its natural membrane environment. The second step tested for the effect of varying $Mg^{2+}$ ion on GDP/GTPγS exchange in the absence and in the presence of the hormone glucagon. In the third step, the $G_{s\alpha}$–GTPγS complexes formed were extracted and quantified in a standard reconstitution assay. The figure shows the effect of the glucagon-activated receptor on the $Mg^{2+}$ required for G-protein activation. Note that (1) hormone was not necessary for $G_s$ activation as long as a high enough (supraphysiologic) concentration of $Mg^{2+}$ was present during incubation with GTPγS and (2) in the presence of the hormone, the $Mg^{2+}$ concentration that was required to activate $G_s$ was ca. 1000-fold lower than in its absence. (Inset) Same as main panel but with an expanded $Mg^{2+}$ concentration scale. Adapted from Iyengar and Birnbaumer (1987).

## B. Structural Determinants

At the molecular level, mutational analysis has shown that amino acids in the third intracellular loop of GPCRs are involved in the ability of a GPCR to activate a G-protein. Swapping intracellular loops between receptors that bind preferentially to different G-proteins, such as switching the loops of the M1 and M2 muscarinic receptors or switching those of the β- and α1-adrenergic receptors, also points to the third intracellular loop as being responsible for defining G-protein specificity. Furthermore, most of the receptor mutations that are of the gain-of-function type are in the distal (C-terminal) end of the third intracellular loop. It is not known, however, why some mutations are activating and others inactivating. Moreover, it is also not known whether these amino acids actually contact the G-protein and, if so, which G-protein subunit they contact.

Mutational analysis and sequence swapping experiments with G-protein α-subunits indicate that receptors interact with the very C-terminus of the G-protein α-subunit. Indeed, swapping as few as 3 of the last 10 amino acids between two α-subunits can lead to a switch in the type of receptor that activates the G-protein. The C-terminus is not the only region of interaction of an α-subunit with a receptor. Multiple sites have been identified by mutational analysis, including the α3β5 and α4β6 loops of the GTPase domain (Figs. 2 and 10). The α-subunit C-terminus and the α3β5 and α4β6 loops are part of the same face of the molecule presumed to be immediately juxtamembranous. Receptors do not interact with only the α-subunit of a trimeric G-protein. Free $G_\alpha$ subunits are not recognized by receptors; they are recognized only in the context of the heterotrimer. In agreement with this conclusion, injection of subunit-specific antisense oligonucleotides or subunit-specific antibodies leads to the loss of receptor-mediated effector regulation, including not only the suppression of $G_\alpha$ but also $G_\beta$ or $G_\gamma$ subunits, all in a gene-specific manner. It has been shown that in pituitary cells the M4 muscarinic receptor activates a $G_o$ protein of subunit composition αo1β3γ4, whereas the somatostatin receptor activates a $G_o$ of subunit composition αo2β1γ3. It follows that receptors "proofread" the subunit subtypes that make up the particular trimer that they contact.

Figure 5 shows that only one of the two faces of the $G_\beta$ propeller is exposed to the milieu; the other faces the α-subunit's Switch II region. The exposed face and the sides of the propeller are therefore available for interaction with the receptor. In turn, since the receptor interacts with $G_\beta$ and $G_\gamma$, it can reasonably be expected that receptors may affect the $G_{\beta\gamma}$ interaction with $G_\alpha$. One model based on these considerations, as well as on the results shown in Fig. 9, is that whereas the interaction of the receptor with the C-terminus of $G_\alpha$ is essential for selection of the type of $G_{\alpha\beta\gamma}$ that will be activated through the GDP/GTP exchange reaction, the "activating" effect of the receptor may in fact be mediated by $G_{\beta\gamma}$. In this sense, the $G_{\beta\gamma}$ dimers would have a receptor-dependent GEF activity. Thus, although $G_{\beta\gamma}$ alone prevents GDP dissociation by acting as a

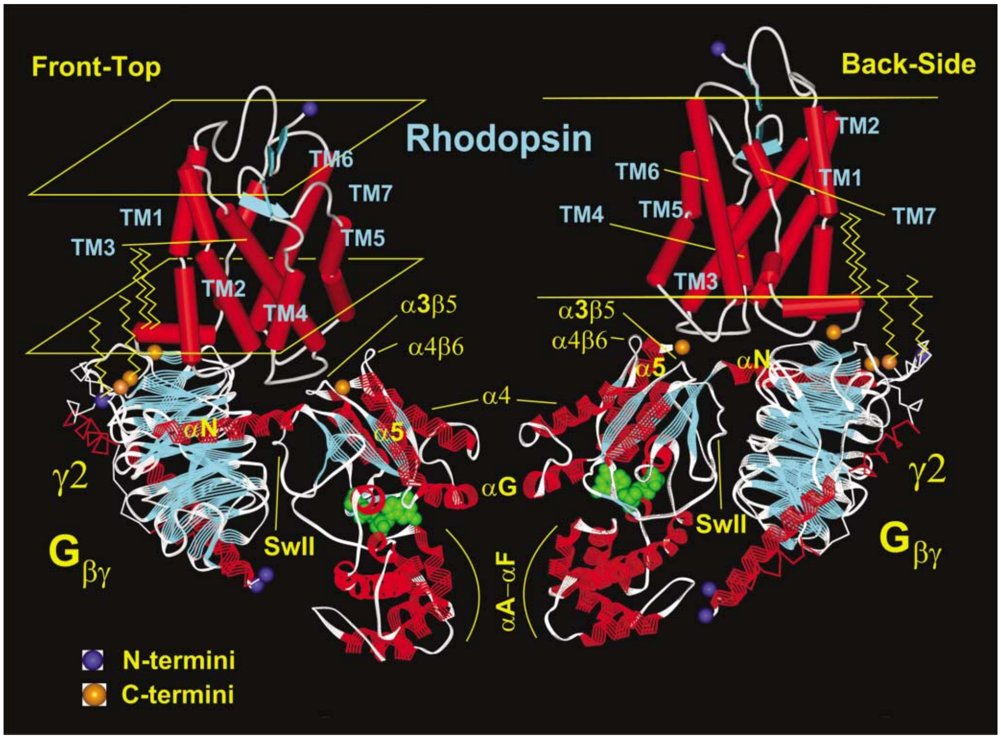

**FIGURE 10** Model of orientation of a $G_\alpha$ subunit with respect to both the inner plane of the plasma membrane and a membrane-embedded GPCR. Based on the crystal structures of bovine rhodopsin (PDB Accession No. 1F88) and the GDP-occupied $G_{i1}$ trimer (PDB Accession No. 1GG2). The molecules are shown in a near-docking situation with the receptor in its "inactive" conformation and the G-protein with GDP bound to it. Two views are shown and the main structural features are annotated. Vertical zigzag lines denote the α-subunit N-terminal myristoylation and/or palmitoylation and the γ-subunit C-terminal polyisoprenylation. Positions of lipids are approximate because the modified N- and C-terminal amino acids were not resolved in the crystal structure of $G_{i1}$. Double zigzag approximately 13 amino acids after the TM7 of rhodopsin denotes double palmitoylation of the C-terminus at that position. Single or double palmitoylation of GPCR C-termini is a common but not universal feature of GPCRs.

guanine nucleotide dissociation inhibitor (GDI), the $G_{\beta\gamma}$–receptor complex has GEF activity, facilitating binding of GTP to its site on $G_\alpha$. Artificial "bending" of the N-terminus, mimicking what $G_{\beta\gamma}$ might do if it were acting as a GEF, does indeed lead to an apparently constitutively active G-protein. It has been speculated that $G_{\beta\gamma}$ may be the site of action of the $Mg^{2+}$ ion. If so, the role of the receptor would be simply to promote binding of the $Mg^{2+}$ ion to $G_{\beta\gamma}$. The $Mg^{2+}$ ion would then be responsible for changing the activity of $G_{\beta\gamma}$ from a GDI to a GEF.

Regardless of the final outcome regarding the events responsible for G-protein activation by a receptor at the submolecular level, the overall reaction for a receptor activating a G-protein is facilitation of the action of the $Mg^{2+}$ ion to promote GDP/GTP exchange, followed by dissociation of the trimer into $G_\alpha$–GTP plus $G_{\beta\gamma}$.

## C. RGSs or Regulators of G-Protein Signaling

As there are receptors that by virtue of their GEF activity promote activation of the heterotrimeric G-proteins, there are also GAPs that accelerate the GTPase activity of activated, GTP-liganded $G_\alpha$ subunits. Two types of $G_\alpha$ GAPs have been identified. One type consists of the RGSs. RGSs accelerate GTPase activity by 100-fold or more and exhibit $G_\alpha$ subunit selectivity. Sixteen RGSs are known and many of them are multidomain, multifunction proteins and thus not only affect the $G_\alpha$ subunits but also aid in the organization of multicomponent "signaling complexes." The second type of GAP includes some of the effectors regulated by the α-subunits. GAP activities of effectors increase the $k_{cat}$ of the $G_\alpha$–GTP complexes by only 10- to 20-fold. In both instances, increased GTP hydrolysis ensures not only prompt deactivation of the signaling protein but also a faster approach to equilibrium, therefore increasing the rate at which responses to an extracellular stimulus can be obtained. Indeed, for RGS proteins, faster on/off rates of the regulated function may be a primary raison d'être. In contrast, when effectors act as GAPs, the primary purpose may be to ensure that they are indeed affected by the activated $G_\alpha$–GTP complex. The intrinsic GTPase activity of $G_\alpha$ subunits is very low, on the order of 4–8 per second, giving them a rather long half-life, ensuring that they "find" their effector(s) while still in their GTP state. Once the effector has been found and the receptor message is delivered, the recipient of the message "kills" the messenger through activation of its deactivating mechanism. Thus, continued stimulation of effector, if this is desirable, requires the continued presence of receptor, agonist and constant reactivation of the G-protein.

Figure 11 illustrates the effect of GAPs on accelerating the rate at which equilibrium is established in a simple on/off reaction, such as binding of a hormone (H) to its receptor (R). The three panels show (1) the basic rate at which a bimolecular reaction reaches equilibrium; (2) the effect of increasing the $k_{off}$ rate on the rate at which equilibrium is reached, expressed as HR formed in absolute concentrations; and (3) the fact that although the number of complexes at equilibrium decreases with increasing values of $k_{off}$, the rate at which equilibrium is reached increases with increasing values of $k_{off}$. It follows that, given the low intrinsic GTPase activity of $G_\alpha$ subunits, the need for a rapid response can be satisfied only by both the existence of a GAP and a very high concentration of reactants so that the amplitude of the read-out signal (regulated effector) is large enough upon activation of only a small fraction of the GTPase. This is in fact the case with the activation of transducin (heterotrimeric G-protein) by rhodopsin. The GTPase of transducin is stimulated by RGS9, a GGL RGS. This ensures that the physiological rapid turn-off occurs within a tenth of a second as opposed to having a half-life of 10 s ($t_{1/2} = \ln 2/k_{off} = 10$ s, where $k_{off}$ corresponds to the published intrinsic GTPase activity of $G_\alpha$ subunits of 4/min). However, to ensure that sufficient active transducin will be formed, Mother Nature endowed the visual system with the highest known concentrations of receptor (rhodopsin: 40% of disc membrane protein) and regulated G-protein (transducin: 10% of disc protein).

## D. Other Forms of G-Protein Modulation: Activators of G-Protein Signaling—The GoLoco Domain

As described above, the free $G_\alpha$–GTP and the $G_{\beta\gamma}$ released on G-protein activation are both active regulators of effector systems. RGSs and effectors acting as GAPs shorten the lifetime of the active $G_\alpha$ with formation of $G_\alpha$–GDP. Due to the high affinity of $G_\alpha$–GDP for $G_{\beta\gamma}$, a GAP activity accelerates the deactivation of not only $G_\alpha$-regulated effectors, but also the $G_{\beta\gamma}$-regulated effectors from which $G_{\beta\gamma}$ is sequestered by $G_\alpha$–GDP. Activators of G-protein signaling (AGSs) were discovered in a search for molecules that potentiate the effect of α-factor in baker's yeast. α-Factor acts by activating an αβγ

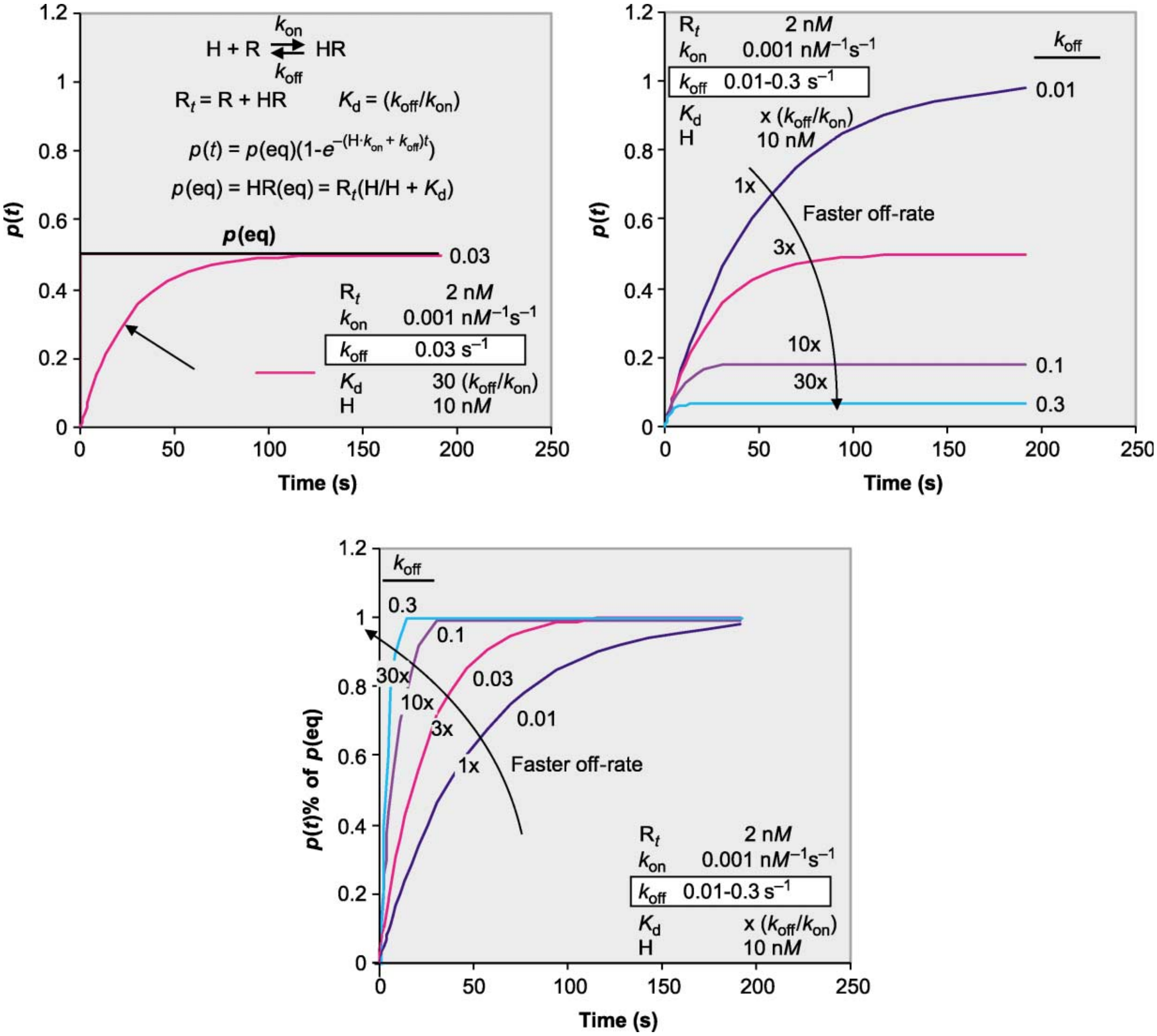

FIGURE 11 Approach to equilibrium of a reversible bimolecular reaction, $H + R \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\Longleftrightarrow}} HR$, HR = 0 at $t_o$. The panels illustrate the effect of increasing the $k_{\mathrm{off}}$ rate, equivalent to the effect of introducing a GTPase-activating RGS into a regulatory GTPase cycle. $R_t$, total receptor concentration.

heterotrimeric G-protein and initiating a $G_{\beta\gamma}$-mediated cascade of reactions that leads to growth arrest and preparation for mating to the opposite mating type. One of the AGSs, AGS3, was found to potentiate $G_{\beta\gamma}$ signaling by binding preferentially to $G_\alpha$–GDP and to do so via a domain found in unrelated proteins and referred to as GoLoco. In mammalian systems, GoLoco domains act as they do in yeast: they bind preferentially to the GDP-liganded $G_\alpha$ subunit of the $G_i/G_o$ type, thus prolonging $G_{\beta\gamma}$ signaling. In addition to AGS3, proteins with a GoLoco domain include several RGSs and Rap–GAPII. This hints at a role that transcends its direct function of binding the GDP forms of $G_i/G_o$ and involves participation in the integration of multicomponent signaling pathways.

Even though they were identified in the same type of bioassay, AGS1, AGS2, and AGS3 differ in their mode of action. AGS1 (also Rasdex1) is a ras-related protein that appears to act as a GEF, whereas AGS2 interacts with $G_{\beta\gamma}$ and AGS3 binds to α–GDP.

Why a $G_{\beta\gamma}$-interacting protein (AGS2) would enhance the effectiveness of either a $G_{\beta\gamma}$ or a $G_\alpha$–GTP remains to be determined. Another $G_{\beta\gamma}$-interacting protein, phosducin, serves to attenuate the action of transducin in the retina. The existence of $G_{\beta\gamma}$- and $G_\alpha$-interacting proteins (RGSs, GoLoco proteins, phosducin, and non-GoLoco AGSs) points to the fact that fine-tuning of the basic regulatory G-protein cycle is required for proper cell homeostasis. These fine-tuning mechanisms are therefore responsible for the ultimate ability of a cell to live a productive life that is in concert with the needs of the whole organism.

## Glossary

**ANF (atrial natriuretic factor)** A peptide produced by the heart that causes the kidney to excrete sodium.

**cGMP (3′,5′-guanosine monophosphate)** A cyclic nucleotide that promotes the intracellular reactions that generate a visual signal in the brain.

**EGF (epidermal growth factor)** A small mitogenic protein involved in regulating normal cell growth, oncogenesis, and wound healing.

**NGF (nerve growth factor)** A neurotrophic factor that promotes a wide range of responses in its target cells. These include neuronal differentiation, maintenance of survival, and regulation of metabolic activity.

**PDGF (platelet-derived growth factor)** A dimeric protein that acts as a mitogen for almost all mesenchymally derived cells.

**TGF-β (transforming growth factor-β)** A cytokine capable of regulating cell growth, extracellular matrix protein synthesis, and immune cell functions.

## See Also the Following Articles

**Epidermal Growth Factor (EGF) Family • GPCR (G-Protein-Coupled Receptor) Structure • Multiple G-Protein Coupling Systems • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Receptor–Receptor Interactions • Signaling Pathways, Interaction of**

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# HGF (Hepatocyte Growth Factor)/MET System

S. Giordano, S. Corso, P. Conrotto, A. Petrelli, and P. M. Comoglio

*University of Torino School of Medicine, Italy*

I. INTRODUCTION
II. HGF AND THE FAMILY OF SCATTER FACTORS
III. SCATTER FACTOR RECEPTORS
IV. SCATTER FACTOR RECEPTORS INDUCE INVASIVE GROWTH
V. DEVELOPMENTAL ROLE OF SCATTER FACTORS
VI. SCATTER FACTOR RECEPTOR RELATIVES: PLEXINS AND SEMAPHORINS
VII. ALTERATION OF THE INVASIVE GROWTH PATHWAY: THE MALIGNANT PHENOTYPE

**Hepatocyte growth factor belongs to the scatter factor protein family; these proteins act through tyrosine kinase receptors of the Met family to control a genetic program that regulates cell detachment, repulsion, protection from apoptosis, invasiveness of extracellular matrices, and proliferation. This genetic program equates to a pleiomorphic response that is defined as “invasive growth.”**

## I. INTRODUCTION

Under physiological conditions, the process of “invasive growth” leads to morphogenic cell movements through the matrix and to ordered building of epithelial tubules. Dysfunctions in this program cause enhanced proliferation, uncontrolled migration into surrounding tissues, and failure to differentiate, events that promote tumor growth and invasiveness. Hepatocyte growth factor, a member of the scatter factor family, involved in promotion of invasive growth, acts through the *Met* tyrosine kinase receptor. During the past few years, a deeper knowledge of the mechanisms through which these receptors exert their physiological activities has enhanced the understanding of how alterations of the tyrosine kinases or of their signal transduction pathways are responsible for cancer onset and progression toward metastasis.

## II. HGF AND THE FAMILY OF SCATTER FACTORS

Hepatocyte growth factor is the prototype of the scatter factor family, so far encompassing two members: hepatocyte growth factor/scatter factor 1 (HGF) and macrophage-stimulating protein/scatter factor 2 (MSP) (Fig. 1). HGF, a heterodimer composed of a 62-kDa α-chain disulfide linked to a 32/34-kDa β-chain, is produced mainly by cells of mesenchymal origin. The α-subunit is characterized by the presence of an N-terminal "hairpin" loop, which is involved in low-affinity binding, and by four repeated domains, named "kringles," which are homologous to those found in plasminogen. The high-affinity receptor for HGF is the transmembrane tyrosine kinase encoded by the *Met* protooncogene. Low-affinity receptors for HGF are heparan sulfate proteoglycans, membrane-bound molecules that allow accumulation of HGF in the extracellular matrix, in the proximity of target cells. The binding sites for both high-affinity and low-affinity receptors, located in the kringles, are known to act as protein–protein interaction motifs. The HGF β-chain is highly homologous to serine protease of the blood-clotting cascade (40% homologous to plasminogen), but it lacks enzymatic activity due to the replacement of critical amino acids in the catalytic site.

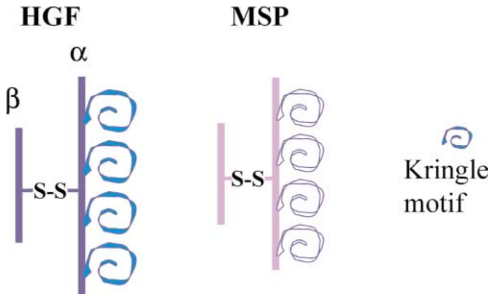

**FIGURE 1** Schematic representation of the two known members of the scatter factor family, hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP). Scatter factors are disulfide-linked (–S–S–) α/β heterodimers. The α-subunit contains four kringles, which are highly conserved protein–protein interaction motifs. The β-subunits (sharing a 50% homology) are similar to serine proteases, although mutations abrogate the catalytic activity. Scatter factors are synthesized as single-chain precursors (pro-HGF and pro-MSP) and are then cleaved in the extracellular environment by specific proteases.

The heterodimeric HGF originates from a 728-amino-acid (pro-HGF) biologically inactive single-chain precursor that is secreted and bound by proteoglycans. In the extracellular environment, pro-HGF is converted in its bioactive form by a single proteolytic cleavage between Arg-494 and Val-495. This cleavage is performed in vitro by several proteases, including tissue-type and urokinase-type plasminogen activators (uPAs), coagulation factor XII, and one homologous serine protease (XII-like factor). Interestingly, uPA, which is capable of extracellular matrix degradation, is a powerful pro-HGF convertase and is also transcriptionally induced by HGF.

The second member of the scatter factor family, macrophage-stimulating protein, so named because it enables peritoneal macrophages to respond to chemoattractants, is highly homologous to HGF and displays similar biological activities (Fig. 1). It is a heterodimer composed of a 53-kDa α-chain containing four kringle motifs that are disulfide linked to a 25-kDa β-chain; the β-chain domain that is homologous to serine protease is devoid of enzymatic activity. In mammalians, the high-affinity MSP receptor is the tyrosine kinase encoded by the *Ron* protooncogene. It has been shown that the avian tyrosine kinase Sea can bind MSP; it has not been formally proved yet that Sea is the avian homologue of mammalian Ron. MSP is secreted as an inactive single-chain precursor that is converted into the fully active dimeric form via proteolytic cleavage by members of the coagulation cascade, including kallikrein.

## III. SCATTER FACTOR RECEPTORS

The products of the protooncogenes *Met* (the HGF receptor; Met) and *Ron* (the MSP receptor; Ron) form a distinct family of protein tyrosine kinase receptors sharing a highly homologous structure. The protooncogene *Sea*, once listed as a third member of the family, is likely to be the avian counterpart of *Ron*. The prototype of this family, encoded by the *Met* gene, is the HGF receptor, a dimeric molecule composed of a 50-kDa α-chain disulfide linked to a 145-kDa β-chain in an α/β complex of 190 kDa. The α-chain is exposed at the cell surface and the β-chain

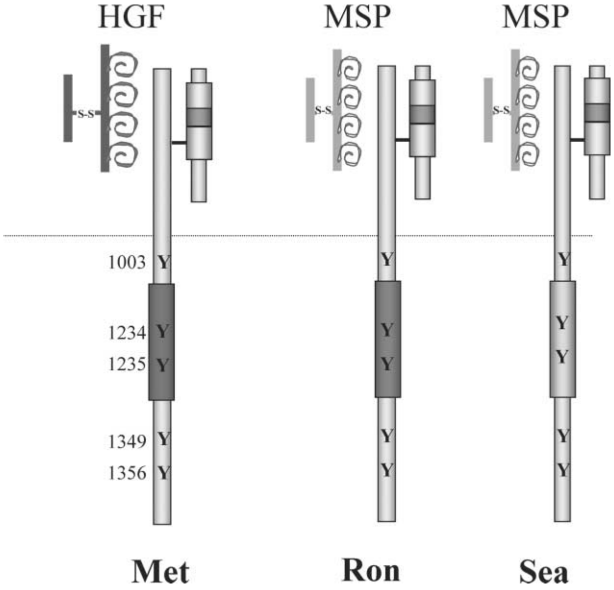

**FIGURE 2** The tyrosine kinase family of scatter factor receptors. The two mammalian members, Met (HGF receptor) and Ron (MSP receptor), share a 63% overall percentage homology. Met and Ron are disulfide-linked heterodimeric proteins formed by an extracellular α-chain and by a β-chain that spans the membrane. The extracellular domains of Met and Ron contain the sema domain [common to the signaling molecules (semaphorins) and to their receptors (plexins)]. The intracellular β-chain contains the two autocatalytic tyrosines in the tyrosine kinase domain (dark gray shaded segment in the cytoplasmic domain), a juxtamembrane tyrosine that is responsible for the negative regulation of the enzyme, and two tyrosines in the C-terminal tail, forming a multifunctional docking site that recruits the full spectrum of signal transducers. The *Sea* gene, found only in avians, is likely to encode the avian counterpart of Ron, which also binds to MSP.

spans the plasma membrane (Fig. 2). The HGF receptor, synthesized as a large precursor (pr170) that includes both the α-chain and the β-chain, undergoes cotranslational glycosylation and is further cleaved by proteases of the furin family to form the mature subunits. Both the α-subunit and the β-subunit are necessary for biological activity. The intracellular portion of the receptor can be divided into three functional domains: a juxtamembrane domain, a tyrosine kinase catalytic domain, and a C-terminal tail.

The juxtamembrane domain negatively regulates Met-mediated activity. Two residues are responsible for the inhibitory effects of the juxtamembrane region: Ser-975 and Tyr-1003. The negative effect of Ser-975 depends on its phosphorylation, which results from activation of protein kinase C and of a Ca$^{2+}$/calmodulin-dependent kinase. The negative effect exerted by Tyr-1003 is less understood and may involve phosphorylation-dependent recruitment to the receptor of a cytosolic tyrosine phosphatase. Moreover, very recently it has been shown that Tyr-1003 plays a critical role in receptor down-regulation. Once phosphorylated, in fact, this tyrosine interacts with Cbl, an ubiquitin ligase that drives Met ubiquitination and, by recruiting a complex formed by CIN85 and endophilins, activates ligand-dependent Met down-regulation.

The tyrosine kinase catalytic domain contains the major phosphorylation site, represented by tyrosine residues 1234 and 1235, which are essential for full activation of the enzyme. On phosphorylation of these residues, the enzymatic activity of the Met kinase is strongly up-regulated in an autocatalytic fashion. Substitution of one of the tyrosine residues with a negatively charged residue results in increased receptor activity, probably due to a conformational change that mimics the one usually observed on phosphorylation of the normal residue.

The C-terminal domain of the Met receptor is crucial for its biological activity; the tail comprises a short sequence containing two tyrosines that are phosphorylated on HGF binding and are responsible for mediating high-affinity interactions with multiple SH2-containing cytoplasmic effectors (Fig. 3). Unlike the docking sites identified in other tyrosine kinase receptors, the Met sequence $Y^{1349}$*VHV*NATY$^{1356}$*VNV* is able to interact with Grb2, p85/phosphatidylinositol 3-kinase (PI3K), phospholipase

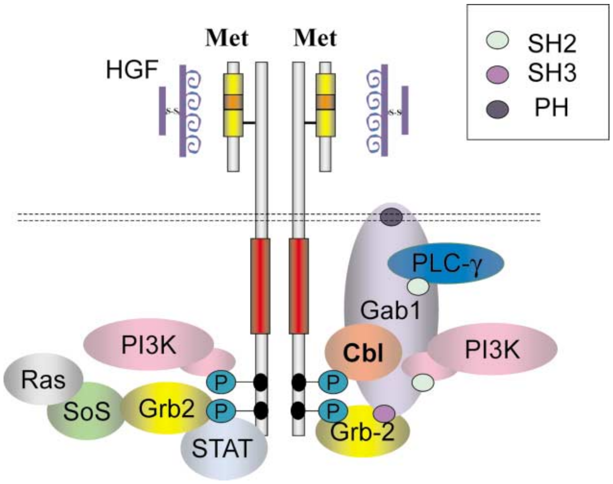

**FIGURE 3** Met signaling machinery. On binding to HGF, Met undergoes dimerization, becomes active, and phosphorylates the two tyrosines in the C-terminal tail of the receptor. These tyrosines then recruit SH2-containing intracellular transducers (e.g., Gab1, Grb2, Stat, PI3K, and Cbl).

C-γ (PLC-γ), Stat3, Shc, and Gab1 and is therefore referred to as the HGF receptor "multifunctional docking site." The multifunctional docking site is essential for Met-mediated biological responses. Substitution of both tyrosines with phenylalanine does not affect the receptor kinase activity but completely abolishes proliferation, motility, invasion, and tubulogenesis. Recent genetic evidence has confirmed the crucial role of the multifunctional docking site for Met signaling *in vivo*.

The components of the signal transduction machinery implicated in individual Met-mediated biological responses have been partially elucidated. Coupling of the receptor to the Ras-mitogen-activated protein kinase (MAPK) pathway is both essential and sufficient for proliferation. Conversely, activation of PI3K as well as the Ras/Rac/Rho pathways is required to induce motility. Notably, concomitant activation of Ras-MAPK and PI3K is both necessary and sufficient for the HGF receptor-induced invasion. The morphogenetic response, which is specifically induced by Met and cannot be elicited by other receptor tyrosine kinases, involves Gab1 and Stat3.

## IV. SCATTER FACTOR RECEPTORS INDUCE INVASIVE GROWTH

Invasive growth is a complex cellular response to a program specifically evoked by scatter factors. Because these ligands and their receptors are widely expressed throughout the body, invasive growth takes place physiologically in almost every tissue. This can be observed during epithelial morphogenesis as formation of tubular structures (such as gland ducts) or in endothelial cells in the process of angiogenesis. Scatter factors stimulate cells to form ramified tubules, sprouting like branches from a tree. By arranging themselves into tridimensional structures, epithelial and endothelial cells develop to their terminal differentiation and become polarized. In the bone marrow, scatter factors stimulate early erythroid cells to dissociate from their stem colonies and to enter the blood circulation. During bone remodeling, invasive growth is observed when osteoclasts migrate into the mineralized matrix and recruit the osteoblasts that will form osteogenic centers. In the embryo, myoblast precursors migrate from myotomes to their peripheral destination following a gradient of scatter factors. A particular form of invasive growth, more recently discovered, takes place in neural tissues, wherein scatter factors stimulate the process of axon guidance when neurites sprout and give origin to ramifications that will reach distant targets.

The accomplishment of the process of invasive growth implies different steps. The first, which is observed *in vitro* within a few hours after stimulation, is cell–cell dissociation. In early studies, this phenomenon was originally described in epithelial cells as "cell scattering." Loss of intercellular contacts allows cells to migrate through the surrounding tissues and across the basement membrane. Active migration requires cytoskeletal reshaping, digestion of the matrix by secreted proteases, and remodeling of cell–matrix contacts. In this process, scatter factors enable cells to survive "anoikis," which usually occurs when cell adhesion is impaired. Cell proliferation and acquisition of polarity are the final steps of this process. Deregulated execution of this process can sustain the invasive behavior of tumor cells.

## V. DEVELOPMENTAL ROLE OF SCATTER FACTORS

### A. Control of Cell Migration in Early Embryogenesis

During gastrulation, HGF and its receptor are expressed in the endoderm and in the mesoderm. Subsequently, the expression of HGF is limited to the mesenchymal cells, whereas that of the receptor is induced in the surrounding ectoderm. At this stage, approximately embryonic day 13 (E13), HGF contributes to the development of several epithelial organs (e.g., lungs, liver, and gut) and induces migration of myogenic precursor cells. HGF, in fact, is expressed in limb mesenchyme and can thus provide the signal for migration, which is received by c-Met expressing myogenic precursors. In c-Met homozygous mutant (−/−) mouse embryos, the limb bud and diaphragm are not colonized by myogenic precursor cells and, as a consequence, skeletal muscles of the limb and diaphragm do not develop. It is also interesting to note that during this process HGF must dissociate the epithelium lining the lateral plate of dermomyotomes to allow the migration of the myogenic precursors. Moreover, transgenic mice overexpressing HGF exhibit ectopic skeletal muscles and melanocytes in the central nervous system, suggesting that HGF has scatter activity *in vivo* and that tight regulation of HGF expression is critical for the correct development. Collectively, these observations are consistent with a role of c-Met and HGF as key regulator molecules in the migration of myogenic cells.

## B. Mesenchymal–Epithelial Interactions

Several observations indicate that c-Met and HGF play an important role in the process of epithelial morphogenesis. One of the best examples is given by the formation of kidney tubules from metanephric tissue and ureteric buds. Metanephric mesenchymal cells secrete HGF whereas epithelial cells of the ureteric bud express the receptor. When the latter contact the metanephros, they form branching tubules; in turn, some of the metanephric cells start to express the receptor for HGF and—stimulated by the autocrine loop—undergo a process known as "mesodermic–epithelial transition." They acquire epithelial features, form cell–cell junctions, polarize, and eventually form tubules. It is interesting to note that, although all of the mesenchymal cells of the renal cortex express HGF, the receptor is found only in those cells that give origin to the renal tubules and not in the cells forming the glomeruli. In the adult, only mesangial cells, which probably represent a poorly differentiated compartment for regeneration, co-express HGF and its receptor.

The mammary gland is a good example of an organ that undergoes major morphological changes after birth and in which branching development takes place during pregnancy and lactation. This program of differentiation relies on sequential stimulation of the epithelium by HGF and neuregulin, which are secreted by the mesenchyme in different developmental stages. HGF is expressed at puberty and promotes branching of the ductal tree, whereas neuregulin is responsible for alveolar budding and the production of milk proteins during pregnancy and lactation.

## C. Signal Transduction Studies in Met Knockout and Knock-in Mice

Genetic analysis in the mouse has elucidated the developmental functions of scatter factors and their receptors. Mutant embryos lacking Met or HGF die *in utero* due to hyponutrition, caused by a reduction of the labyrinth layer of the placental trophoblast, which is normally responsible for controlling the invasion of maternal tissues to expand the placenta. Moreover, these embryos display impaired development of the liver and lack skeletal muscles deriving from long-range migrating precursors.

To analyze the requirement of *Met*-activated signal transduction pathways in development and function of different tissues and organs, a Met receptor impaired in its signal transduction properties was used to construct knock-in mice. Loss of the Met multifunctional docking site ($met^{D/D}$ construct) resulted in embryonic lethality and produced a phenotype coincident with that observed in Met null mutants. In contrast, embryos with a Met docking site modified only to prevent Grb2 binding were viable. In these animals, muscles deriving from migratory precursors were heavily affected, indicating a requirement for Grb2-mediated signaling for survival and proliferation of myoblasts en route to their final destination.

These engineered mice have been also very useful in elucidating the role played by *Met* in the development of the nervous system. It is known that both Met and HGF are expressed in the developing nervous system and that HGF promotes neural induction, stimulates Schwann cell proliferation, and promotes axon outgrowth from P19 embryonal carcinoma cells. Moreover, HGF is a limb mesenchyme-derived chemoattractant for motor axons. Analysis of Met signaling mutants showed that mice carrying the inactivating $met^{D/D}$ alleles have intercostal nerves that are much reduced in length and elaborate fewer terminal branches. Interestingly, Met signaling via Grb2 is not required for sensory neuron development but is critical for survival of sympathetic neuroblasts.

## VI. SCATTER FACTOR RECEPTOR RELATIVES: PLEXINS AND SEMAPHORINS

The search for receptors homologous to Met and Ron has led to identification of the *plexin* gene family, comprising at least nine different genes in humans. These genes are grouped into four subfamilies (*plexins A* to *D*) and encode transmembrane proteins that have extracellular segments containing regions of homology with scatter factor receptors. The cytoplasmic domain of plexins is highly conserved within the family, but, surprisingly, it does not contain any known functional domain. However, this domain includes a number of tyrosine residues that can be phosphorylated and a long α-helix motif, common to protein–protein interaction modules.

Plexins are the receptors for semaphorins, a large family of related proteins, encompassing at least 20 members, that are either secreted or membrane bound. A distinctive feature of these molecules is the presence of the sema domain, a protein–protein interaction domain that mediates receptor-binding specificity. The observation that this domain is present also in plexins and in scatter factor receptors

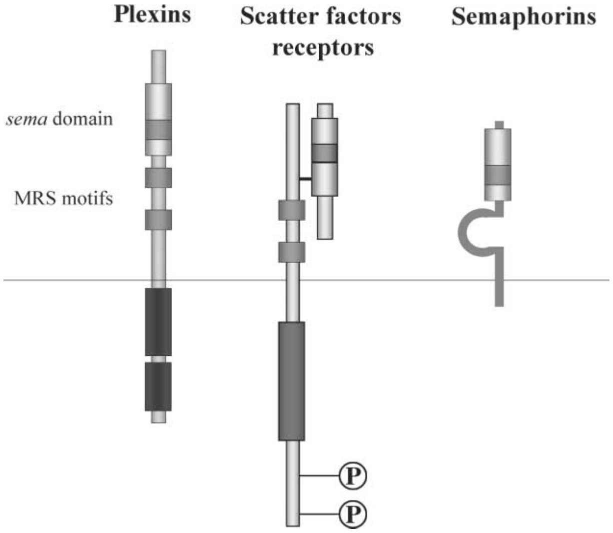

FIGURE 4 The scatter factor, semaphorin, and plexin superfamily. Scatter factor receptors are part of a larger family of molecules, characterized by the presence of the sema domain and of the Met-related sequence (MRS) in the extracellular domain. Semaphorins are signaling molecules that regulate neuronal axon guidance and plexins are their receptors. Plexins are single-pass transmembrane molecules that have a peculiar intracellular domain, conserved among the different plexins, endowed with still unknown functions. The overall homology between scatter factor receptors and plexins is confined to their extracellular domains.

(Fig. 4) raises the possibility that it can mediate homophilic interactions, establishing a interplay with plexins and scatter factor receptors. This view is consistent with the partial overlapping of the biological functions of scatter factors and semaphorins. In fact, semaphorins were first identified as repelling cues for axon guidance during the development of nervous system. More recently it has been discovered that plexins can mediate repelling signals in almost all tissues. Repulsion results in dissociation of packed cells, a process very reminiscent of scatter. Interestingly, semaphorins are overexpressed in invasive and metastasizing tumors, in which they can sustain motility, invasion, and cell survival.

## VII. ALTERATION OF THE INVASIVE GROWTH PATHWAY: THE MALIGNANT PHENOTYPE

Scatter factor receptors are associated with, and in some cases directly responsible for, a wide variety of human cancers. The most convincing evidence of the involvement of Met in human cancer arose from analysis of patients affected by hereditary papillary renal carcinoma (HPRC), a form of inherited cancer, characterized by the presence of multiple, bilateral tumors. These patients bear germ-line missense mutations of Met that are also found in somatic cells of patients affected by sporadic renal carcinomas or childhood hepatocellular carcinoma. Some mutations are homologous to those found in the oncogenes *Ret* or *Kit*, which are responsible for inherited multiple endocrine neoplasia (type 2) or for hematological disorders, respectively. *In vitro* studies show that these mutated forms of Met display an increased tyrosine kinase activity, are transforming, and confer to cells in nude mice the ability to form tumors and to overcome apoptosis. Unexpectedly, it has been demonstrated that tumorigenesis is strictly dependent on stimulation by the ligand and that it is abolished in the presence of HGF specific inhibitors. Conceivably, the onset of HPRC depends both on the presence of inherited Met mutations and on the availability of HGF.

Mutated forms of Met have also been shown to play a role in the metastatic process in human tumors. It has been reported that somatic mutations of Met are selected during the metastatic spread of head and neck squamous carcinoma. One of these point mutations results in substitution of Tyr-1235, critical for the autocatalytic activation of the kinase, with an aspartate, conferring a permanent negative charge that mimics the presence of a phosphate group and is responsible of constitutive kinase activation.

Met overexpression in the absence of missense mutations is the most common alteration found in human tumors and is often associated with metastatic tendency and poor prognosis. In the case of colorectal carcinoma, amplification of the Met gene, associated with protein overexpression, confers a selective advantage for acquiring metastatic potential to the liver. In some instances, tumorigenesis is associated with ectopic expression of Met. Although absent in adult skeletal muscle, Met is expressed in a significant proportion of human sarcomas. Because these cells express HGF, aberrant Met expression results in the formation of an autocrine loop, which confers invasive properties.

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## Glossary

**gene knock-in** Technique in which the coding sequences of one gene are replaced with those of another.

**gene knockout** Technique for selectively inactivating a gene in the germ-line of an animal by replacing the gene with a mutant allele.

**mesenchyme** Embryonic tissue from which are formed the connective tissues, blood, and lymphatic vessels.

**Src homology 2 (SH2) domains** Protein segments able to recognize and bind to phosphorylated tyrosines. They are present in several intracellular proteins acting as signal transducers.

**tyrosine kinases** Enzymes able to transfer the terminal $\gamma$-phosphate groups from ATP to tyrosines that reside in intracellular substrates.

**ubiquitin** Small protein that becomes covalently linked to lysine residues in other intracellular proteins. This reaction is catalyzed by ubiquitin ligases. Ubiquitinylated proteins are usually degraded in the proteasome.

## See Also the Following Articles

**Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • Nerve Growth Factor (NGF) • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor B (VEGF-B)**

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# Human Growth Hormone

See *Growth Hormone*

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# Humoral Hypercalcemia of Malignancy

Arthur E. Broadus and Lauren H. Golden
*Yale University*

I. INTRODUCTION
II. MECHANISMS OF MALIGNANCY-ASSOCIATED HYPERCALCEMIA

III. PARATHYROID HORMONE-RELATED PROTEIN
IV. FEATURES OF HUMORAL HYPERCALCEMIA OF MALIGNANCY
V. MANAGEMENT

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**Parathyroid hormone-related protein is a factor that structurally and functionally resembles parathyroid hormone. When produced by normal tissues, parathyroid hormone-related protein plays an important role in physiological development. When secreted into the circulation by malignancies, parathyroid hormone-related protein is the principal factor responsible for the development of humoral hypercalcemia of malignancy.**

## I. INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was identified in 1987, as investigators strove to unravel the underlying pathophysiologic mechanisms of malignancy-associated hypercalcemia. Hypercalcemia is a common, life-threatening metabolic complication of malignancy, affecting 10–20% of patients with advanced cancers of varying etiologies. Patients present with classic signs and symptoms, including polyuria, polydipsia, nausea and vomiting, constipation, and impairment of mental status. Volume contraction and dehydration often occur as a direct result of hypercalcemia-induced diuresis, and often precipitate renal compromise. Malignancy-associated hypercalcemia progresses and decompensates rapidly, compared to the more gradual onset of hypercalcemia associated with primary hyperparathyroidism. As a result, malignancy-associated hypercalcemia is commonly associated with dramatic symptoms and carries a very poor prognosis for survival.

Two distinct mechanisms have been implicated in the pathogenesis of malignancy-associated hypercalcemia: local osteolytic hypercalcemia (LOH) and humoral hypercalcemia of malignancy (HHM). Osteoclastic bone resorption is the final common pathway leading to hypercalcemia in both LOH and HHM.

## II. MECHANISMS OF MALIGNANCY-ASSOCIATED HYPERCALCEMIA

Local osteolytic hypercalcemia involves hypercalcemia induced as a consequence of direct tumor involvement of the skeleton. Malignant cells metastatic to bone produce bone-resorbing cytokines, including tumor necrosis factor β (TNFβ), tumor necrosis factor α (TNFα), interleukin-6, interleukin-1, and transforming growth factor-α (TFG-α). Local production of these cytokines in the bone microenvironment results in cytokine-induced activation of osteoclasts and, ultimately, bone resorption. LOH occurs most commonly in the setting of breast cancer, multiple myeloma, and other hematologic malignancies such as leukemia and lymphoma (Table 1). Overall, LOH accounts for approximately 20% of clinical cases of malignancy-associated hypercalcemia.

In contrast to local osteolytic hypercalcemia, humoral hypercalcemia of malignancy is mediated by tumor-induced production of humoral factors that are secreted into the general circulation in the presence or absence of skeletal metastases. Historically, the identification of HHM hinged on recognition by clinicians that not all patients with malignancy-associated hypercalcemia suffered extensive skeletal involvement. Thus, local lysis of bone with a resultant release of calcium could not be implicated as the sole pathogenic factor underlying the metabolic derangement. In addition, these patients displayed an associated hypophosphatemia, which could not otherwise be explained.

In 1941, Dr. Fuller Albright described one such patient, a man with a renal carcinoma and a single skeletal metastasis. The clinical association of hypercalcemia and hypophosphatemia, a paucity of bony metastases, as well as the reversal of the metabolic derangement upon resection of the primary tumor, prompted Albright to hypothesize that the tumor was secreting a "humoral" calcemic phosphaturic factor. He speculated that the proposed factor resembled

**TABLE 1 Tumors Commonly Associated with Hypercalcemia**

| Local osteolytic hypercalcemia | Humoral hypercalcemia of malignancy |
|---|---|
| Multiple myeloma | Squamous cell |
| Breast cancer | Lung |
| Lymphoma | Esophagus |
| | Cervix |
| | Head and neck |
| | Genitourinary tract |
| | Renal |
| | Bladder |
| | Ovary |
| | Breast cancer |
| | HTLV-1 lymphoma |

parathyroid hormone in its actions, capable of inducing bone resorption and of modulating calcium homeostasis.

## III. PARATHYROID HORMONE-RELATED PROTEIN

In 1987, the principal tumor-derived factor responsible for HHM was isolated, purified, and identified. The gene for this new factor was localized to the short arm of chromosome 12 and its intron–exon organization was found to resemble that of the PTH gene located on the short arm of chromosome 11. Three gene products of varying lengths and molecular weights were identified, the results of alternative splicing. The total lengths of the three protein products are 139, 141, and 173 amino acids, respectively. Comparison of the amino acid sequence of the new peptide with that of parathyroid hormone revealed striking homology at positions 1–13 of the amino-terminal end, with the first eight amino acids being identical to those of native PTH, and with divergence of the sequences thereafter. It was thus given the name parathyroid hormone-related protein. Posttranslational processing of PTHrP produces N-terminal [PTHrP(1–36)], midregion, and C-terminal peptides in a cell-specific fashion.

It is the amino-terminal portion of the PTH molecule that binds to the PTH receptor. Activation of the classical PTH receptor by PTHrP(1–36) was soon confirmed, a consequence of the above-mentioned amino-terminal homology. In addition to partial sequence homology and an ability to activate the PTH receptor, PTHrP exhibits many physiological actions similar to those of native parathyroid hormone, namely, an ability to stimulate bone resorption, renal tubular calcium reabsorption, and renal phosphate wasting.

## IV. FEATURES OF HUMORAL HYPERCALCEMIA OF MALIGNANCY

Humoral hypercalcemia of malignancy accounts for approximately 80% of cases of malignancy-associated hypercalcemia. Tumors that are often associated with HHM include those of squamous cell origin (lung, esophagus, cervix, and head/neck) and those of genitourinary tract origin (renal, bladder, and ovary), as well as tumors of the breast. HHM is also associated with adult T-cell leukemia/lymphoma, an uncommon malignancy resulting from chronic infection with human T-cell leukemia virus type 1 (HTLV-1). The presence of hypercalcemia in these clinical settings reflects the capacity of particular malignant cells to elaborate quantities of PTHrP sufficient to elevate levels in systemic circulation and induce bone resorption. It is important to note that some malignancies possess the capacity to induce hypercalcemia through both LOH and HHM. For example, it has been demonstrated that the majority of breast cancer skeletal metastases elaborate PTHrP, whereas the majority of nonskeletal metastases do not.

From a biochemical standpoint, HHM shares many features with primary hyperparathyroidism, namely, hypercalcemia, hypophosphatemia, and hypercalciuria. Close examination of the physiologic actions of the two hormones, however, reveals features of HHM that are, in some ways, unlike those of PTH excess (primary hyperparathyroidism), resulting in distinct metabolic profiles. (Table 2).

At the level of bone, the two hormones exert distinct actions. Although both PTH and PTHrP stimulate intense bone resorption, PTH stimulates coupled bone turnover, an increase in bone resorption that is accompanied by an increase in bone formation. In contrast, HHM stimulates uncoupled bone turnover. That is, in HHM there is a marked increase in bone resorption, accompanied by suppression of bone formation. Bone biopsies from patients with HHM reveal increased numbers of osteoclasts, mediators of bone resorption, few or no osteoblasts, and a paucity of osteoid, a premineralized bony substrate that reflects new bone formation (Fig. 1).

In addition, the action of PTHrP at the level of the kidney differs from that of PTH. Nephrogenous

**TABLE 2 Biochemical and Metabolic Features of Primary Hyperparathyroidism and Humoral Hypercalcemia of Malignancy**

| Feature | Primary hyperparathyroidism | Humoral hypercalcemia of malignancy |
|---|---|---|
| Serum calcium | ↑ | ↑ |
| Serum phosphorus | ↓ | ↓ |
| $1,25(OH)_2D_3$ | ↑ | ↓ |
| PTH | ↑ | ↓ |
| PTHrP | ↓ | ↑ |
| Urinary calcium excretion | ↑ | ↑ ↑ |
| Nephrogenous cAMP | ↑ | ↑ |
| Bone resorption | ↑ | ↑ ↑ |
| Bone formation | ↑ | ↓ |

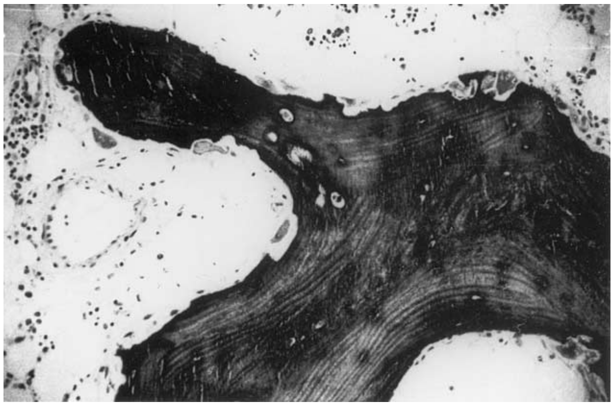

**FIGURE 1** Photomicrograph of a bone biopsy specimen from a patient with humoral hypercalcemia of malignancy. Note the increased numbers of osteoclasts, the absence of osteoblasts, and the paucity of osteoid.

(kidney-derived) cyclic adenosine monophosphate (cAMP) has been established as a sensitive and specific index of parathyroid hormone bioactivity. As mentioned above, PTHrP interacts directly with the PTH receptor in the kidney, and its activity is reflected by the generation of nephrogenous cAMP. Like PTH, PTHrP stimulates renal tubular calcium reabsorption, although to a lesser degree than native PTH. Thus, urinary calcium excretion, increased in both primary hyperparathyroidism and HHM, tends to be elevated to a greater extent in HHM. Like PTH, PTHrP also stimulates renal phosphate excretion, resulting in hypophosphatemia.

The renal actions of PTH and PTHrP appear to differ with regard to the production of $1{,}25(OH)_2D_3$, the activated form of vitamin D. Infusions of both PTH and PTHrP have been shown to induce increases in plasma levels of $1{,}25(OH)_2D_3$. However, levels of $1{,}25(OH)_2D_3$ are inappropriately normal or frankly low in patients with HHM, whereas they are elevated in patients with primary hyperparathyroidism. The mechanisms responsible for this suppression of $1{,}25(OH)_2D_3$ in HHM remain to be elaborated. Because activated vitamin D contributes to absorption of calcium by the gut, absorption of dietary calcium is not a significant contributor to the hypercalcemia associated with HHM.

## V. MANAGEMENT

The hypercalcemia of HHM induces a diuresis that results in dehydration and often precipitates renal compromise. Management of HHM involves three targeted steps: (1) correction of the dehydration, (2) enhancement of calciuresis (renal calcium excretion), and (3) inhibition of further osteoclastic bone resorption.

Rehydration restores intravascular volume and optimizes glomerular filtration rate. Initial frontline therapy in the management of symptomatic hypercalcemia involves aggressive volume repletion with isotonic saline. The rate of intravascular fluid administration is dictated by the severity of the hypercalcemic symptoms and the degree of hemodynamic compromise. Re-expansion of intravascular volume with a sodium load not only corrects the dehydration associated with hypercalcemia, but also increases renal blood flow, enhancing filtration and calciuresis. A modest decline in serum calcium levels occurs as a result. Once intravascular volume has been effectively repleted, the addition of a loop diuretic (i.e., furosemide) to the treatment regimen may further enhance calciuresis by inhibiting both calcium and sodium reabsorption in the ascending loop of Henle.

The mainstay of the pharmacologic treatment of hypercalcemia of malignancy is a class of antiresorptive agents known as bisphosphonates. Bisphosphonates disrupt osteoclastic bone resorption via direct inhibition of osteoclast activity. They also inhibit osteoclastogenesis and promote increased osteoclast apoptosis. Thus, in the setting of HHM, bisphosphonates block the key end-organ effect of PTHrP, blunting the osteoclastic resorptive response and the resultant release of calcium into the systemic circulation.

Bisphosphonates have a core phosphorus–carbon–phosphorus structure. They are rapidly deposited into bone, where they bind to hydroxyapatite crystals. Once deposited into bone, their skeletal half-life is long, on the order of years. Although a variety of bisphosphonates have been used successfully in the treatment of HHM, intravenous pamidronate disodium, a second-generation bisphosphonate, is presently used as firstline treatment. Improvements in serum calcium are not immediate and the duration of response is variable.

More recently, a third-generation bisphosphonate, zoledronic acid, has been shown to have increased antiresorptive potency. Zoledronic acid is administered over a shorter infusion time and demonstrates increased efficacy and prolonged response times compared to pamidronate.

## Glossary

**cytokines** Small, secreted proteins or glycoproteins that function as chemical messengers and regulate growth, differentiation, and activation of cells; may exert influence on the cell of origin, on contiguous cells, or on cells in a distant location, i.e., they may exert autocrine, paracrine, or endocrine effects.

**osteoblasts** Bone-lining cells that synthesize bone matrix; responsible for bone formation.

**osteoclasts** Bone-lining cells responsible for bone resorption.

## See Also the Following Articles

**Calcium Signaling • Parathyroid Hormone • Parathyroid Hormone-Related Protein (PTHrP)**

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# Hypercalcemia

See *Humoral Hypercalcemia of Malignancy*

# Hypertension

See *Mineralocorticoids and Hypertension*

# Hypoglycemia in Diabetes

Salima Mithani and Elizabeth R. Seaquist
*University of Minnesota*

I. INTRODUCTION
II. GLUCOSE HOMEOSTASIS
III. GLUCOSE COUNTERREGULATION

IV. SYMPTOMATIC RESPONSES TO HYPOGLYCEMIA
V. HYPOGLYCEMIA IN DIABETES
VI. CONCLUSION

**Blood glucose levels are maintained within a narrow range by a careful balance between insulin and the hormones that counteract its effects. Hypoglycemia occurs when serum glucose concentrations fall below the normal range. To prevent hypoglycemia, a complex and redundant system of neuroendocrine responses is activated, but these responses are altered in patients with diabetes. Acute hypoglycemia and its accompanying neurogenic and neuroglycopenic symptoms have a profound impact on the lives of patients with diabetes and can cause significant physical and psychosocial morbidity and mortality in these patients.**

## I. INTRODUCTION

The brain requires glucose as its metabolic fuel. In normal subjects, serum glucose concentrations are maintained within a narrow range by a system of coordinated neuroendocrine mechanisms. Whenever hypoglycemia occurs, counterregulatory hormonal responses are activated to ensure an adequate supply of glucose to the brain. However, maintaining euglycemia in persons with diabetes can present a therapeutic challenge. Hyperglycemia often requires treatment with insulin or oral hypoglycemic drugs but many patients develop defective counterregulatory mechanisms and ultimately suffer from hypoglycemia.

The incidence of hypoglycemia in patients with diabetes is probably much greater than appreciated. Severe hypoglycemia is usually defined as an episode of low blood sugar that requires the assistance of another for recovery. In the Diabetes Control and Complications Trial in which more than 700 patients were provided intensive insulin therapy, the frequency of severe neuroglycopenia was 0.62 episodes/patient/year. The frequency of hypoglycemia in people with type 2 diabetes treated with insulin is approximately 2% per year, but when patients with type 2 diabetes and pancreatic beta-cell failure are compared to subjects with type 1 diabetes with a similar duration of insulin treatment, the frequency of severe hypoglycemia is similar.

Acute hypoglycemia causes significant physical and psychosocial morbidity and mortality in patients with diabetes. As many as 4% of deaths of patients with type 1 diabetes have been attributed to hypoglycemia. These deaths may occur because of hypoglycemia-induced cardiac arrhythmias and myocardial ischemia. Acute neuroglycopenia can also lead to coma, convulsions, cerebral ischemia, and transient hemiplegia. The cognitive impairment that can result from hypoglycemia may result in accidents and physical injury. Acute and chronic changes in mood can also occur in diabetic patients with hypoglycemia. Some investigators have found that frequent hypoglycemia increases the risk of developing depression in patients with type 1 diabetes.

Restoration of euglycemia is an essential therapeutic goal in patients with diabetes because good glycemic control has been shown to reduce the risk of microvascular disease in patients with both type 1 and type 2 diabetes. Unfortunately, patients who follow an intensive therapeutic plan are at increased risk for severe hypoglycemia. Indeed, hypoglycemia is the limiting factor in the treatment of diabetes. This article reviews what is known about neuroendocrine responses to hypoglycemia and how these responses are changed in patients with diabetes.

## II. GLUCOSE HOMEOSTASIS

The maintenance of normal plasma glucose concentrations requires a precise balance between insulin and the hormones that oppose insulin's actions. Insulin acts to reduce plasma glucose concentrations by enhancing glucose disposal into muscle and fat and by inhibiting endogenous glucose production from the liver and kidney. The most important hormone to oppose insulin's action under normal homeostatic conditions is glucagon. This hormone serves to increase endogenous glucose production. Insulin and glucagon are both produced in the islets of Langerhans in the pancreas. Each islet consists of a few hundred cells. The insulin-producing beta cells constitute approximately 60% of islet mass and form the core of the structure. These cells are surrounded by the glucagon-producing alpha cells, which constitute approximately 25% of islet mass. After a meal or whenever plasma glucose concentrations begin to rise, insulin is released from the beta cell into the blood that perfuses the islet. Insulin first reaches the alpha cell, where it inhibits glucagon secretion, and then enters the general circulation. As plasma glucose concentrations fall, insulin secretion is reduced and the inhibitory effects on glucagon secretion are removed. Glucagon can then be released into the circulation to maintain glucose homeostasis.

The brain is one organ that is particularly dependent on the maintenance of normal glucose homeostasis. The energy requirements for the brain are very high, approximately 1.0 mg/kg/min or 100 g/day in an adult. Glucose oxidation normally provides more than 90% of the energy needed for brain function and although some glucose is stored in the brain as glycogen, the vast majority of glucose used for cerebral metabolism originates in the blood and passes into the brain across the blood–brain barrier. Under euglycemic conditions, glucose transport is essentially saturated because serum glucose concentration remains well above the 1 to 3 mM concentration that is equal to the $K_m$ defined for this process.

## III. GLUCOSE COUNTERREGULATION

Because of the importance of providing a continuous source of fuel to the brain, the body has developed an elaborate system of compensatory responses to maintain glucose delivery to the brain when blood glucose levels begin to fall. The first step in these compensatory responses is the inhibition of insulin secretion, which serves to reduce insulin-mediated glucose uptake into tissues and to relieve the inhibitory effects of insulin on endogenous glucose production. If serum glucose continues to fall to below 60–65 mg/dl (3.3–3.6 mM), glucagon, epinephrine, norepinephrine, cortisol, and growth hormone are all released into the circulation. These counterregulatory hormones serve to oppose the action of insulin and cause the elevation of serum glucose concentrations. The coordination of glucose counterregulation relies on the interaction of organs that sense a reduction in plasma glucose concentrations and initiate a defense against hypoglycemia, organs that respond to signals from glucose-sensing organs by secreting hormones and neurotransmitters that are important in modulating plasma glucose concentrations, and organs that respond to these hormonal and neural signals by releasing glucose into the blood.

### A. Glucose-Sensing Organs

The brain is of key importance in sensing hypoglycemia. The ventromedial hypothalamus, amygdala, and brainstem are particularly important is sensing a reduction in plasma glucose. The liver and the portal vein also have sensors that convey a drop in plasma glucose to the central nervous system, although these sensors are probably of lesser importance than the glucose-sensing regions in the brain. In addition, hypoglycemia is sensed in the pancreatic beta cell, where it leads to a reduction in insulin secretion. Finally, a drop in plasma glucose elicits a glucagon secretory response from the pancreatic alpha cell, although whether the alpha cell independently senses this reduction in glucose or whether it is responding to neural input is not completely understood.

### B. Counterregulatory Hormones and Neurotransmitters

Once hypoglycemia is detected, the glucose sensors initiate hormonal and neural responses to restore euglycemia. These responses are elicited at different levels of glycemia (see Fig. 1) and provide a redundant system through which to maintain euglycemia. In normal circumstances, the first response to a drop in plasma glucose is the inhibition of insulin release at a plasma glucose concentration of approximately 80 mg/dl (4.7 mM). When plasma glucose falls to 65 mg/dl (3.6 mM), glucagon is released from the pancreatic alpha cell, epinephrine is secreted from the adrenal medulla, and growth hormone is released from the pituitary. Cortisol is secreted from the adrenal cortex when plasma glucose falls below 60 mg/dl (3.3 mM). The sympathetic nervous system also responds to a reduction in plasma glucose and increases norepinephrine release from sympathetic postganglionic neurons when plasma glucose falls below 60 mg/dl (3.3 mM). All of these counterregulatory agents increase plasma glucose by increasing endogenous glucose production and reducing glucose utilization by muscle and fat.

The relative importance of these counterregulatory agents was studied extensively by Cryer and associates in a series of experiments in which glucose recovery following insulin administration was examined in subjects with selected deficiencies in the counterregulatory hormones. In these experiments, secretion of glucagon and growth hormone was suppressed by the infusion of somatostatin, the effects of epinephrine and norepinephrine were blocked by α- or β-adrenergic blockade, or epinephrine secretion was absent as a result of bilateral adrenalectomy. The results of these investigations are depicted in Fig. 2. They demonstrate that subjects with isolated deficiencies in epinephrine, norepinephrine, or growth hormone recover from hypoglycemia as well as controls do but that subjects with isolated glucagon deficiency recover from hypoglycemia more slowly than unmanipulated controls.

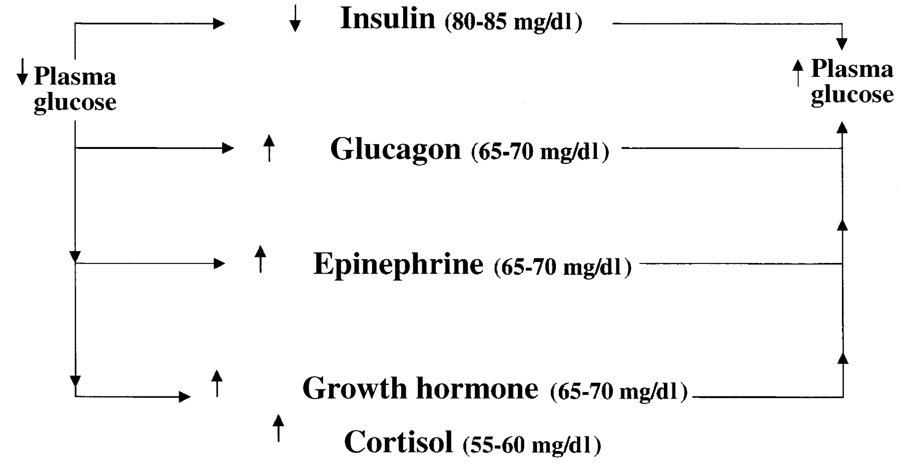

FIGURE 1 Hormonal responses to hypoglycemia. As plasma glucose concentrations fall, insulin secretion is reduced and the release of the counterregulatory hormones glucagon, epinephrine, growth hormone, and cortisol is stimulated to maintain glucose homeostasis.

However, when glucagon secretion was suppressed in adrenally insufficient subjects or in subjects with α- or β-adrenergic blockade, glucose recovery was completely prevented. These observations demonstrated that glucagon secretion is of primary importance in glucose recovery following insulin administration but that catecholamine secretion is also necessary for normal counterregulation to occur.

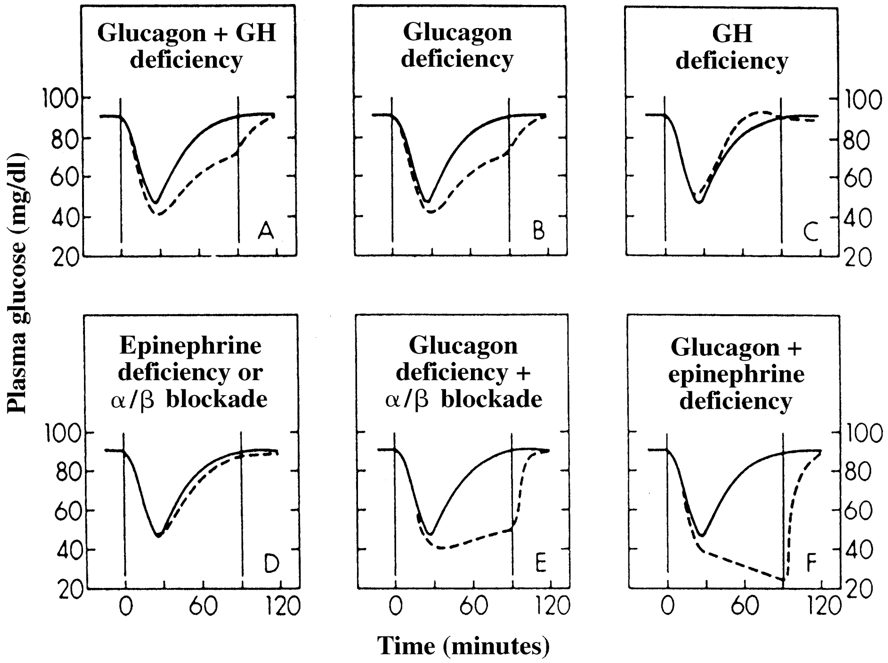

FIGURE 2 Plasma glucose levels during insulin-induced hypoglycemia in normal subjects in control studies (solid lines) and as modified (dashed lines) by (A) somatostatin infusion; (B) somatostatin plus growth hormone infusion; (C) somatostatin plus glucagon infusion; (D) phentolamine plus propranolol infusion or studies in bilaterally adrenalectomized patients; (E) somatostatin plus phentolamine and propranolol infusion; and (F) somatostatin infusion in bilaterally adrenalectomized patients. Reprinted from P. E. Cryer (1981), Glucose counterregulation in man. *Diabetes* 30, 261, with permission from the American Diabetes Association.

### C. Organs Responding to Hypoglycemia-Induced Counterregulation

Many organs respond to the counterregulatory signals elicited by hypoglycemia. The liver and the kidney respond by increasing gluconeogenesis and glycogenolysis. Muscle responds to the proteolytic signals of glucagon and cortisol, thereby providing amino acid substrates for gluconeogenesis. Adipose tissue releases free fatty acids in response to increases in glucagon and catecholamines. Catabolism of these free fatty acids creates energy that can be used to support hepatic glucose production.

## IV. SYMPTOMATIC RESPONSES TO HYPOGLYCEMIA

Experiments in which normal subjects were given insulin and their blood sugar levels were allowed to fall in a controlled fashion have defined the symptoms that accompany the counterregulatory response to hypoglycemia. Although glucagon release is not associated with any distinct symptomatology, the release of catecholamines and activation of the autonomic nervous system cause specific neurogenic symptoms in healthy subjects subjected to experimental hypoglycemia. As listed in Table 1, adrenergic symptoms include rapid heart rate, shakiness, tremulousness, and anxiety. These symptoms result from the release of epinephrine from the adrenal medulla and release of norepinephrine at the sympathetic postganglionic nerves. Cholinergic symptoms, which occur as a result of acetylcholine release from the sympathetic postganglionic nerves, include sweating, hunger, and tingling. Neurogenic symptoms begin to appear when blood glucose drops to 50–55 mg/dl (2.8–3.1 mM). When blood glucose is allowed to drop to a lower level, symptoms of neuroglycopenia occur (see Table 1). These symptoms include dizziness, headache, blunted mental activity, loss of fine motor skills, confusion, abnormal behavior, seizures, and loss of consciousness. Because the neurogenic symptoms are elicited at a higher blood glucose level than the neuroglycopenic complaints and because they are not associated with impaired judgment or mental capacity, they usually prompt the patient to eat something to increase their blood sugar. If the neurogenic symptoms are not treated before neuroglycopenia occurs, the patient becomes unable to help himself and must rely on the assistance of another to overcome the episode of hypoglycemia.

**TABLE 1 Symptoms of Hypoglycemia**

| Type of symptoms | Symptoms |
|---|---|
| Adrenergic symptoms | Rapid heart rate<br>Shakiness<br>Tremulousness<br>Anxiety |
| Cholinergic symptoms | Sweating<br>Hunger<br>Tingling |
| Neuroglycopenic symptoms | Dizziness<br>Headache<br>Blunted mental activity<br>Loss of fine motor skills<br>Confusion<br>Abnormal behavior<br>Seizures<br>Loss of consciousness |

## V. HYPOGLYCEMIA IN DIABETES

In healthy human beings, severe hypoglycemia is prevented by the counterregulatory mechanisms discussed above. However, patients with diabetes who are treated with insulin and/or drugs that enhance endogenous insulin secretion may lack some of the normal counterregulatory responses that prevent hypoglycemia in healthy individuals (Fig. 3). Perhaps most importantly, patients receiving such treatments are unable to reduce the amount of circulating insulin when their blood sugar drops to below 80–85 mg/dl (4.4–4.7 mM). In addition, patients with type 1 diabetes lose their ability to secrete glucagon in response to hypoglycemia after 5 or more years of disease. As a result of the loss of hypoglycemia-induced glucagon secretion, patients with long-standing type 1 diabetes must rely on their hypoglycemia-induced catecholamine responses to detect a fall in their blood glucose and to attempt to restore euglycemia. However, many patients with diabetes and recurrent hypoglycemia may not experience hypoglycemia-induced catecholamine secretion and neurogenic symptoms until blood glucose has fallen below the level that causes neuroglycopenia and consequently develop the syndrome of hypoglycemia unawareness.

It is still uncertain which mechanisms are responsible for the pathogenesis of hypoglycemia unawareness. Some investigators have suggested that elevations in serum cortisol levels during and in response to hypoglycemia may somehow alter neuronal function so that the brain does not perceive additional reductions in glycemia. Other scientists

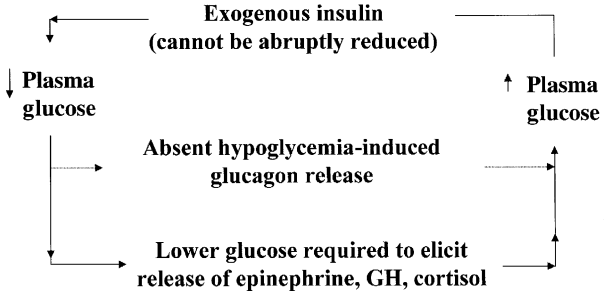
Exogenous insulin
(cannot be abruptly reduced)
↓ Plasma glucose
↑ Plasma glucose
Absent hypoglycemia-induced glucagon release
Lower glucose required to elicit release of epinephrine, GH, cortisol

FIGURE 3 Hormonal responses to hypoglycemia in long-standing type 1 diabetes. Recovery from hypoglycemia is hampered in patients with long-standing type 1 diabetes by the inability to reduce blood insulin concentrations and an altered counterregulatory response.

have made observations that suggest that recurrent hypoglycemia may up-regulate brain glucose transport across the blood–brain barrier. If this does occur, the brain may maintain normal rates of glucose delivery and become unaware of the reduction in glucose experienced by the rest of the body during hypoglycemia until plasma glucose levels drop so low that glucose transport becomes the factor limiting brain metabolism. Regardless of the mechanism, which will eventually be defined, recurrent episodes of hypoglycemia within a sufficient short period of time appear to be of key importance. Many laboratories have demonstrated that recurrent hypoglycemia raises the glucose threshold (that is, lowers the plasma glucose concentration) at which counterregulatory hormone responses to hypoglycemia are elicited. Even brief episodes of hypoglycemia on the order of minutes have been shown to alter the counterregulatory response to an episode of hypoglycemia that occurs days later. Interestingly, the threshold at which cognitive dysfunction occurs is not altered as much by recurrent hypoglycemia as is the hormonal response, so patients can manifest symptoms of neuroglycopenia before they develop the adrenergic symptoms that should prompt the immediate ingestion of carbohydrate. Consequently, patients with hypoglycemia unawareness often become dependent on the assistance of others to overcome episodes of severe hypoglycemia.

Patients who are at particular risk for hypoglycemia can be identified by certain clinical characteristics, such as intensive insulin management, previous episodes of hypoglycemia, and a low hemoglobin A1c. However, none of these characteristics are very sensitive or specific in predicting which patients are at greatest risk for severe hypoglycemia. Although educating patients about the importance of carefully adjusting insulin doses for changes in meals and exercise is certainly of benefit in helping some patients avoid low glucose values, many episodes of hypoglycemia occur without any clearly defined departure from the usual management plan. If patients experience enough recurrent hypoglycemia to develop the hypoglycemia unawareness syndrome, relaxing the intensity of the insulin management and strictly avoiding any episodes of low glucose values will restore hypoglycemia-induced catecholamine secretion after a few weeks. Such patients also regain their neurogenic symptoms of hypoglycemia and thereby restore their ability to recognize and treat low blood glucose values before the development of neuroglycopenia.

Patients with type 2 diabetes appear to be at lower risk than patients with type 1 diabetes for the development of hypoglycemia. Such patients, at least those in the early stages of the disease who maintain adequate beta-cell function, retain the capacity to reduce their own endogenous insulin secretion when blood glucose levels fall. Consequently, they may be able to abort an episode before it begins, just as healthy subjects are able to do in response to mild hypoglycemia. Hypoglycemia-induced glucagon secretion also remains active in patients with long-standing type 2 diabetes so they may be able to restore euglycemia through this mechanism before neurogenic symptoms develop. Finally, patients with type 2 diabetes often are not managed as intensively as patients with type 1 diabetes and their average blood sugar levels may never even approach a normal level of glycemia. As patients and their health care team begin to appreciate the importance of controlling glycemia in type 2 diabetes, the risk of hypoglycemia in these patients may rise.

## VI. CONCLUSION

Serum glucose values are maintained within in a narrow range by a careful balance between insulin and the hormones that oppose insulin's actions. To prevent hypoglycemia, the body has developed a complex and redundant system of responses that include a reduction in insulin secretion, an increase in the secretion of glucagon, epinephrine, growth hormone, and cortisol, and activation of the sympathetic nervous system. These responses are altered in patients with diabetes. The inability to protect oneself from hypoglycemia has a profound impact on the lives of patients with diabetes. Not only do hypoglycemia and its attendant symptoms interfere with daily activities, severe hypoglycemia can cause death from arrhythmias and accidents. Fear of hypoglycemia has been called the primary factor limiting the intensity with which diabetes management can be applied. The fear is more than trivial. Multiple studies have now demonstrated that the risk of developing the microvascular complications of diabetes, and probably the macrovasular complications of diabetes as well, can be reduced by achieving near normoglycemia. Therefore, fear of hypoglycemia prevents patients from working to normalize their blood sugar levels and places them at risk for developing all of the long-term complications of the disease.

## Glossary

**counterregulatory responses** Hormonal and neural responses to hypoglycemia that serve to restore blood glucose levels to the normal range.

**epinephrine** A catecholamine hormone released from the adrenal medulla in response to hypoglycemia.

**glucagon** A hormone secreted by the pancreatic alpha cell in response to hypoglycemia.

**hypoglycemia** A decrease in blood glucose concentration to below the normal level, which can generally be considered 65 mg/dl (3.6 mM).

**hypoglycemia unawareness syndrome** Occurs in some insulin-treated patients with diabetes who experience frequent bouts of hypoglycemia. Such patients are unable to mount a counterregulatory response to hypoglycemia or develop symptoms of hypoglycemia until blood glucose falls below the level at which neuroglycopenia occurs (generally below 40 mg/dl or 2.2 mM).

**insulin** A hormone secreted by the pancreatic beta cell in response to an increase in plasma glucose. It is used as a therapy for diabetes and can cause hypoglycemia.

**neurogenic symptoms** Occur as a result of hypoglycemia-induced epinephrine secretion and activation of the sympathetic nervous system and include rapid heart rate, tremulousness, and sweating.

**neuroglycopenic symptoms** Occur as a result of inadequate glucose delivery to the brain and include dizziness, confusion, seizures, and loss of consciousness.

## See Also the Following Articles

**Diabetes Type 1 • Diabetes Type 2 • Glucagon Action • Glucagon Processing • Glucagon Secretion, Regulation of • Insulin Actions • Insulin Processing • Insulin Resistance in PCOS • Insulin Secretion**

## Further Reading

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target cells, replacing tissue lost at menstruation (in human; only a few other species menstruate). This corresponds to the proliferative phase of the endometrial cycle. Estrogen also triggers the expression of progesterone receptors in the endometrium. Luteinizing hormone rises to a peak, and approximately 36 h later ovulation occurs. In the luteal (postovulatory) phase of the ovarian cycle, both progesterone and estrogen are produced by steroidogenic cells comprising the corpus luteum. Progesterone, rising to a maximum in the midluteal phase, binds to receptors, producing changes in endometrium that prepare it to accept an implanting blastocyst. This part of the endometrial cycle is known as the secretory phase.

In humans, up to this point, the endocrine patterns of conception and nonconception cycles are similar, though circulating luteal phase estrogen and progesterone levels are somewhat higher in implantation than nonimplantation cycles. However, the next stages are quite distinct. If no pregnancy has occurred, progesterone begins to decline and the corpus luteum collapses, leading to menstruation, in which over three-quarters of the endometrium is shed. Alternatively, if an embryo is present, the trophectodermal cells and their immediate progeny (trophoblast) produce the polypeptide hormone chorionic gonadotropin, and this binds to receptors in the corpus luteum, rescuing it and ensuring a continuing rise in progesterone and the survival of the endometrium. In other species, survival of the corpus luteum is achieved by means of different signaling pathways.

The endometrial cycle is less well understood, at least as regards the key requirements for implantation. Rat and mouse endometria permit implantation for only approximately 24 h at day 4–5 of pregnancy, a period known as the implantation window. It is clear that this is maternally controlled, because unimplanted blastocysts recovered by flushing and transferred to receptive mothers give normal pregnancies. The precise hormonal requirements for implantation have been defined by replacing embryos in ovariectomized animals under various regimes of exogenous steroid. The required sequence is estrogen (E) followed by progesterone (P) and then estrogen plus progesterone. The E/P-primed uterus supports the survival of blastocysts in its cavity for a considerable time as long as daily progesterone is administered (this is known as delayed implantation), and a single dose of E during P stimulation is an absolute requirement for their implantation. This corresponds to the nidatory estrogen pulse in natural cycles. The postreceptive uterus is hostile to unimplanted embryos, which degenerate.

In human, assisted reproduction programs have generated much deductive data on implantation rates and optimal timing. It appears that the endometrium is receptive for approximately 4 days, from 7 to 11 days after the luteinizing hormone peak. This may well end when corpus luteum rescue is no longer feasible; that is, it is probably not controlled from the uterus. Thus, the risk of early pregnancy loss increases with later implantation from 13% on day 9 after ovulation to 82% on day 12 or later. It remains unclear whether luteal-phase estrogen production is required for implantation in human. In artificial cycles in women with premature ovarian failure, precise replication of the natural pattern of steroid is not required; for example, the follicular phase can be extended, and constant daily doses of estrogen and progesterone are often used. Furthermore, estrogen followed by progesterone alone is sufficient to support pregnancy, suggesting that the nidatory estrogen required in rodents may not be an absolute requirement for human implantation. However, the human embryo expresses the enzyme aromatase and may therefore be capable of producing its own estrogen, which could act locally on maternal cells at the implantation site. Rather low rates of pregnancy are achieved in women after embryo replacement, however (rarely over 50% and more usually 25% even in younger women). The reasons are discussed below.

Histological changes in the endometrium indicate the tissue's response to ovarian hormones, with the production in glands of glycogen and glycoproteins destined for secretion evident as early as 3 days after the luteinizing hormone (LH) peak. Secretion reaches a maximum 7 days after the LH peak, and bulbous apical plasma membrane swellings known variously as pinopodes or uterodomes appear at the surface of endometrial epithelial cells, surviving for approximately 2 days, dependent on expression of the homeobox gene Hoxa-10. At about this time, the stroma begins to become edematous. However, endometria from as many as 30% of normally fertile women show histological features that vary from the normal midluteal-phase phenotype. Provided that embryo quality is high (see below), some variation in the differentiation state of the endometrium is compatible with implantation. Numerous molecular changes that correlate with the receptive phase have been reported, but there is little evidence in either human or other animal species to throw light on the molecular interactions that attach the implanting embryo to the endometrium. Indeed, this is one of the important unsolved problems of contemporary biology.

## III. THE IMPLANTING EMBRYO

The development of assisted reproductive technology (ART) has led to the widespread practice of oocyte recovery, *in vitro* fertilization (IVF), embryo culture, single-cell preimplantation diagnosis, and embryo transfer. As a result, it has become evident that embryo quality is a major determinant of pregnancy outcome.

For reasons that are not understood, humans produce a high proportion of karyotypically abnormal embryos. Thus, it is estimated that 32% of oocytes, 8% of sperm, and 37% of embryos are aneuploid. By the first trimester of pregnancy, 8–10% of surviving embryos are aneuploid, and by term, the figure is only 0.6%. These figures indicate that a substantial proportion of aneuploid embryos fail to implant, fail to rescue the corpus luteum, or at least fail to progress beyond the earliest stages of development. Some specific chromosomal abnormalities—the monosomies and trisomy 1 are examples—have never been observed in postimplantation stages. Other abnormal karyotypes form blastocysts and implant but do not survive beyond the first trimester. Still others, such as trisomy 21, can survive to term, though even this relatively common abnormality shows approximately 80% prenatal selection. Women who suffer recurrent miscarriage produce higher numbers of aneuploid embryos than normal controls, pointing to ovarian function as a major element in reproductive failure.

Other embryonic attributes are also of major significance in pregnancy outcome. A high proportion of embryos fertilized *in vitro* suffer arrested development. Embryos that do reach the blastocyst stage are variable in terms of the total number of cells, their allocation to inner cell mass and trophectoderm, and the rates of apoptosis in these two compartments. Light microscopic techniques are not available to distinguish cell number and viability in living embryos. Though a low rate of apoptosis appears to be a common feature, it is clear that high levels cannot be compatible with normal development.

There is great interest in the issue of how cell number and partitioning are controlled in the developing embryo. Culture techniques have improved with increased understanding of the metabolic changes that occur during early development, from the relative quiescence of the cleavage stages to the metabolically active blastocyst with its high rate of glucose uptake. Replacement of embryos at the blastocyst stage has become a more widespread approach as it also allows preimplantation genetic studies to be carried out after removal of one or two cells at the cleavage stages. A two-stage culture protocol has been adopted with richer nutrient provision for later stages of development. If culture is carried out continuously in lower-nutrient medium, some embryos do develop to blastocysts, but implantation rates are reduced. Just as with the karyotype, the metabolic characteristics of morphologically normal blastocysts vary greatly, providing yet another strong indication that the ability of an embryo to develop to this stage does not guarantee successful pregnancy.

These issues are not the exclusive preserve of IVF practitioners wishing to improve pregnancy rates. Defining the influence of the maternal reproductive tract environment on the embryo's preimplantation development is just as important for pregnancies conceived without laboratory assistance. Studies of women who were pregnant during the Dutch famine of 1945 have shown that inadequate food intake in early pregnancy leads to small offspring. When female mice are exposed to a low-protein diet before mating and during the first few days of pregnancy, they produce small blastocysts (that is, with fewer cells). Even if the mothers are transferred to a normal diet for the remainder of pregnancy, these small embryos give rise to undergrown pups. Small size at birth, in addition to being associated with elevated childhood morbidity and mortality, increases the risk of high blood pressure, heart disease, and diabetes in adult life.

Superovulation cycles, frequently used in both ART and laboratory animals, produce lower pregnancy rates in both human and mouse in the context of much higher estrogen and progesterone. Though this has been taken as evidence of endometrial compromise (for example, histological abnormalities have been reported), blastocysts from superovulated mice are developmentally delayed and produce fewer and smaller pups after transfer to normally cycling foster mothers. High levels of estrogen also compromise the development of human embryos.

## IV. THE ENDOMETRIUM AT IMPLANTATION

The classical model of estrogen acting on endometrial cells to stimulate progesterone receptor expression, with progesterone then inducing differentiation and preparation for implantation, is an oversimplification, because receptor expression varies in a complex fashion in different cell types and tissue compartments in the uterus. The basal endometrium differs from the superficial or functional layer, and this is particularly significant in humans because it is the former from which postmenstrual regeneration occurs. These differences arise at least in part because of site-specific paracrine interactions between the epithelium and the stroma in regulating endometrial function, as established most elegantly in studies of mice lacking estrogen receptors. Epithelial proliferation in response to estrogen does not occur in estrogen receptor-α (ER-α)-negative stroma recombined with wild-type epithelium, but occurs normally if the receptor is present in the stroma and not in the epithelium. However, the control of expression of epithelial secretory substances such as mouse lactoferrin relies on the presence of the receptor in both the stromal and the epithelial compartments. The same principles apply to human endometrium; for example, in the secretory phase, progesterone stimulates progesterone receptor (PR)-bearing stromal cells to produce transforming growth factor-β (TGF-β), and this in turn suppresses the production of matrix metalloproteinase 7 (MMP7) by the epithelium in a progesterone-independent step. At the end of the cycle, progesterone levels fall and MMP7 contributes to extracellular matrix (ECM) remodeling during menstruation.

Endometrium in both mouse and human expresses the estrogen receptor variant ER-β, though at lower levels than ER-α. Ablation of ER-β in mice compromises reproductive function, and current evidence suggests that it may act to modulate ER-α. Similarly, both progesterone receptor variants A and B, which arise by alternative splicing, are expressed and function as distinct transcription factors. PRB appears to have a role in epithelial proliferation.

Endometrial cells also express the androgen receptor (AR) and luteinizing hormone receptor (LHR). The AR can be expressed in stromal as well as epithelial cells but its functions in endometrial physiology have not been defined. Human chorionic gonadotropin (hCG) is a ligand for LHR, raising the possibility of a paracrine interaction with the embryo. Mice genetically altered to lack the LHR develop a very small uterus. When steroid replacement is undertaken, the uterus grows back to a normal size and histological features are fairly normal, though fewer glands are present. Embryos replaced into these mice, however, produce no pregnancies. At present, the explanation for this infertility is not clear. In human, there is evidence to suggest that hCG treatment in the luteal phase advances endometrial differentiation.

Glands are a feature of the endometrium in most species and are responsible for secreting substances that act either on the endometrium itself or on the embryo. This is discussed further below.

Angiogenesis occurs in endometrium during postmenstrual regeneration, and then the differentiation of spiral arteries occurs in the late secretory phase or during decidualization. These arteries are further modified during pregnancy to become wide, passive channels for the uninterrupted supply of large amounts of maternal blood to the placenta.

## V. THE EMBRYO–ENDOMETRIAL DIALOGUE

Growth factors secreted in response to estrogen and progesterone from both oviductal and endometrial epithelia contribute to the milieu of the developing embryo. Numerous factors, including insulin, leukemia inhibitory factor (LIF), and heparin-binding epidermal growth factor (HB-EGF) have been shown to increase either the rate of embryo development to blastocyst stage or the size of the blastocyst during culture *in vitro*. The complexity of biological fluids and the large range of factors that have been detected make it difficult to establish the contribution made by individual components *in vivo*, and detailed discussion lies beyond the scope of this article. However, studies in genetically modified mice are proving effective in overcoming this problem. Granulocyte/macrophage colony-stimulating factor (GM-CSF) is produced by the uterine epithelium. Secretion is stimulated by estradiol, but more strongly by TGF-$\beta_1$, which is abundant in seminal plasma, suggesting a role for male tract secretions in the preparation of the female tract for pregnancy. The embryo expresses a GM-CSF receptor, indicating the potential for a preimplantation interaction with maternal secretions. Indeed, mice genetically altered to lack GM-CSF produce blastocysts with fewer cells in the ICM, and these give rise to small pups. The most extreme phenotype is associated with the absence of GM-CSF from both the maternal and the embryonic genotypes, indicating that partial rescue is possible from either direction. The defect can be rescued by culturing null preimplantation embryos in medium supplemented with GM-CSF before replacement. Human embryos also contain GM-CSF receptors, indicating the possibility of a similar mechanism.

Delayed implantation is a phenomenon observed in a wide variety of animal species in which the embryo develops as far as the blastocyst stage, but then enters metabolic quiescence, sometimes for periods of several months. This is useful in seasonally breeding species; for example, in American black bears, ovulation occurs in June, with the blastocyst remaining in the uterine cavity until early December. Thus, quiescence is induced in the blastocyst for approximately 5 months. The dormant blastocyst is released by a process known as activation, so that implantation is timed to allow the offspring to maximize their growth before the arrival of the next winter season. There is little clear evidence that delayed implantation occurs in human, though sporadic reports of follicular phase implantation have prompted speculation to this effect.

Studies of delayed implantation in mouse are important for the light they have thrown on the subtle maternal–embryonic dialogue of early pregnancy (see Table 1). In the presence of progesterone, dormant blastocysts remain closely apposed to the uterine epithelium but do not attach. Estrogen treatment produces a receptive state in the uterus and also activates the embryo. However, the latter occurs by an indirect mechanism. Estradiol induces uterine receptivity, whereas the embryo is activated by a catecholestrogen, 4-hydroxyestradiol-17β, produced locally in the endometrium as a metabolite of estradiol.

Calcitonin is an endometrial gland cell secretory product expressed in response to progesterone just prior to implantation, and it is important for pregnancy outcome in rodents, as indicated by a reduction in implantation rates if maternal expression in rats is blocked using antisense oligonucleotide. Calcitonin acts on the preimplantation embryo by binding a G-protein-coupled receptor, triggering an increase in intracellular calcium, activation of adenyl cyclase, and expression of the fibronectin receptor, integrin α5β1. Binding to fibronectin in the maternal stroma is likely to be important in trophoblast invasion in the postimplantation phase.

The epidermal growth factor family is involved in the embryo–endometrial dialogue. This includes six related ligands that interact with four erbB receptor tyrosine kinases. The ligands amphiregulin, epiregulin, β-cellulin, neuregulin, and HB-EGF are all induced in the endometrial mouse lumenal epithelium in the preimplantation phases of pregnancy. HB-EGF appears in epithelial cells immediately adjacent to the implanting embryo just 6–7 h before the embryo attaches. In delayed implantation, HB-EGF appears only after the nidatory estrogen stimulus is given. HB-EGF has been suggested to play two different roles; in addition to accelerating embryo development, it has a membrane-bound variant that can mediate intercellular attachment by binding to erb-B4 and heparan sulfate proteoglycan, both of which are present on the blastocyst.

## VI. ATTACHMENT AND INVASION MECHANISMS

Before an embryo can attach to the uterine surface, it must hatch from the zona pellucida. This is an outer glycoprotein capsule that is present from the time the oocyte is ovulated. Two serine proteases, ISP1 and ISP2 (implantation serine proteases 1 and 2), have been implicated in hatching. ISP1 is produced by the embryo, whereas ISP2 is produced by uterine glands. These enzymes may also be required for local proteolysis of maternal tissue as part of the process of invasion.

The maternal surface is specifically nonreceptive except during the window phase. Like other epithelial surfaces, it contains an apical glycocalyx

**TABLE 1 Molecular Pathways Identified in Mouse Implantation**

| Stage of implantation | Molecular signals |
|---|---|
| Embryo development or activation (see text) | Catecholestrogen, calcitonin, EGF family ligands? LIF, GM-CSF |
| Barrier withdrawal | Loss of Muc1 (and Muc4 in rats) |
| Apposition | Cell surface glycans? HB-EGF? |
| Attachment | Unknown |
| Decidual stimulus | LIF, prostaglandin (PG12), EP3, EP4, PPARδ, Hoxa-10, Hoxa-11, Hmx3, IL11R |
| Epithelial apoptosis | Fas? |
| Early trophoblast invasion | β1 integrins, maternal ECM, MMPs |

(thick glycoprotein coat) that allows the diffusion of small molecules but inhibits cell adhesion. In this way, it acts as a component of the innate immune system, protecting the upper female tract from infectious agents. The cell surface mucin MUC1 is a component of the uterine glycocalyx. In mice and rats, Muc1 is down-regulated precisely at the time of implantation under the control of maternal steroids. There is evidence that another mucin, Muc4, is also down-regulated in rodents. In contrast, humans express high levels of MUC1 throughout the receptive phase. Experimental studies of human implantation in which embryos hatch and attach to endometrial epithelial cells in monolayer culture have shown that MUC1 disappears from a small area of cells that surround the attached embryo (Fig. 1). Maternal cells farther away continue to express the glycoprotein, thus presumably maintaining their barrier property and minimizing the access of pathogens to the underlying tissue and vasculature. The mechanism by which MUC1 disappears has not been established, but the phenomenon indicates that there is a highly localized dialogue that occurs between embryonic and maternal cells.

Apposition of the embryo to the epithelium and intercellular attachment between the trophectoderm and the uterine epithelial cells then occur. Though several adhesion molecules have been identified at these two surfaces, and it is likely that a cascade of adhesion events is required, the molecular details have not been worked out. In species including farm animals, the embryo attaches to the uterine epithelial surface and remains in the uterine cavity. In contrast, mouse and human have interstitial implantation, in which the embryo crosses the epithelium, and the placenta, once developed, makes direct contact with maternal blood (hemochorial). In mice, attachment to the maternal epithelial surface is followed by apoptosis of the epithelial cells, thus allowing the trophoblast direct access to the underlying basement membrane and stroma. In human, it is thought that intrusive penetration of the epithelium is followed by its displacement, but there is also evidence for apoptosis triggered by interaction between the Fas ligand on the trophectoderm and Fas on the uterine lumenal epithelium.

Binding to the extracellular matrix in the maternal stroma is important at the next stage; the trophoblast expresses integrins, cell surface adhesion molecules that act as receptors for ECM ligands. Mouse embryos genetically modified to lack integrin β1, a polypeptide subunit that dimerizes with a family of α chains to produce a wide repertoire of ECM

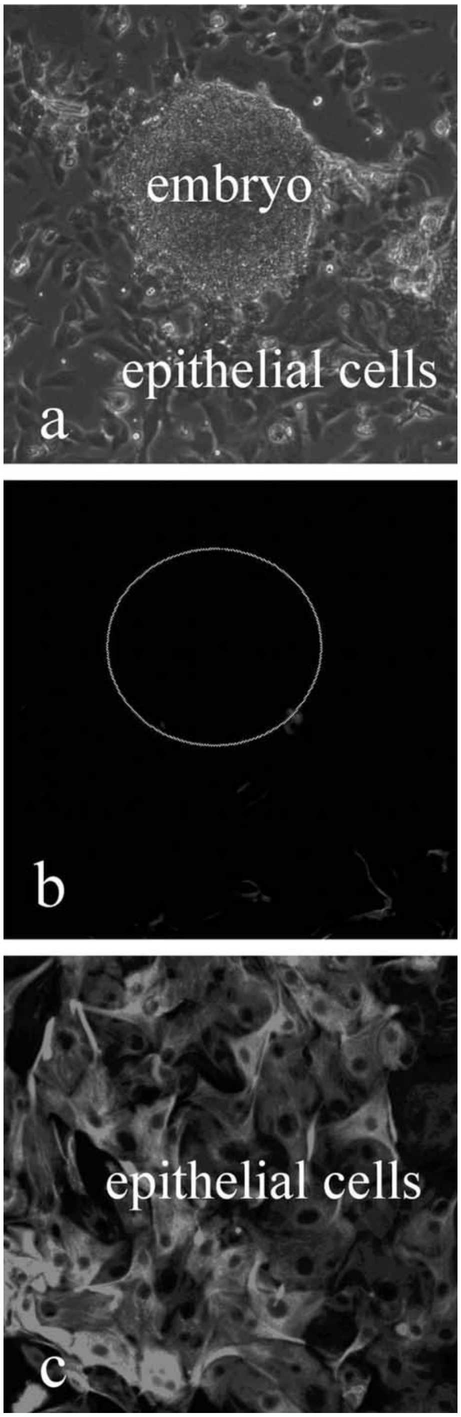

**FIGURE 1** (a) Human embryo attached to a cultured endometrial epithelial monolayer with (b) MUC1 imunolocalization in the same field. (c) MUC1 localization in the same culture but distal to the site of embryo attachment. Clearance of MUC1 from epithelial cells in the vicinity of the embryo is evident. Reproduced with permission from Aplin *et al.* (2001) *Biochem. Soc. Trans.* **29**, 153–156, © the Biochemical Society.

receptors, fail to complete implantation, probably because they lack the ability to bind the maternal ECM. Local tissue remodeling is also necessary, and a balance between matrix metalloproteinase activity and its inhibition is established. If disturbed by experimental elevation of TIMP activity at the implantation site, morphological abnormalities of placentation result, though the effects on pregnancy outcome have not been tracked.

## VII. DECIDUALIZATION: THE POSTIMPLANTATION ENDOMETRIUM

Decidualization is a change that occurs in the endometrial stroma of pregnancy in which the resident fibroblasts enlarge, extend processes to contact neighboring cells, acquire an increased cytoplasmic-to-nuclear ratio, and produce a distinctive range of secretions. Glandular epithelial cells become hypotrophic. The extracellular matrix undergoes remodeling, with the loss of bundles of fibrillar collagen and (in human) the appearance of capsular basement membrane around the decidualizing stromal cells. The proportion of immune cells increases; after decidualization, as many as 35% of the cells present are derived from bone marrow, and these comprise predominantly macrophages and an unusual type of large granulated lymphocyte with some natural killer cell-like features. There are also a few T cells and mast cells. Steroid hormones govern the abundance and phenotype of immune cells by regulating local cytokine and chemokine synthesis. Tolerance is required in the maternal immune system to paternal antigen in the embryo, but it must continue to provide resistance to pathogens. Several mechanisms operate in parallel to achieve these aims, but their description lies outside the scope of this article.

By and large, stromal decidualization occurs only in species that undergo interstitial implantation, and the timing and extent of the changes are species-dependent too. In human, decidual changes are evident in the late secretory phase of nonpregnant cycles, and under chronic contraceptive progesterone stimulation, the endometrium becomes fully decidualized. Thus, decidualization can occur independently of embryonic signals. Embryos implant into an undecidualized stroma, with decidualization occurring during the following few days. In contrast, in the mouse and rat, attachment of the embryo to the anti-mesometrial uterine lumenal epithelium initiates a signaling process in steroidally sensitized endometrium that triggers decidualization. Similar changes (known as deciduomata) can be elicited in hormonally sensitized mice, by a mechanical insult to the uterine lumen or by instillation of an irritant such as oil. During days 5–8 of pregnancy, decidual changes spread from the subepithelial anti-mesometrial stroma into deeper anti-mesometrial regions and then spread laterally, eventually including the mesometrial stroma. The anti-mesometrial and mesometrial decidual cells have distinct secretory repertoires.

It is likely that the early decidual changes create a maternal environment in which paracrine support is provided for the conceptus by local secretory activity, before a hemochorial placenta is established with the capacity to extract nutrients directly from maternal blood. Several maternal decidual secretory components—prolactin (PRL) and TGF-β are examples—are found in amniotic fluid. Much remains to be done to define the precise function of decidua in relation to the developing conceptus. However, genetic manipulation of mice has given useful insights into the signaling pathways through which decidualization is mediated, as well as providing clear evidence that decidual differentiation in early pregnancy is a critical requirement for successful outcome (Table 1).

Cox-2 is one of two cyclooxygenases that catalyze the rate-limiting step in prostaglandin production. The uterine stroma does not decidualize in mice lacking Cox-2, and implantation fails. This phenotype appears to result from lack of prostacyclin, the effects of which are mediated by activation of the nuclear hormone receptor peroxisome proliferator-activated receptor-δ (PPAR-δ).

Hoxa-10 and Hoxa-11 are adjacent homeobox genes that are expressed in the developing genitourinary tract and induced by progesterone in the adult uterus during early pregnancy. Defective implantation and decidualization occur in the absence of either maternal Hoxa-10 or Hoxa-11. Hoxa-10 expression is required for the development of epithelial pinopodes, suggesting an early role in the implantation cascade. Hoxa-11 expression is normally regulated by progesterone in Hoxa-10 null mice. Steroid-dependent stromal cell proliferation is reduced in Hoxa-10 knockouts, with a lower level of cyclin D3 in stroma 1 day after the initiation of decidualization. Cox-2 induction in stroma is significantly reduced in Hoxa-10 null mice; however, decidualization cannot be restored by prostaglandin treatment, probably because two prostaglandin receptors, EP3 and EP4, are aberrantly expressed and inappropriately regulated by progesterone in the stroma. This suggests that

Hoxa-10 mediates steroidal regulation of EP3 and EP4. However, expression of several other progesterone-regulated gene products is identical in wild-type and Hoxa-10 mutant mice.

Ablation of expression from another homeobox gene, Hmx3, results in the failure of decidualization and infertility, despite normal ovulation and fertilization. The major site of expression of Hmx3 is in myometrium, where localization is largely anti-mesometrial on day 4.5 of pregnancy, immediately before the onset of decidualization in the overlying anti-mesometrial stroma. These data raise interesting speculation about the possibility of cross talk between the myometrium and the endometrium in regulating differentiation.

Uterine cells in LIF null mice do not decidualize, either in pregnancy or in response to artificial stimuli applied in pseudo-pregnancy, and the females are infertile. Thus, wild-type embryos transferred to LIF null females fail to implant, and LIF null blastocysts transferred to wild-type females progress to term and produce −/− adults. LIF up-regulation in glandular epithelium is abolished in Hmx3 and Hoxa-11 knockout mice, indicating that LIF is downstream of these two gene products. Expression of LIF, in contrast, is unaffected in Hoxa-10 nulls. The LIF and prostaglandin pathways converge in the initiation of decidual changes in stromal cells.

IL-11 is important at a slightly later point in mouse decidualization. Inactivation of the gene encoding the unique $\alpha$ chain of the interleukin-11 (IL-11) receptor produces a phenotype in which the extent of decidualization is much reduced. At day 4.5 postcoitus, IL-11 is expressed in the primary decidual zone (the anti-mesometrial stromal region beneath the site of attachment) and the IL-11R$\alpha$ is in the surrounding deeper stroma that later becomes secondary decidua. Both in pregnancy and in deciduoma, the null endometrium differentiates to form primary decidua but rather little secondary decidua, implicating IL-11 in the signaling pathway that amplifies and regulates the spatiotemporal expression of the decidual phenotype. As a result, hemorrhage occurs into the uterine lumen at day 6 and embryos are necrotic by day 9. However, the secondary trophoblast giant cell population expands to occupy the area that normally contains the mesometrial decidua. Trophoblast overgrowth in the absence of secondary decidua provides support for the hypothesis that the decidua plays some role in the restriction of trophoblast invasion.

Human endometrium contains Hoxa-10 transcript, IL-11, LIF, PRL, and prostaglandins but it is important in comparing the two species to bear in mind that although both require progesterone priming for decidualization, in the mouse it is initiated by a signal transmitted from the blastocyst to the uterine epithelium and then into the superficial anti-mesometrial stroma, whereas in human, decidualization occurs throughout the endometrium even in the absence of a conceptus. Thus, mechanisms for spatial and temporal regulation may be species-specific.

## VIII. CONCLUSION

Is the improvement of implantation rates a suitable goal for medical research?

Miscarriage imposes a less severe biological burden than the evasion of natural selection by a fraction of abnormal embryos. High rates of early pregnancy failure in human reflect the high rate of embryo abnormality in our species and the fact that the optimal time period in which to select a healthy conceptus is the preimplantation and early postimplantation phases.

The quality of the preimplantation embryo is critical for optimal fetal development and the health of offspring. In mice, blastocysts that have larger numbers of cells go on to produce larger pups. Epidemiological evidence shows that low birth weight is a risk factor for high blood pressure (in rats and humans) and heart disease and diabetes (in humans). Facilitating the implantation of poor-quality embryos would be counterproductive if sustained good health into old age is an agreed aim. On the other hand, with high-quality embryos, pregnancy is possible in both infertile and postmenopausal women. This is not to argue that the uterus is a passive vehicle; instead, it is an active participant in a two-way molecular dialogue with the embryo, and this process itself contributes to embryo selection. Both uterine receptivity and embryo quality are influenced by locally produced growth factors and cytokines, and these in turn depend on cyclic alterations in steroid hormone production by the ovary. Improved endometrial receptivity can become a reasonable therapeutic objective when defective receptivity can be unequivocally specified as the cause of infertility.

## Glossary

**blastocyst** Stage of embryo development at which implantation occurs.

**decidua** Endometrium of pregnancy.

**endometrium** Mucosal lining of the uterus.

**estrogen** One of the two major ovarian steroid hormones upon which implantation depends.

**implantation** Establishment of a stable tissue interface between the maternal endometrium and the embryo.

**implantation window** A restricted period of time during which the endometrium permits implantation of a competent blastocyst.

**progesterone** One of the two major ovarian steroid hormones upon which implantation depends.

## See Also the Following Articles

**Calcitonin • Decidualization • Endometrial Remodeling • Endometriosis • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • *In Vitro* Fertilization • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Ovulation • Progesterone Action in the Female Reproductive Tract**

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# Inhibin Receptor Signaling

Daniel J. Bernard

*Center for Biomedical Research, Population Council and Rockefeller University, New York*

I. INTRODUCTION
II. STRUCTURE OF THE INHIBINS
III. ACTIVIN SIGNALING MECHANISMS
IV. INHIBIN ANTAGONISM OF ACTIVIN SIGNALING
V. ALTERNATE MECHANISMS OF INHIBIN ACTION
VI. EXTRAPITUITARY ROLES OF THE INHIBINS
VII. SUMMARY

**Inhibins are dimeric endocrine hormones produced by the gonads that suppress follicle-stimulating hormone (FSH) production and release by the anterior pituitary gland. Activins are closely related proteins that stimulate pituitary FSH. Whereas the activins are known to bind a serine/threonine kinase receptor complex and to signal through intracellular proteins in the Smad family of transcription factors, the mechanisms of inhibin action are less well understood. Recent data indicate that the inhibins can bind with high affinity to activin receptors in the presence of a co-receptor protein, betaglycan. Thus, inhibins appear to act, at least in part, by blocking activin binding and thereby disrupting downstream signal transduction events.**

## I. INTRODUCTION

Inhibins are endocrine hormones produced in the gonads that regulate the synthesis and secretion of pituitary follicle-stimulating hormone. Two forms of inhibin, inhibin A and B, are produced and secreted by granulosa cells of ovarian follicles in female mammals. Inhibin B is also produced by testicular Sertoli cells and is the major circulating form of inhibin in adult males.

Although some data indicate that inhibins are synthesized within the adrenal gland, castration

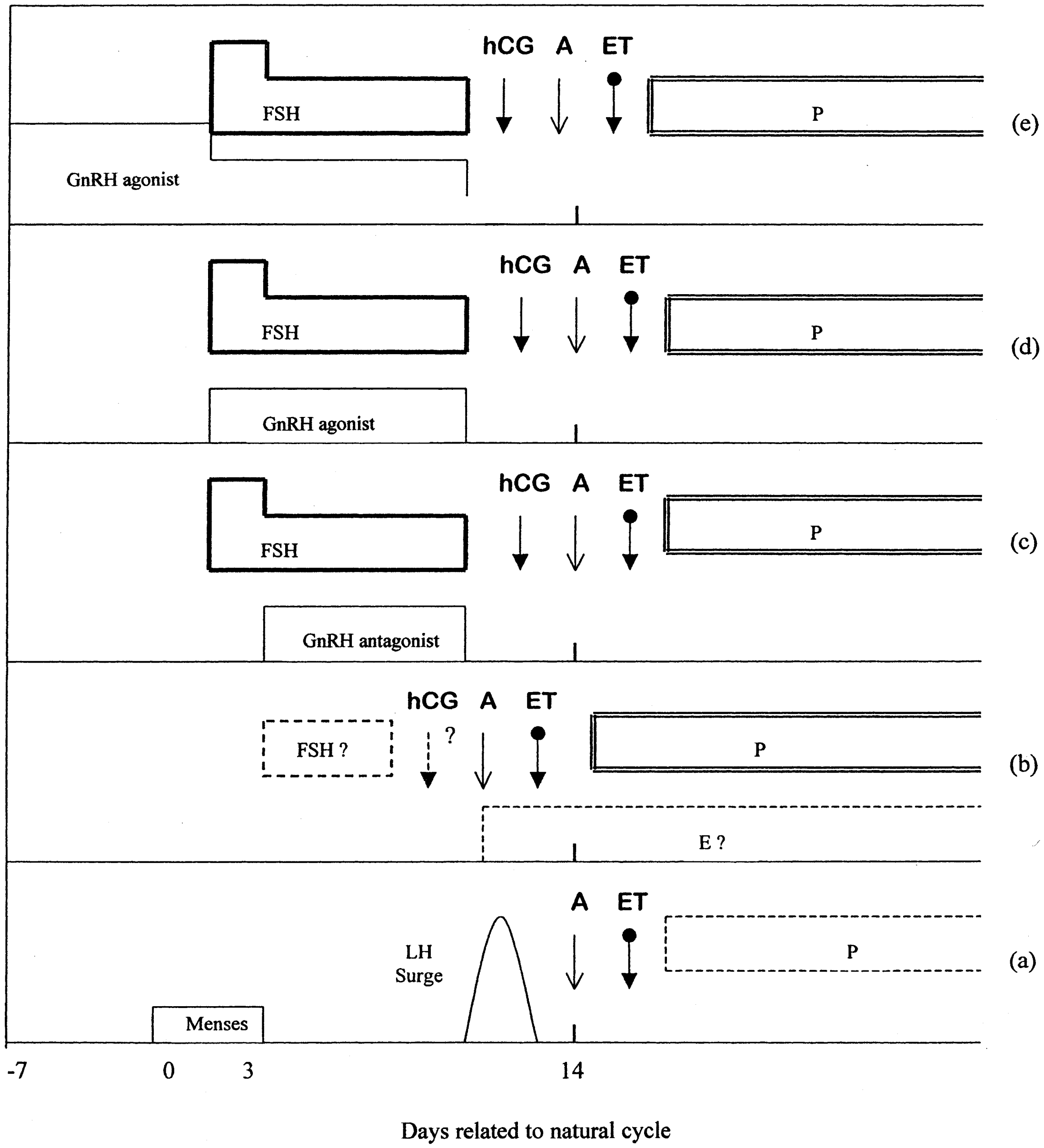

FIGURE 1 Five protocols for recovering oocytes for IVF and embryo transfer (ET): (a) the natural cycle requires the spontaneous LH surge to be detected before the single dominant follicle is aspirated (A); (b) *in vitro* maturation of oocytes (IVM) is based on a natural cycle, but may include mild gonadotropin stimulation and supplementation with estrogen (E) and progesterone (P); (c) GnRH antagonists and GnRH agonists using either the short protocol (d) or the long protocol (e) provide control over endogenous gonadotropins while exogenous FSH and hCG/LH are being administered.

mediators, such as insulin-like growth factor-I. Serum concentrations of FSH control the number of follicles surviving and are regulated by feedback from follicular estrogen and inhibin. FSH also promotes estrogen production by stimulating aromatase activity in granulosa cells, with androgen substrates being produced in the follicular theca under the influence LH ("two-cell theory"). Since follicle rupture occurs approximately 28 h after a natural LH surge or 36 h after administration of a luteinizing agent [LH or human chorionic gonadotropin (hCG)], oocyte collection must be timely; otherwise the oocyte and its cumulus cell envelope may be lost.

## III. CONTROLLED OVARIAN STIMULATION

The use of drugs to induce multiple follicle development for IVF was pioneered in several centers, notably at Monash University in Australia and the Jones Institute in Virginia. Initially, clomiphene citrate was used to raise endogenous FSH levels by alleviating hypothalamic feedback suppression. This selective estrogen receptor modulator was replaced by injections of human menopausal gonadotropin, a semipurified urinary preparation containing approximately equal proportions of FSH and LH. When the additional follicles have grown to full size, they undergo preovulatory maturation in response to LH/hCG. In young ovaries, a crop of 8–10 follicles is normally generated, although patients with low follicular reserves respond as well to gonadotropins. Women with polycystic ovaries produce exaggerated responses, requiring careful monitoring and protocols with mild or no stimulation (Fig. 1b). Hormonal stimulation has undergone a technical evolution from the introduction of impure urinary FSH preparations to highly purified preparations to recombinant gonadotropins, and a meta-analysis confirmed the overall advantages of purified FSH.

## IV. HYPOTHALAMIC DOWN-REGULATION

One of the drawbacks of superstimulation is that raised serum estradiol secretion can trigger a premature surge of pituitary LH and preovulatory follicular changes. As a consequence, treatment cycles may have to be cancelled, fewer oocytes are recovered, and pregnancy rates are lower. This problem has been overcome by suppressing the pituitary gland using long-acting gonadotropic hormone-releasing hormone analogues (GnRHa), and three types of protocol have now emerged (Figs. 1c–1e). The first GnRHa compounds that became available clinically were superactive agonists, which produce a “flare” release of LH and FSH before down-regulating the GnRH receptor and causing hypogonadotropism. In the long protocol, therapy is initiated in the luteal phase of the preceding cycle, and this has become the standard treatment (Fig. 1e). In the short protocol, treatment begins on day 2 or 3 of the cycle, around the time when FSH injections begin (Fig. 1d). Recently introduced GnRH antagonists lack serious allergic side effects and produce immediate inhibition of LH and FSH release. Treatment with antagonists can be shorter and avoid triggering hot flashes and, because the pituitary gland is less profoundly suppressed, less FSH may need to be administered. GnRH antagonists may eventually replace agonists in this role, although pregnancy rates have not improved.

## V. COMPLICATIONS OF OVULATION INDUCTION

The hypothalamic–pituitary–ovarian axis has evolved feedback control mechanisms to prevent polyovulation and multiple pregnancy, which is risky for both mother and child. Administration of exogenous FSH bypasses these safeguards and carries the serious additional risk of ovarian hyperstimulation in which increased vascular permeability associated with multiple corpora lutea and excessive vascular endothelial growth factor causes an accumulation of ascitic fluid within the peritoneal cavity and, later, in the pleural cavity. Since this accumulation occurs at the expense of the intravascular space, there is a tendency toward hypercoagulability, thrombosis, oliguria, decreased organ perfusion, and alterations in electrolytes that can cause fatal cardiac arrhythmias. Another adverse side effect of ovulation induction and ovarian enlargement is the risk of ovarian torsion, possibly requiring surgical intervention and even the need to remove a gangrenous ovary.

## VI. OOCYTE AND EMBRYO QUALITY

The oocyte is often the prime biological factor limiting IVF success. Oocytes and embryos have remarkably high incidences of chromosome abnormalities, including aneuploidy, polyploidy, mosaicism, and chaotic karyotypes. What is more, the frequency of aneuploidy rises with maternal age. The etiologies of these defects are not well understood, and it is doubtful whether changes in hormonal stimulation or embryo culture conditions will radically improve cellular defects originating during gametogenesis. Identifying euploid embryos with high implantation potential is a priority for the aim of single embryo transfer to be realized and for multiple pregnancy to be avoided. Preimplantation genetic diagnosis using fluorescence *in situ* hybridization for screening embryos after blastomere biopsy is becoming more widely used (Fig. 2). Another strategy is to prolong culture so that vigorous embryos with higher survival and implantation rates are self-selected.

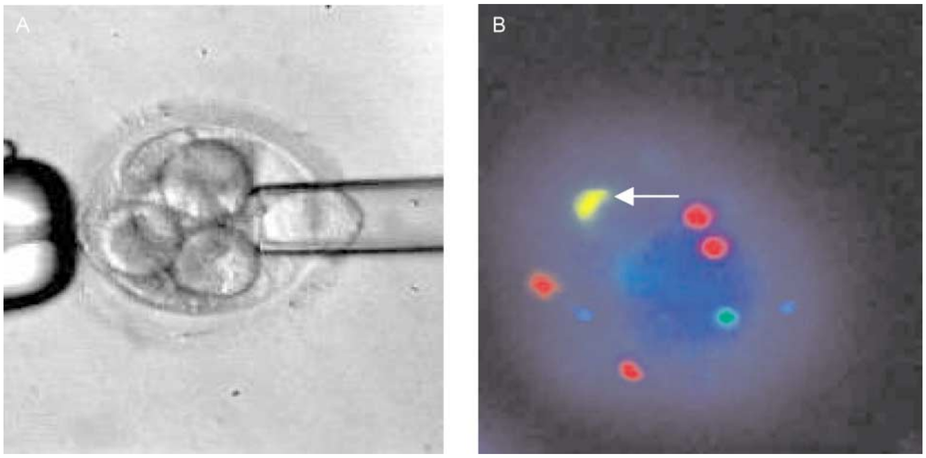

**FIGURE 2** (A) A seven-cell human embryo, laser hatched using the Zona Laser Treatment System (Hamilton Thorne Research, Beverly, MA), demonstrating a biopsy of a nucleated blastomere. (B) Fluorescence photomicrograph of a nucleus from human blastomere (counterstained with DAPI). The nucleus has been evaluated by fluorescence *in situ* hybridization using alpha-satellite DNA-specific probes for chromosomes 13/21, 18, X, and Y (arrow).

## VII. THE FUTURE

Assisted reproductive technology has expanded enormously in the past two decades and the year 2002 is reckoned to mark the birth of the millionth baby conceived by IVF. But pregnancy success rates have risen gradually, and the procedure remains costly and complex and carries some risks. Research is addressing all of these issues. After the recent introduction of recombinant FSH and LH, it is likely that long-acting gonadotropins will emerge from advances in molecular engineering. These agents will not require being administered as frequently and may, in turn, be succeeded by orally active molecules, which could completely eliminate the need for injections.

These innovations are unlikely to radically improve the control over multifollicular development and avoid ovarian hyperstimulation syndrome. However, women with polycystic ovaries, and perhaps others, could benefit from a procedure that is routinely used in animal breeding—*in vitro* maturation (IVM) of oocytes. Oocytes recovered early in the cycle at the germinal vesicle stage undergo spontaneous maturation and can be fertilized *in vitro*. IVM is being tested in clinical trials and has resulted in the birth of several dozen healthy babies. There is no standard protocol, and both unstimulated and hormonally primed cycles have been tested (Fig. 1b). Before the advent of clinical IVF and controlled hormonal stimulation, infertility pioneers envisaged that IVM might be necessary to generate oocytes for IVF, and this procedure could well play a larger role in the future if pregnancy rates are satisfactory.

Advances in cell biology are more likely than in endocrinology to underpin improvements in the quality of embryos so that fewer will need to be transferred and the multiple pregnancy rate can fall. Cytogenetic screens and biomarkers of embryo viability will become more important in the future, but it is less certain whether it will be possible, or safe, to rescue poor-quality oocytes or engineer them from somatic cells. The answers will emerge from ongoing studies of cytoplasmic transfer from donor eggs and induction of haploidy in somatic cell nuclei. Reproductive aging has become a key issue for many women who are deferring child-bearing until the less fertile years of life. Technology may enable them to elect to cryopreserve their immature oocytes as an insurance against infertility or premature menopause. Whatever happens, it is certain that the revolutionary conquest of infertility is continuing and will further increase individual freedom to control fertility.

## Glossary

**dominant follicle** The ovulable follicle emerging during the menstrual cycle as a result of stimulation by follicle-stimulating hormone.

**follicle stimulating hormone** A pituitary polypeptide hormone that acts on granulosa cells to promote follicle survival and estrogen production.

**gonadotropic hormone** Polypeptide hormones that stimulate gonadal function.

**gonadotropin-releasing hormone analogue** A synthetic peptide molecule that binds to the gonadotropin hormone-releasing hormone receptor and possesses either antagonist or agonist activity.

**intracytoplasmic sperm injection** Fertilization of an oocyte by direct injection of a sperm into its cytoplasm.

**luteinizing hormone** A pituitary polypeptide hormone that promotes ovarian steroidogenesis and triggers ovulation.

**oocyte retrieval** A procedure for recovering oocytes by follicular aspiration using a needle guided by transvaginal ultrasound.

**ovarian hyperstimulation syndrome** A potentially life-threatening condition that is triggered by superovulation treatment of women with polycystic ovaries.

**polycystic ovary** An ovary containing numerous antral follicles, which is associated with a clinical profile varying from regular ovulatory cycles to anovulation with virilization (polycystic ovarian syndrome).

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Folliculogenesis • Gonadotropin-Releasing Hormone (GnRH) • Implantation • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Ovulation • Polycystic Ovary Syndrome (PCOS) • SERMs (Selective Estrogen Receptor Modulators)**

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# Iodine: Symporter and Oxidation, Thyroid Hormone Biosynthesis

KRISTA M.D. LA PERLE AND SISSY M. JHIANG
*The Ohio State University*

I. INTRODUCTION
II. SODIUM/IODIDE SYMPORTER: BASOLATERAL IODIDE UPTAKE
III. PENDRIN: APICAL IODIDE TRANSPORT
IV. THYROGLOBULIN: PROHORMONE
V. THYROID PEROXIDASE: IODINATION AND COUPLING
VI. ENDOCYTOSIS, PROTEOLYSIS, AND HORMONE RELEASE
VII. THYROID-STIMULATING HORMONE AND OTHER REGULATORY FACTORS

**The thyroid hormones T3 and T4 are assembled in the thyroid follicular lumen through iodination of tyrosine residues within thyroglobulin by thyroid peroxidase and hydrogen peroxide. Iodide, an essential component of thyroid hormone, is delivered to the follicular lumen through the sequential actions of the sodium/iodide symporter at the thyrocyte–blood interface and the apical transporter pendrin. Numerous factors, either stimulators such as thyroid-stimulating hormone or inhibitors such as follicular thyroglobulin, regulate hormone biosynthesis and release. Defects occurring in any of these key molecules significantly**

removes all detectable forms of inhibins. Thus, the gonads appear to be the primary source of circulating inhibins, at least under normal physiologic conditions.

In women, inhibins A and B are differentially regulated throughout the menstrual cycle (Fig. 1). During the luteal to follicular phase transition, follicle-stimulating hormone (FSH) secretion increases. This FSH signal is critical for recruitment of ovarian follicles that may ovulate during a subsequent cycle. Developing follicles produce inhibin B and estradiol, which feed back to the gonadotrope cells of the anterior pituitary to suppress FSH release. Whereas inhibin B levels are elevated during the follicular phase, inhibin A levels are low and increase just prior to the midcycle surges of FSH and luteinizing hormone (LH). Both inhibin A and B levels increase in association with the primary gonadotropin surges. Thereafter, inhibin B levels decline, but inhibin A levels remain elevated throughout much of the luteal phase. Increased secretion of inhibin A and progesterone suppresses FSH secretion during this stage of the cycle. In the absence of pregnancy, the corpus luteum degenerates, and inhibin A and progesterone levels decline. Circulating FSH levels begin to increase in the absence of these potent negative feedback signals and the cycle repeats as a new follicular phase commences.

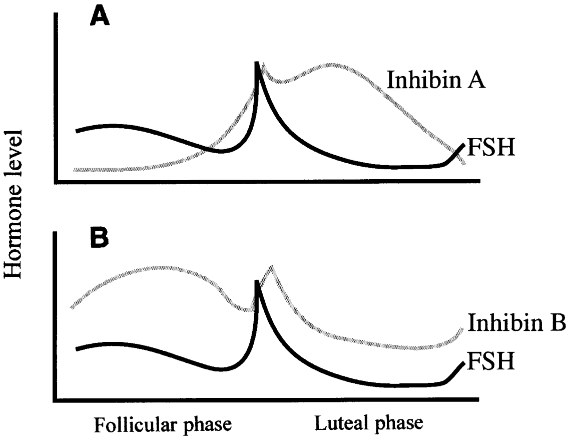

**FIGURE 1** Schematic representation of circulating inhibin A (A), inhibin B (B), and FSH levels across the human menstrual cycle. FSH levels are shown in both plots as a solid black line. Inhibin levels are in gray. Inhibin A is elevated during the luteal phase and, along with progesterone, suppresses FSH secretion during this stage of the cycle. Inhibin B levels are produced by developing ovarian follicles in response to FSH stimulation and are elevated during the follicular phase. The secondary "surge" of FSH observed during this stage of the cycle is suppressed by inhibin B and estradiol.

Similar to the situation in women, female rats exhibit differential patterns of inhibin A and B secretion throughout their reproductive cycles. During the first 2 days of the 4-day estrous cycle (metestrus and diestrus), inhibin B levels are elevated and FSH levels are low. Inhibin B levels begin to decline the following day on proestrus, prior to primary gonadotropin surges (which occur on the afternoon of proestrus). Inhibin A levels are low on metestrus and increase gradually until their peak on the afternoon of proestrus. Both inhibin A and inhibin B are low on the morning of estrus, allowing a secondary surge of FSH to occur. This selective increase in FSH is necessary for recruitment of ovarian follicles that may subsequently ovulate and is analogous to the follicular-phase FSH rise observed in humans.

In adult men, inhibin B appears to be the most potent endocrine regulator of FSH secretion. Indeed, FSH and inhibin B levels are negatively correlated and FSH stimulates inhibin B production. Sertoli cells are thought to be the main source of circulating inhibin B, and germ cells are clearly involved in inhibin B regulation. Sperm count and inhibin B levels are positively correlated and men with spermatogenic defects show diminished inhibin B secretion in response to elevated FSH.

## II. STRUCTURE OF THE INHIBINS

Inhibins are members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors. They are produced through the dimeric assembly of an α-subunit (inhibin α) with one of two β-subunits (inhibin $\beta_A$ or inhibin $\beta_B$) (Fig. 2). Dimers of α-$\beta_A$ and α-$\beta_B$ form inhibin A and inhibin B, respectively. Inhibin α is produced as a pre-prohormone (Fig. 2). In humans, the carboxy-terminal 134 amino acids of the 366-amino-acid precursor constitute the "mature" α-subunit (αC). There are three potential N-linked glycosylation sites within the α-subunit, one in the pro region (N146) and two in the mature domain (N268 and N302), and biologically active inhibins appear to be mono- or diglycosylated. The α-subunit can be secreted in multiple forms both alone and in association with β-subunits. In fact, free α-subunit isoforms (e.g., pro-αC and pro-αN-αC) appear to be secreted in excess of inhibin dimers but are biologically inactive. Biologically active forms of inhibin are composed of the mature region of inhibin α disulfide linked to the mature region of one of the two β-subunits (see below and Fig. 2).

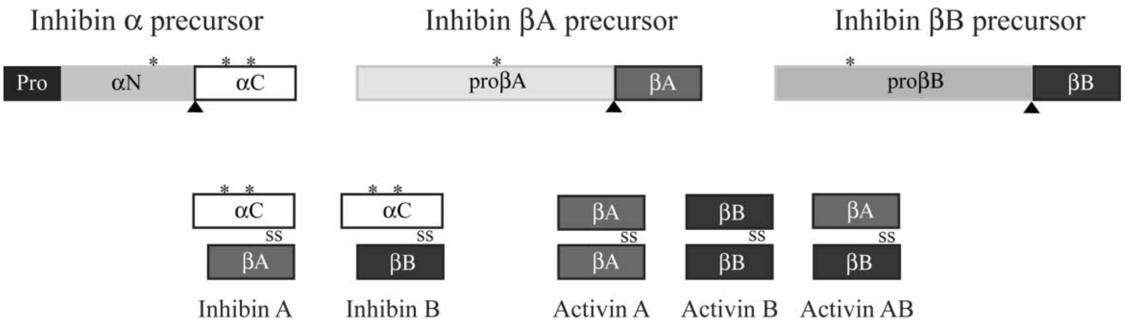

**FIGURE 2** Structure of inhibins and activins. Inhibins and activins are dimeric proteins produced through the assembly of subunit proteins. Inhibin A and inhibin B are produced through the disulfide bonding of the mature region of the inhibin α-subunit (αC) with the mature regions of the inhibin βA- and inhibin βB-subunits, respectively. The inhibin β-subunits are produced as prohormones. The mature regions are cleaved from the carboxy-termini of the precursor proteins (shown at the top). Activins A, B, and AB are produced through dimerization of the mature regions of the β-subunits. The pro regions of the precursors appear to be critical for dimerization but must be cleaved for biological activity of the hormones. Arrowheads indicate where the mature domains are cleaved from the pro regions. Asterisks indicate putative N-linked glycosylation sites. Disulfide bonds are indicated by "ss." Note that other (intermediate) dimeric forms of the inhibins and activins, which include the pro and/or αN domains, are also produced but are not pictured here.

The β-subunits share high sequence similarity with one another but are transcribed from different genes. Both β-subunit proteins are produced as prohormones and are proteolytically cleaved to produce the mature β-subunits (Fig. 2). As described for the α-subunit, the C-terminal domains of the β-subunits (115 or 116 amino acids) represent the mature parts of the proteins and cleavage of the pro region is necessary for biological activity. Both β-subunits can be glycosylated in the pro regions, but not in the mature regions, of the proteins.

The mature β-subunits possess the nine conserved cysteines characteristic of ligands within the TGF-β superfamily. Eight of the cysteines form four intrachain disulfide bonds and the ninth cysteine is involved in dimerization, forming an interchain disulfide bond. The mature α-subunit contains seven cysteines, six of which likely form three intrachain disulfide bonds, with the seventh involved in dimerization. The crystal structures of the activins and inhibins have not yet been solved, but based on structures of other superfamily ligands as well as mutagenesis analyses, cysteine 95 of α, cysteine 80 of $\beta_A$, and cysteine 79 of $\beta_B$ are implicated in interchain disulfide bonding.

The β-subunits can also homo- and heterodimerize to form activin A, activin B, and activin AB (Fig. 2). Activins were first identified during the purification of inhibins and were shown to stimulate pituitary FSH secretion. Thus, despite sharing β-subunits, activins and inhibins have opposing actions, at least at the level of the pituitary gonadotrope. Activins were subsequently shown to have diverse biological functions both during development and in adulthood. Although inhibins appear to function outside of the reproductive axis, their best understood role continues to be that of FSH regulation.

Two additional β-subunits, $\beta_C$ and $\beta_E$, have been described in mammals. These subunits can homodimerize to form activin C and activin E and can heterodimerize with each other and the $\beta_A$- and $\beta_B$-subunits to form various activin isoforms. However, there is no evidence that either $\beta_C$ or $\beta_E$ can dimerize with the inhibin α-subunit. Moreover, these subunits are most highly expressed in liver and do not appear to play any role in mammalian reproduction.

## III. ACTIVIN SIGNALING MECHANISMS

Since the molecular characterization of the inhibins in 1985 and of the activins the following year, much more has been learned about the mechanisms of activin, than of inhibin, signal transduction (Fig. 3). The relative disparity in knowledge stems mainly from the 10-year gap between the identification of the first activin receptor and the identification of the first inhibin receptor (see below). In 1991, the first activin type II receptor (now referred to as ActRIIA) was cloned from a mouse corticotropic cell line. The receptor was shown to be a membrane-bound serine/threonine kinase and its identification facilitated the rapid characterization of similar receptors for other

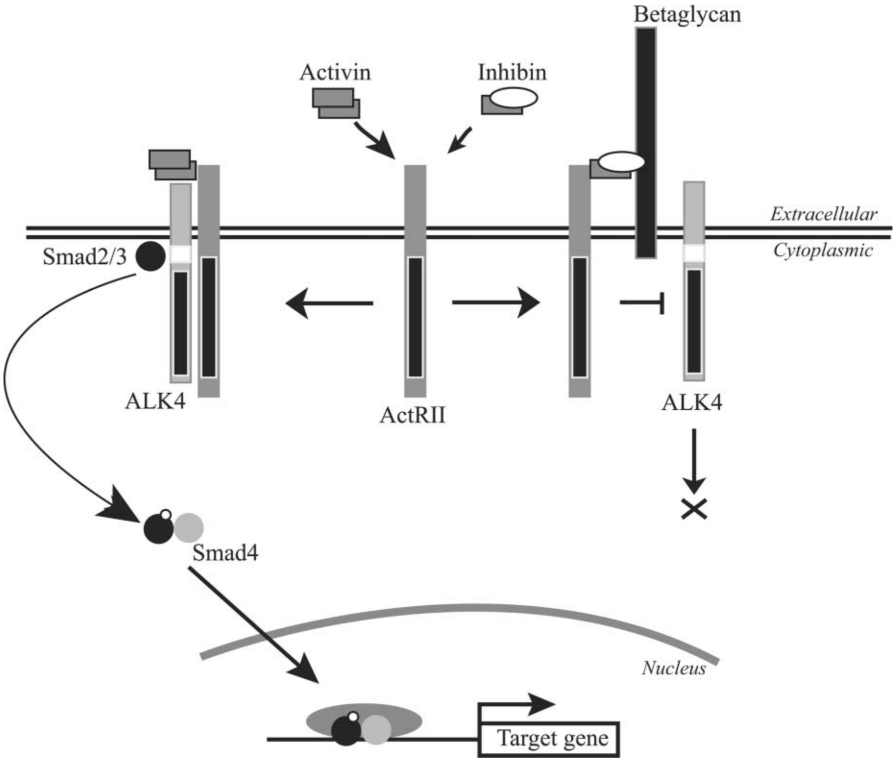

**FIGURE 3** Mechanisms of activin signaling and inhibin antagonism. Activins bind one of two activin type II receptors (shown generically as ActRII in the figure). Upon activin binding (left part of the figure), the activin type IB receptor (ALK4) is recruited into the complex and phosphorylated by the serine/threonine kinase domain of ActRII. ALK4, also a serine/threonine kinase, then phosphorylates the intracellular signaling molecules, Smad2 or Smad3. Phosphorylated Smad2/3 dissociates from ALK4 and associates with the co-Smad4 in the cytoplasm. The activated Smad complex then translocates to the nucleus where it interacts with co-factor proteins to stimulate or repress target gene transcription. Inhibins also bind ActRII but do so with lower affinity than the activins. In the presence of the co-receptor betaglycan, inhibins bind ActRII with high affinity. ALK4 is not recruited into the complex and downstream Smad-dependent signaling does not occur. This provides a powerful mechanism for inhibins to antagonize the actions of the activins. The black rectangles in ActRII and ALK4 denote the approximate locations of the kinase domains. The white box in ALK4 indicates the glycine-/serine-rich region in which ALK4 is phosphorylated (and activated) by ActRII. The white circle on Smad2/3 indicates that the protein has been phosphorylated.

ligands within the TGF-β superfamily, although not for the inhibins.

There are two types of ligand-binding activin type II receptors, ActRIIA and ActRIIB, which are produced by different genes. At least five splice variants of ActRIIB and two of ActRIIA have been described. These receptors bind activin A with high affinity (~ 100–400 pM). Upon ligand binding, the activin type IB receptor (also known as activin receptor-like kinase 4, ALK4) is recruited into the complex and is phosphorylated in a glycine-/serine-rich region of its intracellular domain by the constitutively active kinase domain of the type II receptor. This phosphorylation activates the catalytic domain of ALK4, which also has serine/threonine specificity. ALK4 then phosphorylates serine residues at the carboxy-termini of two members of the Smad family of transcription factors, Smad2 or Smad3, which are anchored in the vicinity of the type I receptor by membrane-associated proteins (not pictured in Fig. 3).

Phosphorylation of the Smads causes a conformational change that precludes their association with both ALK4 and the anchoring proteins, and they are "released" within the cytoplasm. Phosphorylated Smad2 or Smad3 then associates with the partner or co-Smad, Smad4. The Smad complex translocates to the nucleus to affect target gene transcription. The carboxy-terminal domain (also known as the Mad homology 2 or MH2 domain) of Smad2 and Smad3 has transactivation function and associates with the transcriptional co-activator proteins CREB binding protein and p300. The N-terminal MH1 domains of Smad3 and Smad4, but not Smad2, can bind DNA, although they do so with low affinity and low specificity. Instead, Smads interact with DNA-binding transcription factors that recruit the activated Smad complex to distinct promoter elements within target genes. Although many of the details of the "generic" activin signaling pathway have been characterized, the specific mechanisms through which activins stimulate FSH have not yet been described.

Other members of the TGF-β superfamily, including TGF-$\beta_{1-3}$, Müllerian inhibiting substance, and bone morphogenetic proteins, use a similar dual-receptor and Smad signaling system to convey signals to target cells. To date, there is no evidence to suggest that inhibins signal in this way, and attempts to identify receptor serine/threonine kinases that specifically bind inhibins have been unsuccessful. As a result, one current model of inhibin signaling posits that inhibins do not generate intracellular signals but rather generate their effects by blocking the actions of activins through competition for binding to the activin type II receptors (see below and Fig. 3). In the case of FSH synthesis and release, activin B produced locally within gonadotropes appears to stimulate FSH production through an autocrine or paracrine loop. Inhibins from the gonads may therefore act by blocking the intrinsic stimulatory effect of activin B.

## IV. INHIBIN ANTAGONISM OF ACTIVIN SIGNALING

One way that inhibins may exert their biologic effects is by competing with activins for binding to the activin type II receptors. Inhibins can bind both ActRIIA and ActRIIB. This binding appears to occur through the β-subunits of the inhibins. At least in the case of mouse ActRIIA, activin A and inhibin A have been shown to bind to the same hydrophobic amino acids of the receptor (phenylalanine 42, tryptophan 60, and phenylalanine 83). Unlike the case for activins, when inhibins bind the type II receptor, ALK4 is not recruited to the complex nor is it phosphorylated. As a result, when inhibins are bound to the type II receptors, activin signaling can be blocked.

This model, although appealing in its parsimony, does not account for all inhibin actions, however. In particular, inhibin appears to have 10-fold lower affinity for activin type II receptors than does activin A, suggesting that inhibins must be in molar excess of activins to exert their antagonistic effects. Yet, inhibins are known to antagonize activin actions in some contexts where the two ligands are present at equimolar levels. In addition, in other situations, inhibins are unable to antagonize activin actions, even when present in large molar excess quantities. Finally, several cell types, including ovine pituitary cells and testicular Leydig cells, express proteins that bind inhibins but not activins. Collectively, these data indicate that either inhibin receptors independent of activin type II receptors exist or co-receptor proteins are expressed that increase the affinity of the inhibins for activin type II receptors.

To date, there is no evidence that inhibins bind unique signal-transducing receptors. In 2000, however, betaglycan (also known as the TGF-β type III receptor) was shown to bind inhibin A, but not activin A, with high affinity. This membrane-tethered protein lacks any known intracellular kinase domains and therefore cannot generate intracellular signals. This protein is indispensable, however, for some forms of TGF-β signaling. There are three forms of TGF-β in mammals. Unlike TGF-$\beta_1$ and TGF-$\beta_3$, TGF-$\beta_2$ does not bind the TGF-β type II receptor, TβRII, with high affinity. However, in the presence of betaglycan, TGF-$\beta_2$ binds TβRII with an affinity similar to that of the other TGF-β ligands. Remarkably, in the presence of betaglycan, inhibin A binds ActRIIA with an affinity comparable to that of activin A, and the ternary complex between inhibin A, betaglycan, and ActRIIA appears to be resistant to disruption by activin A. Therefore, betaglycan provides a mechanism for high-affinity ActRIIA binding and, as a consequence, activin A antagonism. Whether this model of antagonism applies to inhibin B or to ActRIIB has not yet been reported.

Inhibin A binds to a juxtamembrane domain of betaglycan that shares high sequence conservation with the highly excreted protein uromodulin (the U-domain). This large domain is conserved in other proteins, including the sperm receptors ZP2 and ZP3. Betaglycan is expressed in a variety of tissues and cell types, including some not known to be inhibin responsive. Importantly, however, it is expressed in the anterior pituitary gland and specifically within FSH-producing gonadotropes. Thus, betaglycan is expressed in a manner consistent with its putative role in inhibin's *in vivo* regulation of FSH; however, such a role has not yet been confirmed experimentally.

## V. ALTERNATE MECHANISMS OF INHIBIN ACTION

It is not yet known whether inhibins act exclusively via betaglycan and activin type II receptors to exert all of their cellular effects. It is possible that (1) inhibins bind other co-receptors that increase binding affinity to activin type II receptors or (2) inhibins bind novel receptors that transduce intracellular signals. Thus far, there are no reports in the literature that support the latter possibility. However, inhibin-binding proteins that are distinct from the activin type II receptors and betaglycan have been observed in pituitary, adrenal, testicular, and bone cells. Neither the identities of these proteins nor their mechanisms of action have yet been described.

At the same time that betaglycan was demonstrated to be an inhibin co-receptor, a second candidate inhibin receptor was also described. Inhibin-binding protein or p120 (InhBP/p120) was identified as an inhibin A-binding moiety by affinity chromatography from bovine pituitary membrane extracts. InhBP/p120 was further shown to be expressed highly in the pituitary gland in a variety of species, including rat, mouse, and rhesus monkey. Messenger RNA levels for InhBP/p120 in the pituitary fluctuate throughout the rat estrous cycle, so that levels are highest when circulating FSH levels are lowest and vice versa. Moreover, InhBP/p120 was shown to interact with ALK4 *in vitro* and to antagonize activin A signal transduction in the presence of inhibin B. Nonetheless, more recent analyses have failed to confirm that InhBP/p120 binds inhibin A or inhibin B directly. It is therefore unclear what role, if any, InhBP/p120 plays in inhibin biology *in vivo*.

## VI. EXTRAPITUITARY ROLES OF THE INHIBINS

Although the inhibins are best understood in terms of their pituitary FSH regulation, they also play roles in the other parts of the body. In the ovary, inhibin A can stimulate follicle growth and can increase LH-stimulated androgen production by theca cells. Early reports indicated that inhibin A potentiated LH-stimulated testosterone production by testicular Leydig cells, but these results have not been observed consistently. In humans, inhibins are produced and secreted by the placenta throughout much of pregnancy, although the biological functions of placental inhibins have not been thoroughly described. Outside of the reproductive axis, inhibin A binds bone marrow cells *in vivo*, and *in vitro* it appears to inhibit both osteoblastogenesis and osteoclastogenesis. Adrenocortical cells can also produce inhibins, but the function of adrenal inhibins has not been elucidated.

Inhibins have been implicated as tumor suppressors, particularly in the mouse gonads and adrenal gland. Genetically modified mice that lack the ability to produce the inhibin $\alpha$-subunit (and therefore inhibin A and inhibin B) developed sex cord stromal tumors. If these mice were castrated before the onset of gonadal tumorigenesis, they developed adrenocortical tumors with nearly 100% penetrance. A role for inhibins as tumor suppressors in human ovarian and adrenal cancers is less clear. Moreover, the effect of inhibin deficiency on tumor growth in mice may be indirect. Mice deficient in both inhibins and the gonadotropins (FSH and LH) fail to develop gonadal or adrenal tumors. These data suggest that dysregulated gonadotropin production (FSH production in particular) rather than inhibin deficiency per se may lead to tumor development in mice.

## VII. SUMMARY

The gonadal inhibins suppress pituitary FSH synthesis and secretion. Inhibins A and B are released in discordant fashion and suppress FSH during the luteal and follicular phases of the human menstrual cycle, respectively. Inhibin B alone is produced by testicular Sertoli cells and is arguably the most potent gonadal regulator of FSH in men. Activins are structurally related to inhibins but have opposing actions. That is, activins stimulate pituitary FSH. Although the mechanisms by which activins affect FSH have not been thoroughly characterized, they appear to exert their effects through dual-receptor serine/threonine kinases and Smad proteins. Inhibins, on the other hand, do not generate intracellular signals. Rather, they appear to act by abrogating activin signaling. Inhibins bind the activin type II receptors with high affinity in the presence of the co-receptor, betaglycan. This prevents activins from binding to their receptors and generating intracellular signals.

## Glossary

**betaglycan** The TGF-$\beta$ type III receptor that increases the affinity of inhibin A for the activin type II receptor.

**dimeric protein** A protein formed by the joining of two similar or identical proteins (or subunits).

**follicular phase** The approximately 2-week-long stage of the human menstrual cycle, prior to the luteinizing hormone surge, during which ovarian follicles develop prior to subsequent ovulation.

**luteal phase** The approximately 2-week-long stage of the human menstrual cycle, after ovulation has occurred, during which the corpus luteum produces progesterone and inhibin A.

**receptor serine/threonine kinase** A cell surface protein that can catalyze the phosphorylation of serine or threonine residues in target proteins.

**Smads** Intracellular proteins that convey signals from activated receptors in the TGF-β superfamily to the nucleus.

**TGF-β superfamily** A group of more than 30 structurally related dimeric proteins involved in diverse processes, including cell growth, differentiation, homeostasis, and hormone secretion.

## See Also the Following Articles

**Activin Receptor Signaling • Corpus Luteum in Primates • Follicle Stimulating Hormone (FSH) • Folliculogenesis • Luteinizing Hormone (LH) • Luteinizing Hormone Receptor Signaling • Protein Kinases**

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# Inhibins

David J. Phillips and David M. de Kretser

*Monash University, Melbourne, Australia*

I. DISCOVERY
II. INHIBIN GENES AND PROTEIN ASSEMBLY
III. BINDING PROTEINS, RECEPTORS, AND SIGNALING
IV. TISSUE DISTRIBUTION
V. PHYSIOLOGY OF INHIBIN
VI. CLINICAL AND/OR COMMERCIAL APPLICATIONS
VII. SUMMARY

**The inhibins belong to the transforming growth factor-β superfamily of growth and differentiation factors. They are somewhat unusual for this family in that they have a relatively restricted tissue distribution and function. Inhibins are recognized as the specific negative feedback signals for follicle-stimulating hormone. However, other functions, such as the roles they play in the adrenal gland and as a tumor suppressor gene, have emerged recently. In this article, the basic structure and function of the two forms of inhibin will be outlined, in addition to their effects on target cells and the clinical relevance of these actions.**

## I. DISCOVERY

The concept of inhibin (from the Latin "*inhibeo*," to restrain) first originated in the 1930s, when

D. Roy McCullagh postulated the existence of a nonsteroidal factor produced by the testis that would suppress the function of the pituitary and adrenal glands. A key point was that, unlike androgens, the yet-to-be-isolated inhibin would not affect the function of the secondary sex glands, such as the prostate and seminal vesicles. Fifty-three years after the publication of McCullagh's hypothesis, inhibin was isolated, but surprisingly, from the follicular fluid of ovarian follicles. The inhibin entity was found to be a protein made up of two subunits, one of approximately 18 kDa, termed the α-subunit, and a smaller 14 kDa subunit, termed the β-subunit, joined by disulfide bonds. Moreover, two forms of the β-subunit were detected by sequencing, and these were given the nomenclature $\beta_A$ and $\beta_B$. Because inhibin was made up of an α component and a β component, the $\alpha-\beta_A$ dimer became known as inhibin A and the $\alpha-\beta_B$ dimer as inhibin B. The two forms of inhibin are clearly separate entities and have different patterns of expression and function.

## II. INHIBIN GENES AND PROTEIN ASSEMBLY

Inhibins arise from three gene products: the α subunit is on human chromosome 2q35, the $\beta_A$ subunit gene is on 7p14.1, and the $\beta_B$ gene is on 2q14.2. As in other related proteins, the subunits are first synthesized by cells as larger precursor forms. These are subsequently cleaved by enzymes into the mature subunits, but dimers can form from precursor subunits as well as from the cleaved, mature forms (Fig. 1). It is important to be able to recognize the precursor and intermediate forms because they are known to be present in significant quantities in body fluids and tissues, and some tumors secrete these variants in preference to mature inhibin dimers. It is also a key point that the β-subunits can form dimers with each other independently of the α-subunit. In this case, the dimers are known as activins; their actions counteract that of inhibin and affect a wider range of physiological processes. The balance between the amounts of α- and β-subunits will determine how much inhibin versus activin is formed; more α-subunit favors the formation of inhibin.

## III. BINDING PROTEINS, RECEPTORS, AND SIGNALING

The mechanism by which cells respond to inhibin has been the subject of extensive yet frustrating research. Many groups have sought an inhibin "receptor," but to date, a true receptor has not been identified. What can be said about inhibin and how it acts? It is known that inhibin often has the property of blocking the actions of its sister protein, activin. This is partly due to the ability of inhibin to bind to activin receptors but the inability, once bound, to initiate intracellular signaling pathways. More recent research has identified two proteins, betaglycan and inhibin-binding protein, that enhance the ability of inhibin to couple with activin receptors and make the receptors unstable. Further research is needed to determine whether these mechanisms fully account for the actions of inhibin or whether additional elements remain to be elucidated.

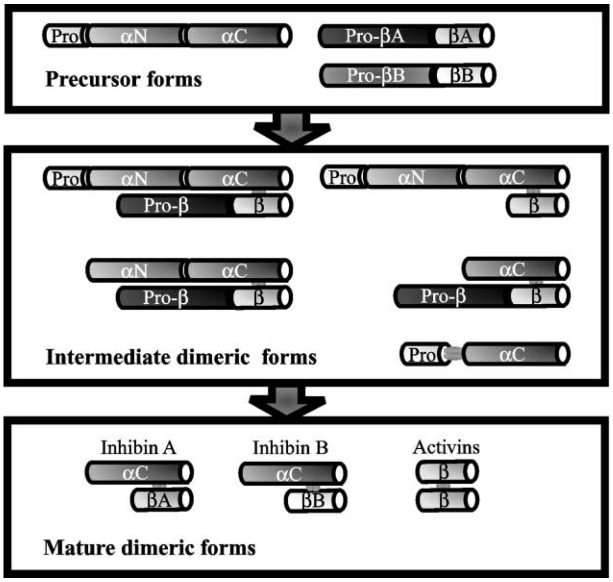

FIGURE 1 Schematic representation of inhibin-containing proteins. Note that several of the precursor and intermediate forms, such as the free α-subunit, are secreted and may be found in serum and other biological fluids. For simplicity, the intermediate forms are depicted as containing a generic β-subunit, which may be either the $\beta_A$ or the $\beta_B$ forms. The disulfide bonds linking subunits are depicted as two parallel lines.

## IV. TISSUE DISTRIBUTION

Low levels of inhibin are made by many tissues in the body, but those that express significant amounts of inhibin are the ovary, placenta, testis, prostate gland, pituitary gland, brain, and adrenal gland. Of these, the testis and ovary are responsible for the majority of inhibin present in the circulation, as blood levels of inhibin plummet to almost undetectable levels when the ovaries or testes are removed. The adrenal gland is a minor source of inhibin in the blood.

## V. PHYSIOLOGY OF INHIBIN

### A. In the Female

Specific assays for the inhibin A and B forms have highlighted the different intraovarian sources of these hormones, although both forms fulfill the role of feedback hormones, suppress follicle-stimulating hormone (FSH) secretion from the pituitary gonadotrope cells, and have a negative correlation with FSH in the circulation. During the menstrual cycle, inhibin A in the bloodstream is low during the follicular phase, begins to rise at the midcycle luteinizing hormone (LH) peak, and then remains high during the luteal phase. The ovulatory follicle and its transformation into the corpus luteum are responsible for this profile. In contrast, inhibin B is highest during the follicular phase and immediately after the LH peak but then drops to low levels during the luteal phase. In this case, inhibin B is a product of the cohort of recruited follicles that are maturing in size and function during the follicular phase. In women approaching menopause, the reduction in the function of follicles being recruited is reflected in a decline in blood levels of inhibin B compared with younger women. Inhibin A levels remain similar to those in younger women and then decline late in the menopausal transition, when increasingly the largest follicle does not proceed to ovulation and menstrual cycles eventually cease.

A potential function has been suggested for inhibin in the maturation of the oocyte around the time of ovulation. However, studies have reported both stimulatory and inhibitory effects on the breakdown of cumulus–oocyte complexes and blastocyst development. It is currently unclear whether the conflicting reports relate to species differences in response, the type of inhibin preparation used, or other experimental conditions.

The placenta is an important source of inhibin during pregnancy. The majority of inhibin arising from the placenta itself, the corpus luteum, the trophoblast, and the developing fetus is inhibin A; inhibin B is almost undetectable throughout most of pregnancy. Inhibin A levels are detectable by approximately week 4 of pregnancy and reach an initial peak in the first trimester before decreasing again in the second trimester. In the third trimester, inhibin A increases steadily to reach at term the highest concentrations of pregnancy. Although the physiology behind the release of inhibin in pregnancy is still not clearly understood, inhibin A levels have been assessed as a marker of abnormal pregnancy or fetus, as is discussed in a later section of this article.

### B. In the Male

Use of the specific inhibin assays has highlighted that in the male the relevant form is inhibin B and that inhibin A is undetectable. Inhibin B is negatively correlated with FSH levels in adult men and is largely the product of Sertoli cells in the testis. The testicular Leydig cell may also make small amounts of inhibin B, but this is not an important contribution to the circulating pool.

Inhibin B levels are detectable in the cord blood of male newborns, rise to levels that are higher than the adult range by 6 months of age, and then decline to low levels at 3–6 years. The initial peak in inhibin is positively correlated with FSH and most likely reflects the increase in Sertoli cell numbers and function that occurs during this period. During puberty, inhibin B levels increase again to adult levels and become negatively correlated with FSH levels. Furthermore, inhibin B in the adult reflects the spermatogenic status of the individual, so that inhibin B is inversely related to FSH concentrations but is directly correlated with sperm counts. In this way, inhibin B fulfills the original hypothesis of McCullagh by signaling to the pituitary gland the status of sperm production by the testis.

The fluid component of the ejaculate, seminal plasma, has high concentrations of inhibin B derived from the testis, since vasectomized men have undetectable levels in seminal plasma. Although inhibin B in seminal plasma generally correlates with sperm production, there are some men with normal sperm counts who have low to undetectable inhibin B levels in this fluid.

The prostate gland is another organ with the capacity to produce inhibin but that may fulfill a paracrine role and not contribute to levels in the circulation or in seminal plasma. The α- and β-subunits are made by epithelial cells in the normal prostate tissue and also in the glands of men with benign prostatic hyperplasia. However, in the development of prostate cancer, the epithelial cells appear to lose the ability to synthesize the α-subunit, resulting in a shift from production of both inhibins and activin in the normal gland to the exclusive production of activin. These clinical findings, in addition to results from mice with a congenital absence ("gene knockout") of the inhibin α-subunit, which also develop tumors, have led to the concept that the α-subunit may be a tumor suppressor gene.

### C. In the Adrenal Gland

The adrenal gland has been known as an inhibin-producing organ for a number of years, but the role of inhibin in this gland is not well understood. The α-subunit is localized to the inner zones of the cortex, whereas the β-subunits are found in all regions of the adrenal gland. Mice in which the α-subunit has been knocked out first develop gonadal tumors, but if the gonads are removed, adrenal tumors develop. This again is consistent with the concept that inhibin may function as a tumor suppressor gene.

## VI. CLINICAL AND/OR COMMERCIAL APPLICATIONS

Potential applications of inhibin in clinical medicine center around its use in two broad areas: as a tool in diagnosing reproductive abnormalities and in defining various types of tumors. In terms of the former, the most widely adopted application has been the use of inhibin A as a marker for the detection in the second trimester of Down's syndrome pregnancies. Inclusion of inhibin A as a screen of maternal serum along with α-fetoprotein and human chorionic gonadotropin (hCG) enables approximately 75% of Down's syndrome pregnancies to be detected, compared with approximately 53% when only α-fetoprotein and hCG are used. Other situations in which inhibins may have utility include early pregnancy. Low inhibin B and high FSH levels have been shown to indicate a poor ovarian response in assisted reproductive technology procedures. However, this index has not been widely adopted in clinical practice because of the difficulty in setting threshold limits for inhibin B that are reliable predictors of a poor outcome. Inhibin A provides an excellent index of corpus luteum function (and number) and has also been assessed as a marker for miscarriages. Although it is able to identify very early miscarriages from nonviable pregnancies, it is not a useful marker for predicting a subsequent miscarriage in pregnancies that proceed beyond approximately 6 weeks.

The most remarkable predictive index for inhibin is in granulosa cell tumors of the ovary. Total α-inhibin levels (not discriminating between the A and the B forms) are used to detect 100% of these tumors; these cancers, however, are very rare and constitute only 5–10% of ovarian tumors. In terms of the more common epithelial ovarian cancers, α-inhibin levels are elevated in approximately 80% of mucinous tumors but in only 5–35% of serous tumors. The assessment of inhibin in defining these subtypes of ovarian cancers is ongoing and may lead to useful tests to detect malignancy.

In the adult male, inhibin B is a useful indicator of Sertoli cell function and germ cell complement, but it is not absolutely predictive. Currently, it does not offer additional diagnostic discrimination beyond the traditional assessment of semen analysis, testicular histology, and FSH levels in the blood. One application may be in the detection of nonpalpable testes in disorders such as cryptorchidism or ambiguous genitalia.

Previous sections have also touched on the use of inhibin as a marker of certain types of cancers. The role of inhibin as a tumor suppressor is suggested from mouse models where gonadal and adrenal tumors develop in mice in which the inhibin α gene has been knocked out. The ovarian tumors in these mice derive from the sex cord stroma, making it difficult to reconcile this origin with the large quantities of α-subunit secreted by human sex cord stromal tumors (which include granulosa cell tumors). As discussed, however, total α-inhibin has been evaluated as a serum marker for a number of human ovarian cancers, with particular use in detecting these granulosa cell tumors.

Histological assessment of tissue sections using α-subunit-specific antibodies has also been informative for some cancers. Loss of α-subunit expression has been recorded in prostate cancers compared with normal prostate tissue, and indeed prostate cancer cell lines do not express the α-subunit either. The use of α-subunit staining on tissue sections has been particularly useful in differentiating between adrenal gland corticocarcinomas (negative for α-inhibin) and renal cell carcinoma (positive for α-inhibin), both of which may be detected as abdominal masses and for which fine-needle biopsies are often taken to help diagnose, based on pathology, the two conditions.

## VII. SUMMARY

Significant leaps in the biology of the inhibins have been made in the 15 years since they were isolated. The concept that inhibins provide a mechanism through which the gonad communicates with the pituitary gland is indubitable, but additional functions in the establishment and maintenance of pregnancy, in the adrenal gland, and in tumor biology have emerged. The development of highly specific immunoassays for the two forms of inhibin has been a vital component in this process. A key area yet to be clarified is exactly how inhibin affects cells: through blocking activin signaling, through its own receptor pathways, or through a combination of the two. Finally, although some of the clinical applications for inhibin have emerged in the arena of reproductive physiology and some cancers, clearly continuing assessment of inhibin is needed in other conditions, for instance, in other significant malignancies such as breast cancer.

## Glossary

**endocrine** Describes the action of a factor that is produced by one tissue and circulates through the bloodstream to reach another target organ on which it has an effect. This is the classical definition of all hormones.

**negative feedback** The inhibition of the action or function of a factor, cell, or organ by another factor or hormone. This type of system is often described as a feedback loop because of its autoregulatory nature.

**paracrine** Describes the action of a factor produced by a cell or groups of cells that acts on neighboring cells of the same type or different types within the same tissue or organ.

**pituitary gonadotrope cells** The cells in the anterior pituitary gland responsible for producing follicle-stimulating hormone and luteinizing hormone, which regulate the function of the gonad. The gonadotropes are one of the primary sites of action for inhibin.

## See Also the Following Articles

**Activin Receptor Signaling • Activins • Follicle Stimulating Hormone (FSH) • Inhibin Receptor Signaling • Inhibins, Activins, and Follistatins**

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# Inhibins, Activins, and Follistatins

David M. de Kretser, David J. Phillips, and Kate L. Loveland

*Monash University, Melbourne, Australia*

I. INHIBIN, ACTIVIN, AND FOLLISTATIN
II. THE PITUITARY–TESTICULAR AXIS: ENDOCRINE EFFECTS
III. PARACRINE EFFECTS WITHIN THE PITUITARY
IV. PARACRINE AND AUTOCRINE EFFECTS WITHIN THE TESTIS
V. EFFECTS WITHIN OTHER ORGANS OF THE MALE TRACT
VI. SUMMARY

**This article discusses the physiology of three groups of proteins, the inhibins, activins, and follistatins, specifically regarding their role in fine-tuning the cross-talk between the gonad and the pituitary gland to regulate male reproductive function. In addition, aspects relating to functions within the testis itself and in the male accessory organs will be covered. Not covered herein are some other aspects of inhibin, activin, and follistatin physiology that relate to additional important roles for these proteins in other tissue systems, including the female reproductive tract.**

## I. INHIBIN, ACTIVIN, AND FOLLISTATIN

Inhibin, activin, and follistatin were originally isolated for their property of modifying the release of follicle-stimulating hormone (FSH) from the pituitary gland. Although purified as separate entities, these proteins are intertwined in terms of their biochemistry and mode of action. Inhibin consists of two subunits (α and β) and, as its name implies, inhibits the production of FSH. Activin, on the other hand, is formed from two β-subunits and stimulates FSH production. It is now believed that inhibin exerts its effect by antagonizing (i.e., blocking) the ability of activin to bind to its cell surface receptors and transduce an intracellular signal. Recent findings have also unveiled the potential for a distinct inhibin-specific mode of action. Follistatin, like inhibin, is a negative regulator of FSH but mediates its effects through physically binding to and preventing activin access to its receptor. It acts as a "sink" for activin, in contrast to an antagonistic receptor occupant, such as inhibin.

Although the above simplistic definitions introduce these three proteins, in fact, each of them consists of a larger set of variants arising from different subunit structures. Four mammalian inhibin/activin β-subunit genes have been isolated, although currently only two of these, βA and βB, are regarded as relevant to male reproductive physiology. The combination of these β-subunits with an α-subunit yields two forms of inhibin, inhibin A and inhibin B. In male mammals, inhibin A is undetectable in serum and tissues and inhibin B is the active form. An exception appears to be the sheep, in which only inhibin A is detectable, although both β-subunits are synthesized by the testis. For activin, combinations of β-subunits give rise to activin A (a homodimer of βA subunits), activin AB (a dimer of βA/βB), and activin B. Follistatin is present in two major forms that vary in both size and function, and more recently, another protein with structural similarity to follistatin and that shows some follistatin-like properties, follistatin-related protein, has also been isolated. In subsequent sections, where relevant to explaining biological processes, the specific forms of activin or follistatin are mentioned, otherwise the generalized effects of these proteins are meant.

## II. THE PITUITARY–TESTICULAR AXIS: ENDOCRINE EFFECTS

Control of male reproductive processes is mediated predominantly by interactions among the hypothalamus, pituitary gland, and testis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which drives the pituitary production of gonadotropin hormones, luteinizing hormone (LH), and FSH. The hypothalamus is responsive to negative feedback signals from the testis in the form of steroids, with testosterone being the primary component. The pituitary gland is also responsive to testicular factors, with testosterone affecting gonadotropin output. Inhibin, activin, and follistatin were proposed to be nonsteroidal feedback factors produced by the testis and that acted at the pituitary gland to specifically regulate FSH (their effects on LH are negligible). The case for inhibin is well established. In some species, such as monkeys, inhibin is the principal testicular hormone responsible for FSH regulation, whereas in other species, such as the sheep, both inhibin and testosterone are involved. Unlike inhibin, in normal circumstances activin and follistatin produced in the testis do not play a significant role in the pituitary feedback system. As discussed in subsequent sections, however, they fulfill important roles as paracrine regulators within the pituitary, testis, and male accessory glands.

## III. PARACRINE EFFECTS WITHIN THE PITUITARY

Inhibin, activin, and follistatin are all synthesized in the pituitary gland. The α- and βB-subunits are specifically produced by gonadotrope cells, the cell type that produces LH and FSH. Minor amounts of the βA-subunit are synthesized by other cell types in the pituitary, and follistatin is produced in most pituitary cells, including gonadotropes. Activin B produced by the gonadotropes drives stimulatory signals for increased FSH production by gonadotrope cells, tempered by the inhibitory influences of inhibin and follistatin. Inhibin can originate from testicular sources via the feedback mechanism discussed above or from locally produced sources (the gonadotrope); for follistatin, pituitary cells are the predominant source. Additional inputs from the hypothalamus and testis are also important in this local network. The nature of GnRH pulses released from the hypothalamus and testosterone levels from the testis influence the pituitary expression levels of inhibin, activin, and follistatin genes.

## IV. PARACRINE AND AUTOCRINE EFFECTS WITHIN THE TESTIS

Functional interactions of these factors within the testis have been difficult to dissect because of the apparent importance of both paracrine and autocrine effects on the dynamic assortment of somatic and germ cell populations. It is clear that the major production of the inhibin α-subunit within the testis is within Sertoli cells, though Leydig cells may also make inhibin. The Sertoli cells produce the large amounts of circulating inhibin protein that control FSH secretion, and the level of Sertoli cell secretion of inhibin B is used as an indicator of testicular function in adults. Men with severe impairment of spermatogenesis have dramatically reduced serum inhibin B levels, including those men with Sertoli cell-only syndrome. Surprisingly, boys with Sertoli cell-only syndrome have normal inhibin B levels for their age, but they have undetectable levels of inhibin in adulthood.

At least two distinct receptor-like proteins for inhibin have been identified on Leydig cells and Sertoli cells, betaglycan and p120, and it has been suggested that each of them detects a specific inhibin isoform. These findings hint at a paracrine or an autocrine effect of inhibin that remains to be elucidated.

In contrast to the inhibin α-subunit, the activin β-subunit is made in several somatic and germ cell types, including Sertoli cells, Leydig cells, peritubular myoid cells, macrophages, gonocytes, and spermatocytes, so each of these cell types can produce the various types of activin. Specific activin receptor subunits have been detected on all somatic and germ cell types in the adult testis. Deletion of the mouse gene encoding one of the activin receptors, which is normally found on Sertoli cells, spermatogonia, and spermatocytes, results in animals with smaller testes and reduced fertility, due to the presence of lower than normal numbers of Sertoli cells and germ cells. This observation of reduced Sertoli cell numbers suggests that activin, directly or through its action on FSH, affects Sertoli cell proliferation in the juvenile animal, a result that is supported by *in vitro* culture experiments with testis fragments from developing animals. Although a similar phenotype occurs in mice that fail to synthesize FSH, it is apparent that breeding these mice to produce a "double-knockout" phenotype causes a further reduction in sperm production without a significant additional change in testis size. Therefore, the two factors function both together and independently to regulate testis development, and again, this observation is supported by *in vitro* experimental data.

Follistatin production has been observed in Sertoli cells and spermatogonia of the adult, and local accumulation of extracellular protein is evident in the interstitial regions of the immature and adult testis. This accumulation of follistatin protein in the extracellular matrix surrounding seminiferous tubules and Leydig cells suggests that it plays a role in regulating activin bioactivity by modulating activin access to its receptor on Leydig, Sertoli, and spermatogonial cells.

As described above, variation in activin isoform bioactivity has important functional implications for germ cell maturation. The production of activin $\beta_A$ by gonocytes is limited to a discrete window of germ cell development, with its mRNA synthesized in the fetal testis and the $\beta_A$ protein stored in the germ cells until they mature into spermatogonia after birth. There appears to be a switch at this stage of germ cell development, so that spermatogonia make follistatin in both juvenile and adult animals. However, as they enter meiosis, germ cells once again synthesize activin, but now producing activin $\beta_B$. Activin A is regarded as the more potent form and appears therefore to be required for the initial stages of spermatogenesis. In other experiments, overexpression of activin or follistatin in spermatocytes causes spermatogenic failure, further indicating that local control of activin bioactivity is important for normal testicular function.

Regulation by activin of both somatic and germ cell proliferation has been demonstrated in cell culture experiments, and its impact appears to change as the testis matures. At birth, Sertoli cells multiply in response to FSH alone, but thereafter both FSH and activin are required. In contrast to the effect on Sertoli cells, activin appears to inhibit division of germ cells when FSH is present. By puberty, when the Sertoli cells stop dividing and begin to differentiate, neither of these agents alone or in combination can provoke further division. These results highlight the dynamic situation that exists in the developing testis at the onset of spermatogenesis. Although there is less information concerning the local role of activin in the adult testis, it is clear that spermatogenesis can proceed to completion in mice that produce reduced levels of functional activin.

## V. EFFECTS WITHIN OTHER ORGANS OF THE MALE TRACT

Development of external genitalia involves the regulation of the actions of activin and related family members. Activin $\beta_A$ is made in the fetal genitalia during development, and replacement of this subunit by activin $\beta_B$ in transgenic mice leads to genital enlargement in adulthood.

In the adult, production of inhibin α- and activin β-subunits occurs within the epididymis, though whether their role is local or hormonal is not clear. Early in development, local interactions between activin and follistatin are implicated in prostate growth, affecting development of the characteristic branches that form the backbone of the secretory epithelium. In the adult prostate, the synthesis of these factors appears to be required for homeostasis, as the loss of inhibin α expression has been associated with the development of high-grade cancer pathologies. Secretion of high levels of follistatin by the prostate gland or seminal vesicles into seminal fluid may modulate the downstream effects of activin derived from the testis or the epididymis.

## VI. SUMMARY

The concept that male reproductive function is regulated not only by steroid hormones but also by protein factors was first proposed approximately 70 years ago. It is only recently with the development of highly specific assays for the various forms of inhibins, activins, and follistatins, new molecular tools, and transgenic animal models that our understanding has moved beyond this initial concept. It is clear the inhibin B is a major regulator of male reproductive function and is of use in the clinical diagnosis of male infertility. Important roles for activin and follistatin as paracrine and autocrine factors within the pituitary, testis, and accessory male glands are suggested from transgenic mouse models and sophisticated *in vitro* studies. Their precise function within these tissues is becoming apparent with the development of tools that can dissect how these molecules direct the complex interactions among various cell types in the normal process of spermatogenesis and in pathological states.

## Glossary

**autocrine** Describes the action of a factor produced by a cell that has an action on that cell itself.

**endocrine** Describes the action of a factor that is produced by one tissue and circulates through the bloodstream to reach another target organ on which it has an effect. This is the classical definition of all hormones.

**germ cells** Cells that can grow to form the reproductive cells (sperm or eggs). In the testis, these are the gonocytes, spermatocytes, spermatids, and spermatozoa.

**paracrine** Describes the action of a factor produced by a cell or groups of cells that acts on neighboring cells of the same type or different types within the same tissue or organ.

**somatic cells** All cells of the body except the germ cells. In the testis, the somatic cells include the Sertoli cells, peritubular myoid cells, Leydig cells, and macrophages.

## See Also the Following Articles

**Activin Receptor Signaling • Activins • Follicle Stimulating Hormone (FSH) • Follitropin (Follicle-Stimulating Hormone) Receptor Signaling • Inhibin Receptor Signaling • Inhibins**

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# Inositol Phosphate Signaling

James W. Putney, Jr.
*National Institute of Environmental Health Sciences,*
*National Institutes of Health, North Carolina*

I. METABOLISM OF INOSITOL PHOSPHATES
II. ACTIONS OF INOSITOL PHOSPHATES
III. INOSITOL PHOSPHATE RECEPTORS
IV. CONCLUSIONS

**In many cell types, hormone activation of surface membrane receptors is associated with a rise in cytoplasmic calcium ($[Ca^{2+}]_i$). This rise in $[Ca^{2+}]_i$ is the initial trigger for a cascade of short-term responses, such as secretion or muscle contraction, and long-term responses, such as cell differentiation, cell division, and even cell death. In most cell types, there are two sources of signaling $Ca^{2+}$. $Ca^{2+}$ is initially released into the cytoplasm from intracellular organelles and also enters the cytoplasm across the plasma membrane. Thus, at least for the intracellular release of $Ca^{2+}$, a signaling messenger is required. There may be several different kinds of $Ca^{2+}$-mobilizing messengers in different cell types. The first of these to be discovered was D-*myo*-inositol 1,4,5-trisphosphate [$(1,4,5)IP_3$]. $(1,4,5)IP_3$ is formed when receptor stimulation is coupled to the activation**

**of a polyphosphoinositide-specific phospholipase C enzyme that degrades a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate. The products of this reaction are diacylglycerol and the head group of the lipid, (1,4,5)$IP_3$. Diacylglycerol functions in signaling pathways by activating a specific protein kinase (protein kinase C), and (1,4,5)$IP_3$ signals the release of intracellular $Ca^{2+}$.**

## I. METABOLISM OF INOSITOL PHOSPHATES

Virtually all cellular signals are terminated either by transport mechanisms or by metabolic degradation. Following phospholipase C activation and formation of (1,4,5)$IP_3$, the molecule is rapidly degraded by a 5-phosphatase producing the inactive product inositol 1,4-bisphosphate. This is in turn sequentially dephosphorylated back to inositol, which is used in resynthesis of phosphatidylinositol, the precursor of phosphatidylinositol 4,5-bisphosphate.

The degradative pathway for (1,4,5)$IP_3$ metabolism is only one aspect of the complex network of inositol phosphate metabolism in cells. (1,4,5)$IP_3$ is also rapidly phosphorylated at the three position by a 3-kinase producing inositol 1,3,4,5-tetrakisphosphate [(1,3,4,5)$IP_4$]. There is evidence, albeit controversial, that (1,3,4,5)$IP_4$ may function as a signal in its own right (discussed below); nonetheless, it is clear that this molecule is the starting point of a complex synthetic pathway generating a variety of inositol phosphates whose functions are not yet completely understood.

The major synthetic and degradative pathways for inositol phosphates in mammalian cells are summarized in Fig. 1. In lower organisms, inositol phosphates can be synthesized by direct phosphorylation of inositol, but the available evidence in mammalian systems suggests that (1,4,5)$IP_3$ is the precursor for all of the major inositol phosphates. A key step in the synthetic pathway is the phosphorylation of inositol 1,3,4,6-tetrakisphosphate to inositol pentakisphosphate [(1,3,4,5,6)$IP_5$ or simply $IP_5$]. This step apparently proceeds at near saturation even under resting, unstimulated conditions. Thus, phospholipase C activation will increase the levels of inositol phosphates at this step and at subsequent steps but does not directly increase the synthesis of higher inositol phosphates. In general, the inositol phosphates with five or more phosphates turn over very slowly, and their functions are not yet fully understood. A breakdown product of $IP_5$, (3,4,5,6)$IP_4$, increases when phospholipase C is activated, probably because lower inositol phosphates inhibit its degradation. This inositol tetrakisphosphate may have signaling roles, especially in controlling chloride channels in some cell types.

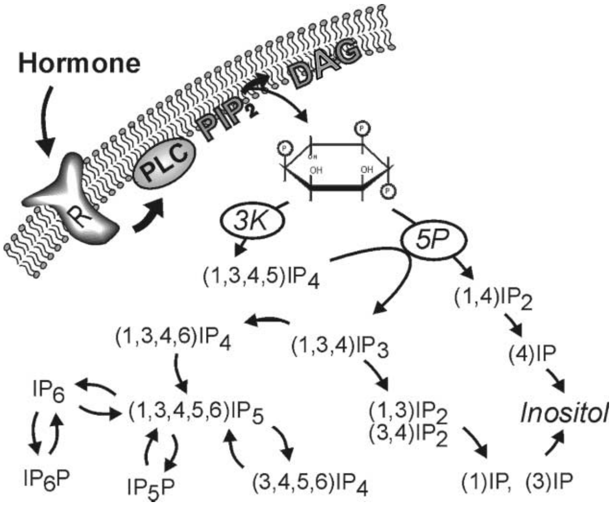

FIGURE 1 Pathways of inositol phosphate metabolism. Agonist activation of a surface receptor (R) activates, usually through a G-protein, a polyphosphoinositide-specific phospholipase C (PLC), which catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into the $Ca^{2+}$-signaling messenger, (1,4,5)$IP_3$ (whose structure is shown), and the protein kinase C activator, diacylglycerol (DAG). (1,4,5)$IP_3$ is metabolized by two enzymes, a 5-phosphatase (5P) and a 3-kinase (3K), initiating complex degradative and synthetic pathways of inositol phosphate metabolism.

The higher inositol phosphates, $IP_5$ and phytic acid ($IP_6$), can be phosphorylated on existing phosphate residues, resulting in pyrophosphates. There is considerable evidence that these pyrophosphates turn over more rapidly than their precursors and that their levels are regulated by other signaling pathways. Although it is likely that these interesting molecules have important cellular roles, as yet, their precise functions are not known.

## II. ACTIONS OF INOSITOL PHOSPHATES

Two of the inositol phosphates, (1,4,5)$IP_3$ and (1,3,4,5)$IP_4$, have been shown, or suggested to play roles in cellular calcium signaling. Of the two, the role of (1,4,5)$IP_3$ as a $Ca^{2+}$-mobilizing messenger is well established. The role of (1,3,4,5)$IP_4$ is less certain, and will be discussed only briefly.

As discussed briefly above, (1,4,5)$IP_3$ was the first $Ca^{2+}$-mobilizing second messenger to be discovered. When receptors coupled to phospholipase C are activated, (1,4,5)$IP_3$ is formed at the plasma membrane and diffuses to the endoplasmic reticulum where it activates (1,4,5)IP$_3$ receptors. These receptors are ion channels, and their activation leads to the discharge of stored Ca$^{2+}$ into the cytoplasm. The endoplasmic reticulum is capable of accumulating and storing significant amounts of Ca$^{2+}$ because of the presence of SERCA (sarcoplasmic–endoplasmic reticulum calcium ATPase) Ca$^{2+}$ pumps, and because the endoplasmic reticulum contains a number of low-affinity, high-capacity Ca$^{2+}$-binding proteins, such as calreticulin and calsequestrin. Some subcellular fractionation studies have shown that the site of Ca$^{2+}$ storage has somewhat different properties from classical endoplasmic reticulum, and the term ‘calciosome’ has been proposed for the critical Ca$^{2+}$-storing organelle involved in receptor-dependent Ca$^{2+}$ signaling. At present, it is not clear whether the calciosome is an organelle clearly distinct from endoplasmic reticulum or a specialized component of the endoplasmic reticulum. It is clear, however, that Ca$^{2+}$ is released from stores that utilize SERCA pumps for Ca$^{2+}$ accumulation, because drugs that poison SERCA pumps in most instances release the same store of Ca$^{2+}$ that is released by (1,4,5)IP$_3$. It is also clear that Ca$^{2+}$ is not released from mitochondria, as was once thought. Rather, there exists an intimate association between mitochondria and Ca$^{2+}$ release sites [i.e., (1,4,5)IP$_3$ receptors] in the endoplasmic reticulum to facilitate the conveyance of [Ca$^{2+}$]$_i$ signals to the mitochondrial matrix where they serve to regulate Ca$^{2+}$-sensitive metabolic pathways.

In many cell types, activation of the (1,4,5)IP$_3$ pathway results in complex temporal and spatial behavior of Ca$^{2+}$ signals, resulting in the appearance of repetitive [Ca$^{2+}$]$_i$ spikes; spatially these spikes are often represented as propagated [Ca$^{2+}$]$_i$ waves (Fig. 2). The significance of this discontinuous behavior is not known for certain, but a favored view is that it provides better signal-to-noise ratio by utilizing a digital or frequency-encoded signal. The cellular and molecular mechanism underlying these oscillatory signaling patterns may result from properties of (1,4,5)IP$_3$ receptors, which are susceptible to complex regulation by Ca$^{2+}$. Low concentrations of Ca$^{2+}$ activate (1,4,5)IP$_3$ receptors, and higher concentrations inhibit them. In addition, the time course of the development of inhibition appears to be slower than for the activation. These kinetic parameters may contribute to the all-or-none behavior often seen in the generation of intracellular Ca$^{2+}$ spikes. In addition, the concentration of Ca$^{2+}$ in the lumen of the endoplasmic reticulum also regulates the (1,4,5)IP$_3$ receptor in a positive fashion; thus, filled stores are more sensitive to (1,4,5)IP$_3$ than are empty ones. This may contribute to the triggering of each new [Ca$^{2+}$]$_i$ spike as intracellular stores become recharged.

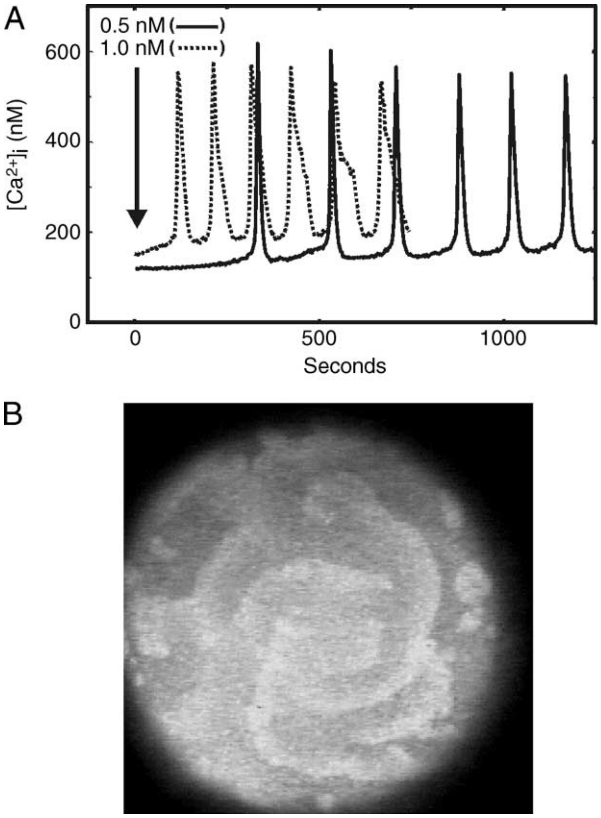

**FIGURE 2** Calcium waves and oscillations. (A) A single rat hepatocyte treated with vasopressin exhibits low-frequency baseline spikes of [Ca$^{2+}$]$_i$. The latency to the first spike and the frequency of spiking are related to the concentration of hormone. (B) A *Xenopus* oocyte was microinjected with ~1 μM IP$_3$. The discontinuous fluorescence depicts the characteristic calcium waves and spirals seen in this preparation.

An alternative mechanism for the production of regenerative [Ca$^{2+}$]$_i$ signals involves cyclical activation and inactivation of the enzyme generating (1,4,5)IP$_3$, resulting in oscillations in (1,4,5)IP$_3$ levels. Calcium is an activator of phospholipase C, which could produce the necessary regenerative force, and the diacylglycerol that is formed can inhibit the enzyme through activation of protein kinase C. It is difficult to demonstrate such a mechanism directly because, unlike the case for Ca$^{2+}$, there are no fluorescent indicators that can track (1,4,5)IP$_3$ levels at the single-cell level in real time. Nonetheless, there is strong evidence for such a regulatory mechanism in some instances. It is possible that in some situations,

$[Ca^{2+}]_i$ oscillates in response to fluctuating $(1,4,5)IP_3$ levels, and in other situations, $[Ca^{2+}]_i$ oscillates at constant $(1,4,5)IP_3$ levels due to cyclical $Ca^{2+}$ regulation of the $(1,4,5)IP_3$ receptor activity.

Activation of cells through the phospholipase C pathway almost inevitably results in an increased flux of $Ca^{2+}$ across the plasma membrane. There are a few instances in which this regulated flux of $Ca^{2+}$ occurs through $(1,4,5)IP_3$ receptors present in the plasma membrane. However, in the majority of instances, the activation of $Ca^{2+}$ entry is a more indirect process. The fall in $Ca^{2+}$ in the lumen of the endoplasmic reticulum in some manner signals to plasma membrane channels, leading to their activation. The process is known as capacitative calcium entry, or store-operated calcium entry. The electrophysiological current associated with capacitative calcium entry is very small and difficult to detect, but it has been characterized in some cell types. In hematopoietic cells, and perhaps some others, the current is highly calcium selective, distinguishing calcium from ions of similar size and charge such as barium and strontium. This highly selective current has been designated $I_{crac}$ for calcium release-activated calcium current. Other cell types may have channels with differing ionic selectivity, including some with store-operated nonselective cation channels. This suggests molecular diversity among store-operated channels in different cell types.

The obvious role of $(1,4,5)IP_3$ in capacitative calcium entry is to deplete calcium from the endoplasmic reticulum, which in turn initiates the signaling to the plasma membrane. However, $(1,4,5)IP_3$ may also play a more direct role in the signaling mechanism for capacitative calcium entry. Currently, there are two general theories regarding the mechanism of signaling capacitative calcium entry. One idea holds that the depleted endoplasmic reticulum releases an unknown signal that diffuses to the plasma membrane and activates channels. The alternative idea, termed conformational coupling, suggests that $(1,4,5)IP_3$ receptors in the endoplasmic reticulum interact directly with plasma membrane channels. In some systems, maintenance of store-operated channels in an active state has been shown to require $(1,4,5)IP_3$ as well as the $(1,4,5)IP_3$ receptor; in other systems, capacitative calcium entry proceeds normally in the complete absence of $(1,4,5)IP_3$ receptors. At present, the issue of the mechanism for signaling capacitative calcium entry is far from resolved. It is possible that more than one signaling mechanism exists, depending on the cell type.

## III. INOSITOL PHOSPHATE RECEPTORS

The biological actions of inositol phosphates depend upon their interaction with specific receptor sites in cells. There is little information about inositol phosphate receptors, other than the receptor for $(1,4,5)IP_3$. This is because, as discussed above, the evidence for biological actions of other inositol phosphates is sketchy at best. For the case of $(3,4,5,6)IP_4$, which seems to regulate chloride channels, direct interaction with the channels or an accessory protein has not yet been demonstrated. It is likely that such an interaction will be found in the not too distant future. For $(1,3,4,5)IP_4$, the phosphorylation product of $(1,4,5)IP_3$, a specific binding protein was isolated and appears to be a Ras-GAP; that is, a protein that activates the GTPase activity associated with the small G-protein, Ras. The significance of this protein to $Ca^{2+}$ signaling is unknown. One problem with understanding the function of an $(1,3,4,5)IP_4$ receptor is that the biological role of $(1,3,4,5)IP_4$ is not well established. A second problem is that the putative $(1,3,4,5)$ $IP_4$ receptor also binds and is activated by the 3-phosphorylated lipid, phosphatidylinositol 3,4,5-trisphosphate, and some believe that this may be the physiological ligand for the receptor.

Much more is known about the structure and function of $(1,4,5)IP_3$ receptors. The best characterized function of $(1,4,5)IP_3$ receptors is as a $Ca^{2+}$ release channel in response to the formation of $(1,4,5)IP_3$. However, as discussed above, they may also function to link endoplasmic reticulum to plasma membrane $Ca^{2+}$ channels, supplying a signal for capacitative calcium entry. There is also evidence from a number of studies that $(1,4,5)IP_3$ receptors play a role in programmed cell death, or apoptosis. It is not known whether this is due to the ion channel function of the molecule or to some other role of the receptor molecules. To date, there are three known genes for $(1,4,5)IP_3$ receptors designated type I, type II, and type III, each sharing considerable sequence homology. The different forms are similar in both sequence and behavior, although there are some functional differences, especially with regard to regulation by $Ca^{2+}$. Although many cell types express all three types of receptors, there are some cell types that express two subtypes or only one subtype. The predicted molecular weight of the receptors is ~300 kDa. In native membranes, a functional receptor is a tetramer of four receptor molecules; these may be of the same or distinct subtypes. Although it is clear that *in situ* $(1,4,5)IP_3$ receptors

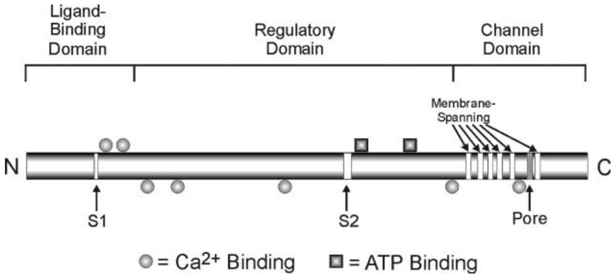

**FIGURE 3** Structure of the type I (1,4,5)$IP_3$ receptor. The (1,4,5)$IP_3$ receptor is composed of ligand-binding, regulatory, and channel domains. The positions of two of the three known splice sites, the membrane-spanning regions, and the putative channel pore region are shown. Also shown are the positions of putative ATP- and $Ca^{2+}$-binding sites.

associate with other proteins, purified recombinant proteins form (1,4,5)$IP_3$-regulated ion channels, indicating that the basic receptor and channel functions are contained within the receptor molecule.

As expected, all three types appear to be localized in the endoplasmic reticulum, as well as in the nuclear envelope, an extension of the endoplasmic reticulum. In the endoplasmic reticulum, they function to release $Ca^{2+}$ to the cytoplasm following activation of the phospholipase C pathway. There is also evidence for phosphoinositide metabolism and specialized release of $Ca^{2+}$ in the nucleus, where $Ca^{2+}$ signaling may play a specific role in gene regulation. In many cells, biochemical evidence suggests the presence of a minor fraction of (1,4,5)$IP_3$ receptors in the plasma membrane. However, there are few data on the functional significance of these receptors, as the major route of $Ca^{2+}$ entry during signaling appears to be the capacitative calcium entry pathway.

Structural analysis of (1,4,5)$IP_3$ receptors by deletions of specific sequences has revealed three major domains of the (1,4,5)$IP_3$ receptor (Fig. 3): an N-terminal (1,4,5)$IP_3$-binding domain, a long regulatory or coupling domain, and a somewhat shorter C-terminal hydrophobic, channel domain. The type I form has three splice sites (two are shown in Fig. 3), giving rise to a large number of splice variants. The N-terminal region contains the $IP_3$-binding region, the S1 splice site, and two $Ca^{2+}$-binding sites. The regulatory domain contains additional $Ca^{2+}$ sites; presumably, some or all of these sites are responsible for the complex regulation of (1,4,5)$IP_3$ receptor function by cytoplasmic $Ca^{2+}$. The regulatory domain is so named because in this region, regulatory molecules such as calmodulin and FK-binding protein have been shown to interact. In addition, sites for regulatory phosphorylation by cyclic nucleotide-dependent protein kinases are found within this region. The regulatory domain of the type I receptor also contains two positive regulatory binding sites for ATP.

The C-terminal domain contains six hydrophobic sequences that would be predicted to be membrane spanning from hydropathy plots. Accordingly, the C-terminus is predicted to be cytoplasmic. Between the fifth and the sixth membrane-spanning sequences is a hairpin loop that may constitute the ion channel pore. There is also a $Ca^{2+}$-binding site within this region; however, its affinity for $Ca^{2+}$ appears to be too high for it to be regulated within the range of $[Ca^{2+}]$ expected within the endoplasmic reticulum. Deletion of the C-terminal domain results in a soluble protein that does not form tetramers; thus, the C-terminus is thought to contain sequences important for both localization and appropriate oligomerization.

## IV. CONCLUSIONS

A variety of hormones and neurotransmitters regulate cell function by activation of a polyphosphoinositide-specific phospholipase C, resulting in the liberation of the signal, (1,4,5)$IP_3$. (1,4,5)$IP_3$ in turns binds to specific (1,4,5)$IP_3$ receptors in the endoplasmic reticulum that function as ion channels, causing the release of stored $Ca^{2+}$ to the cytoplasm (see Fig. 4).

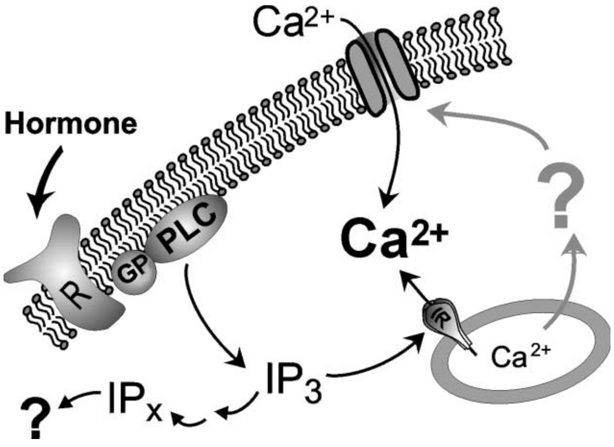

**FIGURE 4** Schematic depiction of inositol phosphates and calcium signaling. Agonist activation of a surface receptor (R) leads to activation of a polyphosphoinositide-specific phospholipase C (PLC), usually through the intermediate action of a heterotrimeric G-protein (Gp). (1,4,5)$IP_3$ is formed and activates a (1,4,5)$IP_3$ receptor channel (IR), causing a rapid release of $Ca^{2+}$. This release in turn signals positively to a capacitative calcium entry channel in the plasma membrane. (1,4,5)$IP_3$ also serves as precursor for a number of inositol polyphosphates, some of which may function in other aspects of $Ca^{2+}$ signaling.

$(1,4,5)IP_3$ also serves as the precursor of a number of higher inositol phosphates and pyrophosphates, some of which may also have functions in various signaling pathways. Coordinated with the intracellular release of $Ca^{2+}$ by $(1,4,5)IP_3$ is an accelerated entry of $Ca^{2+}$ into the cell across the plasma membrane. The signal for this plasma membrane entry is not well understood. It involves a process known as capacitative calcium entry, whereby the signal is somehow initiated by the depletion of the endoplasmic reticulum stores by $(1,4,5)IP_3$. In some instances, $(1,4,5)IP_3$-mediated $Ca^{2+}$ signaling results in the generation of repetitive spikes of intracellular $Ca^{2+}$, sometimes referred to as $Ca^{2+}$ oscillations. The varied and complex patterns of $Ca^{2+}$ signaling initiated by $(1,4,5)IP_3$ control multiple cellular functions, from immediate to long-term responses.

## Glossary

**calciosome** A theoretical organelle, or subcellular fraction, that is specifically involved in storing and releasing $Ca^{2+}$ during $Ca^{2+}$ signaling.

**calcium influx factor** A hypothetical factor released from the endoplasmic reticulum following $Ca^{2+}$ depletion, which diffuses to the plasma membrane and activates $Ca^{2+}$ channels.

**calcium oscillations** Cycles of regenerative, all-or-none rises in cytoplasmic $Ca^{2+}$ superimposed on a stable baseline. Also called calcium spikes. Calcium feedback properties of the $(1,4,5)IP_3$ receptor may underlie these oscillations, at least in some cell types.

**capacitative calcium entry** A process by which the depletion of intracellular endoplasmic reticulum $Ca^{2+}$ stores, either artificially or by the signal $(1,4,5)IP_3$, initiates a signaling process to open channels in the plasma membrane.

**conformational coupling** A theory for signaling capacitative calcium entry whereby depletion of intracellular $Ca^{2+}$ stores causes a conformational change in $(1,4,5)IP_3$ receptors, which in turn interact with plasma membrane $Ca^{2+}$ channels, resulting in their activation.

**inositol phosphates** A series of phosphorylated derivatives of the hexitol, D-*myo*-inositol. In mammalian cells, inositol phosphates are derived from inositol 1,4,5-trisphosphate, formed by cleavage of phosphatidylinositol 4,5-bisphosphate by phospholipase C.

**inositol 1,4,5-trisphosphate [$(1,4,5)IP_3$]** An inositol phosphate that is one of the initial products of phospholipase C cleavage of phosphatidylinositol 4,5-bisphosphate. $(1,4,5)IP_3$ is an important signaling molecule that acts by binding to a specific receptor on the endoplasmic reticulum, triggering release of $Ca^{2+}$ to the cytoplasm. It is also the precursor for higher inositol phosphates, some of which may have roles in various signal transduction pathways.

**$(1,4,5)IP_3$ receptor** A high-molecular-weight protein, located primarily in the endoplasmic reticulum, that forms a tetrameric channel. The channel opens in response to binding of $(1,4,5)IP_3$, resulting in the release of $Ca^{2+}$ to the cytoplasm.

**phospholipase C** An enzyme that cleaves phospholipids at the glycerol–head group diester bond, yielding a phosphorylated head group and diacylglycerol. Some phospholipases C are specific for certain phospholipids, for example, for polyphosphoinositides, which yield the signaling inositol phosphates.

**sarcoplasmic-endoplasmic reticulum calcium ATPase pump (SERCA pump)** The predominant active $Ca^{2+}$ transport molecule responsible for concentrating $Ca^{2+}$ in the sarcoplasmic and endoplasmic reticulum.

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**Calcium Signaling**

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as do vertebrates. Also, neurosecretions from the brain regulate the function of the various endocrine organs just as the hypothalamus of the brain releases the cascade of endocrine gland functions in vertebrates.

## II. INSECT BODY PLAN AND DEGREES OF METAMORPHOSIS

An overview of a general insect body plan and the types of metamorphosis will provide a framework for the description of the anatomy of the endocrine system in insects and the functions that they regulate. The head is a fusion of the first 6 segments of the embryo. It bears the eyes, antennae, and mouth parts that include the labrum and labium (above and below the mouth), mandibles, and maxillae, variously modified in different orders of insects. The thorax is composed of 3 segments, prothorax, mesothorax, and metathorax; each bears a pair of legs, whereas wings are on the last 2 segments. The abdomen, which usually bears no locomotory appendages except in larval forms, typically consists of 11 segments. The terminal segments bear the reproductive structures.

The internal anatomy reflects the segmentation pattern (Fig. 1). The brain of three fused segments (proto-, deuto-, and tritocerebrum) is connected by circumesophageal connectives to the ventral subesophageal ganglion, also composed of three fused segments. Paired connective nerves join this ganglion to those of the ventral nerve cord. A ganglion serves each thoracic and abdominal segment. In addition to the brain and ventral ganglia of the CNS, there is a stomatogastric nervous system with connections to the brain and subesophageal ganglion. It consists of several ganglia and nerve tracts along the digestive tract.

The major organ for movement of blood in insects is a long dorsal tube, called the dorsal vessel or heart. Blood moves into segmental openings of the heart and as a general rule is pumped anteriorly. The function of gas transport is carried out by a system of trachea rather than by respiratory pigments. The trachea are air-filled tubes lined with cuticle that lead to all of the tissues of the body from external openings, called spiracles. The major organ of intermediary metabolism and storage is voluminous fat body tissue. The Malpighian tubules and the gut, primarily the anterior and posterior parts of the hindgut, are the major organs regulating water and ion balance.

The degree of metamorphosis in insects varies from little to extreme. The Ametabola (little or no metamorphosis) are ancient wingless insects of which there are only a few species. They change little during the life cycle except for size as they grow and shed their exoskeleton (molt). As wingless adults, they reproduce and continue to grow and molt. The Hemimetabola (incomplete metamorphosis) have

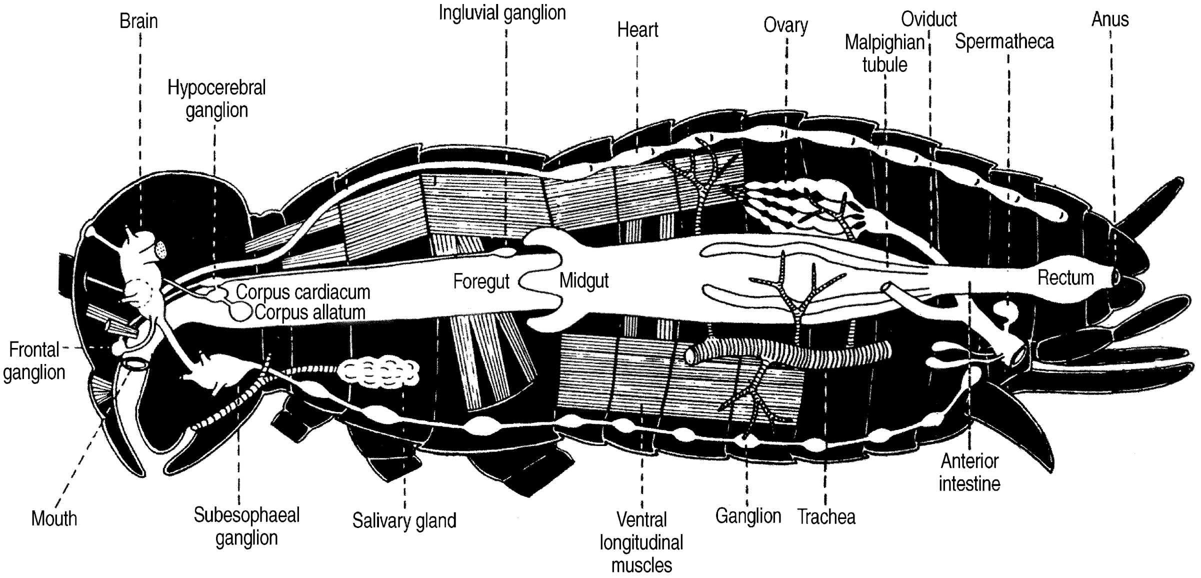

**FIGURE 1** Major internal organs of a generalized insect. The frontal, hypocerebral, and ingluvial ganglia contain the cell bodies of the stomatogastric nervous system. The anterior intestine and rectum are hindgut structures. Modified from Du Porte, E. M. (1959). "Manual of Insect Morphology," p. 152. Reinhold, New York, with permission.

larval (also called nymphal) stages that resemble the adults in form. The larvae are wingless but have external wing pads on thoracic segments that become larger at each larval molt. The adult has wings, is reproductive, and no longer grows and molts. The Holometabola, which constitute the majority of insect species, have larval forms that are specialized for feeding. They show reduced appendages, possess internal imaginal discs (clusters of embryonic cells that will form appendages and external reproductive structures of the adult), and do not resemble the adult in form or life style. After the larva stops feeding, it molts to another form, the nonfeeding pupa. During this stage, the imaginal discs differentiate and larval tissues are degraded or rearranged into the adult form. The winged adults, now specialized for dispersal and reproduction, no longer grow and molt.

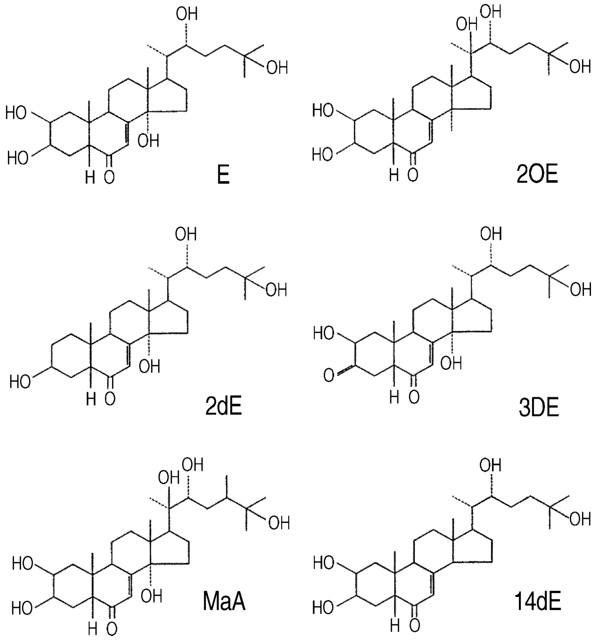

**FIGURE 2** Structural formulas of some major ecdysteroids of insects. E, ecdysone; 20E, 20-hydroxyecdysone; 2dE, 2-deoxyecdysone; 3DE, 3-dehydroecdysone; MaA, makisterone A (24Me20E); 14dE, 14-deoxyecdysone. Reprinted from Gäde *et al.* (1997). *Physiol. Rev.*, p. 997, with permission.

## III. ANATOMY OF INSECT ENDOCRINE SYSTEM: ORGANS AND PRODUCTS

### A. Prothoracic Glands

The prothoracic glands produce ecdysteroids, steroid hormones with multiple functions, the most notable of which is to promote the growth that precedes a molt. Consequently, ecdysteroids are often referred to as molting hormones and the endocrine organs that produce them are ecdysial glands. Ecdysteroid is the general term for the several forms found in insects and other invertebrates (Fig. 2). 20-Hydroxyecdysone is the effective form in most insects; in some insects, however, the active form is the homologue, 24-methyl-20-hydroxyecdysone, known as makisterone A (e.g., the Heteroptera, bugs).

Prothoracic glands are paired glands derived from lateral ectoderm in the head region. Their final position and form vary among different orders of insects. Generally, they become located ventrally in the prothorax associated with a tracheal branch. Most often the form is ribbon-shaped (Fig. 3A), but in Lepidoptera and Hymenoptera (e.g., moths and bees), they are diffuse strings of cells (Fig. 3B). These extended shapes make them difficult or impossible to remove with certainty. In the higher Diptera (e.g., flies), the lateral parts of the ring gland that encircles the heart consist of cells that produce ecdysteroid (Fig. 3C).

The prothoracic glands are composed of endocrine epithelial cells of one type enclosed in an extracellular sheath of fibrous material. Volume and organellar composition of the cells change with their cycles of hormone synthesis. The most conspicuous features of the cells that are actively secreting hormone are the processes on the cell surface (Fig. 4), lobulated nuclei, abundant rough endoplasmic reticulum, followed by an abundant smooth endoplasmic reticulum, and large mitochondria characteristic of the steroid-producing cells of vertebrates. The cells do not store hormone but secrete it as it is produced.

Nerve fibers, some of which contain peptidergic secretory vesicles, enter the glands and there is some evidence of stimulation and inhibition of hormone synthesis by this route. However, the principal tropic hormone is a neurohormone, prothoracicotropic hormone (PTTH), produced by neurosecretory cells in the brain and released into the hemolymph from the retrocerebral complex, organs behind the brain, the corpora cardiaca and the corpora allata (see Fig. 6A and Section IV, B1a). Evidence for neuropeptide inhibition of ecdysteroid synthesis has been confirmed recently by the identification of two different inhibitory peptides. In blowflies, a factor that inhibits ecdysteroid synthesis by the prothoracic gland was isolated from both the larval nervous system and the adult ovaries. This is a hexapeptide that also inhibits trypsin-like enzymes in the digestive tract (see Section III, D1). An entirely different

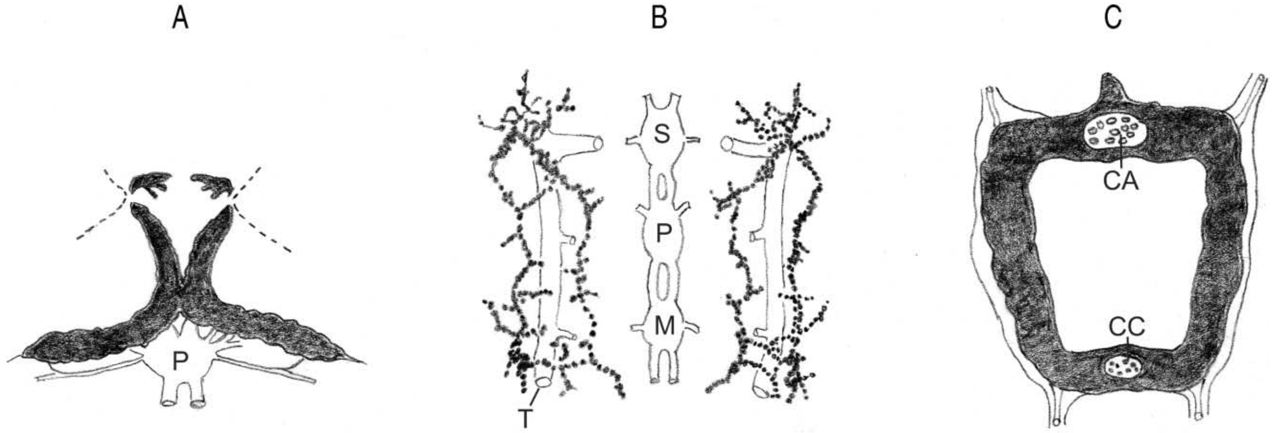

**FIGURE 3** Representative forms of prothoracic glands (black structures). (A) solid ribbon-type from a cockroach, (B) diffuse-type from a moth, (C) ring gland from a fly surrounds the heart. S, subesophageal ganglion; P, prothoracic ganglion; M, mesothoracic ganglion; T, trachea; CA, corpus allatum; CC, corpus cardiacum. Modified from Beaulaton, J. (1990). Anatomy, histology, ultrastructure, and functions of the prothoracic (or ecdysial glands) in insects. *In* "Morphogenetic Hormones of Arthropods" (A. P. Gupta, ed.), Vol. 1, Fig. 7.2, p. 352. Rutgers University Press, with permission.

peptide, identified in *Manduca sexta*, similar to vertebrate galanins also acts as a prothoracicostatic neurohormone. It was first discovered as an inhibitor of muscle contraction. In locusts, this peptide appears to be released from the retrocerebral complex.

As with other endocrine glands, many factors interact to modulate the activity of the prothoracic glands, including juvenile hormone and feedback from ecdysteroid. In adult insects that no longer molt, the prothoracic glands degenerate. This is a programmed cell death that may take one to several days or weeks depending on the insect. The cells involute asynchronously. In the process, many lysosomes and autophagic vacuoles containing cellular organelles

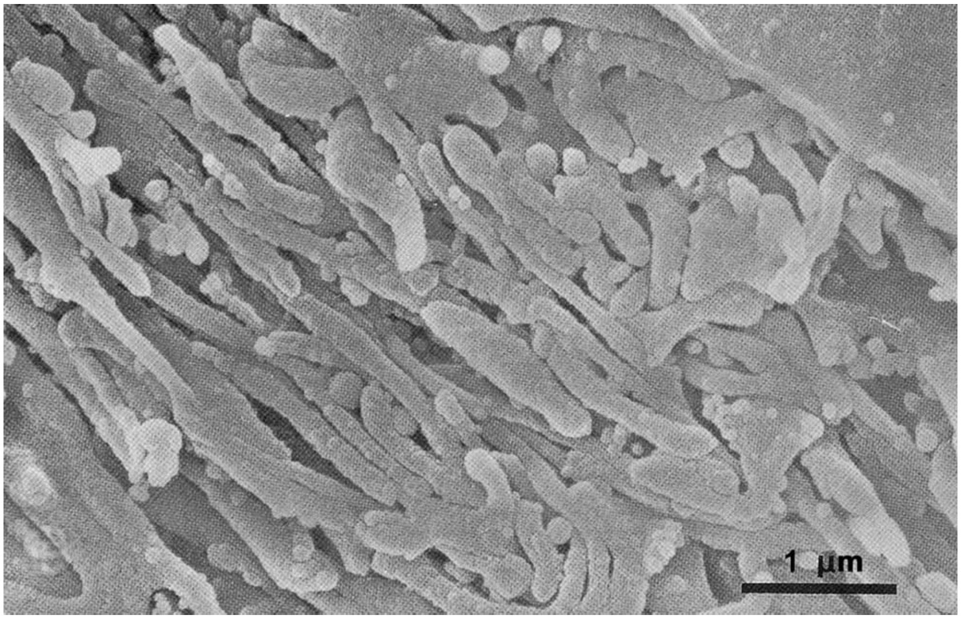

**FIGURE 4** Surface of a portion of a prothoracic gland from a larval moth (*Calpodes*) shows the interdigitation of gland cell processes. Scanning electron micrograph. Reprinted from Locke, M. (1998). Reticular system and intercellular lymph spaces. *In* "Microscopic Anatomy of Invertebrates," (F. W. Harrison and M. Locke, eds.), Vol. 11A, Fig. 15, p. 16. This material is used by permission of John Wiley & Sons, Inc.

are present, and in late stages of degeneration, nuclei become dense and cells fragment within the sheath. In the adult insects, ecdysteroids are again produced by other organs (see Section III, D and F).

## B. Corpora Allata

The corpora allata (CA) produce juvenile hormone (JH), a lipophilic sesquiterpenoid that, like the ecdysteroids, has multiple functions but, as the name of the hormone implies, it plays a major role in morphogenesis. Its presence maintains the immature form and its absence allows adult development to proceed. In most insects, juvenile hormone also regulates reproductive functions. In the 1930s, V. B. Wigglesworth was the first to experimentally demonstrate these functions in the blood-sucking bug *Rhodnius prolixus*, which requires a blood meal to grow and to produce eggs. In most insects, JH is required for the synthesis of vitellogenin (yolk protein) by the fat body and its uptake by oocytes as well as for accessory gland secretions. As with ecdysteroids, there is a family of juvenile hormones, although one juvenile hormone, JH III, is produced in most species (Fig. 5).

The CA are paired glands that arise from lateral ventral ectoderm in the head near the origin of mandibles and maxillae. The small cellular masses migrate dorsally and centrally and come to rest just behind the brain in various positions, some more ventral than others, depending on the insect order. In the more highly evolved orders, the glands are more dorsal and rest on top of the esophagus under the dorsal vessel (Fig. 6A). In some insects, the pair fuse into a single medial gland (Fig. 6B). In the ring gland of the higher Diptera, the fused CA are on the dorsal side of the ring (Fig. 3C).

The CA are innervated by nerves from the brain and subesophageal ganglion in most insects. The nerve fibers from the brain come from cell bodies in clusters of neurosecretory cells in the medial and lateral parts of the protocerebrum and in the tritocerebrum. These axons exit the brain in nerves called corpora cardiacal nerves (NCC) because they pass through and, in some cases, branch in the paired neurohemal release organs called the corpora cardiaca (CC). The NCC I originates in the contralateral medial protocerebrum, NCC II originates in the ipsilateral lateral protocerebrum, and NCC III originates in the tritocerebrum (Fig. 6A). There are also branches of these nerves that enter the stomatogastric nervous system. Together, this assemblage of nerves and the CC and CA are known as the retrocerebral complex (Fig. 6A).

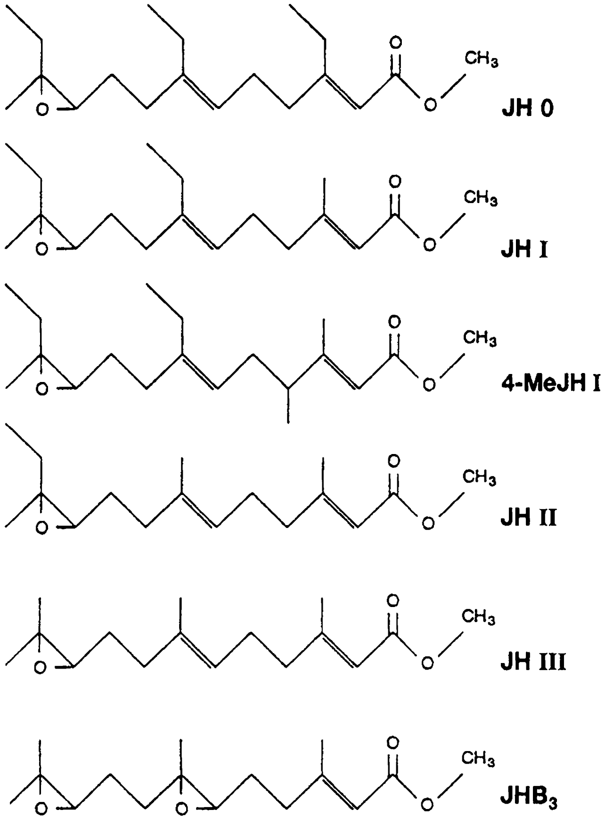

FIGURE 5 Members of the juvenile hormone family. JH 0, I, II, and III, juvenile hormones 0–III; 4-MeJHI, 4-methyl JH I; $JHB_3$, JH III bisepoxide. Reprinted from Gäde *et al.* (1997). *Physiol. Rev.*, p. 986, with permission.

The CA are generally oval in shape and are a single cell type surrounded by an extracellular sheath. Although the description of cell structure varies in different orders of insects, it is clear that cycles of hormone production are accompanied by changes in the morphology of the cells. As with ecdysteroids, the JH is released as it is produced. Increased hormone synthesis is correlated with an increase in plasma membrane projections from the cell surfaces, increase in mitochondrial number and size, and elaboration of much smooth endoplasmic reticulum, characteristics also seen in prothoracic glands. Though not true in all cycles of synthesis of JH, generally the cytoplasmic volume of cells increases as hormone production increases. Involution of the cytoplasmic organelles accompanies low levels of synthesis and cell numbers may decrease. Unlike prothoracic glands, the corpora allata persist in the adult and their hormone assumes new functions such as stimulation of vitellogenin

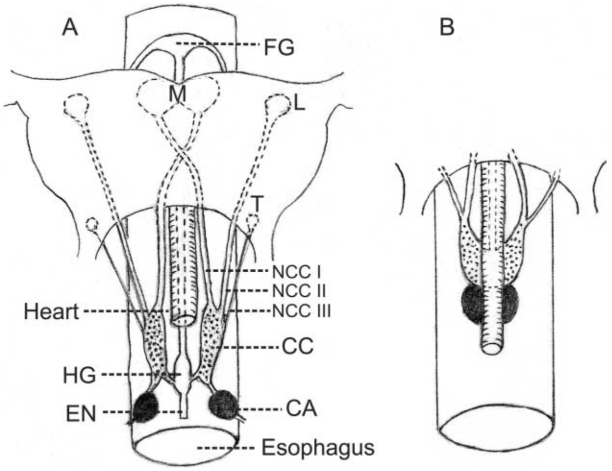

**FIGURE 6** Diagram of two forms of corpora allata (black structures). (A) Paired glands in relationship to the corpora cardiaca (CC), the nerves entering the corpora cardiaca (NCC I, II, and III), and the location in the brain of the soma that give rise to these nerves: medial neurosecretory cells (M), lateral neurosecretory cells (L) in the protocerebrum, and tritocerebral cells (T). FG, frontal ganglion; HG, hypocerebral ganglion; EN, esophageal nerve. (B) Single corpus allatum (fused pair) positioned centrally under the heart. Modified from Cassier, P. (1998). The corpora allata. *In* "Microscopic Anatomy of Invertebrates" (F. W. Harrison and M. Locke, eds.), Vol. 11C, p. 1043. This material is used by permission of John Wiley & Sons, Inc.

synthesis by the fat body and incorporation of this protein into maturing oocytes.

The regulation of hormone production by the CA is undoubtedly multifaceted. A principal pathway occurs through the neurosecretory cell innervation of the glands. Axons in NCC I, II, and probably III enter and arborize in the CA. Their neurosecretory products are released and act at close range (paracrine neurosecretion). In a few insects, the identity and effect of some of these secretions are known. Both allatotropin and allatostatin peptides have been identified for the tobacco hornworm *M. sexta* and related species of insects. A family of allatostatic peptides has been identified that inhibit JH synthesis quickly and reversibly by the CA of crickets and cockroaches following application to the glands in an *in vitro* radiochemical assay. Another family of peptides related to the prothoracicostatic peptide of *M. sexta* was found to inhibit JH synthesis by cricket CA. These peptides act quickly and reversibly *in vitro*. An as yet unidentified neuropeptide inactivates JH synthesis more slowly and for longer periods of time in some species. These peptides may represent only some of several other regulators of CA activity that have yet to be isolated and identified. It is known that JH production by cockroach CA responds indirectly to concentrations of both JH and ecdysteroids in the hemolymph.

## C. Epitracheal Glands

A series of three-celled glands occur attached to the ventral surface of a tracheal branch near the spiracles (Fig. 7). Their function in the larvae of the moth *M. sexta* was discovered in 1996 by Dutsan Zitnan and colleagues, who observed that the one large opalescent cell (termed the Inka cell) of these glands released its contents prior to onset of the behavior associated with shedding of the exoskeleton (ecdysis behavior). Three peptide products of the cell have been identified: preecdysis-triggering hormone (PETH, an 11-amino-acid amidated peptide), ecdysis-triggering hormone (ETH, a 26-amino-acid amidated peptide), and a larger nonamidated ETH-associated peptide. PETH and ETH are part of an intricate cascade of humoral and neurohumoral interactions that lead to successful ecdysis. Eclosion hormone, produced in the brain (released into the blood as well as in the ventral nerve cord), and crustacean cardioactive peptide in cells of the ventral ganglia are part of this cascade. Changing concentrations of ecdysteroid in the blood also condition the production and release of the components of the cascade. Such a cascade is likely to be common to ecdysis in all insects. The gene for both ETH and PETH has been cloned in *D. melanogaster* as well as in *M. sexta*.

## D. Gonads

### 1. *Ovary*

The endocrine function of the insect ovary was not realized until 1974–1975, when D. A. Slaeger and colleagues showed that the ovary was the source of ecdysteroid in mosquito blood, and H. H. Hagedorn and colleagues demonstrated that this ecdysteroid stimulated production of vitellogenin, the precursor of yolk protein, by the fat body. It is known that ovaries of many kinds of insects produce ecdysteroids and that the ovarian ecdysteroids govern processes other than vitellogenin synthesis, e.g., in *Rhodnius*, the ovarian ecdysteroid elicits the release of a brain myotropic factor required for ovulation. In some insects, it has been shown that the source of the ecdysteroid is the follicle cells surrounding the oocytes. In insects in which ecdysteroids are not required for vitellogenin synthesis, the ecdysteroid is transferred from follicle cells to the egg, usually at the

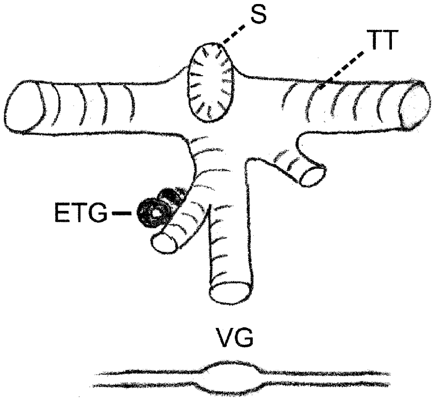

**FIGURE 7** Three cells (Inka cell is the largest) of the epitracheal gland (ETG) on the ventral surface of a tracheal branch above the ventral ganglia near each spiracle (S). VG, ventral ganglion; TT, tracheal trunk. Based on Kingan *et al.* (1997). *J. Exp. Biol.* **200**, 3245–3256 and Truman (1996) *Science* **271**, 40–41.

close of vitellogenesis, and is stored in inactive form for activation at appropriate stages of embryogenesis. Thus, indirectly, the ovary is acting as an endocrine source for embryonic development.

In addition to production of a steroid hormone, the ovary has been demonstrated to produce peptide hormones that play a role in reproductive cycles. In mosquitoes and flies, an ovarian peptide inhibits trypsin synthesis by the gut, thus preventing the digestion of protein required for vitellogenin synthesis. This peptide is called trypsin-modulating oostatic factor (TMOF). The amino acid sequence of TMOF in flies differs from that in mosquitoes, but both affect trypsin synthesis by the gut. The fly TMOF is also an inhibitor of ecdysteroid production by larval and pupal prothoracic glands of flies. Yet another peptide from the fly ovary inhibits JH synthesis by the CA. No doubt other endocrine factors produced by the ovary have yet to be discovered.

As is true of other endocrine glands, the nervous system plays a crucial role in regulating the endocrine activity of the ovary. In mosquitoes, an identified brain neurosecretion of the insulin family known as egg development neurosecretory hormone stimulates the ovary to produce ecdysteroid. Thus, it acts as an ovarian ecdysiotropin. There is evidence that such brain–ovarian axes occur in other insects, although the peptides differ (e.g., ovary-maturing parsin of locusts). In crickets, a brain peptide (of the $XWX_6W_9$ amide family) that also inhibits JH synthesis inhibits *in vitro* production of ecdysteroid by the cricket ovary.

### 2. *Testis*

Endocrine function has also been shown for the testis. The sheath of the larval and pupal testes in *Heliothis* and *Lymantria virescens dispar* (moths) produces ecdysteroid. This in turn promotes production of other growth factors that stimulate growth of the male genital tract. The hormonal activity of the testis is initiated by a peptide produced by the brain. This testis ecdysiotropin is a 21-amino-acid peptide and it differs from the ovarian and prothoracic gland ecdysiotropins. However, the production of ecdysteroid by testes appears also to be influenced by the circulating ecdysteroid concentration. Thus, as in the ovary, there is a web of interactions regulating the hormonal activity of the testis.

## E. Digestive Tract

In vertebrates, a large and important component of the endocrine system is the sum of the cells distributed individually or in groups along the epithelial lining of the stomach and intestine. Many of these gastrointestinal hormones are also found in the central and peripheral nervous system. A parallel localization of nervous system–gut hormones occurs in insects. The endocrine gut cells of insects are similar in structure to vertebrate gut cells and are found in the midgut, the portion of the alimentary canal that also functions in digestion and absorption (Fig. 8). The midgut lies between the foregut and the hindgut. Unlike the latter two parts, the midgut cells do not produce an apical cuticle. Rather, there is usually a delicate detached membrane that protects the epithelial lining, which is

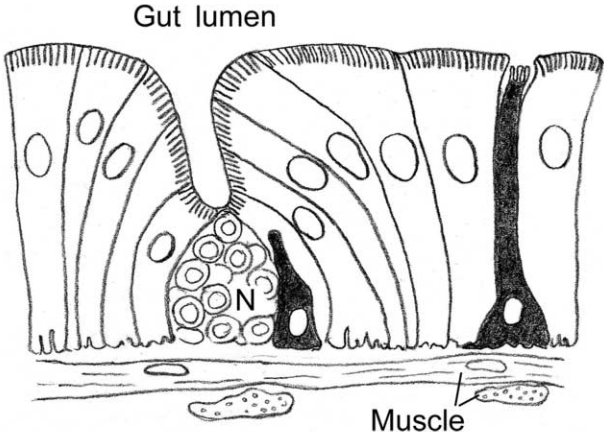

**FIGURE 8** Diagram of a portion of a cross section of midgut. Endocrine cells are black. One lies near a nidus (N), a cluster of regenerative cells; the other lies between the columnar epithelial cells of the midgut.

typically composed of columnar or cuboidal cells with microvilli at their apical surfaces. At the base of these digestive cells are clumps of regenerative cells (nidi) that give rise to new exocrine cells as well as the endocrine cells that become individually dispersed in the epithelial lining (Fig. 8). The extracellular material at the base of the epithelial cells (basal lamina) is continuous with that of the muscle layers surrounding the midgut, a latticework of inner circular and outer longitudinal muscles. The endocrine cells vary in size and shape but usually they are small compared to the exocrine cells. All endocrine cells contact the basal lamina of the epithelium and extend long thin 'necks' toward the lumen of the gut. Some extend all the way to the surface and have microvillar projections at their apical surfaces; some extend only part way to the inner surface (Fig. 8). The endocrine cells may be distributed throughout the midgut but are often found to be more numerous in the posterior part of the midgut.

At the electron microscope level of resolution, these cells exhibit structures consistent with endocrine function. They contain electron-dense membrane-bound vesicles that on occasion have been seen in the process of releasing their contents at the basal surface of the cell. That these vesicles contain humoral factors was confirmed by their immunoreactivity with antibodies against several different neurohormones that also occur in the nervous system. Several of these neurohormones are known to be modulators of muscle function, for example, the tetrapeptide phenylalanine–methionine–arginine–phenyalanine amide (FMRF amide) and other members of this family occur in midgut endocrine cells and in the nerves to the gut in representatives of most insect orders. This peptide is also found in the CNS. Many vertebrate gut hormones have been demonstrated in insect gut by immunocytochemistry, which indicates at least some structural similarity between gut hormones of insects and those of vertebrates. Little is known about how these hormones function in the insects. However, changes in hormone content of the endocrine cells in insects subjected to different conditions of feeding and starvation suggest that they play some role in nutritional state. Experimental studies on gut tissue in short-term culture indicate that some of the hormones may stimulate digestive enzyme secretion and some may act on the muscles surrounding the midgut. The innervation of the midgut muscle layer is far less extensive than that of hind- and foregut. Much remains to be learned about the function of these gut endocrine cells.

### F. Oenocytes and Blood Cells

Oenocytes are large cells that arise in the abdominal epidermis and remain there in some insects but in others they become associated with the fat body or remain in isolated groups. The oenocytes are associated primarily with cuticle formation but they have also been shown to produce ecdysteroids in several insect species. Little is known about the importance of this endocrine function of oenocytes.

A class of granular blood cells in the cockroach, *Diploptera punctata*, was found to synthesize allatostatin, the neuropeptide that inhibits JH synthesis and muscle contraction. The physiological role of the peptide occurring in these cells has not been investigated. There are very likely other neuropeptides that remain to be described in granular blood cells.

## IV. NEUROSECRETORY SYSTEM

### A. Discovery of Neurohormones and Their Abundance

The hormones produced by the nervous system far outnumber those produced by endocrine glands proper and these neurosecretions are involved in the regulation of all aspects of insect biology. The first experimental evidence for a humoral function of the nervous system in any animal was shown in insects. In 1917, Stefan Kopeć demonstrated that the brain of a European silk moth larva was necessary for metamorphosis and that the influence was not through nerves but through the blood. In the 1940s and 1950s, special neurosecretory cells of the nervous system were visualized with specific stains in both vertebrates and invertebrates, including insects, by Ernst and Berta Scharrer. Through the use of several neurosecretory stains, the distribution of different types of neurosecretory cells in the CNS and in specialized release sites were described in many different insects. It was also recognized that in some instances these cells and their release sites could be recognized in living insects because the intracellular granules containing the secretory material scatter light to produce a blue opalescence, known as the Tyndall effect. It was not until 1975, when methods became available for purification of small amounts of material, that the chemical structure of the first insect neuropeptide, the pentapeptide proctolin, was described. Since then, hundreds of neuropeptides have been identified. Antibodies to these identified neurosecretory substances have provided for localization of the nerve cell bodies (soma) producing specific neuropeptides and the projections of their processes. The morphology of neurosecretory cells may be very complex in their ramifications in both the CNS and the periphery.

Some of the neurosecretory cells release their product into the blood from organized release sites that form conspicuous organs, whereas others release their product from varicosities in the axons that project to various organs from which they could be released into the blood or could act locally (paracrine release). Other neurosecretory cells possess axons projecting only within the CNS, suggesting that their secretions act on other nerve cells. Many of the individual neuropeptides have been shown to occur in numerous cells in different parts of the nervous system and release their products in these different ways. Also, the same neuropeptide may occur in cells other than nerve cells (e.g., endocrine cells of the gut and blood cells). Such diverse localization suggests multiple functions for a single type of neuropeptide and indeed this has been shown for many of them. It is also now known that a single cell may produce more than one neuropeptide.

## B. Anatomy of the Neurosecretory System

### 1. *Central Nervous System*

**a. Brain–Retrocerebral Complex**. The brain is a major source of neurohormones. Neurosecretory cells of the brain send axons posteriorly to adjacent paired organs, the CC and the CA. The CC are the major release sites for brain neuropeptides but the CA also serve this function in some species. Together, this part of the neurosecretory system is known as the brain–retrocerebral complex (Fig. 6A). The CC–CA usually lie under the heart and dorsal or lateral to the esophagus. Branches of the axons from the neurosecretory cells of the brain also extend from the CC onto the heart, which also acts as a neurohemal release site. Nerves from the CC also extend into the hypocerebral ganglion and onto the esophagus (part of the stomatogastric nervous system; see Section IV, B2).

In the first segment of the brain, the protocerebrum, there are two conspicuous bilateral groups of neurosecretory cells: a large medial group located at the junction of the two cerebral lobes and a smaller lateral group. Dendrites of these cells have extensive branches within the brain. The third brain segment, the tritocerebrum, also has conspicuous neurosecretory cells. These groups send axons to the corpora cardiaca in nerves called corpus cardiacum nerves NCC I, II, and III, as was described for the innervation of the corpora allata (Section III, B).

The corpora cardiaca are release sites not only for cerebral neurosecretory cells but also for neurosecretions from intrinsic cells (i.e., cell bodies within the CC). The intrinsic cells arise during embryonic development from the esophageal epithelium. In some orders of insects, the intrinsic cells and axon terminals of cerebral cells are intermixed, whereas in others, these compartments are separate lobes, called secretory and storage lobes, respectively. A major product of intrinsic cells is adipokinetic or hyperglycemic hormone, which promotes the release of lipid or carbohydrate, respectively, from the fat body. The storage lobes release many different neurohormones.

The corpora allata have neurosecretory axons in their surrounding sheath and also axons that branch between CA cells. These axons originate in both medial and lateral neurosecretory cells of the brain. The neurosecretory material is released from both the surface nerves and those within the CA. Some of this neurosecretory material has been identified and shown to stimulate JH synthesis (allatotropin) or inhibit JH synthesis (allatostatins). In cockroaches, allatostatin circulating in the hemolymph can inhibit JH production but paracrine release from nerve terminals within the CA is more effective. Surprisingly, in the tobacco hornworm, prothoracicotropic hormone is released from axons that enter the CA, but whether this peptide also affects JH production has not been demonstrated. In this insect, a small form of prothoracicotropic hormone (known as bombyxin in species in which it does not stimulate ecdysteroid production by the prothoracic glands) is released from the nerves surrounding the CA.

**b. Ventral Nerve Cord and Perivisceral Organs**. The ventral nerve cord consists of the subesophageal ganglion in the head, connected to the brain by circumesophageal connectives, and a chain of ganglia in the thoracic and abdominal segments interconnected by paired nerves. Some of the many neurosecretory cells of the subesophageal ganglion release secretions in the retrocerebral complex and others release secretions in more peripheral locations in the head. The release sites for the neurosecretions of the thoracic and abdominal ganglia are in the well-known perivisceral organs (also called perisympathetic organs), which have been described for many insect species. The most conspicuous perivisceral organs occur on the transverse branches of the medial nerves (Fig. 9). Generally, these are dense oval structures consisting of axon terminals of neurosecretory cells of the ventral ganglia. Smaller and less

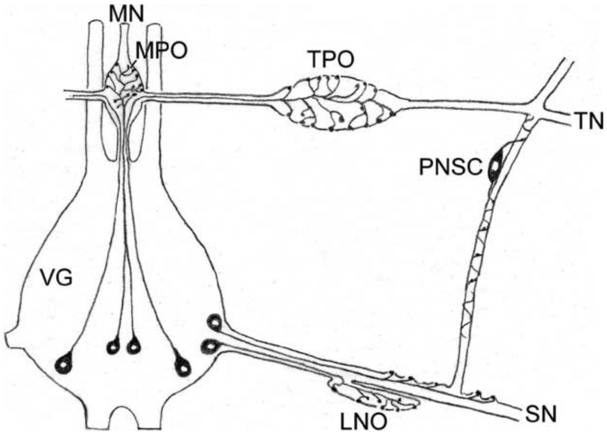

**FIGURE 9** Perisympathetic organs and other neurohemal release sites. Neurosecretory cells (black) in a ventral ganglion (VG) send axons in the medial nerve (MN) to neurohemal release sites in the medial perisympathetic organ (MPO) and to the transverse perisympathetic organ (TPO) along the transverse nerve (TN). Lateral neurohemal organs (LNO) and swellings on the nerve sheath are release sites for neurosecretory cells projecting axons into somatic nerves (SN). Peripheral neurosecretory cells (PNSC) and their release sites are on the surface of nerves. Modified from Schooneveld, H. (1998). Neurosecretion. *In* "Microscopic Anatomy of Invertebrates" (F.W. Harrison and M. Locke, eds.), Vol. 11B, Fig. 18, p. 481. This material is used by permission of John Wiley & Sons, Inc.

conspicuous neurohemal organs occur on the medial nerve proper and on somatic nerves leaving the ganglia (Fig. 9). The transverse nerves continue beyond the perivisceral neurohemal organs with branches to the spiracles and to the nerves that extend along the dorsal heart.

**c. Diffuse Release Sites.** Immunostaining with antibodies against specific neurosecretions has revealed that the perivisceral organs are not the exclusive release areas of the neurosecretory cells of the ventral ganglia. Axons from some of these cells emerge from the peripheral nerves and form networks in the sheath surrounding the nerve. The blebbed or varicose morphology of these networks suggests that they release their product into the hemolymph and thus may constitute a large, diffuse neurohemal area (Fig. 9). In the larvae and pupae of the tobacco hornworm, axons of brain cells that produce eclosion hormone (one of a cascade of hormones involved in molting) travel to the last ganglion of the abdomen and release their hormone into the hemolymph from the surface of the nerve to the hindgut.

**d. Targeted Release.** Many neuropeptides produced by neurosecretory cells of the ventral ganglia that have been demonstrated to circulate in the hemolymph may also function in a paracrine manner; that is, they affect the target organ at close range. For example, nerves carrying myomodulatory peptides innervate the muscles of the gut and probably act locally. As already mentioned, paracrine release of allatostatins is the most effective in inhibition of the production of juvenile hormone.

### 2. *Stomatogastric Nervous System*

In addition to the CNS, there are a series of small ganglia of soma and their interconnecting nerves that are associated with the anterior alimentary canal. Some of the system is illustrated in Figs. 1 and 6A. The frontal ganglion is connected to the tritocerebrum of the brain and in turn to the hypocerebral ganglion near the corpora cardiaca and corpora allata. The esophageal nerve extends between the hypocerebral ganglion and the ingluvial ganglion at the posterior end of the esophagus. Thus, these nerves serve largely the foregut, though some extend over the midgut. This stomatogastric system is interconnected with the CNS. Many different neurosecretory substances have been demonstrated by immunostaining in the nerve cells of this system, and the varicosities of their axon branches on musculature of the anterior gut indicate that neurosecretory material is released from these sites.

### 3. *Peripheral Neurosecretory Cells*

Neurosecretory cells (cell bodies and axons) that lie outside of the CNS are distributed in numerous places in the insect body. They are found on the foregut, on specific nerves where they may penetrate into the nerve and send axons toward the CNS or form an extensive network on the surface of the nerve (Figs. 9 and 10).These individual cells may also constitute a significant area for the release of neurohormones.

### 4. *Identified Neurohormones and Their Functions*

As mentioned in Section IV (Neurosecretory System), many neurohormones affect more than one type of target tissue and their effects on these targets differ. For example, in cockroaches, allatostatins that inhibit JH synthesis by the CA also inhibit gut muscle contraction, and their occurrence in interneurons suggests that they also act as neuromodulators. A second example of a multifunctional neurohormone is the insect kinin family of diuretic hormones that were originally described as stimulators of muscle contraction and later shown to have diuretic activity. In addition, myomodulators may stimulate one type of muscle and inhibit a different type.

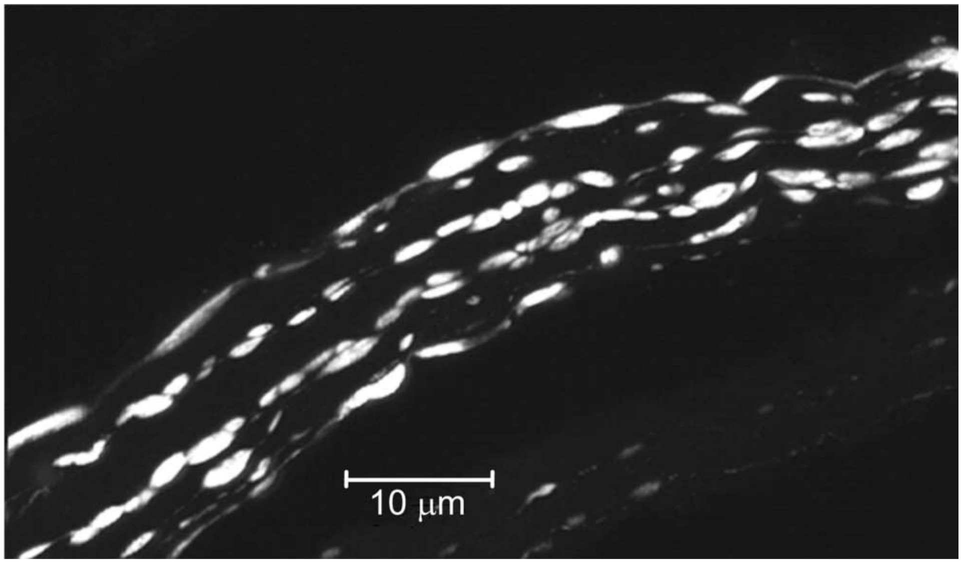

**FIGURE 10** Axons with neurohemal release sites (blebs) of peripheral neurosecretory cells on a somatic abdominal nerve of an unfed larval *Rhodnius prolixus*. The immunofluorescence indicates the presence of leucokinin. Micrograph courtesy of Victoria TeBrugge; antibody against leucokinin I, a myokinin, from D. R. Nassel.

Another generality about neurohormones and their functions is the variability of cross-species effectiveness of a neuropeptide family. Some have conserved sequence structures (at least in part, presumably that part required for biological activity) and maintain the same function in distantly related orders of insects. For example, adipokinetic/hypertrehalosemic hormones, members of the red pigment-concentrating hormone family of crustaceans, have been isolated from more than 30 species from a wide variety of insect orders and found to have a reasonably well-conserved peptide sequence and to play similar roles in mobilization of energy reserves. On the other hand, a neuropeptide family such as the cockroach allatostatins are known to act on CA only in cockroaches and crickets and not in more distantly related insects such as flies, bees, and moths, although these insects produce related peptides that act as inhibitors of muscle contraction, a function also found in crickets and cockroaches.

Reference to the names and functions of several neuropeptides has been made in the description of the anatomy of the neurosecretory system. Table 1 presents some of the major neurosecretory hormones and a presumed principal function, but many of these hormones have multiple functions.

## V. INTEGRATION OF ENDOCRINE FUNCTIONS

The nervous and endocrine systems are functionally integrated in insects as they are in vertebrates. The foregoing description of neurohormones that regulate endocrine glands and neurohormones that directly affect the physiology of other organs indicates clearly that the CNS controls the release of neurohormones and hormones and thus the neurohormonal and endocrine regulation of development, physiology, and behavior of insects. Sensory information that the brain receives from both the external and the internal environments is then integrated to influence this neuroendocrine regulation. For example, environmental conditions, such as temperature and day length, and internal conditions, such as body weight, nutritional state, and juvenile hormone titer, may determine the timing of prothoracicotropic hormone production by the brain that stimulates the production of ecdysteroid required for the next molt.

The tissues themselves also modulate the effect of the hormones through the regulation of receptor abundance, because the response of tissues depends on the presence of receptors for these messengers. Receptor presence and abundance are time- and stage-dependent so that tissues are responsive only when receptors are present. For example, prothoracic glands may be sensitive to PTTH only at specific times, presumably because the receptors are present during sensitive periods and not during the insensitive periods. In addition, different tissues can respond differently to the same hormone and thus multiple functions can be regulated by a single hormone. For example, a single neuropeptide (diuretic hormone) increases urine output by the Malpighian tubules

TABLE 1 Representative Neurohormones and Their Functions

| Type | Hormone | Source | Target | Action |
|---|---|---|---|---|
| **Metabolism** | Adipokinetic hormone | Intrinsic CC cells | Fat body | Lipid mobilization |
| | Hypertrehalosemic hormone | Intrinsic CC cells | Fat body | Carbohydrate mobilization |
| **Ion and water balance** | Diuretic hormones | | | |
| | (a) Corticotropin-releasing hormone-like family | CNS | Malpighian tubules | Urine production (ion transport; $H_2O$ movement into gut) |
| | (b) Kinins | CNS | Malpighian tubules | Urine production (ion transport; $H_2O$ movement into gut) |
| | Anti-diuretic hormones | | | |
| | (a) Neuroparsin | Intrinsic CC cells | Hindgut | Resorption of solutes and water |
| | (b) Ion transport protein | Brain | Hindgut, anterior part | Resorption of solutes and water |
| **Metamorphosis and reproduction** | Prothoracicotropic hormone | Brain | Prothoracic glands | Stimulation of ecdysteroid synthesis |
| | Prothoracicostatic hormone | Brain | Prothoracic glands | Inhibition of ecdysteroid synthesis |
| | Allatotropins | Brain | Corpora allata | Stimulation of JH synthesis |
| | Allatostatins | Brain | Corpora allata | Inhibition of JH synthesis |
| | Eclosion hormone | Brain | Inka cells | Stimulation of ecdysis-triggering hormone |
| | | | Ventral nerve cord | Initiation of motor pattern of ecdysis |
| | Bursicon | Thoracic ganglia | Epidermal cells | Secretion of cuticle tanning agents |
| | Pheromone biosynthesis-activating neuropeptide | Subesophageal and ventral ganglia | Pheromone-secreting gland | Production of sex phermone |
| | Egg development neurosecretory hormone | Brain | Ovary | Stimulation of ecdysteroid synthesis |
| | Ovary-maturing parsin | Brain | Ovary | Stimulation of egg growth |
| | Cricket allatostatin/ecdysiosuppressin | Brain | Ovary | Inhibition of ecdysteroid synthesis |
| | Testis ecdysiotropin | Brain | Testis | Stimulation of ecdysteroid synthesis |
| | Diapause hormone | Subesophageal ganglion | Oocytes | Diapause in embryos |
| **Myomodulatory** | Proctolin | Ventral nerve cord | Visceral and body muscles | Stimulation |
| | Corazonin | Brain, CC | Heart muscle | Stimulation |
| | Cardioactive peptide | Ventral nerve cord | Heart muscle | Stimulation |
| | Kinins | | | |
| | Myokinins; sulfakinins; pyrokinins; tachykinins; periviscerokinins | CNS | Visceral muscle | Stimulation |
| | Myosuppressin and other FLRF amides | CNS | Visceral muscle | Inhibition |

*Note*. CC, corpora cardiaca; CNS, central nervous system; FLRF, phenylalanine–leucine–arginine–phenylalanine; JH, juvenile hormone.

and decreases the output in the hindgut (i.e., acts as an anti-diuretic hormone). Neurohormones can also act synergistically with other neurohormones. For example, a locust diuretic hormone and kinin act to promote more urine formation when administered together than when administered separately (synergism).

A single neuropeptide may be found in neurosecretory cells that are widely dispersed in the CNS, in peripheral nerve cells, and in endocrine cells of the midgut. Such a peptide may be produced at different times in development or as a consequence of different physiological states. These spatial and temporal differences in occurrence of neuropeptides, as well as their limited local action, could provide for local regulation of many different physiological, developmental, and behavioral functions by a single neuropeptide.

## Glossary

**ecdysteroid** A major insect hormone family (a group of related steroids produced by the prothoracic gland primarily) necessary for molting (ecdysis) and growth between stages. In some insects, ecdysteroids regulate reproductive functions and are produced by gonads.

**hemolymph** The fluid that moves in the open circulatory system of insects (i.e., a heart that is open to the body cavity or hemocoel). Also referred to as blood.

**juvenile hormone** A major insect hormone family (a group of similar sesquiterpenoids produced by corpora allata) necessary for maintaining immature characteristics in development and, in most insects, necessary for reproductive functions.

**larva** An immature stage of the life cycle. Nymph is also a larval stage, usually referring to immature stages of insects with incomplete metamorphosis.

**metamorphosis** Changes in form from the immature to the sexually mature stage of the life cycle. The change varies in degree with evolutionary lineage of the orders from primitive to more highly evolved: very little change (ametabolous); some change (incomplete or hemimetabolous); extreme change (complete or holometabolous).

**molting** The process of shedding the exoskeleton between developmental stages. This process is also called ecdysis or eclosion.

**neurohemal organs** Sites of neuronal terminals specialized for release of neurohormones into the hemolymph (circulating body fluid).

**neurosecretions** Peptides, produced by cells of the nervous system, that regulate numerous functions. Some act locally, diffusing to target organs through local extracellular space (paracrine), and others circulate in the body fluid to act at distant target organs (neuroendocrine or humoral).

## See Also the Following Articles

**Crustacean Endocrine Systems • Ecdysone Action in Insect Development • Ecdysteroid Action in Insect Development • Ecdysteroidogenic Pathway • Insect Gut as an Endocrine Organ • Juvenile Hormone Action in Insect Development • Juvenile Hormone Action in Insect Reproduction**

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# Insect Gut as an Endocrine Organ

Mark R. Brown
*University of Georgia*

I. THE INSECT GUT
II. DISTRIBUTION AND MORPHOLOGY OF MIDGUT ENDOCRINE CELLS
III. IDENTIFIED CHEMICAL MESSENGERS
IV. FUNCTION

**The endocrine system of insects has both glandular and "diffuse" components that secrete diverse chemical messengers into the open circulatory system. The major endocrine glands of insects include the corpora allata, prothoracic glands, gonads, and epitracheal glands (Inka cells). The "diffuse" endocrine component exists as tens to thousands of solitary endocrine cells distributed throughout the midgut, the primary organ for digestion, detoxification, and absorption of food in insects. These cells have essentially the same structure as gut endocrine cells in vertebrates, and in both groups of animals, the gut endocrine system is a primary source of numerous peptide hormones and biogenic amines also found in the nervous system. Past studies of the midgut endocrine**

**system in insects focused on cell structure and distribution. More recently, specific peptide hormones originating from this system have been identified, and their roles in the regulation of satiety, digestion, and nutrient absorption and metabolism are being explored.**

## I. THE INSECT GUT

In insects, the alimentary tract consists of a foregut, midgut, and hindgut, and in all life stages, it forms a contiguous sensory and functional interface between the insect and its food. In a generalized insect, the mouth and esophagus empty into the foregut, which differentiates from the embryonic ectoderm, and is covered by a thin protective cuticle on the luminal side. In this gut region, food passes over chemosensory elements en route to the midgut and may be diverted into one or more storage organs. The midgut is formed from anterior and posterior invaginations of embryonic endoderm and, when developed, begins in the thorax at the cardia, where the foregut and midgut join, and ends at the junction of the Malpighian tubules and hindgut. In the immature stages of some insects, the anterior midgut is differentiated into several pouches or gastric ceca. In those insects undergoing metamorphosis in the pupal stage, the larval gut is re-formed into a very different adult gut. The hindgut of ectodermal origin also has an inner cuticular layer and functions to resorb water and other nutrients and to excrete undigested food. Blind-ended Malpighian tubules extend from the junction of the midgut and hindgut into the body cavity. Tubule cells move waste products with water from the hemolymph to the lumen and into the hindgut for excretion. The musculature of the foregut and anterior midgut is innervated by the stomatogastric nervous system, a chain of ganglia connected to the brain and other glands, and the posterior midgut and hindgut by axons from the last two abdominal ganglia of the central nervous system.

The midgut is a tubular monolayer of polarized epithelial cells that is differentiated into several regions. Columnar cells are the largest and most numerous cell type and extend from the outer basal lamina bathed by the open circulatory system to the lumen where food resides. These cells secrete digestive and detoxifying enzymes and facilitate transport of nutrients and ions into the hemolymph. Small endocrine cells and regenerative cells are scattered among these cells. In addition, "goblet" cells specializing in ion transport are present in the midgut of some insect orders. A peritrophic matrix lines the interior of the midgut and is secreted either by the whole epithelium or by specialized cells in the cardia. Air tube cells, circular and longitudinal muscles, and nerve axons intermingle outside the basal lamina of the epithelium.

## II. DISTRIBUTION AND MORPHOLOGY OF MIDGUT ENDOCRINE CELLS

Electron microscopy studies in the early 1970s led to the discovery and first brief descriptions of "clear cells" in the midgut of the silkworm and other insects. Their ultrastructural organization is the same as that of gut endocrine cells in mammals, also first described as clear cells. Only a few studies have described in detail the ultrastructure and distribution of midgut endocrine cells in specific insects, and most reports on midgut ultrastructure simply note the presence of such cells.

In general, endocrine cells are dispersed as solitary cells throughout the midgut, including the cardia and hindgut junction. The cells are positioned basally in the epithelium and are much smaller than the surrounding columnar cells (Fig. 1A). Most endocrine cells have a cone or flask shape due to an apical extension to the midgut lumen that is capped with microvilli and often are termed "open cells" (Fig. 1B). Occasionally, basal extensions are observed on these cells. Other endocrine cells lack an apical extension, as confirmed by serial-section microscopy, and are known as "closed" cells. Notably, both cell types vary in the electron density of the cytoplasm and are seen as clear or dark cells in electron micrographs; the significance of this cytoplasmic condition is not known.

The frequent pairing of endocrine cells and regenerative cells in insect midguts has been noted. Regenerative cells are thought to be stem cells and, depending on the insect group, either form nests or are scattered in the epithelium. These cells typically have a dark cytoplasm and lack secretory granules and apical extensions. In cockroaches, nests of regenerative cells were the first and only ones in the adult male midgut labeled with [$^3$H]thymidine by 7 days postinjection, but by 14 to 21 days, both columnar cells and endocrine cells were labeled. These observations suggest that endocrine cells differentiate from regenerative cells and may even release factors that affect the division and differentiation of regenerative cells into other cell types.

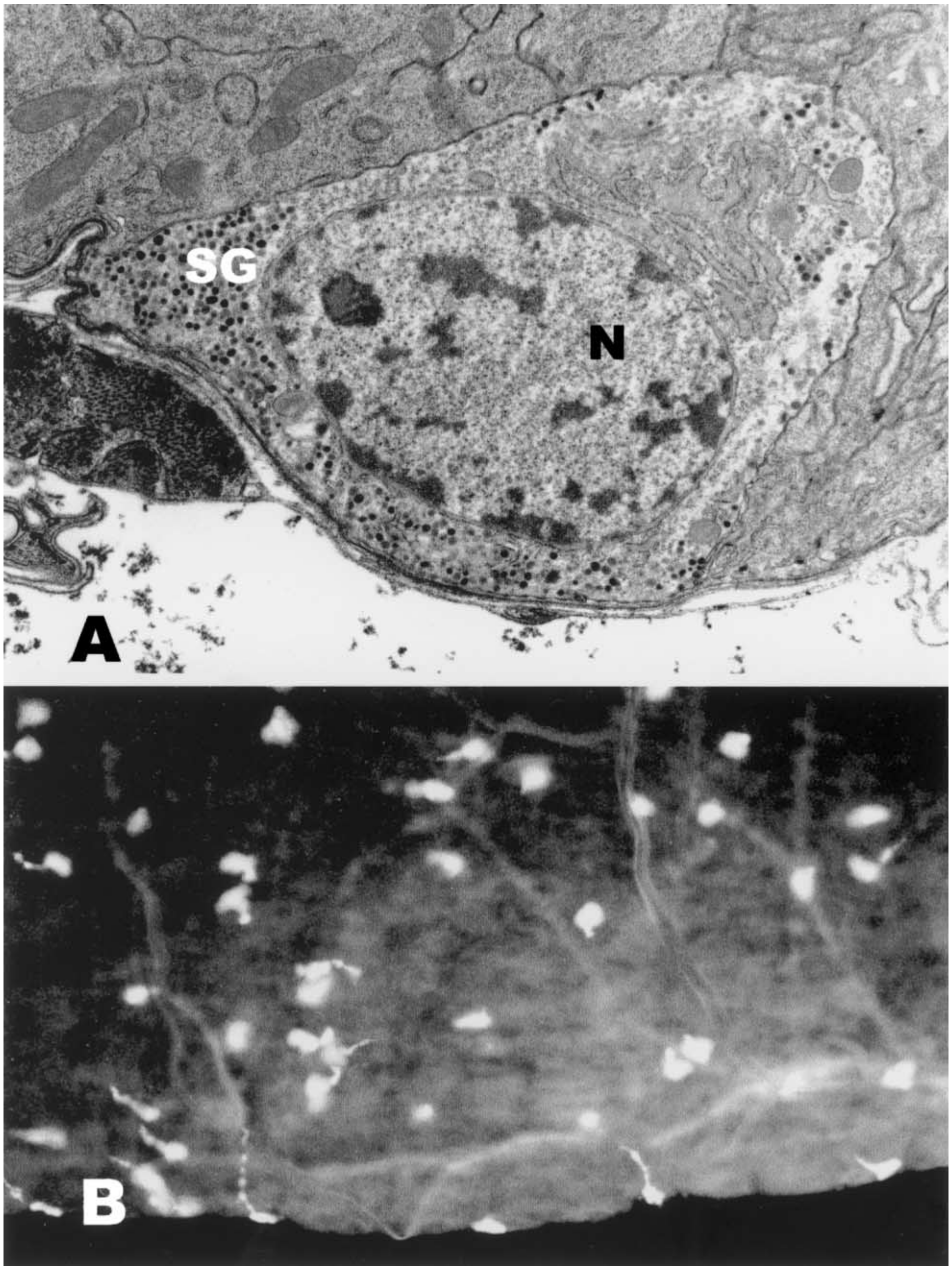

FIGURE 1 (A) Electron micrograph of an endocrine cell positioned basally in the midgut of a female mosquito. N, nucleus; SG, secretory granules. (B) Numerous endocrine cells immunostained with an antiserum to *Drosophila* neuropeptide F and located in the posterior region of a female mosquito midgut. Note stained apical extensions of the endocrine cells on the rim of the midgut.

## A. Formation and Release of Secretory Granules

The most definitive characteristics of midgut endocrine cells are the presence of membrane bounded secretory granules in the basolateral cytoplasm and the absence of a basal membrane labyrinth. In contrast, surrounding digestive cells have only a few such granules and extensive enfolding of the basal membrane. Among the endocrine cells described for insects, secretory granules range in size from 60 to 800 nm and in number from a few to hundreds in a cell (Fig. 1A). In addition, different types of granules have been observed in the endocrine cells of a particular species, but each cell contains only one type. These granule characteristics have been used to classify endocrine cells in the midgut of a few insects, and the different cell types were confined to specific midgut regions.

The ultrastructure of midgut endocrine cells is similar to other cells known to secrete peptide hormones. Peptide precursors or prohormones are transcribed from mRNA into the rough endoplasmic reticulum, after which the precursors move to the Golgi complexes for enzymatic processing and packaging in secretory granules. As demonstrated for midgut endocrine cells, these granules contain peptides, and their contents are released into the extracellular space for diffusion to neighboring cells and into the hemolymph for transport to other target tissues.

### B. Lamellar Bodies

Lamellar bodies (up to 1 μm in diameter) often are present in the apical and lateral regions of midgut endocrine cells. In female mosquitoes, these organelles contain a few secretory granules and lack acid phosphatase, which is present in the degradative organelles (lysosomes) of adjacent digestive cells. Comparable lamellar bodies have been reported for neurosecretory cells in other invertebrate and vertebrate animals and are thought to play a role in the recycling of secretory granules during periods of active secretion.

## III. IDENTIFIED CHEMICAL MESSENGERS

Comparative endocrinology has elucidated two trends in peptide hormone signaling common to insects and vertebrate animals. First, the structure and function of peptide hormones, their processing and degrading enzymes, and their receptors are conserved to a surprising degree. Second, cells that secrete these messengers are found primarily in the nervous system and gut. In both animal groups, discoveries supporting these trends largely were made with immunoassays that detect putative peptide hormones based on their antigenically similar amino acid sequences. Immunocytochemistry stains midgut cells that secrete the peptide of interest (Fig. 1B); thus, the distribution and number of cells and effects of food ingestion or starvation can be determined. Radioimmunoassays or enzyme-linked immunoassays allow for the identification and quantification of related peptides in tissue extracts and are used to monitor the purification of peptides.

Immunocytochemistry most often is used to identify putative peptide hormones secreted by midgut endocrine cells in diverse insects and can be a first step toward the structural and functional characterization of native peptides. Most studies have focused on vertebrate-like peptide hormones because their antisera are readily available. The list of vertebrate peptides found in midgut endocrine cells includes pancreatic polypeptide (PP), enkephalin, endorphin, gastrin, cholecystokinin, corticotropin-releasing factor/urotensin, insulin, and tachykinins. Surprisingly, only one report has shown that the biogenic amine, serotonin, exists in the midgut endocrine system of an insect. Endocrine cells, as well, contain insect-specific peptides, such as allatostatin (an inhibitor of juvenile hormone synthesis by cockroach corpora allata), and different peptides having Arg-Phe-amide (RFa) at the carboxyl-terminus, e.g., myosuppressin. Many studies found that peptide RFa- and PP-like immunostaining is colocalized in the same midgut endocrine cells and the stomatogastric nervous system. These results may be due in part to the fact that RFa and the Arg-Tyr-amide on the PP of higher vertebrates are highly antigenic epitopes and common to many other peptides found in invertebrate and vertebrate animals. Thus, antisera generated to a particular peptide with either of these carboxyl-termini likely recognize unrelated peptides with only this epitope in common. More revealing is the localization of peptide mRNA in midgut cells by *in situ* hybridization. This has been accomplished for neuropeptide F, the invertebrate orthologue of PP, in the midgut of *Drosophila* larvae and for myosuppressin and allatostatin in cockroach midgut.

Although the biological activity of midgut extracts was first reported in the late 1940s and later in the 1960s, there is to date only one report of a midgut peptide that was purified based solely on its bioactivity. The myotropic peptide was isolated from an extract of midguts of tobacco hornworm larvae, and its sequence of five amino acids has little similarity to vertebrate and invertebrate peptides. Allatostatins, myosuppressin, and tachykinins were first isolated from the insect nervous system and later from midgut extracts as immunoreactive peptides that have identical sequences. As synthetic peptides, allatostatins and myosuppressin inhibit muscle contractions of guts and other tissues from a variety of insects, and both peptides stimulate invertase and amylase activity in the lumen of cockroach midguts. Release of tachykinins from midguts has been demonstrated, and these peptides stimulate muscle contractions on many insect tissues.

In all higher vertebrate animals, insulin is produced only in a specialized gut region, and there are several reports on the extraction and purification of insulin-like factors from midguts of insects. Both the immunoreactivity and the bioactivity of these factors have been demonstrated, but to date, insulin peptides have been isolated only from the brains of silk moths and locusts, and they are similarly expressed in *Drosophila*.

At this time, a listing of insect peptide hormones found only in the midgut or nervous system or common to both organs would be highly speculative because it would be derived largely from immunocytochemical studies, which often examine one tissue but not the other of a particular insect and use loosely specific antisera. In the near future, exploration of the genomic and expressed sequence databases for *Drosophila* and other insects likely will produce a reasonably complete list of genes encoding peptide messengers. Only when the tissue and developmental expression of these genes is known will their function as midgut hormones, neuropeptides, or both be defined.

## IV. FUNCTION

Although the regulatory dimensions of the midgut endocrine system have yet to be revealed, a review of the studies to date shows it to be a ubiquitous source of diverse peptide hormones in insects. Identification of additional midgut peptide hormones will be relatively easy in comparison to defining their regulatory roles. Future studies will need to encompass screening for activity in diverse bioassays, receptor identification and tissue localization, and effects of peptide or receptor gene "knockouts" and "overexpression" to identify functions for midgut peptides. Most importantly, these peptides cannot be considered hormones until their release into the hemolymph is shown to occur prior to or accompany the regulated physiological process.

Conceptually, the diffuse distribution of endocrine cells in the midgut likely is an important factor in the system's ability to regulate digestive, absorptive, and metabolic processes in insects. Food in the midgut lumen is "outside" the insect body; thus, endocrine cells with extensions to the lumen would act as "sensory/effector" cells to monitor the quality and quantity of food throughout the midgut. Food molecules or the absence of food would be detected by receptors on the microvilli or by uptake into the cells. Intracellular mediation of these external signals would in turn block or stimulate peptide secretion by endocrine cells. The released peptide hormones may have paracrine effects on neighboring digestive cells that would respond to a titered signal by releasing enzymes in the amount required to digest the food present. Then, as the food was depleted by digestion and absorption, other endocrine cells would signal the muscles to move the food around or out of the midgut. Other gut-specific processes may be regulated, as indicated by the clustering of endocrine cells in the junction of the midgut, hindgut, and Malpighian tubules of locusts that are thought to sense and alter fluid content and flow in the lumen of these tissues relative to hemolymph. Midgut peptide hormones likely have endocrine effects on the fat body, the primary organ of nutrient metabolism and storage, and other tissues by signaling food quality and quantity or its absence. In addition, interactions between the nervous system and midgut endocrine system may coordinate digestive and excretory processes with feeding behavior.

## Glossary

**endocrine cells** Small cells scattered basally in the midgut epithelium that secrete peptide hormones.

**immunocytochemistry** A microscopy technique that identifies cells containing peptides or proteins recognized by specific antibodies, which are labeled.

***in situ* hybridization** A microscopy technique that identifies cells containing messenger RNA for specific peptides or proteins by hybridization to labeled complementary nucleotide probes.

**midgut** The organ for digestion and absorption of food in insects that also has important roles in water balance, nutrient metabolism, and detoxification.

**radioimmunoassay** A solution-based assay for the competition and separation of unlabeled and radioactive isotope-labeled molecules bound to specific antibodies that allows for the identification and quantification of the molecules in tissue extracts and chromatography fractions.

## See Also the Following Articles

**Crustacean Endocrine Systems • *Drosophila* Neuropeptides • Insect Endocrine System**

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# Insulin Actions

Ralph A. DeFronzo and Lawrence J. Mandarino
*University of Texas Health Science Center, San Antonio*

I. INSULIN RESISTANCE AND TYPE 2 DIABETES
II. SITES OF INSULIN ACTIONS AND RESISTANCE
III. CELLULAR MECHANISMS OF INSULIN

**Type 2 diabetes mellitus is characterized by moderate to severe insulin resistance and impaired beta cell function. When the accelerated rate of insulin secretion no longer can compensate for the severity of insulin resistance, overt diabetes ensues. In type 2 diabetic individuals, there is an increase in hepatic glucose production that correlates closely with the elevation in fasting plasma glucose concentration. Muscle and adipose tissue are resistant to insulin and contribute to postprandial hyperglycemia. Impaired insulin signaling (reduced insulin receptor and IRS-1 tyrosinephosphorylation and diminished PI-3 kinase activity and PI-3 kinase activity with IRS-1), decreased glucose transport and phosphorylation, and diminished glycogen synthase all contribute to be insulin resistance in insulin target tissues.**

## I. INSULIN RESISTANCE AND TYPE 2 DIABETES

Both longitudinal and cross-sectional studies have documented conclusively that hyperinsulinemia precedes the development of type 2 diabetes in most ethnic populations with a high incidence of type 2 diabetes. Studies employing the euglycemic insulin clamp, minimal model, and insulin suppression techniques have provided direct quantitative evidence that the progression from normal to impaired glucose tolerance is associated with the development of severe insulin resistance, whereas plasma insulin concentrations, both in the fasting state and in response to a glucose load, are increased (see above discussion on insulin secretion).

Himsworth and Kerr, using a combined oral glucose and intravenous insulin tolerance test, were the first to demonstrate that tissue sensitivity to insulin was diminished in diabetic patients. In 1975, Reaven and colleagues, using the insulin suppression test, found that the ability of insulin to promote tissue glucose uptake in type 2 diabetes was severely reduced. A defect in insulin action in type 2 diabetes also has been demonstrated with limb infusion of insulin using the forearm and leg catheterization techniques, as well as with radioisotope turnover studies, the modified intravenous glucose tolerance test, and the minimal model technique.

DeFronzo *et al.*, using the more physiologic euglycemic insulin clamp technique, have provided the most conclusive documentation that insulin resistance is a characteristic feature of lean type 2 diabetic individuals. Because diabetic patients with severe fasting hyperglycemia (>180–200 mg/dl, 10.0–11.1 mmol/liter) are known to be insulinopenic, and insulin deficiency is associated with the emergence of a number of intracellular defects in insulin action, only diabetics with mild to modest elevations in the fasting plasma glucose concentration (mean = 150 ± 8 mg/dl, 8.3 ± 0.4 mmol/liter) were included in these studies in order to focus on the early stages of the evolution of type 2 diabetes in normal-weight subjects. Insulin-mediated whole-body glucose disposal was reduced by ~40–50% in type 2 diabetic subjects, conclusively demonstrating the presence of moderate to severe insulin resistance. Three additional points are noteworthy: (1) in lean type 2 diabetics with more severe fasting hyperglycemia (198 ± 10 mg/dl), the severity of insulin resistance is only slightly (10–20%) greater than that in diabetic patients with mild fasting hyperglycemia; (2) the defect in insulin action is observed at all plasma insulin concentrations, spanning the physiological and pharmacological range (3) in diabetic patients with overt fasting hyperglycemia, even maximally stimulating plasma insulin concentrations are not capable of eliciting a normal glucose metabolic response under euglycemic conditions. With a few exceptions, the great majority of investigators have demonstrated that lean type 2 diabetic subjects are resistant to the action of insulin. The ability of glucose, i.e., hyperglycemia, to stimulate its own uptake also is impaired in type 2 diabetics.

## II. SITES OF INSULIN ACTIONS AND RESISTANCE

The maintenance of whole-body glucose homeostasis is dependent on a normal insulin secretory response by the pancreatic beta cells and normal tissue sensitivity to the independent effects of hyperinsulinemia and hyperglycemia (i.e., the mass-action effect of glucose) to augment glucose uptake. In turn, the combined effects of insulin and hyperglycemia to promote glucose disposal are dependent on three tightly coupled mechanisms (Table I): (1) suppression of endogenous (primarily hepatic) glucose production; (2) stimulation of glucose uptake by the splanchnic (hepatic plus gastrointestinal) tissues; and (3) stimulation of glucose uptake by peripheral tissues, primarily muscle. Muscle glucose uptake is regulated by flux through two major metabolic pathways: glycolysis (~90% of which represents glucose oxidation) and glycogen synthesis.

### A. Hepatic Glucose Production

In the overnight fasted state, the liver of healthy subjects produces glucose at the rate of ~1.8–2.0 mg·kg$^{-1}$·min$^{-1}$. This glucose flux is essential to meet the needs of the brain and other neural tissues, which utilize glucose at a constant rate of ~1–1.2 mg·kg$^{-1}$·min$^{-1}$. Brain glucose uptake accounts for ~50–60% of glucose disposal during the postabsorptive state and this uptake is insulin independent. Therefore, brain glucose uptake occurs at the same rate during absorptive and postabsorptive periods and is not altered in type 2 diabetes. Following glucose ingestion, insulin is secreted into the portal vein and carried to the liver, where it suppresses hepatic glucose output. If the liver does not perceive this insulin signal and continues to produce glucose, there will be two inputs of glucose into the body, one from the liver and another from the gastrointestinal tract, and marked hyperglycemia will ensue.

In type 2 diabetic subjects with mild to moderate fasting hyperglycemia (140–200 mg/dl, 7.8–11.1 mmol/liter), basal hepatic glucose production (HGP) is increased by ~0.5 mg/kg·min (Fig. 1). Consequently, during the overnight sleeping hours (2200 h to 0800 h), the liver of an 80 kg diabetic individual with modest fasting hyperglycemia adds an additional 30 g of glucose to the systemic circulation. The increase in basal HGP is closely correlated with the severity of fasting hyperglycemia (Fig. 1). Thus, in type 2 diabetics with overt fasting hyperglycemia (>140 mg/dl, 7.8 mmol/liter), an excessive rate of hepatic glucose output is the major abnormality

**TABLE I Factors Responsible for the Maintenance of Normal Glucose Tolerance in Healthy Subjects**

Insulin secretion
Tissue glucose uptake
  Peripheral (primarily muscle)
  Splanchnic (liver plus gut)
Suppression of HGP
  Decreased FFA
  Decreased glucagon
Route of glucose adminstration

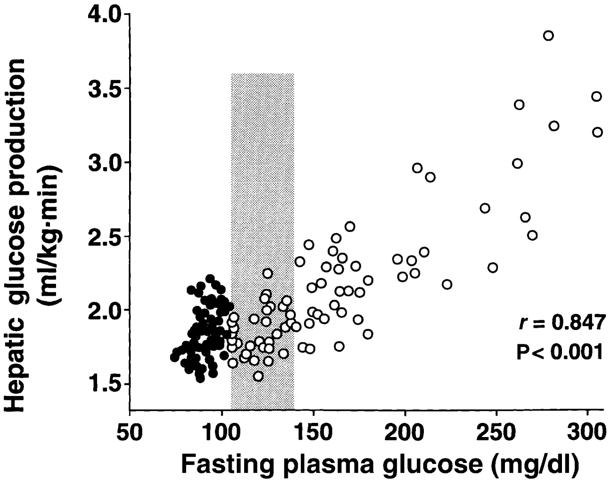

FIGURE 1 Summary of HGP in 77 normal-weight type 2 diabetic subjects (○) with fasting plasma glucose concentrations ranging from 105 to >300 mg/dl; 72 control subjects matched for age and weight are shown by solid circles. In the 33 diabetic subjects with fasting plasma glucose levels <140 mg/dl (shaded area), the mean rate of HGP was identical to that of control subjects. In diabetic subjects with fasting plasma glucose concentrations >140 mg/dl, there was a progressive rise in HGP that correlated closely ($r = 0.847$, $P < 0.001$) with the fasting plasma glucose concentration. Reprinted from DeFronzo *et al.* (1989), *Metabolism* **38**, 387–395, with permission.

responsible for the elevated fasting plasma glucose concentration. The close relationship between fasting plasma glucose concentration and HGP has been demonstrated in numerous studies.

In the postabsorptive state, the fasting plasma insulin concentration in type 2 diabetics is two- to fourfold greater than in nondiabetic subjects. Because hyperinsulinemia is a potent inhibitor of HGP, hepatic resistance to the action of insulin must be present in the postabsorptive state to explain the excessive output of glucose by the liver. Hyperglycemia per se also exerts a powerful suppressive action on HGP. Therefore, the liver also must be glucose resistant with respect to the inhibitory effect of hyperglycemia to suppress hepatic glucose output, and this has been well documented.

Using the euglycemic insulin clamp technique in combination with tritiated glucose, the dose–response relationship between hepatic glucose production and the plasma glucose concentration has been defined. The following points should be emphasized: (1) the dose–response curve relating inhibition of HGP to the plasma insulin concentration is quite steep, with an effective dose for half-maximal insulin concentration ($ED_{50}$) of ~30–40 μU/ml; (2) in type 2 diabetic individuals the dose–response curve is shifted to the right, indicating the presence of hepatic resistance to the inhibitory effect of insulin on hepatic glucose production. However, at plasma insulin concentrations within the high physiologic range (~100 μU/ml), the hepatic insulin resistance can be largely overcome and a near normal suppression of HGP can be achieved; and (3) the severity of the hepatic insulin resistance is related to the severity of the diabetic state. In type 2 diabetic individuals with mild fasting hyperglycemia, an increment in plasma insulin concentration of 100 μU/ml causes a complete suppression of HPG. However, in diabetic subjects with more severe fasting hyperglycemia, the ability of the same plasma insulin concentration to suppress HGP is impaired. These results suggest that there is an acquired component of hepatic insulin resistance and that this defect becomes progressively worse as the diabetic state decompensates over time.

The glucose released by the liver can be derived from either glycogenolysis or gluconeogenesis. Studies employing the hepatic vein catheter technique have shown that the uptake of gluconeogenic precursors, especially lactate, is increased in type 2 diabetic subjects. Consistent with this observation, radioisotope turnover studies, using lactate, alanine, and glycerol, have shown that ~90% of the increase in HGP above baseline can be accounted for by accelerated gluconeogenesis. More recent studies employing $^{13}C$ magnetic resonance imaging (MRI) and $D_2O$ have confirmed the important contribution of accelerated gluconeogenesis to the increase in HGP. An increased rate of glutamine conversion to glucose also has been shown to contribute to the elevated rate of gluconeogenesis in type 2 diabetic subjects. The mechanisms responsible for the increase in hepatic gluconeogenesis include hyperglucagonemia, increased circulating levels of gluconeogenic precursors (lactate, alanine, glycerol), increased FFA oxidation, enhanced sensitivity to glucagon, and decreased sensitivity to insulin. Although the majority of evidence indicates that increased gluconeogenesis is the major cause of the increase in HGP in type 2 diabetic subjects, it is likely that accelerated glycogenolysis also contributes.

Because of the inaccessibility of the liver in human, it has been difficult to assess the role of key enzymes involved in the regulation of gluconeogenesis [pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK)], glycogenolysis (glycogen phosphorylase), and net hepatic glucose output [glucose-6-phosphatase (G-6-Pase)]. However, considerable evidence from animal models of type 2 diabetes and some evidence in humans have implicated increased activity of PEPCK and G-6-Pase in the accelerated rate of hepatic glucose production.

The kidney also has been shown to produce glucose and estimates of the renal contribution to total endogenous glucose production have varied from 5 to 20%. These varying estimates of the contribution of renal gluconeogenesis to total glucose production are largely related to the methodology employed to measure glucose production by the kidney. One unconfirmed study suggests that the rate of renal gluconeogenesis is increased in type 2 diabetics with fasting hyperglycemia. Arguing against this possibility are studies employing the hepatic vein catheter technique that have shown that the entire increase in total-body endogenous glucose production (measured with [3-$^3$H]glucose) in type 2 diabetics can be accounted for by increased hepatic glucose output (measured by the hepatic vein catheter technique).

## B. Peripheral (Muscle) Glucose Uptake

Muscle is the major site of glucose disposal in human. Under euglycemic hyperinsulinemic conditions, approximately 80% of total-body glucose uptake occurs in skeletal muscle. Studies employing the euglycemic insulin clamp in combination with femoral artery/vein catheterization have examined the effect of insulin on leg glucose uptake in type 2 diabetic and control subjects. Since bone is metabolically inert and adipose tissue takes up less than 5% of an infused glucose load, muscle represents the major tissue responsible for leg glucose uptake.

In response to a physiologic increase in plasma insulin concentration (~80–100 μU/ml), leg (muscle) glucose uptake increases linearly, reaching a plateau value of 10 mg/kg leg weight per minute. In contrast, in lean type 2 diabetic subjects, the onset of insulin action is delayed for ~40 min and the ability of the hormone to stimulate leg glucose uptake is markedly blunted, even though the study is carried out for an additional 60 min in the type 2 diabetic group to allow insulin to more fully express its biological effects. During the last hour of the insulin clamp study, the rate of total body glucose uptake was reduced by 50% in the diabetic group. These results provide conclusive evidence that the primary site of insulin resistance during euglycemic insulin clamp studies performed in type 2 diabetic subjects is muscle tissue. Using the forearm and leg catheterization techniques, a number of investigators have demonstrated a decreased rate of insulin-mediated glucose uptake by peripheral tissues. The use of positron emission tomography (PET) scanning to quantitate leg glucose uptake in type 2 diabetic subjects has provided additional support for the presence of severe muscle resistance to insulin in diabetic subjects.

## C. Splanchnic (Hepatic) Glucose Uptake

In humans, it is difficult to catheterize the portal vein, and glucose disposal by the liver has not been examined directly. Using the hepatic vein catheterization technique in combination with the euglycemic insulin clamp, the contribution of the splanchnic (liver plus gastrointestinal) tissues to overall glucose homeostasis has been examined in lean type 2 diabetic subjects with mild to moderate fasting hyperglycemia. In the postabsorptive state, there is a net release of glucose from the splanchnic area (i.e., negative balance) in both control and type 2 diabetic subjects, reflecting glucose production by the liver. In response to insulin, splanchnic glucose output is promptly suppressed (reflecting the inhibition of HGP) and, by 20 min, the net glucose balance across the splanchnic region declines to zero (i.e., there was no net uptake or release). After 2 h of sustained hyperinsulinemia, there is a small net uptake of glucose (~0.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) by the splanchnic area (i.e., positive balance). This uptake is virtually identical to the rate of splanchnic glucose uptake observed in the basal state, indicating that the splanchnic tissues, like the brain, are insensitive to insulin at least with respect to the stimulation of glucose uptake. There was no difference between diabetic and control subjects in the amount of glucose taken up by the splanchnic tissues at any time during the insulin clamp study.

The results of these studies illustrate another important point: namely, that under conditions of euglycemic hyperinsulinemia, very little of the infused glucose is taken up by the splanchnic (and therefore hepatic) tissues. During the insulin clamp, the rate of whole-body glucose uptake averaged 7 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, and of this, only 0.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, or 7%, was disposed of by the splanchnic region. Because the difference in insulin-mediated total-body glucose uptake between the type 2 diabetic and control groups during the euglycemic insulin clamp study was 2.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, from a purely quantitative standpoint it is obvious that a defect in splanchnic (hepatic) glucose removal never could account for the magnitude of impairment in total-body glucose uptake following intravenous glucose/insulin administration. However, after glucose ingestion, the oral route of administration and the resultant hyperglycemia combine to enhance splanchnic (hepatic) glucose uptake and, under these conditions, diminished hepatic glucose uptake has been shown to contribute to the impairment in glucose tolerance in type 2 diabetes (see discussion below).

## D. Summary: Whole-Body Glucose Utilization

Insulin-mediated whole-body glucose utilization during the euglycemic insulin clamp is summarized in Fig. 2. The total height of each bar represents the amount of glucose taken up by all tissues in the body during the insulin clamp in control and type 2 diabetic subjects. Net splanchnic glucose uptake, quantitated by the hepatic venous catheterization technique, is similar in both groups and averaged 0.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. Adipose tissue glucose uptake accounts for less than 5% of total glucose disposal. Brain glucose uptake, estimated to be 1.0–1.2 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the postabsorptive state, is unaffected by hyperinsulinemia. Muscle glucose uptake (extrapolated from leg catheterization data) in control subjects accounts for ~75–80% of the total glucose uptake. In type 2 diabetic subjects, the largest part of the impairment in insulin-mediated glucose uptake is accounted for by a defect in muscle

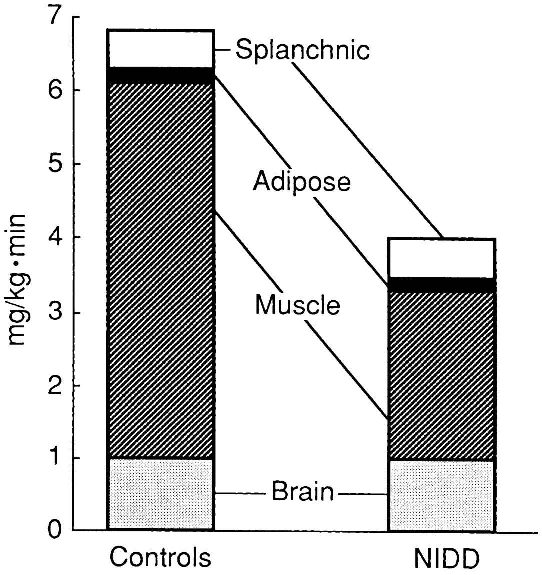

FIGURE 2 Summary of glucose metabolism during euglycemic insulin (100 μU/ml) clamp studies performed in normal-weight type 2 diabetic and control subjects; see text for a more detailed discussion. Reprinted from DeFronzo (1997), with permission.

glucose disposal. Even if adipose tissue of type 2 diabetic subjects took up absolutely no glucose, it could, at best, explain only a small fraction of the defect in whole-body glucose metabolism.

## E. Glucose Disposal During OGTT

In everyday life, the gastrointestinal tract represents the normal route of glucose entry into the body. However, the assessment of tissue glucose disposal following glucose ingestion presents a challenge because of the difficulties in quantitating the rate of glucose absorption, suppression of hepatic glucose production, and organ (liver and muscle) glucose uptake. Moreover, because the plasma glucose and insulin concentrations are changing simultaneously, it is difficult to draw conclusions about insulin secretion or insulin sensitivity.

To address these issues, Ferrannini, DeFronzo, and colleagues administered oral glucose to healthy control subjects in combination with hepatic vein catheterization to examine splanchnic glucose metabolism. The oral glucose load and endogenous glucose pool were labeled with [1-$^{14}$C]glucose and [3-$^{3}$H]glucose, respectively, to quantitate total-body glucose disposal (from tritiated glucose turnover) and endogenous HGP (difference between the total rate of glucose appearance, as measured with tritiated glucose, and the rate of oral glucose appearance, as measured with [1-$^{14}$C]glucose).

During the 3.5 h after glucose (68 g) ingestion: (1) 19 g, or 28%, of the oral load was taken up by splanchnic tissues; (2) 48 g, or 72%, was disposed of by peripheral (nonsplanchnic) tissues; (3) of the 48 g taken up by peripheral tissues, the brain (an insulin-independent tissue) accounted for ~15 g (~1 mg·kg$^{-1}$·min$^{-1}$), or 22%, of the total glucose load; and (4) basal HGP declined by 53%. Similar percentages for splanchnic glucose uptake (24–29%) and suppression of HGP (50–60%) in normal subjects have been reported by other investigators. The contribution of skeletal muscle to the disposal of an oral glucose load has been reported to vary from a low of 26% to a high of 56%, with a mean of 45%. These results emphasize several important differences between oral and intravenous glucose administration. After glucose ingestion: (1) HGP is less completely suppressed, most likely due to activation of local sympathetic nerves that innervate the liver; (2) peripheral tissue (primarily muscle) glucose uptake is quantitatively less important; and (3) splanchnic glucose uptake is quantitatively much more important.

In type 2 diabetic individuals, the disposition of an oral glucose load is significantly altered. The disturbance in glucose metabolism is accounted for by two factors: (1) decreased tissue glucose uptake and (2) impaired suppression of HGP. Splanchnic glucose uptake is similar in diabetic and control groups. Inappropriate suppression of HGP accounted for approximately one-third of the defect in total-body glucose homeostasis, whereas reduced peripheral (muscle) glucose uptake accounted for the remaining two-thirds. Since hyperglycemia per se enhances splanchnic (hepatic) glucose uptake (SGU) in proportion to the increase in plasma glucose concentration, the splanchnic glucose clearance (SGU divided by plasma glucose concentration) is markedly reduced in all type 2 diabetic subjects following glucose ingestion. Using a combined insulin clamp/OGTT technique, an impairment in glucose uptake by the splanchnic tissues in type 2 diabetics has been demonstrated directly.

When viewed in absolute terms, most studies have demonstrated that the total amount of glucose taken up by all tissues of the body over the 4 h period following the ingestion of an oral glucose load is normal or slightly decreased. However, this occurs at the expense of postprandial hyperglycemia. Thus, the efficiency of glucose disposal, i.e., the glucose clearance (tissue glucose uptake divided by plasma glucose concentration), is severely reduced. It should be emphasized that it is not the absolute glucose disposal rate but rather the increment in glucose disposal above baseline that determines the rise in plasma glucose concentration above the fasting value. Every published study has demonstrated that the incremental response in whole-body glucose uptake is moderately to severely reduced in type 2 diabetic individuals. Similar results have been reported for forearm muscle glucose uptake, pointing out the important contribution of diminished muscle glucose disposal to impaired oral glucose tolerance in type 2 diabetes.

In summary, results of the OGTT indicate that both impaired suppression of HGP and decreased tissue (muscle) glucose uptake contribute approximately equally to the glucose intolerance of type 2 diabetes. The efficiency of the splanchnic (hepatic) tissues to take up glucose (as reflected by the splanchnic glucose clearance) also is impaired in type 2 diabetic individuals.

### F. Summary of Insulin Resistance in Type 2 Diabetes

Insulin resistance involving both muscle and liver is a characteristic feature of glucose intolerance in type 2 diabetic individuals. In the basal state, the liver represents a major site of insulin resistance, and this is reflected by an overproduction of glucose despite the presence of both fasting hyperinsulinemia and hyperglycemia. This accelerated rate of hepatic glucose output is the primary determinant of the elevated fasting plasma glucose concentration in type 2 diabetic individuals. Although tissue (muscle) glucose uptake in the postabsorptive state is increased when viewed in absolute terms, the efficiency with which glucose is taken up (i.e., the glucose clearance) is diminished. After glucose infusion or ingestion (i.e., in the insulin-stimulated state), both decreased muscle glucose uptake and impaired suppression of HGP contribute to insulin resistance. Following glucose ingestion, the defects in insulin-mediated glucose uptake by muscle and the suppression of HGP by insulin contribute approximately equally to the disturbance in whole-body glucose homeostasis in type 2 diabetes. However, under euglycemic hyperinsulinemic conditions, HPG is largely suppressed and impaired muscle glucose uptake is primarily responsible for the insulin resistance.

## III. CELLULAR MECHANISMS OF INSULIN

The stimulation of glucose metabolism by insulin requires that the hormone must first bind to specific receptors that are present on the cell surface of all insulin target tissues. After insulin has bound to and activated its receptor, "second messengers" are generated and these second messengers initiate a series of events involving a cascade of phosphorylation–dephosphorylation reactions that eventually result in the stimulation of intracellular glucose metabolism. The initial step in glucose metabolism involves activation of the glucose transport system, leading to an influx of glucose into insulin target tissues, primarily muscle. The free glucose, which has entered the cell, subsequently is metabolized by a series of enzymatic steps that are under the control of insulin. Of these, the most important are glucose phosphorylation (catalyzed by hexokinase), glycogen synthesis (controlled by glycogen synthase), and glycolysis and glucose oxidation (regulated by PFK and PDH, respectively).

### A. Insulin Receptor/Insulin Receptor Tyrosine Kinase

The insulin receptor is a glycoprotein consisting of two α-subunits and two β-subunits linked by disulfide bonds (Fig. 3). The α-subunit of the insulin receptor is entirely extracellular and contains the insulin-binding domain. The β-subunit has an extracellular domain, a transmembrane domain, and an intracellular domain that expresses insulin-stimulated kinase activity directed toward its own tyrosine residues. Insulin

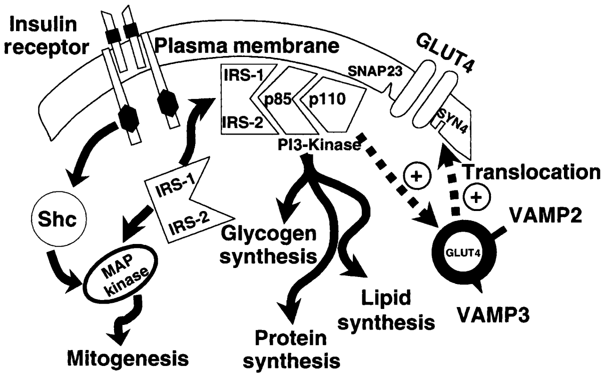

FIGURE 3 Schematic representation of the insulin receptor and the cascade of intracellular signaling molecules that have been implicated in insulin action; see text for a more detailed discussion. SNAP, soluble N-ethylmaleimide sensitive fusion protein attachment protein; VAMP, vesicle-associated membrane protein.

receptor phosphorylation of the β-subunit, with subsequent activation of insulin receptor tyrosine kinase, represents the first step in the action of insulin on glucose metabolism. Mutagenesis experiments have shown that insulin receptors devoid of tyrosine kinase activity are completely ineffective at mediating insulin stimulation of cellular metabolism. Similarly, mutagenesis of any of the three major phosphorylation sites (at residues 1158, 1163, and 1162) impairs insulin receptor kinase activity, resulting in a decrease in the acute metabolic and growth-promoting effects of insulin.

## B. Insulin Receptor Signal Transduction

Following activation, insulin receptor tyrosine kinase phosphorylates specific intracellular proteins, of which at least nine have been identified. Four of these belong to the family of insulin-receptor substrate proteins: IRS-1, IRS-2, IRS-3, and IRS-4 (the others include Shc, Cbl, Gab-1, $p60^{dok}$, and APS). In muscle, IRS-1 serves as the major docking protein that interacts with the insulin receptor tyrosine kinase and undergoes tyrosine phosphorylation in regions containing amino acid sequence motifs (YXXM or YMXM) that, when phosphorylated, serve as recognition sites for proteins containing *src*-homology 2 (SH2) domains (where Y denotes a tyrosine, M denotes a methionine, and X denotes any amino acid). Mutation of these specific tyrosines severely impairs the ability of insulin to stimulate glycogen and DNA synthesis, establishing the important role of IRS-1 in insulin signal transduction. In liver, IRS-2 serves as the primary docking protein that undergoes tyrosine phosphorylation and mediates the effect of insulin on hepatic glucose production, gluconeogenesis, and glycogen formation. In adipocytes, Cbl represents another substrate that is phosphorylated following its interaction with the insulin receptor tyrosine kinase and that is required for stimulation of GLUT4 translocation. Phosphorylation of Cbl occurs when the CAP/Cbl complex associates with flotillin in caveolae, or lipid rafts, containing insulin receptors.

In muscle, the phosphorylated tyrosine residues on IRS-1 mediate an association between the two SH2 domains of the 85 kDa regulatory subunit of PI3-kinase, leading to activation of the enzyme (Fig. 3). PI3-kinase is a heterodimeric enzyme composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The latter catalyzes the 3′ phosphorylation of phosphatidylinositol (PI), PI 4-phosphate, and PI 4,5-diphosphate, resulting in the stimulation of glucose transport. Activation of PI3-kinase by phosphorylated IRS-1 also leads to activation of glycogen synthase, via a process that involves activation of PKB/Akt and subsequent inhibition of kinases such as glycogen synthase kinase-3 (GSK-3) and activation of protein phosphatase 1 (PP1). Inhibitors of PI3-kinase impair glucose transport by interfering with the translocation of GLUT4 transporters from their intracellular location and block the activation of glycogen synthase and HKII expression. The action of insulin to increase protein synthesis and inhibit protein degradation also is mediated by PI3-kinase and involves the activation of mTOR. mTOR controls translation machinery by phosphorylation and activation of p70 ribosomal S6-kinase ($p70^{rsk}$) and phosphorylation of initiation factors. Insulin also promotes hepatic triglyceride synthesis by increasing the transcription factor steroid regulatory element-binding protein-1c and this lipogenic effect of insulin also appears to be mediated via the PI3-kinase pathway.

Other proteins with SH2 domains, including the adapter protein growth factor receptor-binding protein 2 (Grb2) and *Shc*, also interact with IRS-1 and become phosphorylated following exposure to insulin. Grb2 and *Shc* serve to link IRS-1/IRS-2 to the mitogen-activated protein (MAP) kinase signaling pathway (Fig. 3), which plays an important role in the generation of transcription factors. Following the interaction between IRS-1/IRS-2 and Grb2 and *Shc*, Ras is activated, leading to the stepwise activation of Raf, MAP kinase kinase (MEK), and extracellular signal-related kinase (ERK). Activated ERK than translocates into the nucleus of the cell, where it catalyzes the phosphorylation of transcription factors that promote cell growth, proliferation, and differentiation. Blockade of the MAP kinase pathway prevents the stimulation of cell growth by insulin but has no effect on the metabolic actions of the hormone.

Under anabolic conditions, insulin stimulates glycogen synthesis by simultaneously activating glycogen synthase and inhibiting glycogen phosphorylase. The effect of insulin is mediated via the PI3-kinase pathway, which inactivates kinases such as glycogen synthase kinase-3 and activates phosphatases, particularly PP1. It is believed that PP1 is the primary regulator of glycogen metabolism. In skeletal muscle, PP1 associates with a specific glycogen-binding regulatory subunit, causing dephosphorylation (activation) of glycogen synthase. PP1 also phosphorylates (inactivates) glycogen phosphorylase. The precise steps that link insulin receptor tyrosine kinase/PI3-kinase activation to stimulation of PP1 have yet to be defined. Some evidence suggests that p90 ribosomal S6-kinase may be involved in the activation of glycogen synthase. Akt also has been shown to phosphorylate and thus inactivate GSK-3. This decreases glycogen synthase phosphorylation, leading to activation of the enzyme. A number of studies have convincingly demonstrated that inhibitors of PI3-kinase inhibit glycogen synthase activity and abolish glycogen synthesis. From a physiological standpoint, it makes sense that activation of glucose transport and glycogen synthase should be linked to the same signaling mechanism in order to provide a coordinated stimulation of intracellular glucose metabolism.

## C. Insulin Signal Transduction Defects in Type 2 Diabetes

### 1. *Insulin Receptor Number and Affinity*

Both receptor and postreceptor defects have been shown to contribute to insulin resistance in type 2 diabetic individuals. Some but not all studies have demonstrated a modest 20–30% reduction in insulin binding to monocytes and adipocytes from type 2 diabetic patients. This reduction is due to a decreased number of insulin receptors without a change in insulin receptor affinity. In addition to the decreased number of cell-surface receptors, a variety of defects in insulin receptor internalization and processing have been described. However, some caution should be employed in interpreting these studies. Muscle and liver, not adipocytes, represent the major tissues responsible for the regulation of glucose homeostasis *in vivo* and insulin binding to solubilized receptors obtained from skeletal muscle biopsies and liver has been shown to be normal in obese and lean diabetic individuals when expressed per milligram of protein. Moreover, a decrease in insulin receptor number cannot be demonstrated in over half of type 2 diabetic subjects, and it has been difficult to demonstrate a correlation between reduced insulin binding and the severity of insulin resistance. The insulin receptor gene has been sequenced in a large number of type 2 diabetic patients from diverse ethnic populations using denaturing-gradient gel electrophoresis or single-stranded conformational polymorphism analysis, and, with very rare exceptions, physiologically significant mutations in the insulin receptor gene have not been observed. This excludes a structural gene abnormality in the insulin receptor as a cause of common type 2 diabetes mellitus.

### 2. *Insulin Receptor Tyrosine Kinase Activity*

Insulin receptor tyrosine kinase activity has been examined in a variety of cell types (skeletal muscle, adipocytes, hepatocytes, and erythrocytes) from normal-weight and obese diabetic subjects. Most but not all investigators have found reduced tyrosine kinase activity that cannot be explained by alterations in insulin receptor number or insulin receptor binding. However, near normalization of the fasting plasma glucose concentration (by weight loss) has been reported to correct the defect in insulin receptor tyrosine kinase activity. This observation suggests that the defect in tyrosine kinase is acquired and results from some combination of hyperglycemia, defective intracellular glucose metabolism, hyperinsulinemia, and insulin resistance—all of which improved after weight loss. A glucose-induced reduction in insulin receptor tyrosine kinase activity has been demonstrated in rat fibroblast culture *in vitro*. Insulin receptor tyrosine kinase activity assays are performed *in vitro*, and the results of these assays could provide misleading information with regard to insulin receptor function *in vivo*. To circumvent this problem, investigators have employed the euglycemic hyperinsulinemic clamp in combination with muscle biopsies and anti-phosphotyrosine immunoblot analysis. Such an analysis yields a "snapshot" of the insulin-stimulated tyrosine phosphorylation state of the receptor *in vivo*. The results of these studies have demonstrated a substantial decrease in insulin receptor tyrosine phosphorylation in both obese nondiabetic and type 2 diabetic subjects. When insulin-stimulated insulin receptor tyrosine phosphorylation was examined in normal glucose-tolerant or impaired glucose-tolerant individuals at high risk of developing type 2 diabetes, a normal increase in tyrosine phosphorylation of the insulin receptor was observed. These observations are consistent with the concept that impaired insulin receptor tyrosine kinase activity in type 2 diabetic patients is acquired secondarily to hyperglycemia or some other metabolic disturbance.

### 3. *Insulin Signaling (IRS-1 and PI3-Kinase) Defects*

A physiologic increase in the plasma insulin concentration stimulates tyrosine phosphorylation of the insulin receptor and IRS-1 in lean healthy subjects to 150–200% of basal values. In obese nondiabetic subjects, the ability of insulin to activate these two early insulin receptor signaling events in muscle is reduced, whereas in type 2 diabetics insulin has no significant stimulatory effect on either insulin receptor or IRS-1 tyrosine phosphorylation. The association of p85 protein and PI3-kinase activity with IRS-1 also is greatly reduced in obese nondiabetic and type 2 diabetic subjects compared to lean healthy subjects. Insulin also failed to increase the association of the p85 subunit of PI3-kinase with IRS-2 in muscle, indicating that type 2 diabetes is characterized by a combined defect in IRS-1 and IRS-2 function. The decrease in insulin stimulation of the association of the p85 regulatory subunit of PI3-kinase with IRS-1 is closely correlated with the impairment in muscle glycogen synthase activity and *in vivo* insulin-stimulated glucose disposal. Defective regulation of PI3-kinase gene expression by insulin also has been demonstrated in skeletal muscle and adipose tissue of type 2 diabetic subjects. In animal models of diabetes, an 80% decrease in IRS-1 phosphorylation and a greater than 90% reduction in insulin-stimulated PI3-kinase activity have been reported.

In the insulin-resistant, normal glucose-tolerant offspring of two type 2 diabetic parents, IRS-1 tyrosine phosphorylation and the association of p85 protein/PI3-kinase activity with IRS-1 are markedly decreased despite normal tyrosine phosphorylation of the insulin receptor; these insulin signaling defects are correlated closely with the severity of insulin resistance, measured with the euglycemic insulin clamp technique. In summary, a defect in the association of PI3-kinase with IRS-1 and in its subsequent activation appears to be a characteristic abnormality in type 2 diabetics, is closely correlated with *in vivo* muscle insulin resistance, and is unrelated to a disturbance in insulin receptor tyrosine phosphorylation. Several groups have reported that a common mutation in the IRS-1 gene (Gly972Arg) is associated with type 2 diabetes, insulin resistance, and obesity, but the physiologic significance of this mutation remains to be established.

The profound insulin resistance of the PI3-kinase signaling pathway contrasts markedly with the ability of insulin to stimulate MAP kinase pathway activity in insulin-resistant type 2 diabetic and obese nondiabetic individuals. Hyperinsulinemia increases MEK1 activity and ERK1/2 phosphorylation and activity to the same extent in lean healthy controls as in insulin-resistant obese nondiabetic and type 2 diabetic patients. This finding of selective insulin resistance is similar to that recently observed in the vasculature of Zucker fatty rats. Two possible reasons for this difference are alternate insulin signaling pathways and differential signal amplification. With regard to the former, the MAP kinase pathway can be activated through Grb2/Sos interaction with IRS-1/IRS-2 or with Shc. Because IRS-1 tyrosine phosphorylation is dramatically reduced in diabetics, it is possible that insulin activation of the MAP kinase pathway *in vivo* primarily occurs through Shc activation. There is evidence from *in vitro* studies to support this concept. Like ERK and MEK activity, insulin increased Shc phosphorylation to the same extent in lean and obese nondiabetic and type 2 diabetic subjects. These results indicate that, in type 2 diabetes, insulin induces sufficient activation of the insulin receptor tyrosine kinase to increase Shc phosphorylation normally. It also is possible that differential signal amplification in the PI3-kinase and MAP kinase pathways can explain their differing susceptibilities to the effects of insulin resistance.

Maintenance of insulin stimulation of the MAP kinase pathway in the presence of insulin resistance in the PI3-kinase pathway may be important in the development of insulin resistance. ERKs can phosphorylate IRS-1 on serine residues, and serine phosphorylation of IRS-1 and the insulin receptor itself has been implicated in desensitization insulin receptor signaling. Continued ERK activity, when IRS-1 function already is impaired, could lead to a worsening of insulin resistance. Thus, diabetic and obese subjects have inappropriately high MAP kinase activity. One also could postulate that insulin resistance in the metabolic (PI3-kinase) pathway, with its compensatory increase in beta-cell function and hyperinsulinemia, leads to excessive stimulation of the MAP kinase pathway in vascular tissue. This would result in the proliferation of vascular smooth muscle cells, increased collagen formation, and increased production of growth factors and inflammatory cytokines, possibly explaining the accelerated rate of atherosclerosis in type 2 diabetic individuals.

## D. Glucose Transport

Activation of the insulin signal transduction system in insulin target tissues leads to the stimulation of glucose transport. The effect of insulin is brought about by the translocation of a large intracellular pool of glucose transporters (associated with low-density microsomes) to the plasma membrane. There are five major, different facilitative glucose transporters with distinctive tissue distributions (Table II). GLUT4, the insulin regulatable transporter, is found in insulin-sensitive tissues (muscle and adipocytes), has a $K_m$ of ~5 mmol/liter, which is close to that of the plasma glucose concentration, and is associated with HKII. In adipocytes and muscle, its

TABLE II Classification of Glucose Transport and HK Activity According to Their Tissue Distribution and Functional Regulation

| Organ | Glucose transporter | Hexokinase | Classification |
|---|---|---|---|
| Brain | GLUT1 | HKI | Glucose dependent |
| Erythrocyte | GLUT1 | HKI | Glucose dependent |
| Adipocyte | GLUT4 | HKII | Insulin dependent |
| Muscle | GLUT4 | HKII | Insulin dependent |
| Liver | GLUT2 | HKIVL | Glucose sensor |
| GK beta-cell | GLUT2 | HKIVB (glucokinase) | Glucose sensor |
| Gut | GLUT3 symporter | — | Sodium dependent |
| Kidney | GLUT3 symporter | — | Sodium dependent |

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concentration in the plasma membrane increases markedly after exposure to insulin, and this increase is associated with a reciprocal decline in the intracellular GLUT4 pool. GLUT1 represents the predominant glucose transporter in the insulin-independent tissues (brain and erythrocytes), but also is found in muscle and adipocytes. It is located primarily in the plasma membrane, where its concentration changes little after the addition of insulin. It has a low $K_m$ ($\sim 1$ mmol/liter) and is well suited to its function, which is to mediate basal glucose uptake. It is found in association with HKI. GLUT2 predominates in the liver and pancreatic beta cells, where it is found in association with a specific hexokinase, HKIV. In the beta cell, HKIV is referred to as glucokinase. GLUT2 has a high $K_m$ ($\sim 15-20$ mmol/liter) and, as a consequence, the glucose concentration in cells expressing this transporter rises in direct proportion to the increase in plasma glucose concentration. This characteristic allows these cells to respond as glucose sensors. In summary, each tissue has a specific glucose transporter and associated hexokinase, which allow it uniquely to carry out its specialized function to maintain whole-body glucose economy.

Glucose transport activity in type 2 diabetic patients uniformly has been found to be decreased in adipocytes and muscle. In adipocytes from type 2 diabetic humans and rodent models of diabetes, there is a severe reduction in GLUT4 mRNA and protein, and the ability of insulin to elicit a normal translocation response and to activate the GLUT4 transporter after its insertion into the cell membrane is impaired. In contrast, muscle tissue obtained from lean and obese type 2 diabetic subjects exhibits normal or increased levels of GLUT4 mRNA expression and normal levels of GLUT4 protein. Moreover, acute (2–4 h) physiological hyperinsulinemia does not increase the number of GLUT4 transporters in muscle in either healthy or type 2 diabetic subjects. Several studies have demonstrated an increase in muscle GLUT4 mRNA levels in response to insulin in control subjects, but not in diabetics, suggesting insulin resistance at the level of gene transcription. However, the physiological significance of the blunted increase in muscle GLUT4 mRNA levels in type 2 diabetic subjects is unclear, since both basal and insulin-stimulated GLUT4 protein levels are normal. Large populations of type 2 diabetics have been screened for mutations in the GLUT4 gene. Such mutations are very uncommon and, when detected, have been of questionable physiologic significance.

The results summarized above indicate that the gene (GLUT4) encoding the major insulin-responsive glucose transporter and its transcriptional/translational regulation are not impaired in type 2 diabetes. However, in contrast to the normal expression of GLUT4 protein and mRNA in muscle of type 2 diabetic subjects, every study that has examined adipose tissue has reported reduced basal and insulin-stimulated GLUT4 mRNA levels, decreased GLUT4 transporter numbers in all subcellular fractions, diminished GLUT4 translocation, and impaired intrinsic activity of GLUT4. These observations demonstrate that GLUT4 expression in humans is subject to tissue-specific regulation. Although insulin does not increase GLUT4 expression in muscle, it stimulates the translocation of GLUT4 transporters from their intracellular location to the cell membrane. In type 2 diabetic humans, the ability of insulin to stimulate GLUT4 translocation in muscle is impaired. Using a novel triple-tracer technique, the *in vivo* dose–response curve for the action of insulin on glucose transport in forearm skeletal muscle has been examined in nondiabetic and type 2 diabetic subjects. Insulin-stimulated inward muscle glucose transport was severely impaired in type 2 diabetic subjects who were studied under euglycemic conditions. The defect in glucose transport cannot be overcome by repeating the insulin clamp at each subject's normal fasting glucose (hyperglycemia) level. Since the number of GLUT4 transporters in the muscle of diabetic subjects is normal, impaired GLUT4 translocation and decreased intrinsic activity of the glucose transporter must be responsible for the defect in muscle glucose transport. Impaired *in vivo* muscle glucose transport in type 2 diabetics also has been demonstrated using MRI and PET.

## E. Glucose Phosphorylation

Glucose phosphorylation and glucose transport are tightly coupled phenomena. Isoenzymes of hexokinase (HKI–HKIV) catalyze the first committed intracellular step of glucose metabolism, the conversion of glucose to G-6-P (Table II). HKI, HKII, and HKIII are single-chain peptides that have a number of properties in common, including a very high affinity for glucose and product inhibition by G-6-P. HKIV, also called glucokinase, has a lower affinity for glucose and is not inhibited by G-6-P. Glucokinase (HKIVB) is believed to be the glucose sensor in the beta cell, whereas HKIVL plays an important role in the regulation of hepatic glucose metabolism.

In both rat and human skeletal muscle, HKII transcription is regulated by insulin. HKI also is present in human skeletal muscle, but it is not regulated by insulin. In response to physiological euglycemic hyperinsulinemia, HKII cytosolic activity, protein content, and mRNA levels increase by 50–200% in healthy nondiabetic subjects and this is associated with the translocation of hexokinase II from the cytosol to the mitochondria. In contrast, insulin has no effect on HKI activity, protein content, or mRNA levels.

In forearm muscle, insulin-stimulated glucose transport (measured with the triple-tracer technique) has been shown to be markedly impaired in lean type 2 diabetics. However, since the rate of intracellular glucose phosphorylation was impaired to an even greater extent, insulin caused an increase in the intracellular free glucose concentration. By performing the insulin clamp at each diabetic's normal level of fasting hyperglycemia, normal rates of whole-body glucose disposal and a normal rate of glucose influx into muscle were elicited. However, the rate of intracellular glucose phosphorylation increased only modestly; consequently, there was a dramatic rise in the free glucose concentration within the intracellular space that is accessible to glucose. These observations indicate that in type 2 diabetic individuals, although both glucose transport and glucose phosphorylation are severely resistant to the action of insulin, impaired glucose phosphorylation (HKII) appears to be the rate-limiting step for insulin action. A similar pattern of impaired muscle glucose phosphorylation and transport is present in the insulin-resistant, normal glucose-tolerant offspring of two diabetic parents. These results are consistent with dose–response studies using PET to evaluate glucose phosphorylation and transport in skeletal muscle of type 2 diabetics. They also are consistent with $^{31}$P nuclear magnetic resonance (NMR) studies that demonstrate that, during hyperinsulinemia, muscle G-6-P concentrations decline in type 2 diabetic versus control subjects. However, subsequent studies using $^{31}$P NMR in combination with [1-$^{14}$C]glucose suggest that the defect in insulin-stimulated muscle glucose transport exceeds the defect in glucose phosphorylation and is responsible for the decline in muscle G-6-P concentration. Because of methodologic differences, the results of the triple-tracer and MRI studies cannot be reconciled at present. Nonetheless, observations from these studies are consistent in demonstrating that the defects in glucose phosphorylation and glucose transport in muscle are established early in the natural history of type 2 diabetes and cannot be explained by glucose toxicity. Clear evidence that HKII activity is crucial for glucose uptake derives from studies in transgenic mice who overexpress HKII. In this model, HKII overexpression increased both insulin- and exercise-stimulated muscle glucose uptake.

In healthy nondiabetic subjects, physiologic hyperinsulinemia for as little as 2–4 h increases muscle HKII activity, gene transcription, and translation. In lean type 2 diabetics, insulin-stimulated HKII activity and mRNA levels are markedly reduced compared to controls. Decreased basal muscle HKII activity and mRNA levels and impaired insulin-stimulated HKII activity in type 2 diabetic subjects have been reported by other investigators. A decrease in insulin-stimulated muscle HKII activity also has been described in subjects with IGT. Because of its central role in insulin-mediated muscle glucose metabolism, several groups have looked for point mutations in the HKII gene in individuals with type 2 diabetes. Although several nucleotide substitutions have been found, none have been located close to the glucose- and ATP-binding sites and none have been associated with insulin resistance. Thus, an abnormality in the HKII gene is unlikely to explain the inherited insulin resistance in common variety type 2 diabetes mellitus.

## F. Glycogen Synthesis

After glucose is phosphorylated by hexokinase II, it can either be converted to glycogen or enter the glycolytic pathway. Of the glucose that enters the glycolytic pathway, ~90% is oxidized. At low physiologic plasma insulin concentrations, glycogen synthesis and glucose oxidation are of approximately equal quantitative importance. With increasing plasma insulin concentrations, glycogen synthesis predominates. If the rate of glucose oxidation (determined by indirect calorimetry) is subtracted from the rate of whole-body insulin-mediated glucose disposal (determined from the insulin clamp), the difference represents nonoxidative glucose disposal (or glucose storage), which reflects primarily glycogen synthesis. Glucose conversion to lipid accounts for <5% of total-body glucose disposal and less than 5–10% of the glucose taken up by muscle is released as lactate. Reduced insulin-stimulated glycogen synthesis is a characteristic finding in all insulin-resistant states, including obesity, diabetes, and the combination of obesity plus diabetes. Impaired glycogen synthesis also represents the major cause of insulin resistance in obese subjects with normal or only slightly impaired glucose tolerance. Thus, the inability of insulin to promote glycogen synthesis is a characteristic and early defect in the development of insulin resistance in both obesity and type 2 diabetes. The emergence of overt diabetes with fasting hyperglycemia is associated with a major reduction in insulin-mediated nonoxidative glucose disposal (glycogen synthesis) in all ethnic groups. Impaired glycogen synthesis also has been demonstrated in the normal glucose-tolerant offspring of two diabetic parents, in the first-degree relatives of type 2 diabetic individuals, and in the normoglycemic twin of a monozygotic twin pair in which the other twin has type 2 diabetes.

Using NMR imaging spectroscopy, a decrease in insulin-stimulated incorporation of [$^1$H, $^{13}$C]glucose into muscle glycogen of type 2 diabetic subjects has been demonstrated directly. In type 2 diabetics, there was a marked lag in the onset of insulin-stimulated glycogen synthesis that was similar to the delay in insulin-mediated leg muscle glucose uptake. The rate of glycogen synthesis in type 2 diabetic subjects was decreased by ~50%, paralleling the decrease in total glucose uptake by leg muscle, and impaired muscle glycogen synthesis accounted for essentially all of the defect in whole-body glucose disposal.

In summary, an abundance of convincing evidence demonstrates that impaired glycogen synthesis is the major metabolic defect in normal glucose-tolerant obese subjects, in individuals with IGT, and in patients with overt diabetes. Moreover, numerous studies have documented that the earliest detectable metabolic abnormality responsible for the insulin resistance in normal glucose-tolerant individuals who are destined to develop type 2 diabetes is impaired glycogen synthesis.

Glycogen synthase is the key insulin-regulated enzyme that controls the rate of muscle glycogen synthesis. Insulin enhances glycogen synthase activity by stimulating a cascade of phosphorylation–dephosphorylation reactions (see above discussion of insulin receptor signal transduction), which ultimately lead to the activation of PP1 (also called glycogen synthase phosphatase). The regulatory subunit (G) of PP1 has two serine phosphorylation sites, site 1 and site 2. Phosphorylation of site 2 by cAMP-dependent kinase inactivates PP1, whereas phosphorylation of site 1 by insulin activates PP1, leading to the stimulation of glycogen synthase. Phosphorylation of site 1 of PP1 by insulin in muscle is catalyzed by insulin-stimulated protein kinase 1 (ISPK-1), which is part of a family of serine/threonine protein kinases termed ribosomal S6-kinases. Because of their central role in muscle glycogen formation, considerable attention has focused on the three enzymes, glycogen synthase, PP1, and ISPK-1, involved in the pathogenesis of insulin resistance in individuals with type 2 diabetes.

Glycogen synthase exists in an active (dephosphorylated) and an inactive (phosphorylated) form. Under fasting conditions, total glycogen synthase activity in type 2 diabetic subjects is reduced and the ability of insulin to activate glycogen synthase is severely impaired. An impaired ability of insulin to activate glycogen synthase also has been demonstrated in the normal glucose-tolerant relatives of type 2 diabetic individuals. Insulin-mediated activation of glycogen synthase and insulin-stimulated glycogen synthase gene expression have been shown to be impaired in cultured myocytes and fibroblasts from type 2 diabetic subjects. Studies in insulin-resistant nondiabetic and diabetic Pima Indians have documented that the ability of insulin to activate muscle PP1 (glycogen synthase phosphatase) is severely reduced. PP1 dephosphorylates glycogen synthase, leading to its activation. Therefore, a defect in PP1 appears to play an important role in muscle insulin resistance.

The effect of insulin on glycogen synthase gene transcription and translation *in vivo* has been studied extensively. Most studies have shown that insulin does not increase glycogen synthase mRNA or protein expression in human muscle studied *in vivo*. However, glycogen synthase mRNA expression is decreased in muscle of type 2 diabetic patients, explaining in part the decreased glycogen synthase activity in this disease. However, the major abnormality in glycogen synthase regulation in type 2 diabetes and other insulin-resistant conditions is its lack of dephosphorylation and activation by insulin as a result of insulin receptor signaling abnormalities (see previous discussion). The glycogen synthase gene has been the subject of extensive investigations. An association between glycogen synthase gene markers and type 2 diabetes has been demonstrated in Japanese, French, Finnish, and Pima Indian populations. However, DNA sequencing has revealed either no mutations or rare nucleotide substitutions that cannot explain the defect in insulin-stimulated glycogen synthase. Nonetheless, the association between the glycogen synthase gene and type 2 diabetes mellitus suggests that another gene close to the glycogen synthase gene may be involved in the development of type 2 diabetes. The genes encoding the catalytic subunits of PP1 and ISPK-1 have been examined in insulin-resistant Pima Indians and Danes with type 2 diabetes. Several silent nucleotide substitutions were found in the PP1 and ISPK-1 genes in the Danish population; the mRNA levels of both genes were normal in skeletal muscle. No structural gene abnormalities in the catalytic subunit of PP1 were detected in Pima Indians. Thus, neither abnormalities in the PP1 and ISPK-1 genes nor abnormalities in their translation can explain the impaired enzymatic activities of glycogen synthase and PP1 that have been observed *in vivo*. Similarly, there is no evidence that an alteration in glycogen phosphorylase plays any role in the abnormality in glycogen formation in type 2 diabetes.

In summary, glycogen synthase activity is severely impaired in patients with type 2 diabetes mellitus and in insulin-resistant normal glucose-tolerant individuals who are predisposed to develop type 2 diabetes. However, the defect cannot be explained by an abnormality in the genes encoding glycogen synthase or its promoter or by other key genes—PP1 or ISPK-1—involved in the regulation of glycogen synthase activity.

## G. Glycolysis/Glucose Oxidation

Glucose oxidation accounts for ~90% of total glycolytic flux and anaerobic glycolysis accounts for the other 10%. Two enzymes, PFK and PDH, play pivotal roles in the regulation of glycolysis and glucose oxidation, respectively. The glycolytic/glucose oxidative pathway has been shown to be impaired in many individuals with type 2 diabetes. Although one study suggested that the activity of PFK is modestly reduced in muscle biopsies from type 2 diabetic subjects, the majority of evidence indicates that the activity of PFK is normal. Insulin has no effect on muscle PFK activity, mRNA levels, or protein content in either nondiabetic or diabetic individuals. PDH is a key insulin-regulated enzyme whose activity in muscle is acutely stimulated by a physiological increment in the plasma insulin concentration. Three previous studies have examined PDH activity in type 2 diabetic patients. Insulin-stimulated PDH activity is decreased in isolated subcutaneous human adipocytes from patients with type 2 diabetes mellitus and in skeletal muscle from type 2 diabetic subjects undergoing euglycemic hyperinsulinemic clamps. However, when type 2 diabetic patients had muscle biopsies during hyperglycemic hyperinsulinemic clamps, activation of PDH by insulin was normal, in concert with normalized rates of muscle glucose uptake. These results suggest that insulin stimulation of PDH activity is influenced by glycolytic flux.

Both obesity and type 2 diabetes mellitus are associated with accelerated FFA turnover and oxidation, which would be expected, according to the Randle cycle, to inhibit PDH activity and consequently glucose oxidation. Thus, any observed defect in glucose oxidation or PDH activity could be acquired secondarily to increased FFA oxidation and feedback inhibition of PDH by elevated intracellular levels of acetyl CoA and reduced availability of NAD. Consistent with this observation, the rates of basal and insulin-stimulated glucose oxidation have been shown to be normal in the normal glucose-tolerant offspring of two diabetic parents and in the first-degree relatives of type 2 diabetic subjects, whereas it is decreased in overtly diabetic subjects. Studies examining PHD activity in muscle tissue from lean diabetic subjects with mild fasting hyperglycemia are needed before the role of this enzyme in the development of insulin resistance in type 2 diabetes can be established or excluded.

In summary, postbinding defects in insulin action primarily are responsible for the insulin resistance in type 2 diabetes. Diminished insulin binding, when present, occurs in individuals with IGT or very mild diabetes and results secondarily from down-regulation of the insulin receptor by chronic sustained hyperinsulinemia. In type 2 diabetic patients with overt fasting hyperglycemia, postbinding defects are responsible for the insulin resistance. A number of postbinding defects have been documented, including diminished insulin receptor tyrosine kinase activity, insulin signal transduction abnormalities, decreased glucose transport, reduced glucose phosphorylation, and impaired glycogen synthase activity. The glycolytic/glucose oxidative pathway appears to be largely intact and, when defects are observed, they appear to be acquired secondarily to enhanced FFA/lipid oxidation. From a quantitative standpoint, impaired glycogen synthesis represents the major pathway responsible for insulin resistance in type 2 diabetes, and family studies suggest that a defect in the glycogen synthetic pathway represents the earliest detectable abnormality in type 2 diabetes. Recent studies link the impairment in glycogen synthase activation to a defect in the ability of insulin to phosphorylate IRS-1, causing a reduced association of the p85 subunit of PI3-kinase with IRS-1 and decreased activation of PI3-kinase.

## Glossary

**endogenous glucose production** Synthesis of glucose by the internal organs of the body. Approximately 85% of endogenous glucose production is derived from the liver and 15% is derived from the kidneys. Of the total endogenous glucose production, approximately half is derived from glycogenolysis and the other half from gluconeogenesis.

**glucose transport and phosphorylation** Transport of free glucose from the interstitial compartment into the cell via the GLUT4 transporter and its subsequent phosphorylation by hexokinase within the cell.

**impaired insulin secretion** Inability of the pancreatic beta cells to secrete sufficient amounts of insulin to maintain normal glucose tolerance.

**insulin resistance** Impaired ability of insulin to exert its normal effects on insulin target tissues, including muscle, liver, and adipocytes.

**insulin signal transduction** The intracellular events [tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), association of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase) with IRS-1, and activation of PI3-kinase] whereby insulin binding to its receptor stimulates, glucose transport.

**splanchnic glucose uptake** The amount of glucose taken up by the splanchnic tissues (liver plus gastrointestinal tissues) during the postabsorptive state and/or following glucose ingestion. The great majority (~80–90%) of splanchnic glucose uptake occurs in the liver.

**type 2 diabetes mellitus** This form of diabetes is characterized by insulin resistance and, at least initially, by a relative lack of insulin secretion. With time, there is a progressive decline in beta-cell function and absolute insulin deficiency ensues.

## See Also the Following Articles

**Diabetes Type 1 (Insulin-Dependent Diabetes Mellitus) • Diabetes Type 2 (Non-Insulin-Dependent Diabetes Mellitus) • Glucagon Secretion, Regulation of • Hypoglycemia in Diabetes • Insulin Gene Regulation • Insulin Processing • Insulin Secretion**

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# Insulin-Dependent Diabetes Mellitus

**See *Diabetes Type 1***

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# Insulin Gene Regulation

Michael S. German
*University of California, San Francisco*

I. INTRODUCTION
II. INSULIN GENE STRUCTURE
III. INSULIN GENE PROMOTER
IV. TRANSCRIPTION FACTORS
V. TRANSCRIPTION ACTIVATION COMPLEX
VI. METABOLIC REGULATION
VII. SUMMARY

**Although insulin production is limited almost exclusively to the beta cells in adult mammals, most mammalian cells can produce insulin if insulin gene transcription is activated. The mechanisms that control insulin gene transcription therefore restrict insulin production to the beta cells. In addition, beta cells modulate insulin gene transcription in response to medium-term (days to weeks) changes in nutrient status and insulin production requirements. Insight into the regulation of the insulin gene provides a view of how beta cell function is directed at the genetic level.**

## I. INTRODUCTION

Insulin plays a central role in energy metabolism in vertebrates. In response to rising blood glucose levels after meals, the beta cells in the islets of Langerhans in the pancreas synthesize insulin and secrete it into the bloodstream. Circulating insulin binds to specific receptors found on the surface of most cells; insulin-receptor binding alerts the cells to the presence of excess nutrients and stimulates nutrient uptake and metabolism, storage of energy, and growth. Equally important, a decrease in insulin production and secretion in response to fasting results in the release of stored energy and in the synthesis of glucose by the liver and kidney. Impairment of insulin production or function leads to hyperglycemia and metabolic derangement characteristic of diabetes mellitus.

Beta cell insulin production, directed at the genetic level, is under the control of a multicomponent activation complex that forms on the insulin gene promoter. The almost exclusive production of insulin by pancreatic beta cells, despite the presence of the insulin gene in most mammalian cells, is a starting point for analysis of the underlying transcription activation mechanisms.

## II. INSULIN GENE STRUCTURE

The human insulin gene (*INS*) resides on the short arm of chromosome 11, immediately between the genes encoding tyrosine hydroxylase and insulin-like growth factor-II (IGF-II) (see Fig. 1). The gene consists of three exons totaling 446 base pairs that form the mature preproinsulin mRNA. The first intron, IVS1, lies within the 5′ untranslated sequence; IVS2 splits the sequence that codes for the C-peptide (see Fig. 2). The mature preproinsulin mRNA encodes the preproinsulin peptide, which includes a 20-amino-acid prepeptide that is proteolytically removed prior to the packaging of proinsulin in granules.

Adjacent to the sequences encoding the preproinsulin mRNA, 362 bp upstream of the transcription start site, lies a highly polymorphic sequence consisting of a variable number of simple tandem repeats of a G-rich 14 bp sequence [the *INS* gene variable number of tandem repeats (*INS* VNTR)]. Although there is wide variation in the human population, most individual *INS* VNTRs fall into three classes distinguished by having approximately 20, 40, or 80 repeats. The inheritance of longer VNTRs appears to provide some reduction in the risk of developing type 1 diabetes. Although the explanation for this protection is uncertain, it may be related to the demonstrated ability of the VNTRs to affect transcription rates from the adjacent *INS* gene.

The structure of the insulin gene has been remarkably well conserved in vertebrates, and this conservation generally extends to the IGF genes and the insulin/IGF genes found in invertebrates. Rats and mice have two copies of the insulin gene, apparently due to reverse transcription and reintegration of an incompletely processed RNA transcript of the original insulin gene (*insulin2*). The duplicate gene (*insulin1*) lacks IVS2 but retains IVS1 and most of the structural features of the ancestral gene. All of the other known insulin genes and the human gene have retained the same exon/intron structure, although the exact lengths and the internal sequences of both introns have not been conserved. Only primate genes contain the VNTR.

## III. INSULIN GENE PROMOTER

Transcription of the insulin gene depends on untranscribed sequences immediately upstream (5′ flanking sequences) of the transcription start site: the insulin promoter (see Fig. 3). The insulin promoter is defined as all of the 5′ flanking DNA necessary for appropriate initiation and regulation of transcription. The exact 5′ end of the promoter is ill defined, but sequences as far as several kilobases upstream of the start site may contribute. Although the overall sequence of the insulin promoter is not well conserved, the position and sequences of the functional sequence elements have been well conserved in mammals.

Although it normally functions as an integral component of the insulin gene, and may interact with other sequences around the gene such as IVS1, the insulin promoter can function as an independent unit. When isolated from the remainder of the gene and linked to a reporter gene such as firefly luciferase, the promoter can direct expression of the heterologous gene. The isolated promoter can direct expression of a reporter gene specifically to beta cells in both *in vitro* cell lines and *in vivo* transgenic animals. In addition, Studies with such chimeric reporter constructs have permitted the careful mapping of functionally important sequences within the promoter and have provided much of our present understanding of how the insulin gene is regulated at a molecular level.

Deletion and mutation analyses of the human and rat promoters both in cell lines and in transgenic mice have defined a series of functionally critical sequence elements along the proximal promoter, as outlined in Fig. 3. In essence, these sequences form a code that acts as recognition sites for nuclear DNA-binding proteins, the transcription factors. Transcription factors found in the beta cell nucleus bind to these

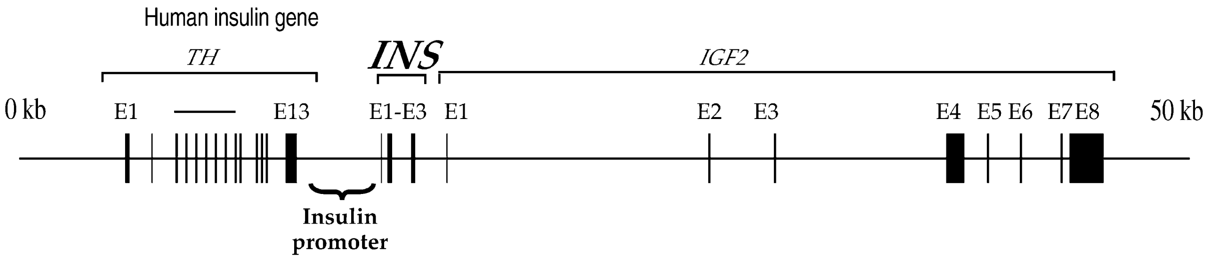

**FIGURE 1** The human insulin (*INS*) gene and surrounding loci on chromosome 11. *TH*, Tyrosine hydroxylase gene; *IGF2*, insulin-like growth factor II gene.

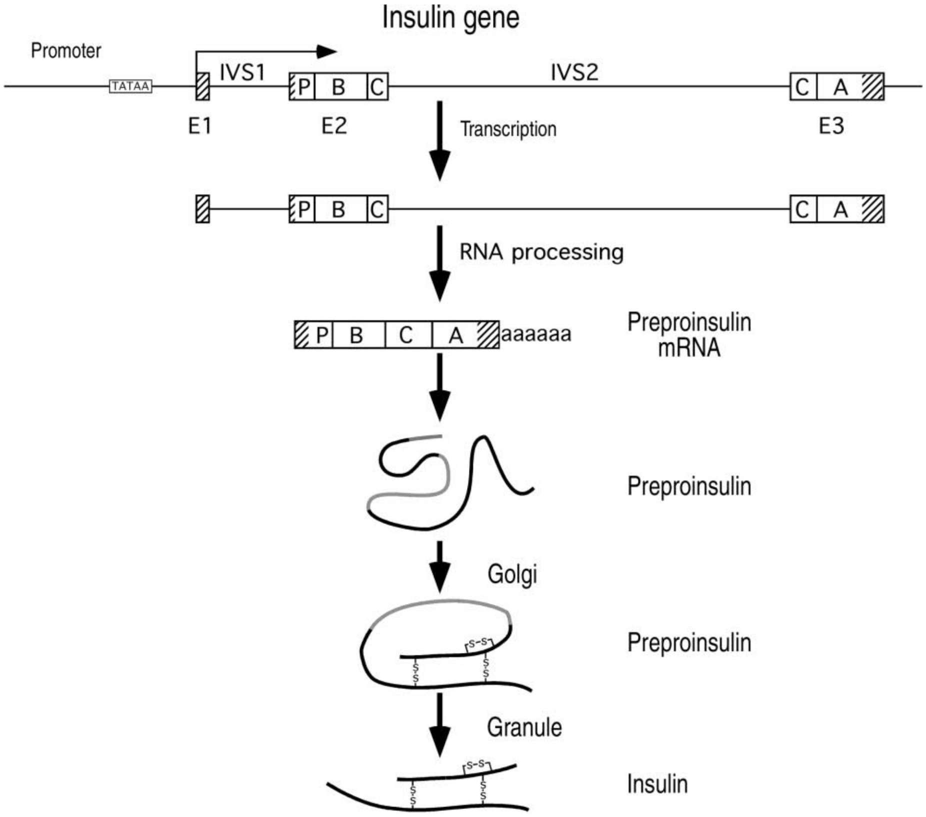
Insulin gene
Promoter
TATAA
IVS1
IVS2
P B C
C A
E1
E2
E3
Transcription
RNA processing
P B C A aaaaaa
Preproinsulin mRNA
Preproinsulin
Golgi
Preproinsulin
Granule
Insulin
S-S

FIGURE 2 Production of insulin from gene to mature peptide. The pathway by which insulin is produced in the beta cell starts with insulin gene transcription and progresses through the processing of proinsulin to mature insulin, the form that is secreted from the beta cell.

sites and form a unique DNA-protein complex that activates transcription. Other cell types with different sets of nuclear proteins cannot form the unique beta cell complex and therefore cannot activate transcription.

## IV. TRANSCRIPTION FACTORS

### A. Basic Helix–Loop–Helix Factors

As shown in Fig. 3, the proximal insulin promoter contains multiple sequence elements, but much of

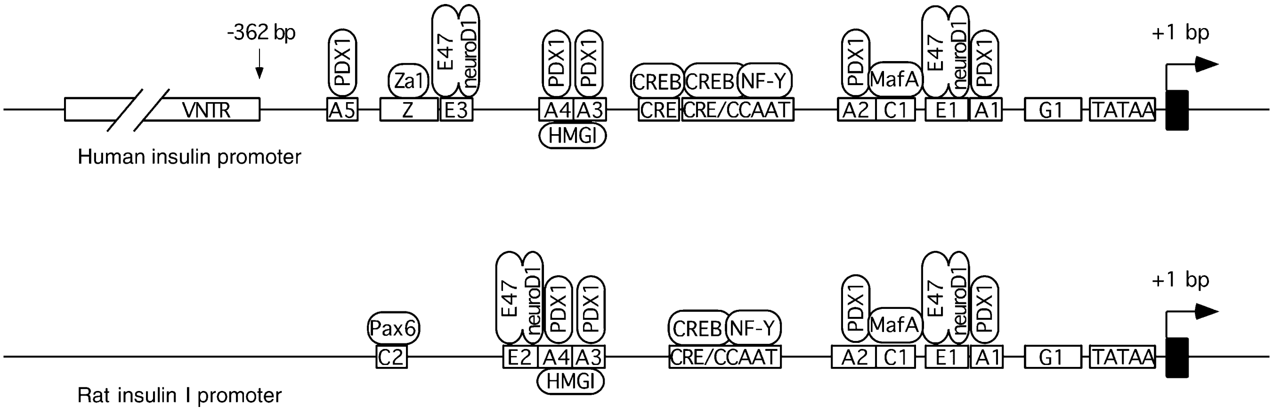
-362 bp
+1 bp
PDX1
Za1
E47
neuroD1
PDX1
PDX1
CREB
CREB
NF-Y
PDX1
MafA
E47
neuroD1
PDX1
VNTR
A5
Z
E3
A4
A3
CRE
CRE/CCAAT
A2
C1
E1
A1
G1
TATAA
HMGI
Human insulin promoter
+1 bp
Pax6
E47
neuroD1
PDX1
PDX1
CREB
NF-Y
PDX1
MafA
E47
neuroD1
PDX1
C2
E2
A4
A3
CRE/CCAAT
A2
C1
E1
A1
G1
TATAA
HMGI
Rat insulin I promoter

FIGURE 3 The human insulin and rat insulin I gene promoters with known sequence elements and binding factors. The boxes represent functionally important sequence elements. Some of the key transcription factors that bind to these sites are shown above or below the promoter.

the activity of the promoter depends on juxtaposed E and A sequences and their cognate binding proteins. The E elements contain the core sequence CANNTG and act as binding sites for basic helix–loop–helix (bHLH) proteins, transcription factors containing a stretch of basic amino acids adjacent to a helix–loop–helix motif. The bHLH proteins bind to DNA as dimers, using the HLH domain for dimerization and the basic domain for DNA binding.

The predominant E-element-binding complex in beta cells consists of a heterodimer formed by a ubiquitous bHLH protein and a cell-type-specific bHLH protein. The role of the ubiquitous partner is filled by one of three proteins, HEB, E12, or E47. The predominant cell-type-specific partner that dimerizes with these ubiquitous bHLH proteins in beta cells is neuroD1 (also called Beta2). NeuroD1 belongs to a large family of neural bHLH proteins and is expressed in neurons and gut endocrine cells as well as all islet cells. Mice homozygous for a targeted disruption in the *neuroD1* gene have a marked defect in insulin production. In addition, absence of neuroD1 also causes a reduction in the number of beta cells due to accelerated apoptosis, demonstrating a role for neuroD1 in beta cell maintenance as well as in insulin gene transcription.

### B. Homeodomain Factors

By itself, ectopic expression of neuroD1 in non-beta cells is sufficient to activate the insulin promoter modestly, but it cannot fully activate the promoter in the absence of an appropriate A-element-binding protein. The A elements contain a consensus TAAT core sequence and function as binding sites for homeodomain transcription factors. The para-hox homeodomain protein PDX1 (also called IPF1, STF1 and IDX1) binds to all of the A sites in the insulin promoter and is expressed in antral stomach, duodenum, and pancreas. Early in development, all or most of the pancreatic cells express PDX1, but with maturation, high-level expression of IPF1 becomes largely limited to the beta cells, delta cells, and duodenum, with lower level expression in some duct cells and acinar cells. Like neuroD1, PDX1 by itself can only weakly activate the insulin promoter in non-beta cells, but in some non-insulin-producing islet cell lines, forced expression of PDX1 can activate the endogenous insulin gene.

Mice homozygous for a targeted disruption of the *pdx1* gene fail to develop a pancreas and die shortly after birth, demonstrating that PDX1 is required for development of the entire pancreas. Mice heterozygous for a targeted disruption of the *pdx1* gene develop normally but have an impaired ability to clear glucose after a glucose challenge. Furthermore, targeted inactivation of the *pdx1* gene selectively in beta cells causes beta cell loss and dysfunction, impaired insulin production, and diabetes, providing additional evidence that PDX1 is specifically required for normal beta cell function. Human genetic studies have demonstrated that PDX1 plays a similar role in humans. Human patients lacking a normal copy of the *IPF1* gene (the human homologue of the mouse *pdx1* gene) also fail to develop a pancreas, and heterozygous mutation of the *IPF1* gene is associated with the development of diabetes in young adults.

Beta cells express multiple homeodomain proteins in addition to PDX1, including Pax6, a member of the paired homeodomain protein family. In addition to the homeodomain, Pax6 shares a second DNA-binding domain, the paired domain, with other paired homeodomain proteins. Pax6 is present in all islet cells as well as some gut endocrine cells and regions of the nervous system. Pax6 binds to the C2 element in the rodent insulin genes and can activate insulin gene transcription. Mice homozygous for a null mutation of the *pax6* gene have severe defects in neural development and both a general defect in islet cell formation and reduced insulin mRNA levels in their remaining beta cells. The role of Pax6 in human insulin gene transcription is uncertain, because the C2 site is not completely conserved in the human insulin promoter.

### C. MafA

Function of the human and rat insulin II promoters depends on the proximal C1 element as well as the E and A elements. MafA, a transcription factor originally identified in the chick eye, has recently been purified from beta cell nuclei and shown to bind to the C1 element. Although the function of MafA in beta cells is unknown, it likely plays an important role in controlling insulin gene expression along with the bHLH and homeodomain proteins that bind to the E and A elements.

## V. TRANSCRIPTION ACTIVATION COMPLEX

The insulin promoter is more than the sum of its independent parts. Each cis-acting element with its cognate binding factors is not an independent unit but functions instead by interacting with the other elements along the promoter to activate transcription synergistically. The result is a promoter that can function properly only in the presence of a minimal set of transcription factors. In the nuclei of beta cells, the insulin promoter forms a large DNA–protein complex, the function of which is dependent on all its parts. This model explains the exquisite cell-type specificity of the pancreatic promoters, because deletion or substitution of any of these factors in non-beta cells will drastically reduce promoter activity.

The physical interactions that produce this complex can be observed as functional cooperativity among factors in assays of promoter activity. For example, although neither neuroD1 nor PDX1 can activate the insulin promoter more than modestly, the two factors together dramatically activate the promoter. This synergistic activation can be further enhanced by including other proteins that contribute to the complex (see Fig. 4). Other members of the complex include the small DNA-binding high-mobility group (HMG) nuclear proteins such as HMGI(Y), which bind to the minor groove of the DNA and limit its flexibility, thus stabilizing interactions among the complex components. The DNA-binding proteins can then cooperatively recruit non-DNA-binding co-activator proteins such as P300, which link the activation complex to the basal transcriptional machinery binding at the TATAA box and activate RNA polymerase II.

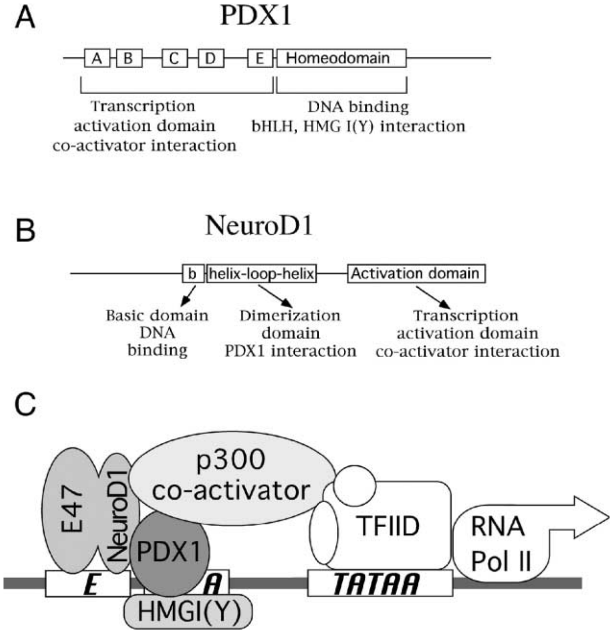
A
PDX1
A
B
C
D
E
Homeodomain
Transcription activation domain co-activator interaction
DNA binding bHLH, HMG I(Y) interaction
B
NeuroD1
b
helix-loop-helix
Activation domain
Basic domain DNA binding
Dimerization domain PDX1 interaction
Transcription activation domain co-activator interaction
C
E47
NeuroD1
p300 co-activator
PDX1
TFIID
RNA Pol II
E
A
TATAA
HMGI(Y)

**FIGURE 4** The insulin promoter transcription activation complex. (A) The functional domains of the PDX1 protein; the regions labeled A–E represent conserved domains within the activation domain. Domains that interact with other components of the complex are indicated. (B) The functional domains of the neuroD1/Beta2 protein are outlined with the domains that interact with other components of the complex indicated. (C) Interactions among PDX1, the bHLH proteins, HMGI(Y), and p300 result in transcriptional activation of the insulin promoter.

The very complexity of these interactions ensures that they cannot be completely replicated in other cell types. In addition, the dependence of the transcription complex on multiple interacting factors provides multiple opportunities for regulation, because regulation of any one factor can affect its interactions with the other components of the complex and thereby alter the overall activity of the promoter and the rate of insulin gene transcription.

## VI. METABOLIC REGULATION

Using the circulating glucose concentration as a gauge of nutritional status, beta cells promptly respond to the feeding state of the organism by rapidly altering the rate of insulin secretion and synthesis. Beta cells sense changes in glucose concentration indirectly through the end products of glucose catabolism. Rapid changes in insulin secretion rates, even in response to modest changes in glucose concentration, hold serum glucose concentrations in a narrow range in healthy mammals. Insulin mRNA levels, however, change much more slowly and contribute very little to meal-to-meal variations in circulating insulin levels. Insulin mRNA in beta cells has an exceptionally long half-life, in the range of several days. The large pool of stable insulin mRNA buffers any rapid changes in insulin gene transcription and prevents rapid swings in mRNA levels on any scale less than many hours.

On the other hand, insulin mRNA levels do vary in response to long-term changes in food intake and probably play an important role in the beta cell response to prolonged fasting, to seasonal changes in diet, or to insulin resistance such as seen in obesity. Over a period of many hours to several days, glucose stimulates a rise in insulin mRNA levels in beta cells due to the combined effects of increased insulin gene transcription, increased insulin preRNA splicing, and decreased insulin mRNA degradation. The effect of glucose on transcription rate in the beta cell is selective for the insulin gene and is due, at least in part, to regulation of the insulin promoter.

Detailed knowledge of the insulin promoter has allowed mapping of sequence elements responsible for glucose activation, and has revealed multiple pathways for glucose regulation of insulin gene transcription. Several regions of the promoter have been found to respond to glucose, including the E/A element combinations, the C1/E1 region, and the human insulin promoter Z element. Further fine mapping has implicated the E and A elements, the C1 element, and the Za1 site. The multiplicity of metabolic regulatory elements demonstrates that, like cell-type-specific transcription, glucose regulation results from the combined interactions of multiple promoter elements and their cognate binding proteins.

Binding of the beta cell bHLH heterodimers to the E elements increases in response to glucose over several days, apparently due, at least in part, to phosphorylation of both dimer partners. Furthermore, overexpression of dominant negative bHLH dimers dampens the glucose response by the insulin promoter. These data suggest a model in which glucose catabolism by the beta cell results in the activation of the E47/neuroD1 dimer. This dimer then binds the E elements and synergizes with PDX1 binding at the A elements to activate insulin gene transcription (see Fig. 5).

Glucose also stimulates an acute increase in PDX1 binding to the A elements. In response to the rise in glucose concentration, PDX1 is phosphorylated through a pathway that involves phosphatidylinositol 3-kinase (PI3K) and possibly the stress-activated protein kinase 2 (SAPK2). Phosphorylation of PDX1 has several effects on its function, possibly due to phosphorylation at multiple sites. First, it increases its affinity for the insulin promoter A sites. Second, phosphorylation stimulates a shift in the cellular distribution of PDX1 to the nucleus. Finally, glucose-stimulated phosphorylation also increases the activation potential of the PDX1 activation domain, presumably by increasing its affinity for co-activator proteins. Together, these glucose-stimulated modifications significantly enhance PDX1-dependent transcription in the beta cell nucleus and increase synergy with the activated bHLH complex binding to the E element (see Fig. 5).

In addition to the activation of the bHLH heterodimer and PDX1, binding of the protein complex that was recently identified as MafA to the C1 element next to the E1 element also increases in response to glucose, and this increase in binding is dependent on phosphorylation. Although the pathway by which MafA is activated remains to be elucidated, it seems likely that activated MafA will synergize with other components of the insulin promoter activation complex, thereby magnifying the overall response to glucose. All of these effects would then combine to give the overall increase in insulin gene transcription observed in response to glucose.

Similar to its effect on insulin secretion, glucose stimulates insulin gene transcription indirectly through the end products of its catabolism. Both the end product (ATP) and the intermediates of glycolysis are required for this response. The rate-limiting step for glycolysis in beta cells is phosphorylation of glucose to glucose 6-phosphate by glucokinase, the high-$K_m$ hexokinase found in hepatocytes and beta cells. As a result, any alteration in the levels or activity of hexokinase in beta cells directly affects insulin gene transcription rates.

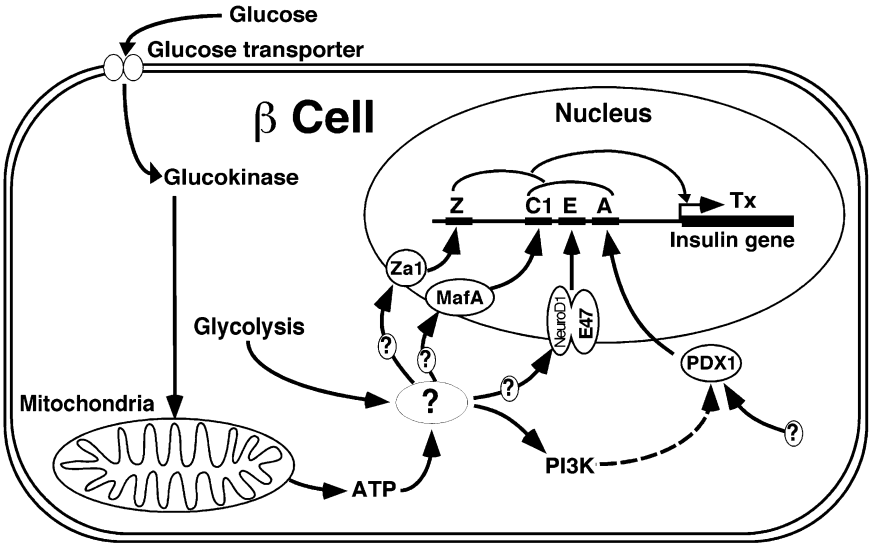

FIGURE 5 Pathways for glucose regulation of insulin gene expression. Question marks indicate unknown steps in the pathway or the probable intersection with other pathways. Dashed lines indicate a multistep pathway.

## VII. SUMMARY

Insulin gene transcription in the beta cell depends on the activity of a multicomponent transcription activation complex that forms on the insulin gene promoter. The very complexity of this activation complex ensures its specificity because other cells lack the correct set of factors to build a functional complex. Glucose affects the formation of this complex by altering the availability of the components and their interactions. As a result, the insulin gene transcription factors play a central role in insulin production and therefore in metabolic homeostasis.

## Glossary

**basic helix–loop–helix proteins** Family of transcription factors; bind to DNA as dimers. DNA binding depends on a stretch of basic amino acids adjacent to the dimerization domain formed by a helix–loop–helix structure.

**co-activator** Non-DNA-binding component of the transcription activation complex; links the transcription factors binding to upstream promoter elements to the basal transcription machinery and promotes transcription of the gene.

**homeodomain proteins** Family of transcription factors; contain a DNA-binding domain with a helix–turn–helix motif originally described in homeotic genes in *Drosophila*.

**MafA** Transcription factor of the Maf family originally described in chick eye development; contains a basic leucine-zipper-type DNA-binding domain and binds to and activates through the insulin promoter C1 element.

**neuroD1** A bHLH protein present in neurons and islet cells; heterodimerizes with ubiquitous bHLH proteins, binds to the E elements in the insulin promoter, and stimulates insulin gene transcription.

**Pax6** Paired-homeodomain transcription factor; contains both paired domain and homeodomain DNA-binding motifs; present in all islet cells and binds to the C2 site in the insulin promoter and stimulates insulin gene transcription.

**PDX1** A para-hox class homeodomain transcription factor; present in duodenum and pancreas, with high-level expression in mature beta cells; binds to the A elements in the insulin promoter and stimulates insulin gene transcription.

**promoter** Untranscribed DNA sequences upstream of the transcription start site; regulates transcription of the linked gene; includes all sequences involved in directing the transcription initiation site and controlling the rate of transcription initiation.

**transcription factor** Nuclear protein that forms part of the transcription activation complex binding to a gene promoter; is involved in regulating transcription from that gene.

## See Also the Following Articles

**Diabetes Type 1 • Diabetes Type 2 • Glucose-Dependent Insulinotropic Polypeptide (GIP) • Insulin Actions • Insulin-like Growth Factor (Igf) Signaling • Insulin Processing • Insulin Receptor Signaling • Insulin Resistance in PCOS • Insulin Secretion**

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**peptide sequence. The conversion of proinsulin to active, mature insulin occurs mostly in the secretory granule of the beta cell by limited proteolysis and is catalyzed by the endopeptidases PC-2 and PC-3 and the exopeptidase carboxypeptidase-H. A defect in proinsulin processing and insulin production leads to hyperproinsulinemia, which is a characteristic feature of type 2 diabetes.**

## I. INTRODUCTION

Normal glucose homeostasis is the result of the coordinated regulation of insulin production and utilization. Insulin is produced and stored in the pancreatic beta cell and released only in response to an appropriate stimulus. Stimulated insulin secretion is accompanied by a parallel increase in proinsulin biosynthesis, primarily at the translational level, in order to replenish the beta-cell insulin stores. Insulin was the first polypeptidic hormone found to be synthesized as a larger precursor, proinsulin. Thereafter, it was established that most polypeptide hormones, neuropeptides, and many other secretory proteins are also synthesized as larger, inactive precursor molecules. The maturation of these precursors involves limited proteolysis at specific sequences of several basic amino acid residues. Correct proinsulin to insulin conversion is essential for coping with an increase in circulatory glucose levels, since proinsulin biological activity is $<5\%$ that of insulin. Proinsulin processing occurs in the insulin secretory granule of the beta cell by the action of two endopeptidases, PC-2 and PC-3, and the exopeptidase, carboxypeptidase-H. A defect in the normal processing of proinsulin and production of insulin results in hyperproinsulinemia, which is a characteristic feature of type 2 diabetes. Hyperproinsulinemia can be the result of (1) mutation of key residues of the proinsulin molecule; (2) a defect in the production or action of the proinsulin processing enzymes; or (3) an increased secretory demand of the beta cell, which may result in defective sorting of proinsulin and/or the proinsulin processing enzymes to the beta-cell compartment where processing normally occurs.

## II. STRUCTURE OF THE PROINSULIN MOLECULE

The proinsulin molecule is a 12 kDa single-chain polypeptide that comprises the B-chain (30 amino acids) and the A-chain (21 amino acids) of insulin joined by the connecting peptide, C-peptide (Fig. 1).

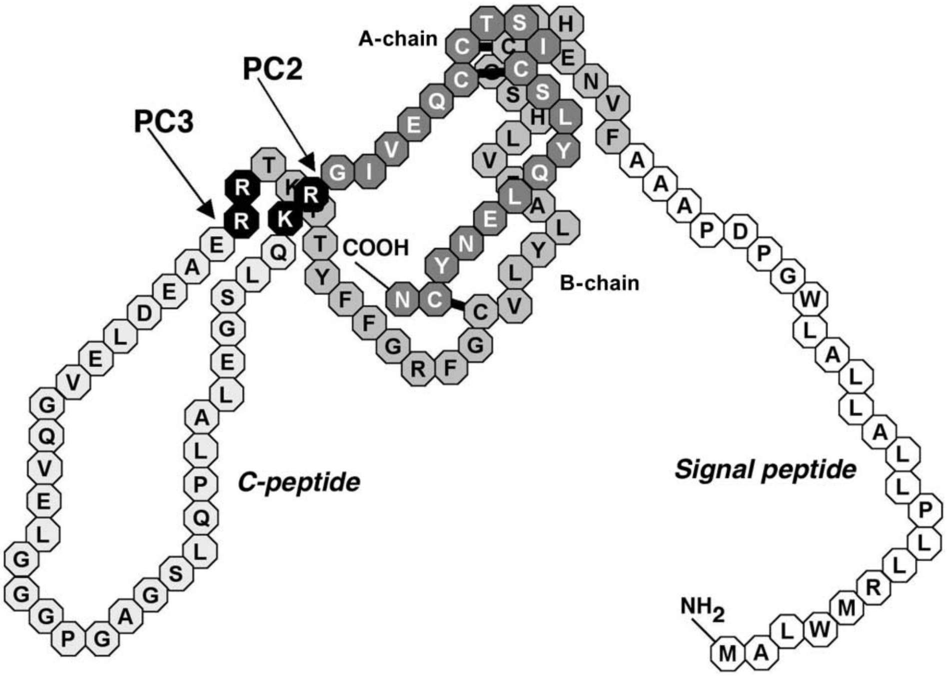

FIGURE 1 The primary structure of preproinsulin indicating the N-terminal signal peptide, followed by the insulin B-chain, the connecting-peptide (C-peptide), and then the C-terminal insulin A-chain. The two dibasic proteolytic conversion sites and the endoproteases that cleave at these are also indicated (PC-3 at Arg31Arg32 and PC-2 at Lys64Arg65).

It contains two disulfide bonds between chains A and B and one A-chain intrachain disulfide bridge. Proinsulin to insulin conversion occurs by cleavage at the B-chain/C-peptide and C-peptide/A-chain junctions and release of the C-peptide. The insulin molecule consists of the two independent chains A and B bound by the disulfide bridges. The proinsulin primary amino acid sequence, mainly that of chains A and B, is highly conserved in higher vertebrates. The sequence of the C-peptide shows greater variation and its length also varies: It contains between 26 and 38 residues in higher vertebrates. The position of the disulfide bonds, as well as the dibasic amino acid residues at the proinsulin to insulin conversion site, is also highly conserved among species.

Insulin is initially synthesized as a preproinsulin precursor, a single polypeptide chain consisting of the sequence of proinsulin flanked at the N-terminus by the signal peptide (24 amino acids). Signal sequences are commonly found in the precursors of secretory, plasma membrane, lysosomal, endoplasmic reticulum (ER), and Golgi proteins. The function of the signal peptide is to direct the protein synthesized in the ribosomal/mRNA complex from the cytoplasm into the lumen of the rough endoplasmic reticulum (RER). Removal of the preproinsulin signal sequence to generate proinsulin occurs rapidly, 1–2 min after synthesis initiation, even before translation is completed. In the RER, the proinsulin molecule folds and the disulfide bridges are formed to give the molecule its secondary structure (Fig. 1). The secondary structure of the proinsulin molecule mostly resembles that of insulin except for the presence of the C-peptide, which shows very little secondary structure. The A-chain of insulin contains two α-helices linked by a certain length of extended polypeptide. The B-chain includes a central α-helix between two stretches of extended polypeptides. In addition, the predicted secondary structure of proinsulin molecule reveals the presence of β-turns enclosing the two processing sites and Ω-loops (sequences of no ordered structure) flanking the β-turn at the C-peptide/A-chain junction. These structures seem to be required for proinsulin to insulin conversion. Moreover, the secondary structure of proinsulin indicates close proximity of the two dibasic processing sites to each other, which also contributes to the efficient proteolytic conversion to insulin.

Mature insulin is stored in the secretory granule of the beta cell in a hexameric crystalline structure. A major characteristic of a pancreatic beta cell, when viewed by electron microscopy, is that it is full of insulin secretory granules (Fig. 2). The electron-dense core of the granule is actually a large hexameric-shaped insulin crystal containing an estimated 250,000 insulin molecules. This high concentration of insulin in the secretory granule, along with the favorable acidic environment and the presence of $Zn^{2+}$ ions, facilitates the hexameric aggregation of insulin molecules. The hexameric structure consists of three insulin dimers bound by interaction with two $Zn^{2+}$ atoms at His10 of the insulin B-chain. This arrangement dictates that $Zn^{2+}$ be localized to the center of the insulin hexamer. The insulin moiety within the proinsulin molecule is also able to form three-dimensional structures similar to that of insulin hexamers. In this instance, the C-peptide is located on the outer surface of the hexamer, making the dibasic processing sites easily accessible to the endopeptidases and hence highly efficient at proinsulin to insulin processing. This is reflected in the fact that the proinsulin content in the beta-cell secretory granule is less than 2% that of insulin.

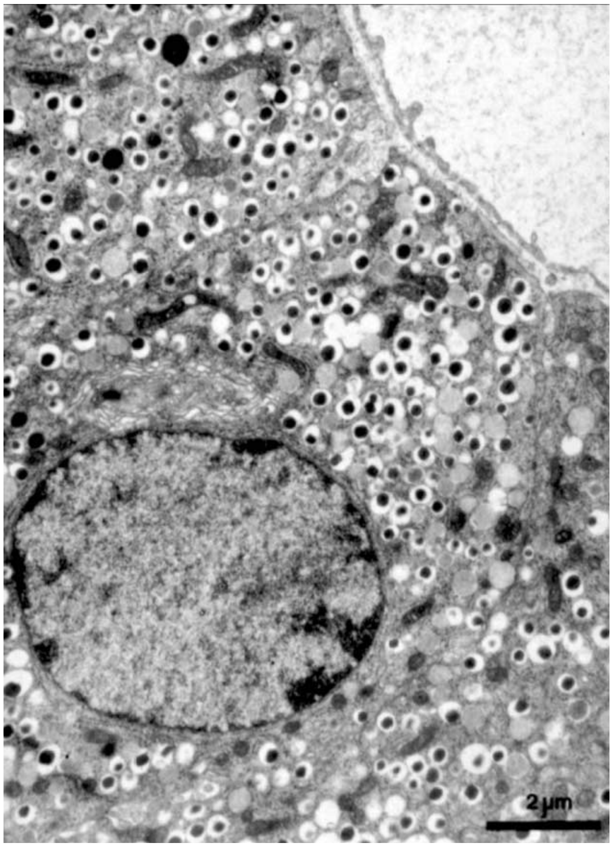

FIGURE 2 Electron micrograph of a pancreatic beta cell indicating the abundance of insulin secretory granules with electron-dense hexameric insulin crystalline cores.

## III. PROINSULIN BIOSYNTHESIS

### A. Translation, Translocation, and Transport through the Secretory Pathway

The first step in the mechanism of proinsulin biosynthesis is the translation of preproinsulin mRNA into preproinsulin polypeptide. The intracellular localization of preproinsulin mRNA translation in the beta cell is on the ribosomes of the RER. Initially, ribosomes are attached to free preproinsulin mRNA in a beta-cell cytosolic storage pool by the process of translation initiation normally applied to most mammalian mRNAs under the control of eukaryotic initiation factors (eIFs). Preproinsulin mRNA translation to polypeptide begins in the cytosol with synthesis of the signal peptide of the preproinsulin molecule. This emerging signal sequence binds the signal recognition particle (SRP), a cytosolic ribonucleoprotein complex. Then, by binding of SRP to the SRP receptor, an integral ER membrane protein, the preproinsulin mRNA/ribosomal complex is localized to the RER. More than one ribosome can scan along a single preproinsulin mRNA to form polyribosomal complexes, the consequence of which is that many preproinsulin polypeptide copies can be produced from a single preproinsulin mRNA template. As the SRP binds to its SRP receptor, the nascent signal peptide of the newly forming preproinsulin dissociates from SRP and is transferred to another RER integral membrane protein called the signal sequence receptor (SSR). The SSR is thought to be part of a "translocation pore" that facilitates transport of the newly forming preproinsulin polypeptide across the RER membrane into the RER lumen. As the elongation phase of preproinsulin mRNA translation continues, increasing the length of the preproinsulin polypeptide, the signal peptide is cleaved by another RER membrane-associated protein, the signal peptidase. Cleavage of the signal peptide from newly forming preproinsulin polypeptide by signal peptidase probably occurs co-translationally before preproinsulin mRNA translation is complete. Preproinsulin polypeptide elongation is halted at the UAG stop codon on preproinsulin mRNA and is mediated by a GTP-binding protein called releasing factor (RF). RF promotes the release of the newly formed (pre)proinsulin polypeptide from the preproinsulin mRNA/ribosomal complex concomitant with GTP hydrolysis and allows recycling of ribosomes for another round of preproinsulin mRNA translation initiation/elongation. The C-terminal portion of the newly formed (pre)proinsulin polypeptide then completes translocation across the RER, resulting in a soluble nascent proinsulin molecule in the RER lumen.

The proinsulin molecule then undergoes appropriate folding so that the intrachain disulfide bond of the insulin A-chain and two A-/B-chain interchain disulfide bridges within the proinsulin molecule can be correctly aligned. This process is likely catalyzed by the molecular chaperonin, BiP, and a disulfide isomerase activity, both of which are found in the RER lumen of beta cells. Newly synthesized proinsulin is then transported from the RER in small transport vesicles to the *cis*-Golgi apparatus. It then passes from stack to stack of the Golgi apparatus in small COP-coated vesicles, eventually arriving at the *trans*-Golgi network (TGN). The newly synthesized proinsulin then aggregates (mostly likely in $Zn^{2+}$-containing dimer/hexameric form) in clathrin-coated regions of the TGN, which are the sites of insulin secretory granule biogenesis. So-called immature, partially clathrin-coated, insulin secretory granules then bud off the TGN. It is in this intracellular compartment that the majority of proinsulin to insulin conversion takes place as granule maturation occurs.

The process of insulin secretory granule maturation includes a progressive intragranular acidification from approximately pH 6.9 to pH 5.0 via activation of the ATP-dependent $H^+$-pump present in the granule membrane. This provides the appropriate environment (pH 5.0–5.5) for optimal proinsulin conversion and formation of the insulin crystal around the isoelectric point of insulin ($pK_i$ 5.3) to occur. The patches of clathrin coat on the immature insulin secretory granule membrane are also removed during the maturation process. Once a mature granule is formed, it is retained in a "storage compartment" in the beta cell for several days and undergoes exocytosis in order to secrete insulin only upon the introduction of an appropriate stimulus. This is characteristic of (pro)insulin being specifically targeted to the beta cell's regulated secretory pathway. Although the mechanism of sorting of proinsulin to secretory granules at the level of TGN is not, for the moment, particularly well understood, it is nonetheless a highly efficient process with $>99\%$ of newly synthesized proinsulin targeted to insulin secretory granules.

### B. Effectors and Inhibitors of Proinsulin Biosynthesis

Proinsulin biosynthesis can be regulated by nutrients and certain hormones, but, among these, glucose is the most physiologically relevant. Long-term glucose exposure to beta cells ($>6$ h) modestly up-regulates preproinsulin gene transcription by approximately 2-fold. In addition, preproinsulin mRNA stability is increased. However, the predominant control mechanism of glucose-induced proinsulin biosynthesis (especially in normal circumstances of glucose stimulation, $<2$ h) is at the translational level (see below). The stimulation of proinsulin biosynthesis by glucose is rapid. Upon an increase from a basal 3 mM glucose to a stimulatory 10 mM glucose, there is a lag period of about 20 min followed by a marked rise in the rate of proinsulin biosynthesis translation that reaches a maximum by 60 min, 20- to 30-fold above the rate at basal glucose, without any change in preproinsulin mRNA levels. The threshold concentration of glucose required to stimulate proinsulin biosynthesis translation is between 2 and 4 mM glucose, which is lower than the glucose threshold of 4–6 mM required to stimulate insulin secretion. Glucose metabolism is required to generate stimulus-coupling signals for proinsulin biosynthesis translation. Currently, it is thought that such a secondary metabolic signal for glucose-induced proinsulin biosynthesis emanates from anaplerosis of the tricarboxylic acid cycle where succinate/succinyl-CoA is the most promising candidate.

In general, most nutrients that increase insulin secretion also control proinsulin biosynthesis in parallel. This ensures that intracellular stores of insulin in the beta cell are maintained at optimal levels. However, there is one notable exception for parallel nutrient-induced control of proinsulin biosynthesis/insulin secretion. Fatty acids are marked potentiators of glucose-induced insulin secretion; however, if anything, fatty acids inhibit glucose-induced proinsulin biosynthesis. As such, there is a failure to replenish beta-cell insulin content at the biosynthetic level when insulin secretion is stimulated by fatty acids that can lead to decreased insulin secretory capacity after prolonged exposure to fatty acids and consequently is a contributing factor to beta-cell dysfunction in the pathogenesis of type 2 diabetes.

It should be noted that the stimulus-coupling signaling mechanism for glucose-induced control of proinsulin biosynthesis translation is distinct from that for insulin secretion. ATP is required for general protein synthesis in the beta cell, but is not a specific coupling signal as for glucose-induced insulin secretion. Indeed, the $K_{ATP}$ channel plays a key role in stimulus-coupling mechanism for glucose-induced insulin secretion, but is not involved in the control of proinsulin synthesis. Neither the agonists of the $K_{ATP}$ channel (e.g., sulfonylureas) that stimulate insulin secretion nor the antagonists (e.g., diazoxide) that inhibit insulin secretion affect glucose-induced proinsulin biosynthesis. Moreover, increased cytosolic $[Ca^{2+}]_i$ is a prerequisite for the mechanism of glucose-induced insulin secretion, but glucose-induced translational control of proinsulin biosynthesis is $Ca^{2+}$-independent. Finally, while certain hormones can either stimulate (e.g., GLP-1) or inhibit (e.g., epinephrine) both glucose-induced insulin secretion and proinsulin biosynthesis in parallel, other hormones (e.g., somatostatin) can markedly inhibit glucose-induced insulin secretion without affecting proinsulin biosynthesis.

## C. Translational Control for Proinsulin Biosynthesis

In general, glucose modestly increases general protein synthesis in the beta cell 1.5- to 2-fold. This seems to occur in response to phosphorylation regulation of certain eIFs, in particular, eIF2α, eIF-2B, and eIF-4E-BP. However, the effect of glucose on proinsulin synthesis translation, as well as the synthesis of a small subset of all beta-cell proteins most of which are components of the insulin secretory granule, is much greater (reaching $>10$-fold stimulation). This indicates a more specific effect of glucose on the translational control of preproinsulin mRNA translation. It is known that glucose increases the rate of "free cytoplasmic" preproinsulin mRNA translocation to ribosome and polyribosome complexes, indicating control at the initiation phase of translational mechanism where 40S and 60S ribosomal subunits are attached to mRNA. Glucose then promotes translocation of preproinsulin mRNA–ribosomal complexes to the RER membrane, most likely via increasing the association of the SRP initiation complex with the SRP receptor. However, this happens to all proteins in the beta cell that possess a signal peptide on entering the secretory pathway and does not account for the specific nature of glucose-induced preproinsulin biosynthesis. This likely occurs via translational control *cis*-elements in the untranslated regions (UTRs) of preproinsulin mRNA itself. Indeed, in the 5′-UTR there is a "stem-loop" secondary structure (not necessarily primary sequence), which is highly conserved across mammalian preproinsulin mRNAs. Moreover, in the 3′-UTR of preproinsulin mRNA, just downstream of the polyadenylation signal is a highly conserved primary sequence containing a UUGAA core. Recent studies have shown that the 5′-UTR stem-loop element of the preproinsulin mRNA is required for glucose stimulation of proinsulin biosynthesis, since the translation of a preproinsulin mRNA variant that lacks this 5′-UTR does not undergo glucose-induced translational regulation. However, when the 3′-UTR UUGAA element is additionally present, the effect of the 5′-UTR on glucose stimulation of proinsulin biosynthesis is augmented. The effect of the 3′-UTR *cis*-element appears to be a glucose-induced alleviation of a translational block, but this occurs only when the 5′-UTR stem-loop is present. Thus, it appears that there is cooperativity between the preproinsulin mRNA 5′- and 3′-UTRs for the *specific* translational control of proinsulin biosynthesis influenced by extracellular glucose concentrations (Fig. 3). In addition, the preproinsulin mRNA 3′-UTR UUGAA *cis*-element also encodes a signal that preserves preproinsulin mRNA stability specifically in beta cells, which in turn could contribute to longer term up-regulation of proinsulin biosynthesis by glucose. It is currently postulated that certain beta-cell-specific *trans*-acting factors associate with the 5′- and 3′-UTRs of preproinsulin mRNA, which will be key to the specific control of proinsulin biosynthesis by glucose. However, the identity of such *trans*-acting factors is not yet known. Notwithstanding, once such factors are identified, a connection between secondary factors emanating from glucose metabolism and control of proinsulin biosynthesis should be made, which in turn will markedly improve our understanding of the control mechanism behind proinsulin production in the beta cell.

## IV. PROINSULIN PROCESSING

### A. Proinsulin Processing Enzymes

Proinsulin to insulin conversion is catalyzed by the action of two endopeptidases, PC-2 and PC-3 (also known as PC-1), and the exopeptidase, carboxypeptidase-H (CP-H). The enzymatic process of proinsulin conversion is outlined in Fig. 4. In 1988, two distinct proinsulin processing enzymatic activities were identified in secretory granules of insulinoma cells and named type I and type II activities. These two activities were $Ca^{2+}$-dependent and had an acidic pH optimum (~pH 5.0). These activities were subsequently cloned in the early 1990s, and the type I and type II proinsulin processing activities were

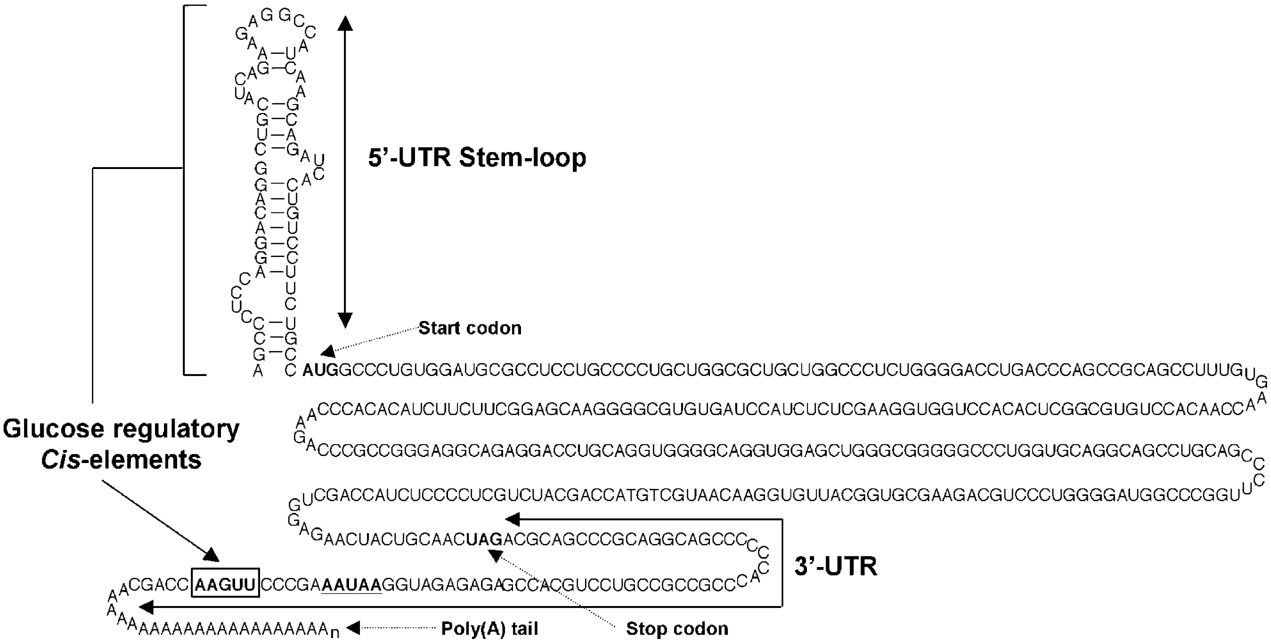
5'-UTR Stem-loop
Start codon
AUGGCCCUGUGGAUGCGCCUCCUGCCCCUGCUGGCGCUGCUGGCCCUCUGGGGACCUGACCCAGCCGCAGCCUUUG
CCCACACAUCUUCUUCGGAGCAAGGGGCGUGUGAUCCAUCUCUCGAAGGUGGUCCACACUCGGCGUGUCCACAACC
CCCGCCGGGAGGCAGAGGACCUGCAGGUGGGGCAGGUGGAGCUGGGCGGGGGCCCUGGUGCAGGCAGCCUGCAG
CGACCAUCUCCCCUCGUCUACGACCATGTCGUAACAAGGUGUUACGGUGCGAAGACGUCCCUGGGGAUGGCCCGGU
AACUACUGCAACUAGACGCAGCCCGCAGGCAGCCC
CGACC AAGUU CCCGAAAUAAGGUAGAGAGAGCCACGUCCUGCCGCCGCC
AAAAAAAAAAAAAAAAAAn
Glucose regulatory
Cis-elements
3'-UTR
Poly(A) tail
Stop codon

FIGURE 3 Schematic representation of human preproinsulin mRNA. The primary sequence of human preproinsulin mRNA is shown highlighting the glucose-responsive *cis*-elements in the 5′- and 3′-untranslated regions (UTRs). The predicted "stem-loop" secondary structure of the 5′-UTR is shown, followed by the AUG start codon (in boldface type) intervening coding sequence ending with the UAG stop codon (in boldface type), followed by the 3′-UTR and poly(A) tail. Within the 3′-UTR, the polyadenylation signal (boldface and underlined) and glucose regulatory/mRNA stability UUGAA *cis*-element (boxed) are shown.

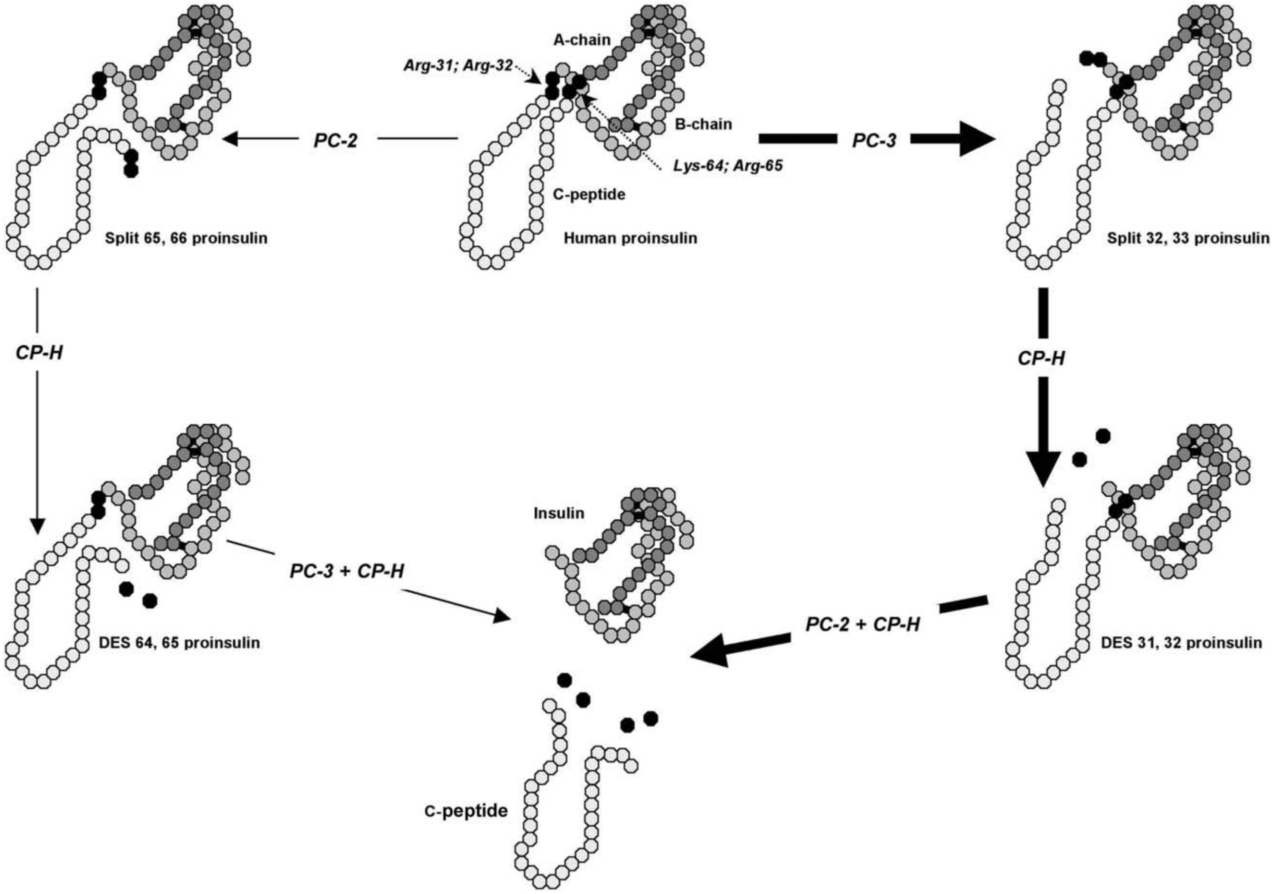

**FIGURE 4** Pathways of proinsulin processing. The two potential pathways of proteolytic conversion of proinsulin by PC-2, PC-3, and CP-H to the final products of mature insulin, C-peptide, and free basic amino acids is shown. However, one of these routes is preferred as indicated by the broader arrows between each catalytic step.

identified as the subtilisin-like endopeptidases PC-3 and PC-2, respectively. Mammalian PC-2 and PC-3 are members of the proprotein processing subtilisin family of serine proteases, which also includes yeast Kex2 and the other mammalian endopeptidases furin/PACE, PC-4, PACE-4, PC-5/PC-6, and LPC/PC-7/PC-8. Proprotein-converting endopeptidases are responsible for the posttranslational conversion of most prohormones, neuropeptide precursors, and other protein precursors into their biologically active forms. Members of this endopeptidase family all share a homology with a subtilisin-like catalytic domain where conserved Asp, His, and Ser residues make up the catalytic triad aligned by similar flanking sequences. Proprotein-converting endopeptidases contain a signal peptide targeting them to the cell's secretory pathway, as well as a propeptide that is likely autocatalytically removed upon maturation in a zymogen-like manner.

The tissue distribution of different proprotein-converting endopeptidases varies. Furin, PACE-4, PC-5, and PC-7 are broadly expressed in a wide number of different tissues. In contrast, expression of PC-2 and PC-3 is restricted to neuroendocrine tissues (e.g., pancreatic islets, pituitary, adrenal medulla, and other brain tissues). PC-4 is highly specialized where its expression is limited to testicular spermatogenic cells. Furin, PACE-4, PC-5, and PC-7 are responsible for the conversion of proprotein precursors generally secreted via the constitutive pathway, including proalbumin, certain clotting factors, some growth factors, and plasma membrane receptor proteins. However, PC-2 and PC-3 generally process precursors of hormones and neurotransmitters that are secreted via regulated pathways. Indeed, proPC-2 and proPC-3 are transported to the secretory granules along with their proprotein substrates and undergo maturation beginning in the TGN.

Intriguingly, proPC-2 and proPC-3 are accompanied by individual chaperonin molecules, 7B2 and proSAAS, respectively, that specifically inhibit the activity of these endopeptidases. Some believe that 7B2 and proSAAS should even be considered regulatory subunits for proPC-2 and proPC-3, respectively. Proteolytic cleavage of 7B2 by PC-2, and proSAAS by PC-3, in the TGN/immature secretory granule compartment alleviates the inhibition of proPC-2 and proPC-3, promoting their maturation and activation to mature PC-2 and PC-3. This then provides a degree of regulating proPC-2 and proPC-3, helping to restrict their activation to the secretory granule compartment. It appears that the 7B2 and proSAAS chaperonins are very important in controlling PC-2 and PC-3 activity *in vivo*, as illustrated by the 7B2 knockout mouse, which has multiple neuroendocrine disorders including hypoglycemia, hyperproinsulinemia, hypoglucagonemia, and Cushing syndrome. Likewise, there is marked defective prohormone conversion in PC-2 knockout mice and multiple endocrine disorders in a human patient deficient in PC-3, both of which have defective proinsulin conversion resulting in hyperproinsulinemia.

The substrate specificity of all endopeptidases is cleavage of proproteins on the carboxylic side of multibasic amino acid sequences. The cleavage site for proproteins of the constitutive pathway is typically Arg-X-Lys/Arg-Arg, whereas those for the regulated pathway proproteins are more often the dibasic sequences Lys-Arg and Arg-Arg. For human proinsulin, PC-2 preferentially cleaves on the carboxylic side of the dibasic Lys64Arg65 site and PC-3 on the carboxylic side of Arg31Arg32 (Fig. 1). Other prohormones processed by PC-2 and/or PC-3 include proopiomelanocortin in the pituitary anterior and intermediate lobes and proglucagon in the pancreatic α-cell and certain gut cells. These particular precursor molecules can be differentially processed to yield different hormone products in a tissue-specific manner, which depends mostly on the relative expression level of PC-2 and PC-3 in that particular cell type.

Once PC-2 and PC-3 have cleaved proinsulin at the dibasic sites Arg31Arg32 and Lys64Arg65, the newly exposed basic residues are removed by the action of the exopeptidase CP-H (also known as CP-E). CP-H is a carboxypeptidase B-like enzyme that is widely expressed in many endocrine and neural tissues including the pancreatic beta cell. The important role of CP-H *in vivo* has been demonstrated in obese *fat/fat* mice, where a single Ser-202 to Pro mutation abolishes CP-H enzymatic activity. These animals have defective proinsulin processing, resulting in hyperproinsulinemia, and indeed there is little insulin present since most of the "insulin" formed is actually diarginyl-[Arg31, Arg32]-insulin due to the failure of CP-H to trim off the dibasic residues after PC-3 cleavage. Diarginyl-insulin has only approximately 50% of the biological activity of native insulin, so that these animals are mildly insulin deficient. However, this does not explain the obese phenotype of the *fat/fat* mice; this is more likely due to deficient processing of neuronal peptides associated with appetite control.

## B. Regulation of Proinsulin Processing

Proinsulin conversion begins in the region of the TGN, but takes place mostly in the immature secretory granule compartment and is ongoing as part of the secretory granule maturation process. As mentioned previously, autocatalytic cleavage of the propeptide on proPC-2 and proPC-3 occurs in this compartment to yield active PC-2 and PC-3. This is accompanied by an alleviation of the inhibition on PC-2 and PC-3 mediated by proteolytic cleavage of the proPC-2 chaperonin, 7B2, and pro-PC-3 chaperonin, proSAAS. However, this complex proteolytic activation process is dictated by generation of the appropriate intraorganellar environment suitable for the optimal activity of PC-2 and PC-3 endopeptidases. PC-2, PC-3, and CP-H require an acidic pH for optimal activity. Intragranular acidification from approximately pH 6.8 in the TGN to pH 5.0–5.5 in the insulin secretory granule occurs as part of the immature insulin secretory granule maturation process. This acidification is a result of activating an ATP-dependent proton pump present on insulin secretory granule membranes. The developing acidic environment in an immature granule ensures that the majority of PC-2, PC-3, and CP-H activation occurs within the secretory granule compartment, confining insulin production and crystallization to the intracellular compartment in which it is stored. In addition, both PC-2 and PC-3 are $Ca^{2+}$-dependent enzymes requiring $>1$ mM [$Ca^{2+}$] for full activity. This is a relatively high $Ca^{2+}$ requirement; however, there is a correspondingly high free [$Ca^{2+}$] present in the insulin secretory granule. Thus, the $Ca^{2+}$ dependency of PC-2 and PC-3 activity also ensures that insulin conversion is confined to the insulin secretory granule compartment.

As indicated above, glucose can markedly increase proinsulin biosynthesis at the translational level, as much as 20- to 30-fold within 60 min. This obviously places an increased demand on the proinsulin conversion process in beta cells. However, this is compensated for by a parallel glucose-induced increase in proPC-2 and proPC-3 biosynthesis at the translational level. Intriguingly, both preproPC-2 and preproPC-3 mRNAs have a conserved stem-loop secondary structure in their $5'$-UTR that is very similar to that in preproinsulin mRNA, which acts as a glucose-regulatory *cis*-element to stimulate proinsulin biosynthesis translation. However, neither preproPC-2 nor preproPC-3 mRNA contains the $3'$-UTR glucose regulatory *cis*-element found in preproinsulin mRNA, which might account for the observation that glucose-induced translational control of PC-2 and PC-3 biosynthesis is not as robust as that of proinsulin and that preproPC-2 or preproPC-3 mRNA is less stable than preproinsulin mRNA in beta cells. Interestingly, CP-H biosynthesis is not regulated by glucose, and preproCP-H mRNA has neither a predicted $5'$- or $3'$-UTR glucose-regulatory *cis*-element. Nevertheless, the constitutively high concentration of CP-H in the beta cell, which comprises 2–5% of the total insulin secretory granule protein content, seems to be sufficient to cope with the requirement of glucose-stimulated increase of proinsulin processing.

### C. Sequential Proinsulin Processing

Proinsulin is coordinately cleaved on the carboxylic side of two sites, Arg31Arg32 and Lys64Arg65 on human proinsulin, to generate the final products of mature insulin, C-peptide, and free basic amino acids (Fig. 4). This can occur by two possible routes. PC-2 may first cleave at Lys64Arg65 to yield a split 65, 66 proinsulin intermediate, followed by CP-H trimming of the newly exposed lysine and arginine residues to yield des 64, 65 proinsulin. PC-3 can then cleave des 64, 65 proinsulin at Arg31Arg32, which together with CP-H trimming of the exposed arginine residues, yields insulin and C-peptide (Fig. 4). Alternatively, PC-3 may first cleave at Arg31Arg32 to yield a split 32, 33 proinsulin intermediate, followed by CP-H trimming of the revealed arginine residues to yield des 31, 32 proinsulin. PC-2 can then cleave des 31, 32 proinsulin at Lys64Arg65, which, together with CP-H trimming of the lysine and arginine residues, yields insulin and C-peptide (Fig. 4). However, although both of these routes can occur in the beta cell *in vivo*, experimental evidence indicates that the latter route (via the des 31, 32 proinsulin intermediate) is predominant. The PC-2 endopeptidase activity has a better affinity for des 31, 32 proinsulin as a substrate than intact proinsulin. In contrast, PC-3 can readily cleave at Arg31Arg32 on intact proinsulin. Nuclear magnetic resonance analysis of proinsulin has indicated an ordered structure around the Lys64Arg65 C-peptide/A-chain junction, termed the CA-knuckle, that seems to affect substrate recognition by PC-2 by acting as a blocking structure to prevent PC-2 access to the cleavage site Lys64Arg65 in intact proinsulin. However, upon PC-3 cleavage of intact proinsulin at Arg31Arg32, to generate des 31, 32 proinsulin (with CP-H trimming), the CA-knuckle structural constraint is alleviated, allowing better access for PC-2 to Lys64Arg65 on its preferred des 31, 32 proinsulin substrate. Thus, the majority of proinsulin conversion is sequential, where PC-3 first cleaves at Arg31Arg32 on proinsulin, followed by PC-2 cleavage at Lys64Arg65 on des 31, 32 proinsulin. This is corroborated by the observation of a marked increase in des 31, 32 proinsulin levels in PC-2 knockout mice. Moreover, there are much higher circulating levels of des 31, 32 proinsulin than of des 64, 65 proinsulin in humans.

## V. SUMMARY

Insulin is initially synthesized in the RER of the pancreatic beta cell as a larger precursor molecule, preproinsulin. Preproinsulin is processed to proinsulin in the RER by removal of the signal peptide sequence. Proinsulin conversion to active, mature insulin occurs mostly in the beta-cell secretory granule by limited proteolysis and is catalyzed by the endopeptidases PC-2 and PC-3 and the exopeptidase CP-H. PC-3 and PC-2 process proinsulin in a preferential sequence. PC-3 first cleaves proinsulin at the pair of basic amino acid residues Arg31Arg32, which after trimming of the newly exposed basic residues by CP-H yields des 31, 32 proinsulin. Then, PC-2 processes des 31, 32 proinsulin at the dibasic pair Lys64Arg65, which, after CP-H trimming of the basic residues, yields mature insulin and C-peptide. Proinsulin conversion can be regulated on multiple levels governed by translational control of proPC-2 and proPC-3 in parallel to their proinsulin substrate; by specific chaperonin molecules 7B2 (for proPC-2) and proSAAS (for proPC-3), which guide zymogen-like activation of proPC-2 and proPC-3 maturation; and by the acidic (pH 5.5) and high free $[Ca^{2+}]$ ($>1$ mM) intragranular environment. This ensures that insulin production is confined to the compartment in which it is stored, a process that under normal conditions in the beta cell is $>99\%$ efficient. However, under abnormal conditions, such as type 2 diabetes where a chronic increased demand for insulin production is placed on the beta cell by hyperglycemia and peripheral insulin resistance, proinsulin production and processing become dysfunctional and hyperproinsulinemia results. This, in turn, further contributes to the insulin-deficient condition of type 2 diabetes, since proinsulin has $<5\%$ of the activity of mature insulin.

## Glossary

**endopeptidases** The enzymes responsible for the limited proteolysis of proproteins, usually on the carboxylic side of dibasic amino acid sequences, to yield the active mature protein. Proprotein convertase 2 (PC-2) and PC-3 are the endopeptidases that catalyze proinsulin to insulin conversion in the insulin secretory granule compartment of the pancreatic beta cell. Also known as convertases.

**exopeptidase** An enzyme involved in the mechanism of proprotein processing. It selectively removes the carboxylic basic amino acids exposed after the endopeptidase cleavage. Carboxypeptidase-H (CP-H) trims the newly exposed arginine and lysine residues after the proteolysis of proinsulin by PC-2 and PC-3.

**insulin secretory granule** The intracellular compartment of the pancreatic islet beta cells where insulin is stored, ready to be released when an appropriate stimulus triggers the beta cell. The insulin secretory granule is formed in the *trans*-Golgi, where proinsulin, the precursor of insulin, is sorted into the clathrin-coated immature insulin secretory granule. Secretory granule maturation comprises clathrin uncoating, granule acidification, and conversion of proinsulin to insulin.

**proinsulin** The precursor of insulin; it has less than 5% of the biological activity of insulin. Proinsulin is processed to mature, active insulin by limited proteolysis.

## See Also the Following Articles

**Diabetes Type 1 • Diabetes Type 2 • Glucose-Dependent Insulinotropic Polypeptide (GIP) • Insulin Actions • Insulin Gene Regulation • Insulin-like Growth Factor (Igf) Signaling • Insulin Receptor Signaling • Insulin Resistance in PCOS • Insulin Secretion**

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# Insulin Receptor Signaling

Suzanne G. Laychock

*State University of New York, Buffalo*

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III. THE INSULIN RECEPTOR
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**Insulin is the principal hormone regulating blood glucose levels and glucose metabolism in mammals; in addition, insulin affects lipid metabolism, protein synthesis, and cell growth. Binding of insulin to the insulin receptor serves as a signal that leads to modification of expression of many enzymes and proteins involved in cellular activity.**

## I. INTRODUCTION

Insulin has a molecular weight of about 6000 and is composed of two chains (A and B) of amino acids joined together by disulfide linkages (Fig. 1). The secondary structure of the insulin protein situates chain A between the amino- and carboxy-terminal ends of the folded B-chain. Insulin is synthesized from proinsulin, a single-chain precursor that is packaged into secretory granules and released from the pancreatic islet of Langerhans beta cells in response to glucose and other secretagogues. After secretion from the beta cell, insulin is carried in the blood to target tissues. The major insulin target tissues are liver, adipocytes, and muscles. However, insulin has effects on many tissues, including the cardiovascular system and the brain.

At target tissues, the insulin ligand binds to and activates the insulin receptor localized in the plasma membrane. The insulin receptor tyrosine kinase activity autophosphorylates the receptor in response to insulin binding. The phosphorylated receptor then binds to and initiates phosphorylation of intracellular substrates that act as signal transduction elements. There are now more than 100 genes targeted by insulin regulation. Insulin modifies the expression of numerous enzymes and proteins in cells that possess

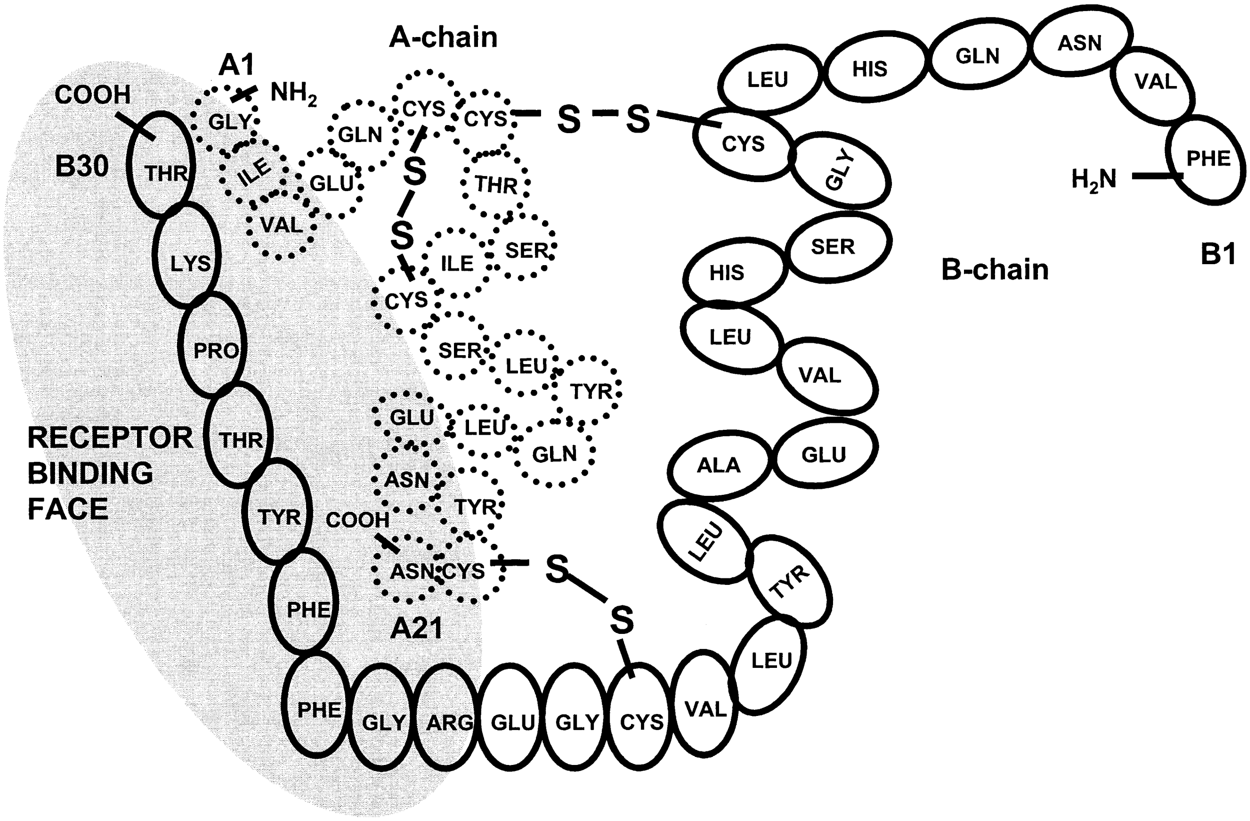
A-chain
A1
COOH
NH2
B30
GLY
ILE
VAL
GLN
GLU
CYS
CYS
S – S
S
S
THR
SER
ILE
CYS
SER
LEU
TYR
GLU
LEU
GLN
ASN
TYR
COOH
ASN
CYS
S
S
A21
RECEPTOR BINDING FACE
THR
LYS
PRO
THR
TYR
PHE
PHE
GLY
ARG
GLU
GLY
CYS
VAL
LEU
TYR
LEU
ALA
GLU
VAL
LEU
HIS
SER
CYS
GLY
LEU
HIS
GLN
ASN
VAL
PHE
H2N
B-chain
B1

FIGURE 1 Insulin molecule amino acids of the A-chain (dotted-line circles) and the B-chain (solid-line ovals), showing terminal amino acids in each chain (numbers) and disulfide bonds (–S–S–). Amino acids in the approximate receptor-binding face of the molecule (shaded area) will bind to the α-subunits of the insulin receptor.

an insulin receptor, indicating that insulin signals the nucleus to increase the transcription of genes needed to carry out pleiotropic cellular activities.

## II. DYNAMICS OF INSULIN RECEPTOR STIMULATION

The action of insulin at its target tissues is to interact at the plasma membrane with a receptor that initiates a series of intracellular responses. The tail of the B-chain and part of the A-chain amino acid sequence constitute the receptor-binding face of the insulin molecule (Fig. 1).

Many of the biochemical responses to insulin receptor stimulation involve protein phosphorylation and dephosphorylation. However, the insulin receptor is an active participant in cellular responses. The ligand-bound insulin receptor autophosphorylates due to an inherent tyrosine kinase activity and migrates within the plane of the plasma membrane of the cell and is internalized (endocytosed) to the cytoplasm, where it can be degraded in lysosomes, or the receptor is recycled back to the plasma membrane from endosomes (Fig. 2). The acidic environment of the endosome leads to insulin dissociation from the receptor and degradation by acidic insulinase (Fig. 2). Insulin-bound receptors in the endosome can phosphorylate substrates spatially distinct from those at the plasma membrane, but once the insulin and receptor are dissociated, phosphorylation events cease and the receptor is dephosphorylated by extralumenal endosomal-associated protein tyrosine phosphatases. The insulin receptor can be recycled to the plasma membrane, and insulin receptor protein synthesis can also contribute to the insulin receptor population (Fig. 2). When circulating insulin levels are

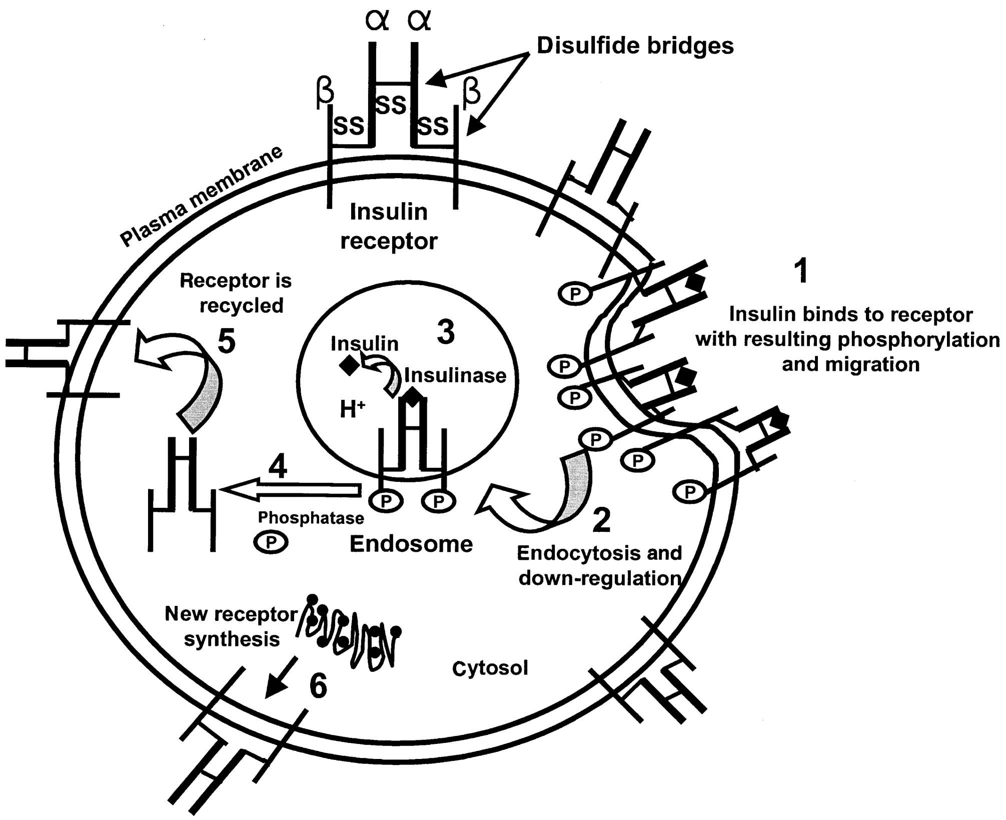

FIGURE 2 Insulin receptor stimulation, endocytosis, and recycling. The insulin receptor heterotetramer is shown with two α-subunits and two β-subunits connected by disulfide bonds (SS). (1) Insulin (◆) binds to insulin receptor α-subunits, initiating insulin receptor autophosphorylation (P) and aggregation within the plane of the plasma membrane. (2) The aggregated insulin receptors undergo endocytosis and (3) within the acidic environment of the endosome the insulin is removed from the receptor by insulinase. (4) Phosphate groups on the β-subunits of the receptor are removed by endosome-associated phosphatase and (5) the receptor is recycled to the plasma membrane, where it can bind to another insulin molecule and begin the cycle again. (6) New insulin receptor molecules are synthesized in the endoplasmic reticulum and added to the plasma membrane population of receptors.

elevated and stimulation of receptors is prolonged, the population of available insulin receptors is down-regulated with degradation of the receptor, and insulin responses can be muted. In non-insulin-dependent (type 2) diabetes mellitus, this down-regulation creates a state of insulin resistance, i.e., the target cells fail to respond adequately to insulin. On restoration of insulin stimulation to normal, insulin receptors will be reinstated at the plasma membrane to mediate insulin stimulatory responses.

## III. THE INSULIN RECEPTOR

The insulin receptor is present in virtually all vertebrate tissues, although the levels of receptor in the membrane can vary from a few per cell to several thousand in the major insulin-responsive tissues. The insulin receptor gene on human chromosome 19 encodes a transmembrane glycoprotein composed of an $\alpha_2/\beta_2$ heterotetramer with a molecular mass of 300–400 kDa. The two α-subunits (135 kDa) are composed of 719 or 731 amino acids, depending on whether there is alternative splicing of messenger RNA (mRNA) surrounding exon 11 in the gene. A disulfide bond links the two α-subunits. The β-subunits are 95 kDa, comprising 620 amino acids. The α- and β-subunits are derived from a single proreceptor molecule by proteolytic processing and are linked in the mature β/α/α/β heterotetramer by disulfide bonds (Fig. 2). The α-subunits are oriented entirely extracellular to the surface of the plasma membrane and contain the insulin-binding domain at the N-terminus. Between one and two insulin molecules bind per receptor. The β-subunits are transmembrane peptides that contain (1) an extracellular portion containing a cysteine that forms a disulfide bridge with an α-subunit cysteine (Figs. 2 and 3), (2) a transmembrane domain, and (3) an intracellular extension that includes the juxtamembrane domain, ATP-binding site, catalytic tyrosine kinase domain, and C-terminal domain (Fig. 3). The intrinsic β-subunit protein tyrosine kinase activity is inhibited by unoccupied α-subunits.

When the receptor is activated by insulin, there occurs a noncovalent oligomerization, resulting in autophosphorylation of several tyrosine residues in the β-subunit intracellular juxtamembrane region, a regulatory region, and the carboxyl terminus of the β-subunit. The binding of insulin to the α-subunits induces a conformational change that brings the α-subunits into closer proximity and enables ATP to bind the β-subunit intracellular domain. Although seven tyrosine residues are phosphorylated in the β-subunit, three main clusters of autophosphorylation sites are recognized to play a functional role in insulin action, including Tyr-972 in the juxtamembrane domain (an important "docking site" in the interaction between the receptor and its intracellular substrates); Tyr-1158, Tyr-1160, and Tyr-1162 in the catalytic domain (essential for receptor kinase activity); and Tyr-1328 and Tyr-1334 in the carboxyl-terminal domain (suggested to mediate mitogenic activity of the receptor) (see Fig. 3, in which tyrosine is designated with the one-letter symbol, Y). Progressive phosphorylation of tyrosine residues in the regulatory region results in parallel changes in biological activity. Phosphorylation and activation of the insulin receptor kinase activity have two important functions: to signal the receptor-ligand complex to be endocytosed at regions of clathrin-coated pits in the plasma membrane and to signal the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and other proteins.

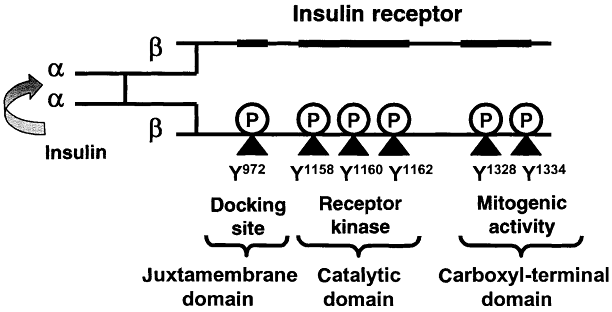

FIGURE 3 The insulin receptor tyrosine phosphorylation sites of regulation. Insulin binds to the receptor and initiates the autophosphorylation of tyrosines within the three major regulatory domains of the receptor. The functionality of the domains is indicated. Numbers refer to the position of tyrosine (Y) in the β-subunit amino acid sequence.

There is also phosphorylation on serine and threonine residues of the insulin receptor under basal and stimulated cell conditions. Stimuli such as insulin, phorbol esters (which stimulate protein kinase C activity), and cyclic AMP (which stimulates protein kinase A) can induce serine and threonine phosphorylation of the insulin receptor. It is possible for serine phosphorylation to decrease insulin-stimulated tyrosine kinase activity. Thus, at least three levels of regulation of the insulin receptor—tyrosine kinase autophosphorylation, serine/threonine phosphorylation, and inhibitory serine phosphorylation—are regulated by extracellular signals and intracellular messengers and other events.

## IV. INSULIN RECEPTOR SUBSTRATES

Insulin receptor substrate-1 (IRS-1) was the first docking protein identified within the IRS class of molecules. Five other related proteins within the family have also been identified, including IRS-2, IRS-3, IRS-4, Gab1, and $p62^{dok}$. Each of the IRS proteins share a common architecture: an $NH_2$-terminal pleckstrin homology and/or a phosphotyrosine-binding (PTB) domain, tyrosine residues that create SH2 (SRC homology 2) protein-binding sites, proline-rich regions for SH3 or WW domain recognition, and serine/threonine-rich regions. Insulin, insulin-like growth factor-I (IGF-I), and certain cytokine receptors can phosphorylate IRSs at specific Y-x-x-M (methionine) motifs that then serve as molecular bonding agents to other signaling molecules capable of transducing a signal to the diverse array of metabolic and biochemical pathways influenced by insulin. The PTB domain provides a coupling site for IRS with the insulin receptor, and binds weakly to the phosphorylated NPXY (asparagine, proline, X, tyrosine) motif in the juxtamembrane region of the insulin receptor. The pleckstrin homology domain may also play a role in IRS protein binding to the insulin receptor.

IRS-1 and IRS-2 are expressed in many tissues, but their levels are regulated independently. IRS-3 is expressed in adipose tissue, liver cells, pancreatic islet beta cells, and fibroblasts, whereas IRS-4 is expressed in kidney, brain, and thymus. Gab1 (Grb2-associated binding protein) is found in many mammalian tissues, and insulin induces tyrosine phosphorylation. In the absence of IRS-4, there is modest growth retardation and insulin resistance, whereas loss of Gab1 activity results in an embryonic-lethal phenotype.

IRS proteins in conjunction with activated insulin receptor become tyrosine phosphorylated and bind to the SH2 domains of various signaling proteins to generate downstream signals (Fig. 4). IRS-1 and IRS-2 associate with phosphatidylinositol 3-kinase (PI3K); the phosphotyrosine phosphatase SHP2; the Src-like kinase fyn; and the adapter proteins Grb2 (growth factor receptor binding protein 2), Nck, and Crk. IRS-1 and IRS-2 have some functional similarities, but also retain unique characteristics. IRS-1

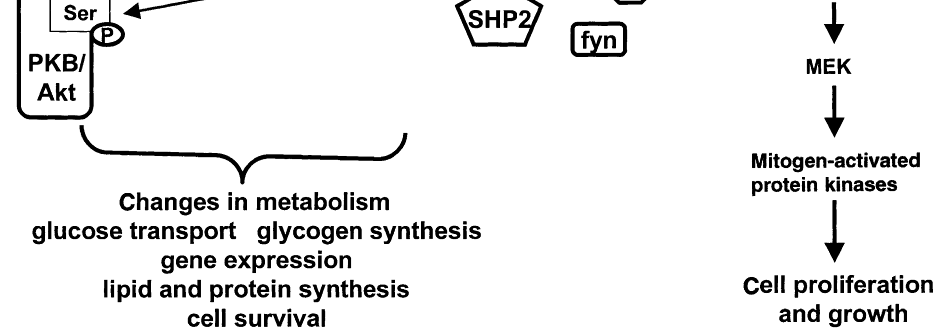
Insulin binds to insulin receptor
Plasma membrane
PIP3
PIP3
PIP3
PIP2
PI
PH
Atypical PKCζ and λ
Class 1a PI3K
P
Thr
Ser
PDK1
PDK2
PKB/ Akt
IRS
Shc
Sos
Grb2
Nck
Crk
SHP2
fyn
Ras
Raf
MEK
Mitogen-activated protein kinases
Cell proliferation and growth
Changes in metabolism
glucose transport glycogen synthesis
gene expression
lipid and protein synthesis
cell survival

FIGURE 4 Activated insulin receptor signal transduction pathways. Insulin binding to the insulin receptor initiates phosphorylation of the receptor and subsequent binding of insulin receptor substrate (IRS) proteins and Shc protein substrates. Adapter proteins Grb2, Nck, and Crk mediate signal transduction events. SHP2 is an IRS phosphatase. Phosphorylated IRS proteins activate PI3K, increasing phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) levels in the plasma membrane. PI3K also activates phosphoinositide-dependent kinases (PDK1 and PDK2) to phosphorylate protein kinase C (PKC) isoforms and/or PKB/Akt. Receptor-mediated binding and phosphorylation of Shc induces association with an adapter protein that activates Sos and the mitogen-activated protein kinase cascade. Insulin activation of PKC and PKB/Akt initiates cell responses unique from those elicited by the mitogen-activated protein kinase pathway.

and IRS-2 coordinate insulin effects on metabolism, islet beta cell function, reproduction, and growth and development. IRS-3, when tyrosine phosphorylated, binds PI3K. It has yet to be proved that IRS-4 behaves in a similar manner, although it contains several potential tyrosine phosphorylation sites. p62$^{dok}$ is unique in its failure to bind PI3K.

In addition to IRS proteins, products of the *shc* gene are also substrates of the insulin receptor. The protein products of this gene contain SH2 domains and are tyrosine phosphorylated in response to insulin receptor activation. Shc and IRS proteins compete for the same binding site on the insulin receptor. Phosphorylated Shc associates with Grb2 and can activate the Ras (superfamily of small guanosine trisphosphatases)/mitogen-activated protein kinase (MAPK) pathway independently of IRS-1 (Fig. 4). Grb2, an adapter protein containing SH3 domains, allows constitutive association with the guanine nucleotide exchange factor Sos (son-of-sevenless) and is part of the cascade including Ras, Raf, and MEK [the dual-specificity MAPK/extracellularly regulated kinase (ERK)] that leads to activation of MAPK and mitogenic responses characterized by gene transcription initiated by transcription factors such as elk1 and c-fos (Fig. 4). Thus, insulin initiates cell proliferation in certain target tissues.

IRS proteins are important loci of signal divergence in insulin signaling. One path leads to IRS-1 and Grb2 activation of the ERK–MAPK cascade, promoting gene transcription. The alternative path leads to IRS protein activation of PI3K class 1a. PI3K class 1a has been implicated in biological responses to insulin and insulin-like growth factor, including glucose transport, DNA synthesis, changes in glycogen metabolism, and cell survival with antagonism of apoptotic responses. IRS-1 seems to be particularly important for somatic growth, especially embryonic and neonatal growth, as demonstrated in mice. Interestingly, IRS-2 cannot substitute for IRS-1 in mediating somatic growth, suggesting unique roles for these IRS proteins. IRS proteins display signal redundancy as well as diversity in the activation of PI3K. IRS-1 and IRS-3 activate PI3K more strongly than does IRS-2, whereas IRS-4 barely activates PI3K in cell-based assays. In liver, IRS-2 plays a major role in PI3K activation.

IRS-1 exerts its greatest effect on metabolism by mediating insulin signals in muscle and adipose tissue, with lesser effects on liver metabolism, for which IRS-2 dominates. In mice with *irs1* or *irs2* gene disruption, there is marked insulin resistance compared with wild-type mice, which have normal gene function. It appears that the lack of IRS-1 in IRS-1$^{-/-}$ knockout mice leads to loss of insulin responsiveness, mainly due to decreased insulin-stimulated peripheral glucose uptake; the lack of IRS-2 in IRS-2$^{-/-}$ mice results in insulin resistance largely associated with decreased peripheral glucose uptake and unsuppressed hepatic gluconeogenesis. The IRS-2$^{-/-}$ knockout mice also show poor suppression of lipolysis in response to insulin. With regard to carbohydrate and lipid metabolism, IRS-1 seems to play a major role in muscle and adipose tissue, whereas IRS-2 has primary importance in liver and muscle.

### A. The Insulin–IRS Pathway and the Pancreatic Beta Cell

The insulin–IRS pathway also plays an important role in the functioning of the insulin-secreting pancreatic beta cells. In mice that lack the IRS-1 gene (IRS-1$^{-/-}$) but that are heterozygous for IRS-2$^{-/+}$, there is no overt diabetes mellitus because an active beta cell compensates for insulin resistance by increasing the amount of insulin secreted. In contrast, homozygous IRS-1$^{-/-}$/IRS-2$^{-/-}$ mice fail to compensate for peripheral insulin resistance, have underdeveloped beta cell mass and hyperglycemia, and die before maturity. There is also evidence that IRS-2 signaling may be important for embryonic development and postnatal growth of pancreatic beta cells. The importance of insulin and the IRS-signaling pathway in normal glucose metabolism is emphasized by the roles these molecules play, not only in general aspects of metabolism but also in beta cell survival and glucose-sensitive insulin secretion.

## V. PHOSPHATIDYLINOSITOL 3-KINASE

An initial step in insulin signal transduction is the IRS-induced activation of PI3K. Phosphorylation of IRS proteins creates recognition/docking sites for the two SH2 domains of an adapter protein complexed with cytosolic class 1a PI3Ks (Fig. 5). Such docking promotes the translocation of the cytosolic PI3Ks to the juxtamembrane region, where they phosphorylate their lipid substrate inositol ring (Fig. 5). Phosphatidylinositol (PI) is a phospholipid with a hydrophobic domain consisting of two fatty acid molecules attached by ester bonds to a three-carbon glycerol moiety in the plane of the plasma membrane; a hydrophilic phosphorylated base group

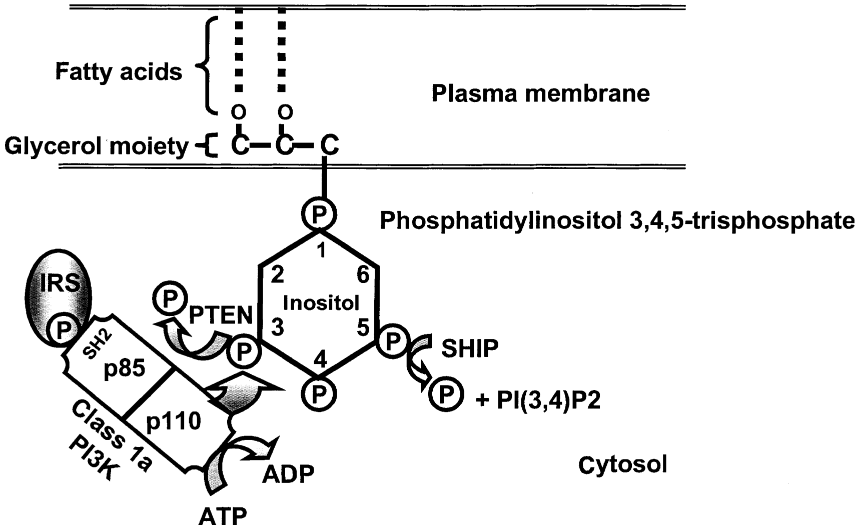

FIGURE 5 Phosphatidylinositol 3,4,5-trisphosphate formation and dephosphorylation. The two fatty acid side chains on C-1 and C-2 of the glycerol backbone of phosphatidylinositol 4,5-bisphosphate [PI(3,4)P2] are situated in the plane of the membrane, whereas the phosphorylated inositol ring base group extends to the cytosol. Phosphatidylinositol 3-kinase (PI3K) class 1a is activated by complex formation with insulin-activated insulin receptor substrate (IRS) protein at the adapter protein p85 subunit. The activation of p85 initiates catalytic activity of the p110 subunit, utilizing ATP and transferring a phosphate (P) group to the 3′ position of the inositol ring, to form phosphatidylinositol 3,4,5-trisphosphate. SHIP and PTEN are phosphatases, removing P and converting phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate and/or phosphatidylinositol 4-phosphate.

(inositol phosphate) extends from the glycerol moiety into the juxtamembrane region of the cytosol (Fig. 5). PI3K catalyzes the phosphorylation of phosphoinositides at the 3′ position of the inositol ring or proteins at serine residues. The predominant class 1a PI3K exists as heterodimers, with an adapter 85-kDa subunit associated with a catalytic 110-kDa subunit. However, diversity within the PI3K family is achieved through the expression of multiple regulatory subunits (p85α, p85β, $p55^{PIK}$, p55α/AS53, and p50α) and multiple catalytic subunits (p110α, p110β, or p110δ). Class 1a PI3K exists as several isoforms and splice variants of the adapter and catalytic subunits. The class 1b PI3K is regulated by G-protein-coupled receptors and not by insulin.

Class 1 PI3Ks *in vitro* have been shown to prefer specific substrates: PI, phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate ($PIP_2$). However, in intact cells PI3Ks seem to prefer $PIP_2$ as substrate. The most abundant product formed by PI3K activation in response to insulin stimulation is phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$). Class 2 PI3K can be activated by insulin stimulation, but it cannot utilize $PIP_2$ as a substrate. Class 3 PI3K seems to use only PI as a substrate. Thus, it is not likely that class 2 or class 3 PI3Ks mediate insulin signaling in large measure. The levels of the phosphatidylinositols are reduced by the actions of specific phosphatases. The lipid phosphatase PTEN (for phosphatase and tensin homologue deleted from chromosome 10) dephosphorylates 3-phosphoinositides specifically at position 3 of the inositol ring and has highest specificity for $PIP_3$ (Fig. 5). SH2 domain-containg phosphatase 2 (SHIP-2), widely expressed in nonhematopoietic cells, is an inositol 5′-phosphatase that hydrolyzes $PIP_3$ to $PIP_2$ (Fig. 5). SHIP-1 is an isoform found in hematopoietic cells. In cells lacking SHIP, there is prolonged activation of $PIP_3$-dependent pathways.

PI3K activation mediates cellular responses to insulin in a variety of ways. Phospholipid products of PI3K recruit serine kinases to the plasma membrane, including phospholipid (3-phosphatidylinositol)-dependent protein kinases (PDK1 and PDK2) and protein kinase B (PKB)/Akt (a product of the *akt* protooncogene) (Fig. 4). PI3K activates PKB/Akt and atypical protein kinase Cs (PKCs) that are not stimulated by diacylglycerol but are responsive to $PIP_3$ (PKC ζ and PKC λ) (Fig. 4). PIP3 also activates salt- and glucocorticoid-induced kinases and wortmannin-sensitive and insulin-stimulated serine kinases, among others.

## VI. PROTEIN KINASE B/AKT

PKB/Akt is a serine/threonine kinase that contains a pleckstrin homology domain, a catalytic domain, and a putative regulatory fragment at the carboxyl terminus. PKB/Akt activation involves interaction with $PIP_3$ and/or $PIP_2$ through binding of the phospholipids at the pleckstrin homology domain (Fig. 4). Three isoforms of PKB/Akt encoded by three separate genes have been identified in mammalian cells. PKB/Aktα is predominantly activated by insulin in muscle, liver, and adipocyte tissue. In adipocytes, it is PKB/Aktβ that is primarily activated, whereas PKB/Aktγ is not activated by insulin in the major target tissues, although there is activation in some cultured cells. The activation of class 1a PI3K is necessary and sufficient for activation of PKB/Akt, and blockade of PI3K activity blocks the stimulation of PKB/Akt. The activation of PKB/Akt by insulin appears to be primarily through phosphorylation on two sites (threonine and serine). The threonine site is associated with the catalytic subunit and the serine phosphorylation site is associated with the regulatory fragment. PDK1 is responsible for phosphorylation at the threonine site in each of the PKB/Akt isoforms, and phosphorylating activity is dependent on the presence of $PIP_3$ *in vitro*. The enzyme responsible for serine phosphorylation in PKB/Akt has yet to be cloned, but has been named PDK2. The inositol–lipid interaction recruits PKB/Akt to the plasma membrane, where it undergoes a conformational change and opens threonine and serine residues to phosphorylation by PDK1 and PDK2, respectively (Fig. 4). Once phosphorylated and activated, PKB/Akt can phosphorylate specific substrates, including glycogen synthase kinase-3 (GSK-3), which results in inhibition of the enzyme and an increase in glycogen synthesis—a major metabolic mechanism of action of insulin in hepatocytes and other tissues. However, PKB/Akt activation mediates several of insulin's pleiotropic responses, including changes in metabolism, glucose transporter synthesis and mobilization, gene expression, and lipid and protein synthesis (Table 1).

The forkhead (FKH) transcription factor family, including FKHR, FKHRL, and AFX, has important effects on gene transcription and may mediate insulin effects on genes, such as those encoding IGF-I, phosphoenolpyruvate carboxykinase, and glucose 6-phosphatase. FKHR enhances transcription of genes that regulate glucose synthesis and cell division as well as cell death (apoptosis). The phosphorylation of these factors leads to inactivation. Insulin induces the phosphorylation of FKHR by $PIP_3$-dependent kinases, such as PKB/Akt and others. Due to the fact that FKHR is inhibited by insulin-induced phosphorylation, this limits transcription of genes

TABLE 1 PKB/Akt-Mediated Signal Transduction Events

| Target | Response | Event |
|---|---|---|
| **PKB/Akt target** | | |
| Phosphofructokinase-2 | Activation | Activates phosphofructokinase-1, increases glycolysis |
| GLUT4 | Translocation | Increases glucose uptake |
| mTOR | Activation | Activates P70 S6 kinase and protein synthesis, activates eIF-4 initiation complex, increases mRNA translation, increases peptide chain elongation |
| eIF-2B | Activation | Activates eIF-2, increases mRNA translation, inactivation of GSK-3 |
| IκB kinase | Activation | Promotes IκB dissociation from NF-κB, activates NF-κB-mediated gene transcription |
| Phosphodiesterase 3B | Activation | Regulates intracellular cyclic AMP levels |
| Cyclic AMP response element binding protein | Activation | Transcriptional activation |
| Glycogen synthase kinase-3 | Inhibition | Increases glycogen synthesis |
| Glycogen synthase kinase-3β | Inhibition | Activates AP-1 and other transcription factors, increases transcription |
| Forkhead factor FKHR | Inhibition | Limits transcription of genes regulating apoptosis, glucose production, and cell division |
| **Gene target** | | |
| GLUT1, GLUT3 | Increased | Increases glucose uptake |
| Fatty acid synthase promoter | Increased | Increases fatty acid synthesis |
| *c-fos* | Increased | Increases transcription |
| Phosphoenolpyruvate carboxykinase | Decreased | Decreases gluconeogenesis |

that are antagonistic to insulin actions (Table 1). PKB/Akt may also affect other transcription factors, such as nuclear factor-κB (NF-κB); cAMP response element binding protein (CREB), which is phosphorylated by PKB/Akt to induce transcriptional activation; and eukaryotic initiation factor eIF-2B. The effects of PKB/Akt on NF-κB are indirect, initiated by activation of IκB kinase with subsequent degradation of IκB and activation of NF-κB.

In addition to transcription factors, PKB/Akt can have effects on messenger RNA translation by affecting other factors. PKB/Akt can activate the mammalian target of rapamycin (mTOR), which activates 4E-BP, which in turn activates eIF-4E (Table 1). Through the activation of mTOR, PKB/Akt may be able to influence the elongation of translation through p70 ribosomal S6 kinase (p70 S6 kinase) and elongation factor activation. Elongation initiation factor activity and mRNA translation may also be mediated through the inhibition of GSK-3.

Metabolism is affected by PKB/Akt at several levels. PKB/Akt increases expression of the glucose transporters GLUT1 and GLUT3 and the translocation of GLUT4 to the plasma membrane. Moreover, glycogen synthase activation and glycogen synthesis follow the inactivation of GSK-3. In cardiac cells, the rate-limiting step in mammalian glycolysis, phosphofructokinase-1, is activated by PKB/Akt (Table 1). PKB/Akt also affects several other genes involved in metabolic control, such as the fatty acid synthase promoter and the phosphoenolpyruvate carboxykinase gene.

Consistent with effects on metabolism, PKB/Akt has been shown to promote cell survival and block apoptosis. Similar to some growth factors, PKB/Akt maintains the integrity of mitochondria and prevents the release of cytochrome *c*, which initiates the apoptotic cascade. These effects may be integral to the antiapoptotic effects of PKB/Akt. It is also likely that the positive metabolic effects of PKB/Akt activation on glycolysis and protein synthesis contribute to cell survival.

## VII. p70 S6 KINASE

Another serine/threonine kinase involved in insulin action is p70 S6 kinase, which requires phosphorylation for activation. Evidence suggests that PI3K, PKB/Akt, and mTOR activation participates in the activation of p70 S6 kinase, because inhibition of PI3K or PKB/Akt blocks its activation. Although there is no direct evidence that p70 S6 kinase is phosphorylated by PKB/Akt, PDK1 phosphorylates one of the threonine residues that activates the enzyme. PI3K-independent mechanisms also regulate p70 S6 kinase, but these entail longer activation times. Because rapamycin can inhibit the activation of p70 S6 kinase, this is evidence that mTOR is in one of the pathways regulating this enzyme.

## VIII. ATYPICAL PROTEIN KINASE Cs

Insulin-induced PI3K activation results in activation of protein kinase C ζ and PKC λ. These are atypical PKCs that do not require $Ca^{2+}$ and are not activated by diacylglycerol or phorbol ester. Blockade of PI3K prevents the insulin-stimulated activation of PKC ζ, whereas the phospholipids $PIP_3$ and $PIP_2$ directly stimulate PKC ζ *in vitro* to mimic the effects of insulin. Likewise, in mutant cells lacking IRS-1–PI3K binding sites, the ability of insulin to activate PKC ζ is lost. PI3K appears to act through the kinase PDK1 to phosphorylate and activate PKC ζ. However, in the cascade, PKC ζ activation does not lead to PKB/Akt, or to mTOR and p70 S6 kinase activation, suggesting that PKC ζ and PKC λ promote effector pathways separate from those responsive to PKB/Akt. Like PKC ζ, the activation of PKC λ is not dependent on PKB/Akt activation in response to PI3K. In addition, atypical PKC ζ and PKC λ have been implicated in insulin-dependent glucose transport and protein synthesis.

## IX. MITOGEN-ACTIVATED PROTEIN KINASE

Another of the signaling pathways for insulin is the mitogen-activated protein kinase series of enzymatic reactions. As a result of insulin stimulation, IRS proteins and Shc bind to the SH2 domains of small adapter proteins (Fig. 4). One such adapter protein, Grb2, links IRS/Shc and Sos. The two SH3 domains on Grb2 associate with proline-rich regions in the guanine nucleotide exchange factor, Sos. Sos stimulates GDP/GTP exchange on Ras. Ras is a key player in recruiting the serine/threonine kinase Raf to the plasma membrane. Once Raf is activated, a cascade of activation begins with the activation of MEK, the dual-specificity MAPK kinase/extracellularly regulated kinase. MEK is activated by phosphorylation at two serine residues, but activates MAPK by phosphorylating both tyrosine and threonine. The p90 ribosomal protein S6 kinase (p90 rsk), phospholipase $A_2$, and many transcription factors are activated as a result of MAPK-induced phosphorylation. Among the signaling pathways identified for insulin, the MAPK pathway is not very sensitive to insulin and is not involved in the major metabolic responses to insulin. It has been proposed that activation of GTP-loaded Ras interacts directly with PI3K; however, the functional interaction of these two players in insulin action remains under investigation.

## X. INHIBITION OF INSULIN SIGNALING

Mechanisms for termination of insulin receptor signal transduction include promoting serine phosphorylation of IRS proteins or the insulin receptor, and dephosphorylating serine or tyrosine. The IRS-1- and Grb2-activated MAPK pathway participates in the former process. Because phosphorylation and dephosphorylation of distinct proteins play major roles in insulin stimulation of cells, phosphatases may be viewed as negative modulators of insulin action—removing phosphate groups from enzymes dependent on them for activation. The phosphatase family of enzymes comprises more than 100 different genes. SHP2 is an SH2-containing phosphatase (also known as Syp or SH-PTP2) negatively modulating insulin receptor activity. The association of SHP2 SH2 domains with tyrosine-phosphorylated IRS-1 during insulin stimulation promotes phosphotyrosine dephosphorylation, thus attenuating insulin signaling by reducing the association with effector proteins in certain signaling pathways. PTPase 1B is another tyrosine phosphatase that is a potential negative modulator of insulin action as it has been demonstrated to dephosphorylate the insulin receptor *in vitro*. The role of PTPase 1B in insulin action *in vivo* remains to be demonstrated, although mice lacking PTPase 1B exhibit a phenotype with enhanced insulin sensitivity.

Phosphatase activation also plays an important metabolic role in insulin actions. Insulin activates protein phosphatase-1 (PP-1), a serine/threonine phosphatase that is part of the signal transduction cascade regulating glycogen metabolism. Phosphorylation is required for PKB/Akt activity, and dephosphorylation is a potential mechanism for cellular termination of an insulin response. Phosphatase 2A may be an important enzyme in the regulation of PKB/Akt. Phosphatase inhibitors such as vanadate and okadaic acid can augment PKB/Akt activity. Moreover, a specific inhibitor of phosphatase 2A, calyculin A, can prevent dephosphorylation and inactivation of PKB/Akt. The activity of PKB/Akt is also modulated by PTEN and SHIP dephosphorylation of $PIP_3$.

## XI. CELLULAR RESPONSES TO INSULIN

### A. Glucose Transport

The major metabolic actions of insulin relate to changes in glucose metabolism. Blood glucose levels are lowered and metabolism is fueled by glucose influx into cells across plasma membranes by GLUT-mediated facilitated diffusion. Glucose transporters are integral membrane-spanning glycoproteins with molecular masses of about 50,000 Da. Insulin–receptor binding initiates the translocation of intracellular vesicles containing glucose transporters (GLUT4 and GLUT1) to the plasma membrane, and glucose transport is proportional to the increase in transporter population in the membrane; insulin also diminishes the rate of dissociation of GLUT back to the cytoplasm (Fig. 6). The mobilization of glucose transporters is reversible on reduction in the insulin stimulus, when the transporters are sequestered back to the intracellular vesicular pool (Fig. 6). GLUTs associate and dissociate from the plasma membrane in the basal state. However, not all GLUTs are insulin responsive; for example, GLUT2 in plasma membranes of pancreatic islet beta cells allows these cells to respond directly to extracellular glucose concentrations.

Two models for GLUT sequestration/mobilization argue either that GLUT-containing vesicles are mobilized in response to insulin receptor stimulation, or that GLUTs are constitutively prevented from cycling to the plasma membrane by some inhibitory factor, and that insulin receptor stimulation removes that inhibition. Certain of the factors supporting the former vesicular sequestration model have been identified. The vesicle containing GLUT4 is proposed to dock onto and fuse with the plasma membrane via a v-SNARE protein (vesicle SNAP receptor) pairing with a target site on the membrane and/or recognizing t-SNAREs (Fig. 6). VAMP-2 is the predominant v-SNARE found in GLUT4 vesicles. Within the plasma membrane of insulin-responsive cells, the main t-SNAREs are syntaxin 4 and SNAP-23. Synip is a syntaxin 4-binding protein that dissociates in response to insulin and allows GLUT4 translocation. Munc18 is another syntaxin 4-binding protein that dissociates after insulin stimulation, thus allowing increased binding of VAMP-2 to syntaxin 4 and constituting docking of the GLUT vesicle to the plasma membrane.

The elements of the insulin receptor signaling cascade that are important for GLUT4 translocation include the tyrosine kinase activity of the receptor

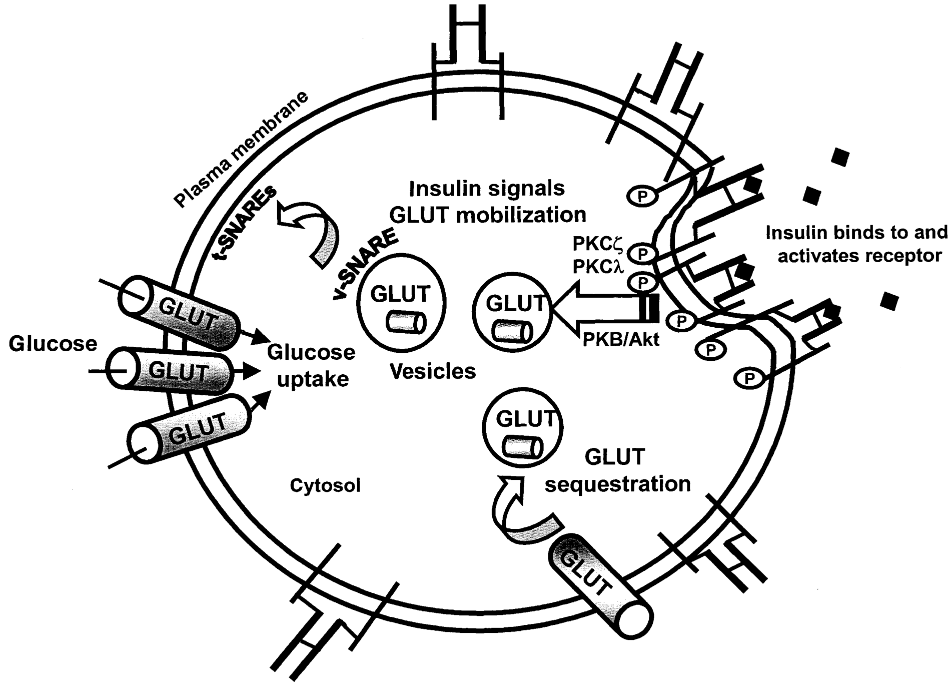

FIGURE 6 The vesicle model of GLUT translocation. Insulin binding activates the insulin receptor and the downstream effector molecules that participate in the mobilization of GLUT-containing vesicles to the plasma membrane. For glucose facilitated diffusion, v-SNARE peptides in the vesicles recognize t-SNARE peptides at the plasma membrane docking sites for GLUT insertion to the plasma membrane. In the absence of insulin receptor binding, GLUTs are sequestered in intracellular vesicles, from which they can be mobilized.

and IRS activation, although IRS-1 may not play an essential role and in fact many mechanisms contributing to GLUT translocation are not known. PI3K has also been implicated in GLUT regulation because PI3K inhibition can inhibit GLUT4 mobilization, whereas overexpression of the PI3K gene increases GLUT4 translocation in adipocytes and increases glucose uptake. However, PI3K does not appear to be a sufficient stimulus for glucose uptake, because stimuli other than insulin that increase PI3K activity do not increase glucose uptake.

PKB/Akt has been implicated in the regulation of glucose transport downstream of PI3K. Active PKB/Akt is associated with GLUT4 translocation to the plasma membrane and increased glucose transport. Moreover, PKB/Aktβ associates with GLUT4-containing vesicles following insulin stimulation of cells. A more specific role for PKB/Akt isoforms in glucose uptake remains to be determined. The atypical PKCs also appear to play a role in regulating glucose uptake. Expression of PKC ζ in cells increases GLUT4 translocation and glucose transport in response to insulin. With the expression of a dominant negative PKC ζ mutant in adipocytes and a muscle cell line there is inhibition of PKC ζ synthesis and partial inhibition of GLUT4 translocation and glucose uptake. Similarly, expression of PKC λ mutants deficient in kinase activity leads to reduced GLUT4 translocation and inhibition of glucose uptake.

Downstream in the insulin response pathway, p70 S6 kinase activation is not required for insulin-stimulated glucose uptake or for GLUT4 translocation. However, p70 S6 kinase mediates long-term effects of insulin on synthesis of GLUT1 transporter in cells. The MAPK pathway also does not seem to play a major role in the rapid stimulation of glucose transport in muscle and fat cells. Ras-microinjected adipocytes do not show increased GLUT4 translocation, nor does Ras inhibition negatively impact insulin-stimulated translocation. Moreover, inhibition of MEK does not inhibit the glucose transporter response to insulin stimulation. In contrast, the long-term effects of insulin on synthesis of GLUT3 in muscle cells appear to be mediated by the MAPK pathway.

## B. Glycogen Synthesis

One of the major actions of insulin is to promote glucose uptake into cells with storage of energy in the form of glycogen, primarily in skeletal muscle and liver cells. Glycogen synthase in skeletal muscle is subject to phosphorylation at multiple sites by many protein kinases. When insulin induces dephosphorylation of glycogen synthase, there is an increase in catalytic activity. Protein phosphatase 1G (PP1G) is a glycogen-bound form of protein phosphatase 1 and is responsible for dephosphorylation of glycogen synthase. Insulin-stimulated phosphorylation of PP1G may contribute to regulation of its activity. Whether p90 rsk2, which phosphorylates PP1G, mediates PP1G activity in intact cells is a subject of debate. Or, in lieu of a phosphatase to remove phosphate groupings, the inhibition of a kinase may regulate glycogen synthase. GSK-3 is phosphorylated in response to insulin, and inactivated. PI3K phosphorylates GSK-3, and the downstream PI3K effectors PKB/Akt, mTOR, and p70 S6 kinase may also play roles in glycogen synthase activation and glycogen storage. The underlying molecular mechanisms regulating glycogen synthase activity continue to undergo investigation.

## C. Protein Synthesis

One of the major actions of insulin is on protein synthesis, involving not only changes in transcriptional activation and messenger RNA production, but also increased translational activity of specific proteins in certain tissues. The mRNA cap-binding protein, eukaryotic initiation factor-4E (eIF-4E), recognizes the mRNA cap structure and regulates translation. Insulin activation induces the phosphorylation of serine in eIF-4E, which in turn increases the affinity of eIF-4E for the mRNA cap structure and increases translation. The eIF-4F initiation complex is formed by the association of eIF-4E, and the RNA helicase eIF-4A, and eIF-4G (which forms a bridge between eIF-4E and eIF-4A) (Fig. 7). During insulin stimulation of adipocytes, there is phosphorylation of eIF-4E-binding protein [4E-BP1; also known as the phosphorylated heat- and acid-stable protein (PHAS-1)], which subsequently dissociates from eIF-4E, allowing translation to commence (Fig. 7). Such a regulatory phenomenon has been observed for skeletal muscle, adipocytes, and myoblasts. Insulin activation of PI3K, with subsequent activation of PKB/Akt and the mTOR pathway, mediates insulin-induced phosphorylation of 4E-BPs. It does not appear that the p70 S6 kinase downstream of mTOR, nor the MAPK pathway, participates in this regulatory process.

eIF2 is another important component of the mRNA translational sequence. A requirement for initiation is the role of eIF2 to bind the initiator Met-tRNA to the 40S ribosomal subunit. eIF-2B is a guanine nucleotide exchange factor that promotes release of bound GDP and binding of GTP to eIF2. In addition, PI3K activation appears to play a role in insulin activation of eIF-2B. The mTOR, p70 S6 kinase, and the MAPK pathways do not appear to play a role in eIF-2B activation. Elongation of peptide chains is crucial for protein synthesis, and elongation factors eEF1 and eEF2 are required. Insulin stimulates the elongation rate and increases the phosphorylation of eEF1, in contrast with a reduction in eEF2 phosphorylation. PI3K acting through the mTOR pathway mediates insulin actions on elongation.

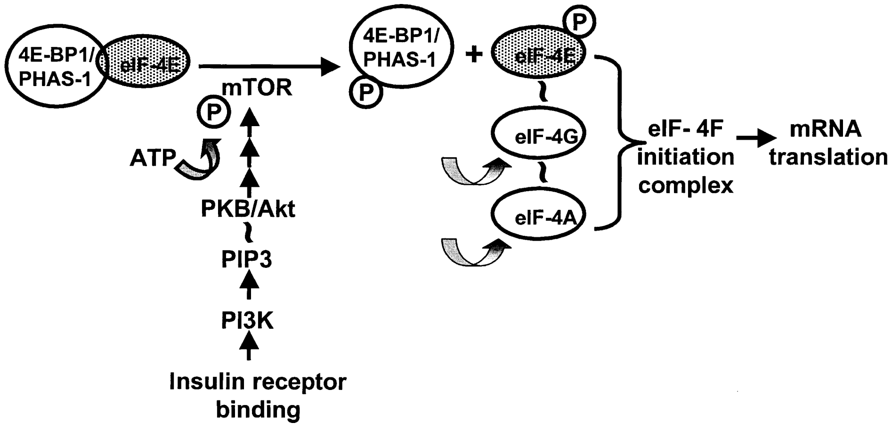

FIGURE 7 Insulin stimulation of mRNA translation. Insulin stimulates PKB/Akt activity, which leads to phosphorylated (P) mTOR; mTOR activation phosphorylates 4E-BP1, which causes dissociation of the inactive 4E-BP1–eIF-4E heterodimer complex. Phosphorylated eIF-4E associates with the bridge factor eIF-4G, which links to eIF-4A to form the eIF-4F initiation complex that mediates increased mRNA translation and protein synthesis.

## XII. SUMMARY

Insulin is notable for the variety of cellular responses that it evokes in target cells and tissues. Accounting for the pleiotropic responses, the insulin receptor signal transduction mechanism represents complex regulatory and counterregulatory pathways and events. The primary and secondary structures of the insulin molecule and insulin receptor have been elegantly sequenced and modeled, providing a window with a view as to how these complex molecules interact at the molecular level. Major players in the insulin receptor signaling cascade, including peptide mediators and adapter proteins, enzymes such as PI3K with their elaborate pathways for regulating metabolic, genetic, and protein synthetic responses in target cells, provide a strong base on which to formulate future hypotheses concerning the regulation of insulin-stimulated activities in cells.

## Glossary

**apoptosis** The process of programmed cell death. Numerous genetic and biochemical pathways contribute to apoptosis, and the process is linked to degradation of nuclear DNA.

**autophosphorylation** Transfer of a phosphate group to an amino acid within a protein (self-phosphorylation). Certain proteins have kinase activity as a part of their molecular structure and have this ability.

**catalytic domain** Amino acid sequence that contains a specific enzymatic activity associated with a protein.

**diabetes mellitus** Disease characterized by high blood glucose levels and low insulin levels and/or insufficient insulin receptor activity (insulin resistance).

**endocytosis** The process of membrane engulfment by a cell. A portion of the plasma membrane "pinches off" into the cytoplasm such that there is formation of an inside-out intracellular vesicle that contains the proteins, etc. that were originally present in the plasma membrane.

**pleiotropic** Having more than one effect; a single stimulus can often evoke several different cellular responses, often mediated by distinct intracellular messengers or pathways.

**protein kinase C** A family of serine/threonine protein kinases characterized by their ability to be activated by $Ca^{2+}$ and diacylglycerol (classical PKCs), by diacylglycerol alone (novel PKCs), or not by diacylglycerol or $Ca^{2+}$ but by phospholipids and phosphorylation (atypical PKCs).

**receptor** Protein that resides in the cell membrane, cytoplasm, or nucleus of cells. A ligand binds and activates the receptor to initiate a cellular biochemical or molecular response unique to each receptor.

**substrate** Molecule that will be altered by the actions of an enzyme.

**transcription** The molecular process of copying a gene to form a messenger RNA molecule; regulated by initiation factors and promoter regions in the nuclear chromatin.

**translation** The processing of information coded in messenger RNA to form amino acid sequences; results in cytoplasmic peptide and protein synthesis.

**translocation** The process of movement within a cell; usually the movement of a subcellular molecule or organelle from one site to another.

## See Also the Following Articles

**Apoptosis • Glucose-Dependent Insulinotropic Polypeptide (GIP) • Insulin Actions • Insulin-like Growth Factor (Igf) Signaling • Insulin Processing • Insulin Secretion**

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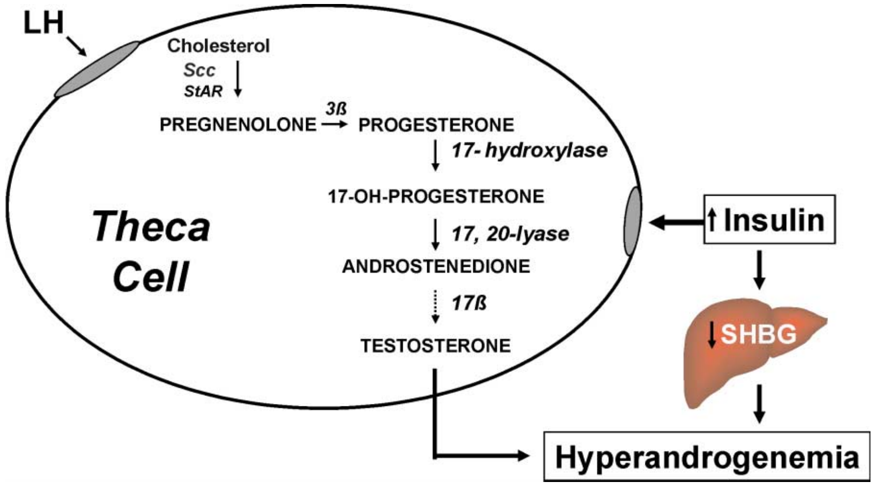

**FIGURE 1** Steroidogenic pathway in theca cells of small antral follicles in the ovary. Biosynthesis of androgen in response to luteinizing hormone (LH) is modulated by activities the dual activities (17-hydroxylase and 17, 20-lyase) of cytochrome P450c17. Insulin acts to suppress hepatic production of sex hormone-binding globulin (SHBG), thereby reducing the circulating levels of free (bioavailable) testosterone. Insulin also appears to regulate theca cell androgen production by activation of its cognate receptor. Scc, side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; 3β, 3β-hydroxysteroid dehydrogenase; 17β, 17β-hydroxysteroid dehydrogenase.

with abnormalities in glucose tolerance, insulin secretion, and lipoprotein profiles.

Obesity contributes to the insulin resistance in PCOS. However, the magnitude of insulin resistance exceeds that which would be predicted on the basis of total or even fat-free body mass. The cause of obesity in PCOS remains elusive. Nonetheless, it has been clearly documented that attenuation of insulin resistance, whether by weight loss or pharmacologically with diazoxide, metformin, or troglitazone, ameliorates many of the metabolic aberrations in women with PCOS.

## A. Impaired Glucose Tolerance and Type 2 Diabetes

Obesity is a well-recognized risk factor for the development of type 2 diabetes, but alone is insufficient to cause glucose intolerance. Thus, although it is generally accepted that women with PCOS are predisposed to type 2 diabetes, the development of diabetes cannot be attributed solely to the obesity that typically accompanies PCOS.

Recent data have established that the prevalence of impaired glucose tolerance (IGT) and type 2 diabetes mellitus among women with PCOS is exceptionally high and consistent across populations of varied ethnic and racial backgrounds. The prevalence of IGT is between 30 and 40% and that of type 2 diabetes is between 5 and 10%. These prevalences approximate those in Pima Indians, who have one of the highest rates of diabetes in the world. In addition, there appears to be a nearly 5- to 10-fold increase in the expected conversion rate from IGT to type 2 diabetes in PCOS with evidence for an enhanced rate of development of diabetes evident from long-term follow-up of women with the disorder.

This predisposition to type 2 diabetes in PCOS is related in part to insulin resistance, the magnitude of which is greater in women with PCOS than in carefully matched controls. A distinct and possibly selective form of insulin resistance may account for these findings.

Yet, although a substantial proportion of women with PCOS develop glucose intolerance, the majority do not. It is therefore reasonable to ask whether the defects in insulin action described above are sufficient to account for the high prevalence of diabetes in this population. Specifically, what factors distinguish insulin-resistant women with PCOS who develop glucose intolerance from those who are able to maintain normoglycemia?

Defects in insulin secretion play an important role in the propensity to develop diabetes in PCOS and are most appropriately expressed in relation to the magnitude of ambient insulin resistance. The product of these measures can be quantitated (the so-called "disposition index") and related as a percentile to the hyperbolic relationship for these measures established in normal subjects. In so doing, researchers have found that a subset of PCOS subjects have beta-cell

TABLE 1 Insulin Lowering Therapies in Polycystic Ovary Syndrome

| Therapy | Mechanism(s) of action | Examples | Use(s)[a] |
|---|---|---|---|
| Weight loss | Reduction in insulin demand, decreased secretion | | |
| Diazoxide | Decreased pancreatic insulin secretion | | |
| Biguanides | Reduce hepatic glucose production, secondarily lowering insulin levels; ?direct effects on ovarian steroidogenesis | Metformin (Glucophage, Glucophage XR) | 1, 2, 3, 4 |
| Thiazolidinediones | Enhance insulin action at target tissue level (adipocyte, muscle); some evidence for direct effects upon ovarian steroidogenesis | Rezulin, Pioglitazone (Actos), Rosiglitazone (Avandia) | 1, 2, 3, 4 |
| D-*Chiro*-inositol | Enhanced insulin action by repletion of specific D-*chiro*-inositol-containing inositolphosphoglycan | Not applicable | 2, 3, 4 |

[a] (1) Hirsutism and/or acne; (2) oligomenorrhea/amenorrhea; (3) ovulation induction; and (4) insulin-lowering therapy.

secretory dysfunction. In absolute terms, women with PCOS had normal first-phase insulin secretion compared to controls. In contrast, when first-phase insulin secretion was analyzed in relation to the degree of insulin resistance, women with PCOS exhibited a significant impairment in beta-cell function. This reduction was particularly marked in women with PCOS who had a first-degree relative with type 2 diabetes. Beta-cell function in PCOS has been additionally quantitated by examining the insulin secretory response to a graded increase in plasma glucose and by the ability of the beta cell to adjust and respond to induced oscillations in the plasma glucose level. Results from both provocative stimuli were consistent: when expressed in relation to the degree of insulin resistance, insulin secretion was impaired in PCOS subjects with a family history of type 2 diabetes when compared to controls. The inability to secrete insulin in quantities adequate for the degree of insulin resistance appears to have a heritable component in PCOS kindreds.

## III. TREATMENT OF HYPERINSULINEMIA IN PCOS

The realization that hyperinsulinemia is a key component in the pathogenesis of PCOS has provided the basis for these recent advances in treatment strategies for women with the disorder. Weight reduction, when it can be achieved, is still an important component in the treatment of PCOS. However, not all women with PCOS are obese, and since the etiology of obesity in PCOS in not known, there is currently no effective way to target this problem in PCOS. Pharmacologic reduction in insulin levels appears to offer another therapeutic modality for PCOS and is one that may ameliorate the sequelae of both hyperinsulinemia and hyperandrogenemia (Table 1). However, additional studies and long-term follow-up of patients so treated are necessary before these agents can be considered first-line treatment for PCOS.

## Glossary

**insulin resistance** The state in which insulin concentrations that are equal to or greater than normal produce less than the expected biologic effect.

**oligomenorrhea** Menstrual cycle pattern consisting of fewer menstrual periods within a given time, typically fewer than nine menstrual periods in 12 months.

## See Also the Following Articles

**Diabetes Type 2 • Hypoglycemia in Diabetes • Insulin Actions • Insulin-like Growth Factor (IgF) Signaling • Insulin Secretion • Polycystic Ovary Syndrome (PCOS) • Sex Hormone-Binding Globulin (SHBG)**

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# Insulin Secretion

Robert A. Ritzel, Darren J. Michael, and Peter C. Butler

*University of Southern California, Los Angeles*

I. OVERALL ROLE OF INSULIN
II. ANATOMY OF THE PANCREAS, BETA-CELL MASS, AND ITS REGULATION
III. INSULIN SYNTHESIS, STORAGE, AND SECRETION AT THE SINGLE BETA-CELL LEVEL
IV. PHYSIOLOGY OF INSULIN SECRETION: ISLETS
V. PHYSIOLOGY OF INSULIN SECRETION *IN VIVO*

**Upon stimulation of pancreatic beta cells, insulin is released into the pancreatic vein, which empties into the portal vein, to reach insulin's primary site of action, the liver. In humans, insulin is secreted in high-frequency pulses with a mean period of 4–6 min.**

## I. OVERALL ROLE OF INSULIN

Insulin is perhaps the most widely studied hormone. The absence of insulin leads to the life-threatening disease diabetes mellitus. Insulin has multiple actions on carbohydrate, fat, and protein metabolism. In the short term, its most important action is to regulate the concentration of glucose in the circulation at a concentration of ~90 mg/dl (~5 mmol/liter) (Fig. 1). Circulating glucose concentrations that are either too low (hypoglycemia) or too high (hyperglycemia) are life-threatening. Therefore, the concentration of glucose in the circulation is the predominant regulator of the insulin secretion rate.

## II. ANATOMY OF THE PANCREAS, BETA-CELL MASS, AND ITS REGULATION

Although the existence of an insulin-secreting organ had been anticipated for many years, it was not until the late 19th century that the pancreas was identified by the German physiologist Minkowski as the

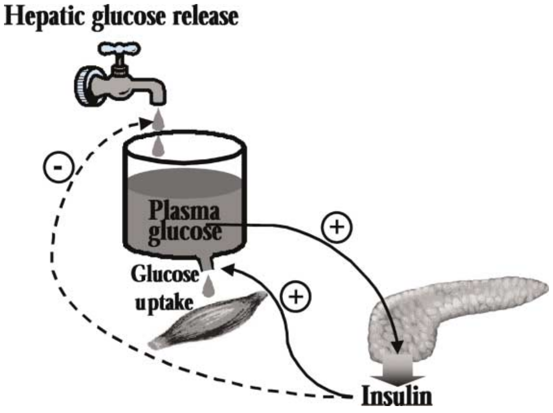

FIGURE 1 After nutrient intake, insulin secretion from the endocrine pancreas is enhanced to maintain physiologic plasma glucose concentrations (normoglycemia). Insulin stimulates glucose uptake into tissues and suppresses hepatic glucose release.

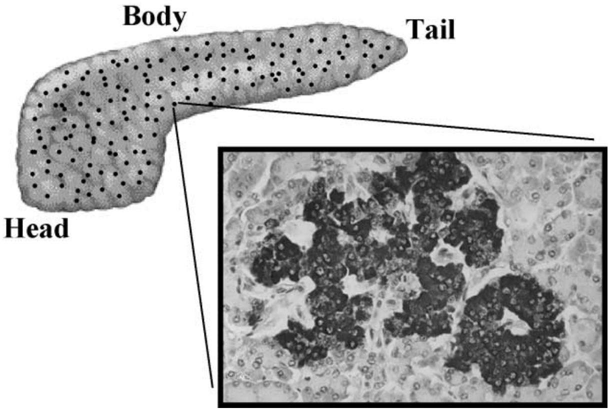

FIGURE 2 Macroscopically, the human pancreas is subdivided into head, body, and tail. The inset shows an islet of Langerhans (immunostained for insulin), which is surrounded by exocrine tissue.

organ from which insulin was secreted. The pancreas is a retroperitoneal organ that consists mostly of exocrine tissue (Fig. 2). This exocrine tissue synthesizes and secretes digestive enzymes for delivery to the small bowel via the exocrine duct in response to food ingestion. The endocrine component of the pancreas is only ~1% of the pancreas by volume and is dispersed throughout the much larger exocrine pancreas in the form of approximately one million clusters of cells called islets of Langerhans, after the German anatomist Paul Langerhans (Fig. 3), who first described them in 1869. Each islet consists of approximately 3000 endocrine cells, the most frequent of which are the insulin-producing beta cells. The beta cells are present in the core of the islet surrounded by a mantle of the other endocrine cells, glucagon-secreting alpha cells, somatostatin-secreting delta cells, and pancreatic polypeptide-secreting PP cells.

Each islet of Langerhans is independently vascularized and innervated. The supplying arteriole penetrates into the core of the islet, from which the capillary network supplies the beta cells first, before reaching the periphery of the islet. In consequence of this specific islet-microanatomy, the insulin concentration in the efferent venule is very high when it reaches the other endocrine cells, such as the glucagon-secreting alpha cells. When the isolated pancreas preparation was perfused via the conventional arterial route versus the reverse venous route, it was confirmed that this local release of insulin into the islet capillary network does have a local regulatory effect on downstream endocrine cells (i.e., alpha cells). Islets of Langerhans also secrete vascular and neurotropic factors, leading to the observation that islets transplanted into the liver regain their independent vascularization and innervation within approximately 1 week and 1 month, respectively.

During embryonic development, new islets bud off from the pancreatic duct. This process continues in adult life. In fact, regulation of beta-cell number (or beta-cell mass) is an important facet of regulation of insulin secretion. Since there is a basal rate of apoptosis of beta cells within islets, there must be replenishment of beta cells or the beta-cell mass would gradually diminish to zero. There are two potential sources for replenishment of beta cells: (1) the formation of new islets, called islet neogenesis, to replace aging and diminishing islets and (2) beta-cell replication within islets.

Beta-cell mass adaptively increases in rodents exposed to either sustained high blood glucose concentrations or insulin resistance resulting from obesity. In rodents, this increase is largely accomplished through the mechanism of increased beta-cell replication and, to a lesser extent, through increased neogenesis. Rodents with an acute decrease in beta-cell mass, induced by a partial pancreatectomy, show an impressive capacity to replace beta-cell mass within a few days by both mechanisms, a marked increase in islet neogenesis and beta-cell replication within the new islets. In humans, neogenesis is also

FIGURE 3 Paul Langerhans (1847–1888). Reprinted from "Diabetes in Medizin- und Kulturgeschichte" (von Engelhardt, ed.), copyright 1989 Springer-Verlag, Berlin, with permission.

present and, as in rodents, it increases in response to obesity by approximately 50%. In contrast, beta-cell replication is almost absent in mature islets in humans. However, human islets cultured *in vitro* and dispersed to a monolayer show some capacity for beta-cell replication. At present, it is unclear whether there are circumstances *in vivo* when beta-cell replication becomes important in mature human islets.

In summary, in humans insulin is secreted by pancreatic beta cells that exist in clusters of ~3000 cells, called islets of Langerhans. Approximately one million of these islets are scattered throughout the pancreas. Islets are created throughout life from stem cells within the exocrine ducts. Regulation of islet turnover (neogenesis and apoptosis) defines the beta-cell mass. In healthy individuals, beta-cell mass adapts to the demand for insulin. The mechanisms involved in the regulation of islet neogenesis and beta-cell apoptosis are currently under active investigation as potential cures for diabetes.

## III. INSULIN SYNTHESIS, STORAGE, AND SECRETION AT THE SINGLE BETA-CELL LEVEL

Soon after the source of insulin was established to be the endocrine pancreas, several efforts were made to isolate an insulin extract from the pancreas for the purpose of injection into people with diabetes. This was finally achieved by the team of Banting, Best, Collip, and MacLeod in 1921. Subsequently, insulin was the first protein to be crystallized to establish its structure. In humans, the insulin gene is on chromosome 11. Insulin is synthesized as a 109-amino-acid preproinsulin that is processed to an 86-amino-acid proinsulin. Proinsulin is predominantly trafficked to the secretory pathway via the Golgi apparatus into secretory vesicles (Fig. 4). Within these vesicles, proinsulin is cleaved into insulin and C-peptide. In beta cells, as examined by electron microscopy, insulin appears to be organized in crystals, in so-called dense core granules. As insulin secretory vesicles mature, they become acidified by a proton pump. It is believed that this acidification is responsible for the formation of insulin crystals although proof that these crystals are present *in vivo*, as opposed to being an artifact of electron microscopy, is missing.

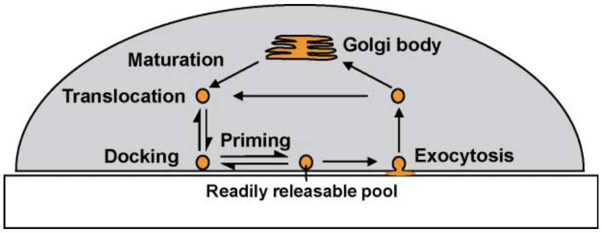

**FIGURE 4** The secretory pathway of insulin secretion in the pancreatic beta cell. Vesicles bud off the Golgi apparatus and undergo maturation, translocation, docking, and priming to enter the readily releasable pool. Upon stimulation of the cell, exocytosis occurs and the vesicles may reenter the pathway.

The rate of proinsulin biosynthesis can be regulated, both at the level of gene transcription and at the level of messenger RNA translation. Insulin within aging vesicles is subject to degradation when mature vesicles fuse with lysosomes (crinophagy). Therefore, the size of insulin stores depends on the rates of proinsulin biosynthesis, insulin degradation, and insulin secretion. Oloffson and colleagues established that in mouse beta cells there are ~9000 insulin vesicles, ~650 of which appear to be in close relation to the beta-cell plasma membrane, potentially in the membrane-docked pool (Fig. 5). In other neuroendocrine cells, such as chromaffin cells, the relationship between the docking (and undocking) of secretory vesicles and subsequent secretion has been elucidated in some detail. Similar studies are now under way with pancreatic beta cells.

Secretion of insulin is tightly regulated (Fig. 6). The most important regulator of insulin secretion, glucose, readily gains access to beta cells through glucose transporter proteins in the cell membrane (glucose-2-transporter) and is phosphorylated to glucose-6-phosphate by glucokinase. The $K_m$ of glucokinase in beta cells is ~10 mmol/liter. This ensures that the rate of glucose-6-phosphate production reflects the concentration of glucose to which the beta cell is exposed. Glucose-6-phosphate undergoes glycolysis to pyruvate, which is then oxidized to carbon dioxide and water via the tricarboxylic acid cycle in the mitochondria. The resulting energy generates a proton gradient across the inner mitochondrial membranes through the electron transport chain. This mitochondrial membrane proton gradient is in turn employed to generate ATP from ADP and phosphate. The increment in ATP closes the ATP--sensitive potassium channel in the beta-cell membrane, resulting in depolarization of the cell membrane. Beta-cell depolarization results in an influx of calcium via voltage-gated calcium channels, which triggers the exocytosis of preformed vesicles to stimulate insulin secretion.

In this widely held scheme for glucose-mediated insulin secretion by beta cells, glucokinase can

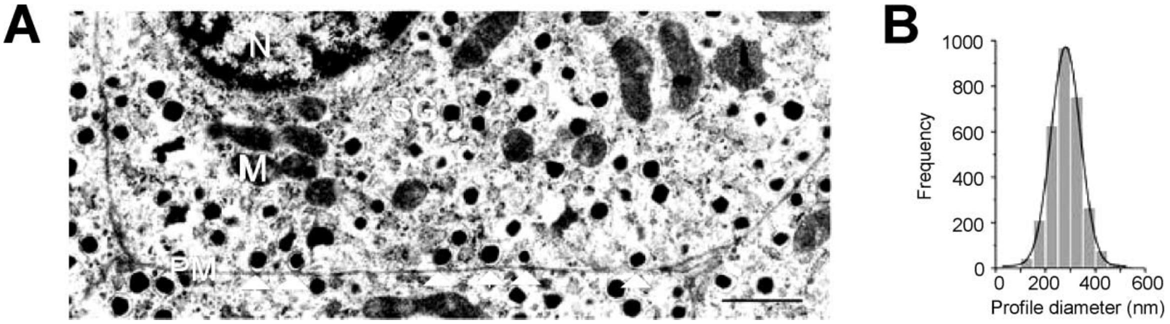

**FIGURE 5** (A and B) Ultrastructure of the pancreatic beta cell. (A) Electron micrograph of a beta cell in an intact islet. The nucleus (N), the plasma membrane (PM), a mitochondrion (M), and a secretory granule (SG) are indicated. The arrows indicate docked granules. Scale bar, 1 μm. (B) Gaussian fit to the distribution of the granule profile diameters. Reprinted from Olofsson *et al.*, *Pflügers Arch.—Eur. J. Physiol.* **444**, 43–51, copyright Springer-Verlag, 2002, with permission.

therefore be considered the signal-sensing component. The increment in cytoplasmic ionized calcium is proportionate to the provision of glucose-6-phosphate and represents the signal effector component for regulated insulin secretion. There are numerous points of potential modulation of the extent of the insulin secretion response to a given increment in glucose within this system. For example, on the sensing side any change in flux of glucose through glucokinase, change in tricarboxylic acid cycling, or change in efficiency in ATP production per oxidized glucose molecule would adjust the signal-to-response ratio. An example of this is the action of uncoupling protein-2 (UCP-2) present in mitochondrial membranes of beta cells. UCP-2 allows the leakage of protons down the proton gradient (generating heat) and so diminishes the signal for insulin secretion at any given glucose concentration. UCP-2 expression increases in response to chronic high glucose and thus attenuates subsequent glucose-mediated insulin secretion. If UCP-2 expression is decreased, insulin secretion increases at any given glucose concentration. On the response arm of this regulatory pathway, alterations in the magnitude of the increment in calcium, such as those mediated through cyclic AMP, can amplify or attenuate the insulin secretory response to a given glucose stimulus. For example, glucagon like-peptide 1 (GLP-1) activates adenylate cyclase to generate cyclic AMP, resulting in an amplified increment in insulin secretion in response to an increment in glucose concentration.

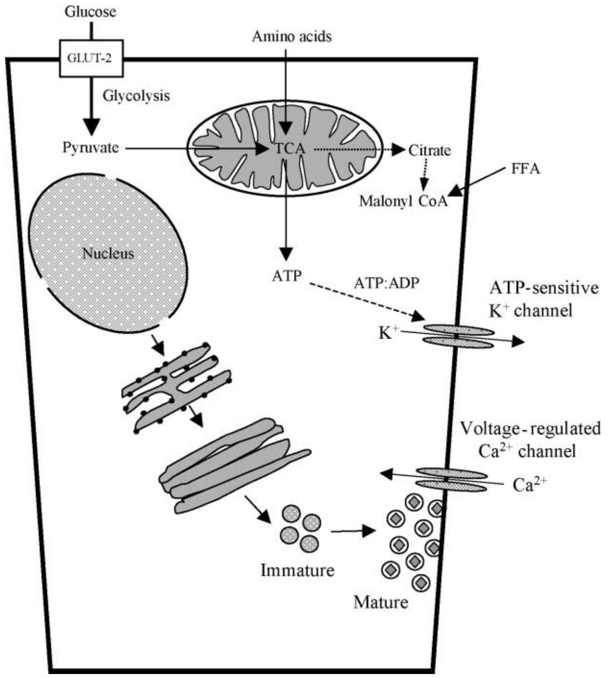

**FIGURE 6** Stimulus–secretion coupling in the pancreatic beta cell.

Other potential metabolic sources that lead to energy production and increased ATP concentrations can also enhance insulin secretion through the same final common pathway. For example, high plasma levels of free fatty acids induce their own oxidation within the mitochondria to increase ATP production and insulin secretion, although more prolonged exposure to elevated free fatty acids can lead to decreased insulin secretion. After meal ingestion, insulin inhibits the lipolysis of stored triglycerides and the circulating levels of free fatty acids decrease. However, the free fatty acid concentration inside the beta cells might be increased, because insulin-sensitive lipoprotein lipase enhances the lipolysis of meal-related triglycerides. The enhanced insulin secretion following meal ingestion is therefore presumably reflective of both the increased circulating levels of glucose and the acute increase in the tissue levels of free fatty acids. In addition to this direct fuel-generated stimulation of insulin secretion, meal ingestion enhances insulin secretion through the mechanism of incretin hormones. The incretin effect describes the phenomenon that glucose ingestion induces a greater insulin response than intravenous glucose administration, either when the same amount of glucose is infused or when the same glucose concentration profile is reproduced. Incretin hormones are released from the gut by oral nutrients and stimulate insulin secretion only at elevated glucose levels. After meal ingestion, the incretin effect contributes to total insulin secretion with 30–60%. Glucose-dependent insulinotropic peptide (GIP) and GLP-1 are examples of incretin hormones.

In summary, the regulation of insulin secretion by individual beta cells is largely dependent on the glucose stimulus but also other circulating sources of energy. They increase the production of ATP, which subsequently depolarizes the beta-cell membrane, leading to the influx of ionized calcium to trigger insulin granule exocytosis. There are numerous additional complexities in the signal transduction pathway superimposed on this scheme that allow both enhanced and attenuated responses to nutrient levels. Although it is convenient to examine the signal transduction pathways for insulin secretion in the simplified scheme of a single beta cell, cell clusters (as in islets) behave quite differently than single beta cells. To examine the regulation of insulin secretion, it is therefore essential to study the intact islet and indeed the whole organism.

## IV. PHYSIOLOGY OF INSULIN SECRETION: ISLETS

Many studies of insulin secretion have been performed at the level of isolated islets. One advantage of this approach is that there are fewer confounding effects than might be present *in vivo*. Changes in insulin secretion or other parameters of islet function can be therefore more directly related to the experimental condition or action of a potential compound. For example, use of a compound that stimulates insulin secretion *in vivo* might be expected to decrease glycemia, which would attenuate the effect of the compound. Another important advantage of islet studies is that they allow measurement of insulin secretion in mouse islets, whereas the measurement of insulin secretion in mice *in vivo* has technical limitations due to a low blood volume. This is important given the frequent use of transgenic, knockout, and knock-in experiments in mice to establish the molecular basis of physiology. The use of isolated islets as an experimental model has the disadvantage that they may very well behave differently than islets *in vivo*, due to the loss of islet innervation and vascularization. There are also technical limitations: small changes in temperature, pH, or osmotic pressure, which inadvertently occur in such studies, may have profound effects on islet secretion. Despite these limitations, studies in isolated islets have contributed substantially to the field of islet biology.

## V. PHYSIOLOGY OF INSULIN SECRETION *IN VIVO*

Insulin secretion *in vivo* has also been extensively studied. As predictable from the studies of single beta cells described above, the most important regulators of insulin secretion are circulating nutrients, in particular, glucose. In the fasting state, insulin secretion is maintained at levels that provide sufficient insulin to constrain hepatic glucose release at rates that match glucose utilization (~2 mg/kg/min) and so the plasma glucose concentration is maintained at normal levels of ~90 mg/dl (~5 mmol/liter). After meal ingestion, glucose concentrations in the circulation rise and stimulate insulin secretion (Fig. 7). Increased delivery of insulin into the circulation causes further suppression of hepatic glucose release (to ~0.5 mg/kg/min) and increased stimulation of glucose uptake by insulin-sensitive tissues such as muscle to restore normoglycemia. Therefore, the simplest model to describe insulin secretion *in vivo* would have two components: a constant basal rate of insulin secretion superimposed on which are meal-related increments. Although this model (concept of basal-bolus insulin therapy) is commonly employed by physicians attempting to replace insulin in patients who secrete insufficient insulin, it is an oversimplification of a very complex dynamic neuroendocrine secretory system.

Even basal insulin secretion in the fasting state can be resolved into several complex secretory patterns. First, there is a circadian rhythm of insulin release with decreased insulin secretion during the night. Second, insulin secretion can also be resolved into an ultradian rhythm with an ~40 min oscillatory period that might reflect the feedback loop between insulin secretion and insulin action through the intermediary of the prevailing plasma glucose concentration.

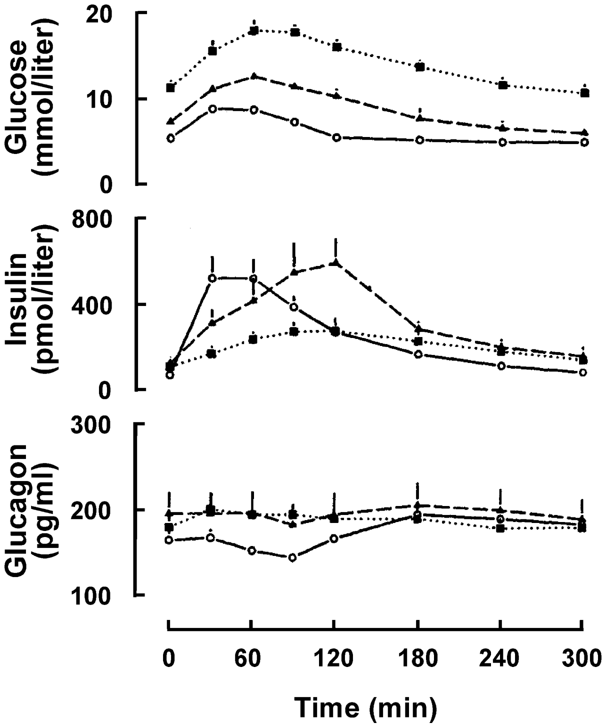

FIGURE 7 Plasma glucose, insulin, and glucagon concentrations after meal ingestion (at 0 min) in nondiabetic (○), glucose-intolerant (▲), and type 2 diabetic (■) subjects. After an initial increase, plasma glucose decreases rapidly in nondiabetic humans, whereas sustained postprandial hyperglycemia occurs in glucose-intolerant and type 2 diabetic subjects. The increase in plasma insulin concentration is delayed and markedly reduced in humans with type 2 diabetes. Reprinted from Butler *et al.*, *Diabetes* 40, 1991, 73–81, with permission. Copyright 1991 American Diabetes Association.

Third, insulin secretion can also be resolved into high-frequency discrete insulin secretory bursts, so-called pulsatile insulin secretion (Fig. 8). These pulses occur approximately once every 4 min and yield an insulin concentration profile in the portal vein that shows dramatic peaks and troughs of as much as 5000 pmol/liter in amplitude. Virtually all insulin is secreted in these discrete 4 min insulin secretory bursts, indicating that regulation of insulin secretion is accomplished through changes in either insulin pulse frequency or pulse mass. In fact, almost all regulation of insulin secretion is accomplished by the modulation of pulse mass (*vide infra*). To understand the mechanisms that result in pulsatile insulin secretion, it is necessary to contemplate both a pacemaker and a pulse coordinator (between islets).

Individual or perfused islets also secrete insulin in ~4 min discrete insulin pulses, indicating that the pacemaker responsible for generation of pulsatile insulin release is present in each islet. Two hypotheses are currently being investigated: one suggests that the pacemaker might arise at the level of the membrane due to intermittent depolarization of the membrane; the other hypothesis is that oscillations in glycolysis drive an oscillatory production of ATP. Perhaps both hypotheses are correct and an intermittent membrane depolarization temporarily decreases ATP concentrations (ATP is consumed, restoring the resting membrane potential). The dip in ATP would be expected to elicit a surge in glycolysis and entrain the natural glycolytic oscillations generated at the level of the reversible interconversion of fructose-1-phosphate and fructose-1,6-diphosphate. Even if the individual islets are potentially independent pacemakers, the question remains how approximately one million islets, scattered throughout the pancreas, are synchronized to secrete insulin in coordinate insulin secretory bursts? The best available evidence suggests that this is mediated through the intrapancreatic neural network. The denervated, isolated-perfused pancreas retains pulsatile insulin secretion, indicating that intrinsic innervation is not required for this process. Islets that are transplanted into the portal

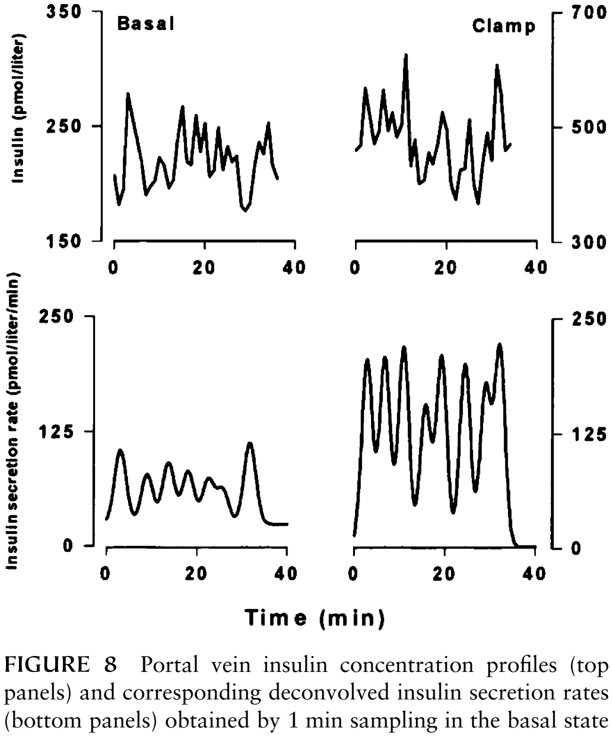

FIGURE 8 Portal vein insulin concentration profiles (top panels) and corresponding deconvolved insulin secretion rates (bottom panels) obtained by 1 min sampling in the basal state (left panels) and during a hyperglycemic clamp (right panels). Reprinted from Song *et al.* (2000), Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. *J. Clin. Endocrinol. Metab.* 85, 4491–4499, with permission. Copyright 2000 The Endocrine Society.

vein secrete insulin in a noncoordinated manner until they become reinnervated and coordinate pulsatile insulin secretion is reestablished. Regulation of insulin secretion is accomplished by modulation of the magnitude of insulin secretory bursts with amplification following meal ingestion, glucose infusion, or GLP-1 infusion and inhibition with somatostatin, a hormone that inhibits insulin secretion.

In numerous studies of insulin secretion *in vivo*, the technique of an acute bolus injection of glucose, arginine, or glucagon (all insulin secretagogues) has been used. Following this approach, insulin is secreted in a biphasic manner with a first phase of insulin secretion over ~15 min generally ascribed to release of insulin from the readily releasable insulin vesicles already docked to the beta-cell membrane. The first-phase insulin release is decreased in the setting of a partial loss of beta-cell mass, as is the magnitude of insulin pulses in response to an increment in glucose. Taken together, these data indicate that the insulin secretory bursts and first-phase insulin release are derived from a physiologically related pool of insulin vesicles.

The second phase of insulin secretion is separated from the first phase by a nadir. It is characterized by a lower amplitude and longer duration and lasts as long as the secretory stimulus is active on the beta cells. The underlying mechanism that leads to the more sustained nature of the second phase is under active investigation. There is evidence from studies at the single-cell level to suggest that vesicle maturation and mobilization from a reserve pool into the membrane-bound readily releasable pool may be the rate-limiting steps determining $V_{\max}$ of the second-phase secretion process. The characteristic physiology of insulin secretion with a biphasic response to a step increase in the ambient glucose concentration and a typical pulsatile pattern is preserved in isolated human islets, which are independent of innervation and vascularization. *In vivo*, insulin secretion follows a circadian rhythm (~24 h period), an ultradian rhythm (~40 min period), and a high-frequency rhythm (~4 min period). Insulin secretion is regulated by modulation of the mass of the high-frequency pulses and pulse frequency remains remarkably stable. Correlative studies at the level of the single beta cell should help establish the basis of regulation of the insulin secretion with each pulse.

## Glossary

**beta cell** Pancreatic beta cells form 70–80% of the endocrine cell mass. They contain insulin, which is formed by proteolytic cleavage from a precursor molecule, proinsulin. The resulting by-product, C-peptide (connecting peptide), is secreted together with insulin at a 1:1 molar ratio.

**beta-cell mass** The total mass of all beta cells in the endocrine pancreas. The beta-cell mass is a functional unit and expands in insulin-resistant states. In humans with type 2 diabetes, the beta-cell mass is reduced.

**insulin** Hormone synthesized in the beta cells of the islets of Langerhans, which form the endocrine pancreas. The main function of insulin is the regulation of carbohydrate metabolism.

**islets of Langerhans** Structures that form the endocrine pancreas (~1% of mean pancreatic volume). There are $10^5$ to $10^6$ islets in the human pancreas. Each islet consists of 2000–3000 cells. Four endocrine cell types can be distinguished in the islets: alpha cells (secrete glucagon), beta cells (secrete insulin), delta cells (secrete somatostatin), and PP cells (secrete pancreatic polypeptide).

**secretory granule** Beta cells contain ~9000 secretory granules. Upon stimulation, their contents are released by exocytosis. These granules contain insulin crystals made up of zinc–insulin hexamers.

## See Also the Following Articles

**Diabetes Type 1 • Diabetes Type 2 • Glucose-Dependent Insulinotropic Polypeptide (GIP) • Insulin Actions • Insulin Gene Regulation • Insulin-like Growth Factor (Igf) Signaling • Insulin Processing • Insulin Receptor Signaling • Insulin Resistance in PCOS**

## Further Reading

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genes produces alternative E peptides, but the physiological significance of the diversity of the prohormones is unclear. The presence of multiple leader exons in mammalian Igf-I genes also results in variation in the signal peptide of the preprohormones, but the physiological consequences of this are also unclear. The organization and splicing of the human and rodent Igf-II genes are also complex, but involve noncoding exons and, therefore, do not affect the structure of the mature Igf-II molecule or its precursor forms.

## B. Igf-I and Igf-II Expression

The expression of the Igf-II gene in rodents is widespread prenatally and diminishes drastically after birth, with the choroid plexus and the leptomeninges being the persistent sites of synthesis in adult animals. Murine expression of Igf-I, on the other hand, is low prenatally and significantly increases during puberty, with hepatic production being a major contributor to overall Igf-I levels in the circulation. Igf-I produced by numerous other organs, including kidney, lung, and bone, contributes to endocrine, paracrine, and autocrine effects. The overall picture of Igf expression in rats and mice initially led to the concept that Igf-II functioned as a fetal growth factor and that Igf-I acted as an adult growth factor. This model is not applicable to humans, however, because both Igf-I and Igf-II are produced throughout life by multiple tissues. In fact, circulating levels of Igf-II are consistently several-fold higher than those of Igf-I, which raises the issue of potentially divergent roles for Igf-I and Igf-II in human physiology.

# III. THE Igf RECEPTORS

## A. The Classical Igf Receptors

The Igf-I and Igf-II ligands interact with an array of cell-surface receptors that may be present singly or in combination on target cells. These are depicted in Fig. 1. It was initially thought that Igf-I primarily activated the type I Igf receptor (Igf-IR), a transmembrane tyrosine kinase that is structurally and functionally related to the insulin receptor (IR). Igf-II, on the other hand, was known to interact with high affinity with the type II Igf receptor (Igf-IIR). Subsequent studies have shown that both Igf-I and Igf-II interact with the Igf-IR, albeit with an ∽ threefold difference in affinity (Igf-I > Igf-II). The cloning of the cDNA encoding the Igf-IIR revealed that it was identical to the previously characterized cation-independent mannose 6-phosphate (M6P) receptor involved in endocytosis and intracellular trafficking of M6P-tagged proteins. Although some early studies proposed an active role for the Igf-IIR in Igf-II signaling based on apparent sequence homology between the cytoplasmic domain of the Igf-IIR and the intracellular loops of G-protein-coupled receptors, subsequent studies ruled out the ability of the short intracellular domain of the Igf-IIR to mediate signal transduction. The function of this molecule in Igf-II action is thought to reflect its ability to serve as a clearance receptor for Igf-II, thereby influencing the extracellular concentration of Igf-II.

## B. Igf-IR and Insulin Receptors

Most, if not all, of the effects of Igf-I result from its activation of the Igf-IR. Igf-I does not cross-react with the IR except at pharmacological doses (i.e., the relative affinity of Igf-I for the Igf-IR vs the IR differs by almost two orders of magnitude). Until recently, it was thought that Igf-II, like Igf-I, bound appreciably only to the Igf-IR, as compared to the IR. Studies in knockout mice lacking various components of the Igf and IR systems suggested that Igf-II acted through the IR in early development, prior to detectable Igf-IR gene expression. The molecular basis for this phenomenon was revealed when it was discovered that a splice variant of the IR displayed high affinity for Igf-II. Specifically, the IR transcript is subject to alternative splicing of exon 11, which encodes a 12-amino-acid segment in the C-terminus of the extracellular α-subunit. Previous studies had shown that the version of the IR encoded by the mRNA lacking the exon 11 sequence (IR-A) displayed a twofold higher affinity for insulin than did the IR-B form specified by the exon 11-containing mRNA. More recently, it has been reported that IR-A, in fact, functions as a high-affinity receptor for Igf-II and exhibits predominantly proliferative effects as compared to the principally metabolic effects elicited by insulin stimulation of IR-B. Thus, Igf-I functions primarily by activating the Igf-IR, whereas Igf-II has the capacity to act through either the Igf-IR or through the A form of the IR.

## C. Hybrid Receptors and the Insulin Receptor-related Receptor

The scope of Igf signaling is made considerably more complex by the existence of hybrid receptors that result from the dimerization of Igf-IR and IR hemireceptors, each consisting of a single α and β subunit linked by disulfide bonds. These hybrid receptors are formed by the formation of intra-α-subunit disulfide bonds in the Golgi apparatus of cells expressing both the Igf-IR gene and the IR gene. Although originally considered to represent a small proportion of the total number of Igf-IRs and IRs in a given cell, some recent evidence suggests that the formation of hybrids is actually preferred over the formation of classical Igf-IR and IR heterotetramers. This may conceivably be due to the preferential formation of disulfide bonds between cysteine residues in the Igf-IR and IR α-subunits. Thus, in some circumstances, hybrid receptors may outnumber "pure" Igf-IR or IR molecules at the cell surface.

With respect to ligand binding, Igf-IR/IR hybrid receptors retain high affinity for Igf-I, but exhibit severely reduced affinity for insulin. It is thought that this reflects the ability of Igf-I to bind efficiently to either Igf-IR α-subunit, whereas tight insulin binding requires its interaction with both of the β subunits found in the IR. As a consequence, the existence of a significant number of hybrid receptors may preferentially diminish cellular responsiveness to insulin, but not to Igf-I. This has, in fact, been proposed as a mechanism through which up-regulation of Igf-IR expression could result in insulin resistance in cells expressing the IR. The situation with hybrid receptors has, of course, been further complicated by the existence of and the Igf-II-binding characteristics of IR-A and IR-B. IR-A/IR-B hybrid receptors undoubtedly occur, because most cells express both splice variants. The difficulty in distinguishing these variants experimentally has precluded an examination of the binding characteristics and signaling capabilities of this particular class of hybrid receptors. It has been demonstrated, however, that Igf-IR/IR-A hybrids bind Igf-I, Igf-II, and insulin, whereas Igf-IR/IR-B hybrids bind Igf-I with high affinity, Igf-II with low affinity, and do not bind to insulin. Thus, the relative expression of the Igf-IR and IR genes and the degree of alternative splicing of exon 11 of the IR gene govern the ability of a given cell to respond to Igf-I, Igf-II, and insulin.

The insulin receptor-related receptor (IRR) is the third member of the Igf-IR/IR family; it does not exhibit binding to Igfs or to insulin. Although still considered an orphan receptor, it has been shown to form hybrids with the IR when both entities are overexpressed in NIH3T3 fibroblasts. The formal possibility exists, therefore, that Igf-IR/IRR, IR-A/IRR, or IR-B/IRR hybrids may occur in the restricted set of tissues that express the IRR gene, and that the formation of such hybrids could, like the formation of Igf-IR/IR hybrids, influence cellular Igf and insulin responsiveness. The characterized and potential receptor hybrids that may be involved in Igf signaling are depicted in Fig. 2.

## D. Igf-IR and IR Signaling Pathways

The particular pathways involved in Igf signaling are, in large part, represented by those identified to date for the Igf-IR (depicted in Fig. 3). On binding of Igf-I or Igf-II to the extracellular α-subunit (specifically, a binding region composed of three domains corresponding to the N-terminus, the internal cysteine-rich region, and the C-terminus of the α-subunit), a conformational change is induced in the transmembrane β-subunits, resulting in trans-autophosphorylation of the tyrosine kinase domain intrinsic to the cytoplasmic portion of the β-subunit. This process fully activates the receptor tyrosine kinase, with the autophosphorylated additional tyrosine residues in the juxtamembrane and C-terminal domains that flank the tyrosine kinase domain. These residues, particularly Tyr-950 in the juxtamembrane domain, then serve as docking sites for members of the insulin receptor substrate (IRS) and the Shc adapter protein families. Subsequent phosphorylation of these proteins by the receptor tyrosine kinase allows IRS and Shc proteins to engage factors such as Grb2/Sos and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), leading to activation of the mitogen-activated protein kinase (MAPK) and PI3K cascades that constitute the major signal transduction cascades emanating from the activated Igf-IR. Among the ultimate targets of the MAPK and PI3K cascades are members of the Ets and forkhead transcription factor families, which provides a mechanism for Igf action at the cell-surface to effect the changes in gene expression that underlie the effects of Igf signaling on cellular proliferation, differentiation, and apoptotic sensitivity.

Although Igf action can clearly be controlled by the levels of extracellular ligand and the number (and types) of receptors at the cell-surface, the relative abundance of receptor targets may be an important factor in determining the effects of Igfs in a given target cell. For example, there are four members of the IRS family, IRS-1–IRS-4, each of which has a similar, yet unique, structure. The presence of different combinations of IRS proteins may result in differential responses to Igf-IR activation. In fact,

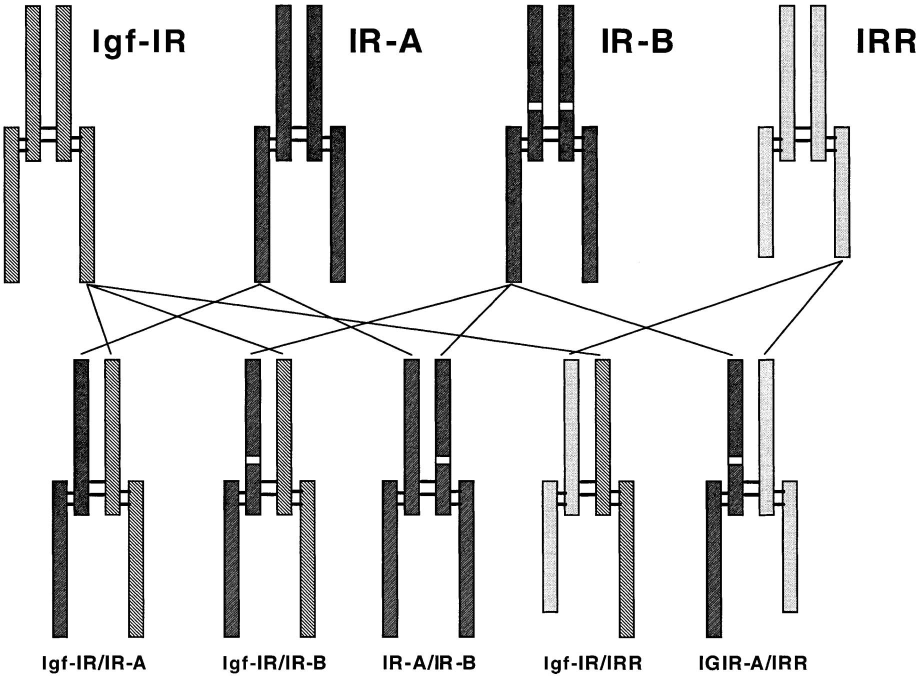

FIGURE 2 Hybrid receptors involved in insulin-like growth factor (Igf) signaling. Shown are hybrids involving the Igf-I receptor (Igf-IR), insulin receptors (IR-A and IR-B), and the insulin receptor-related receptor (IRR) that are involved in Igf signaling. Igf-IR/IRR hybrids have not been demonstrated to date, but their existence is inferred from the ability of the IR and the IRR to form hybrids. IR-B/IRR hybrids are also a formal possibility, but would not be expected to be involved in Igf signaling because they would presumably not bind Igfs or insulin.

recent studies have suggested that IRS-3 and IRS-4 can inhibit processes activated through IRS-1 and IRS-2. The relative levels of Shc and IRS proteins may also be an important factor influencing Igf action, in that they have been shown to compete for binding to Tyr-950 of the activated Igf-IR.

The general characteristics of signaling and the regulatory possibilities for the Igf-IR also apply to the IR (with IR-A and Igf-IR/IR hybrid receptors being relevant here), but there are important differences between the Igf-IR and IR that may have important ramifications for differential actions of Igf-I and Igf-II. In the first place, apart from the conserved tyrosine 950/960 in the Igf-IR and the IR, the position and number of tyrosine residues in the juxtamembrane and C-terminal domains that are subject to autophosphorylation differ between the Igf-IR and IR. In addition, the Igf-IR and the IR have been shown to utilize different heterotrimeric G-proteins as part of their signaling mechanisms, and other proteins have been identified that interact specifically with the C-terminus of the Igf-IR, but not with the IR. A final difference in signaling pathways engaged by the Igf-IR and the IR is the involvement of Stat3 and Stat5 in Igf-IR signaling.

## IV. Igf-BINDING PROTEINS

The biological activities of the Igf ligands are modulated by a family of high-affinity Igf-binding proteins (IgfBP-1 through IgfBP-6) that are found in the circulation and in extracellular fluids (either free or cell-surface associated). IgfBP-3 is the predominant IgfBP in serum, and most circulating Igf-I and Igf-II molecules are not found in a free form, but in a ternary complex with IgfBP-3 and a third component, acid-labile subunit (ALS), in a 1:1:1 molar ratio. IgfBP-5 also forms ternary complexes with Igfs and ALS. Although IgfBP-1–IgfBP-4 have generally similar affinities for Igf-I and Igf-II (within twofold), IgfBP-5 and IgfBP-6 bind Igf-II with 10- and 100-fold greater affinity, respectively, compared to Igf-I. The IgfBPs do not bind insulin. The IgfBPs control Igf action by increasing the half-lives of circulating Igfs, by controlling their availability for receptor binding, and, in the case of cell-surface-associated IgfBPs, by potentially influencing their direct interaction with receptors. Each of the IgfBPs is subject to limited and potentially regulated proteolysis by various proteases. Therefore, ligand-receptor interactions in the Igf signaling systems are subject to complex

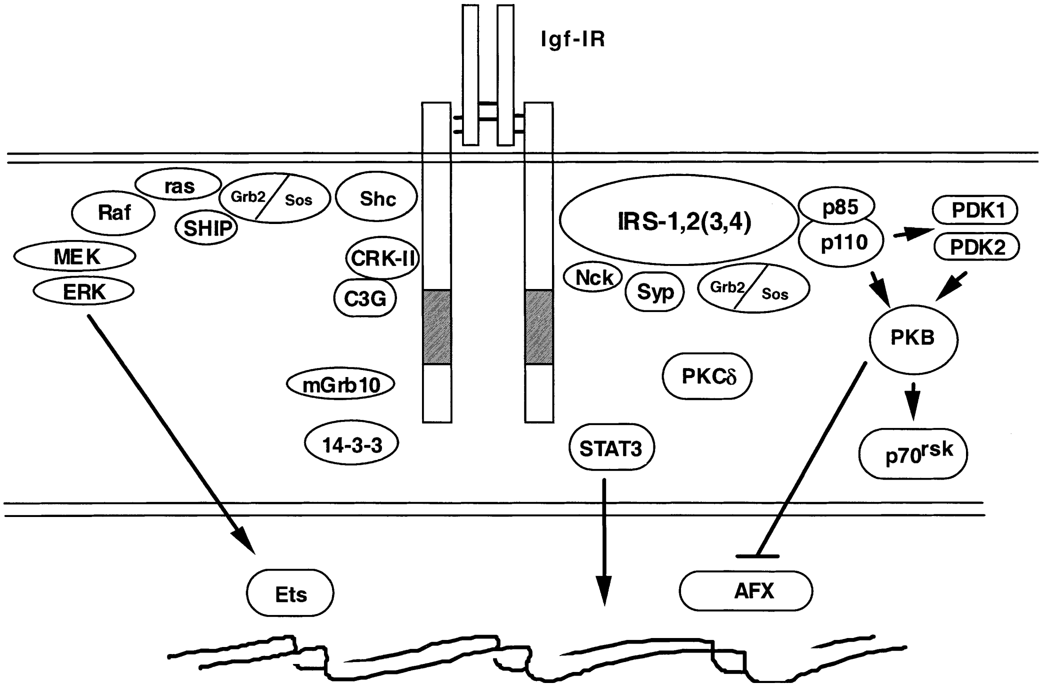

FIGURE 3 Insulin-like growth factor-I receptor (Igf-IR) signaling pathways. The activated Igf-IR initiates signaling through two primary cascades, the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. As shown, insulin receptor substrate (IRS) proteins couple the receptor to the phosphatidylinositol 3-kinase cascade, and Shc couples the receptor to the mitogen-activated protein kinase cascade. In reality, IRS proteins, through their association with Grb2/Sos, also couple to the mitogen-activated protein kinase pathway, whereas Shc, through interaction with the IRS homologue Gab-1, can activate the phosphatidylinositol 3-kinase pathway. Thus, IRS proteins and Shc both integrate Igf activation of these two signaling pathways. A number of other factors, some of which are illustrated, have been shown to interact with, or be in some way involved in, Igf-IR action.

regulation as a result of IgfBP levels, expression profile, degree of cell-surface association, and extent of proteolysis.

A series of studies performed over the past several years has established the concept of Igf-independent actions of some of the IgfBPs. IgfPB-3 and IgfBP-5, in particular, have been shown to exhibit effects on proliferation, migration, and sensitivity to apoptosis that are independent of their effects on Igf signaling per se. Some of these "Igf-independent" effects are still modulated by Igf binding to the responsible IgfBP, so that "Igf receptor-independent action" may be a more accurate term for these novel functions. The cell-surface or intracellular molecules that participate in those effects are poorly characterized, but IgfBP-3 and IgfBP-5 have been identified in the nuclei of cells following exposure to exogenous recombinant protein. This aspect of IgfBP action, when clarified, will add an important dimension to understanding the Igf signaling system in general.

## V. PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF Igf ACTION

The complex Igf signaling system plays an important role in normal growth and development and in a variety of pathological situations, particularly tumorigenesis. The role of Igf action in prenatal growth was first demonstrated by the severe growth retardation seen in knockout mice in which the Igf-I, Igf-II, or Igf-IR genes had been inactivated by homologous recombination. In humans, severe growth retardation is seen in rare individuals with mutations in, or reduced copy number of, the Igf-I, Igf-IR, or IR genes. The increased expression of Igf-I during puberty is thought to be a major contributor to adult height, because this pattern of Igf-I expression is blunted in populations such as African Pygmies, who exhibit constitutional short stature. Igf action is also important in specific organ development, such as in the nervous system, in which Igf signaling regulates neuronal proliferation, apoptosis, and survival.

A major aspect of human disease in which Igf signaling plays a critical role is in cancer development and progression. A growing body of epidemiological data suggests that high levels of circulating Igf-I constitute a risk factor for the development of breast, prostate, colon, and lung cancer. Additionally, the expression levels of Igf-IR and IR are predictive of breast cancer outcome. Experimentally, modulation of Igf-IR activity affects the growth of many types of tumor cells. As a result of these findings, intensive research is being conducted on the utility of the Igf system as both a diagnostic marker and a therapeutic target in cancer therapy.

In summary, the Igf signaling system plays a central role in many aspects of mammalian development and exerts a major influence on both normal and abnormal physiology. The complexity of this system, as outlined in this brief review, is a reflection of the importance of Igf signaling and the attendant need for exquisite regulation of its actions.

## Glossary

**insulin-like growth factor** Member of a class of hormones structurally related to insulin, but exhibiting proliferative and differentiative, rather than metabolic, effects.

**mitogen-activated protein kinase cascade** Signaling pathway involving Ras, Raf-1, MEK, and ERK in a series of tyrosine and serine–threonine phosphorylation events that generally lead to proliferation.

**phosphatidylinositol 3-kinase cascade** Signaling pathway involving phosphoinositide 3′-kinase, PDK1, PDK2, and PKB/Akt in lipid-mediated serine–threonine phosphorylation events leading to differentiation and control of apoptosis.

**receptor tyrosine kinase** Member of a class of cell-surface molecules that includes the insulin-like growth factor and insulin receptors, which respond to binding by extracellular ligands by undergoing autophosphorylation and subsequent phosphorylation of downstream targets to initiate specific signaling pathways.

## See Also the Following Articles

**Cancer Cells and Progrowth/Prosurvival Signaling • Epidermal Growth Factor (EGF) Receptor Signaling • Insulin Receptor Signaling • Placental Development**

## Further Reading

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# Insulin Processing

Cristina Alarcon, Barton Wicksteed, and
Christopher J. Rhodes
*Pacific Northwest Research Institute and University of Washington*

I. INTRODUCTION
II. STRUCTURE OF THE PROINSULIN MOLECULE
III. PROINSULIN BIOSYNTHESIS
IV. PROINSULIN PROCESSING
V. SUMMARY

**Most polypeptidic hormones and neurotransmitters, as well as other secretory and cell constituent proteins, are synthesized as larger, inactive precursors. Insulin is first synthesized in the rough endoplasmic reticulum (RER) of the pancreatic beta cell as the precursor molecule preproinsulin. Preproinsulin is processed to proinsulin in the RER by removal of the signal**

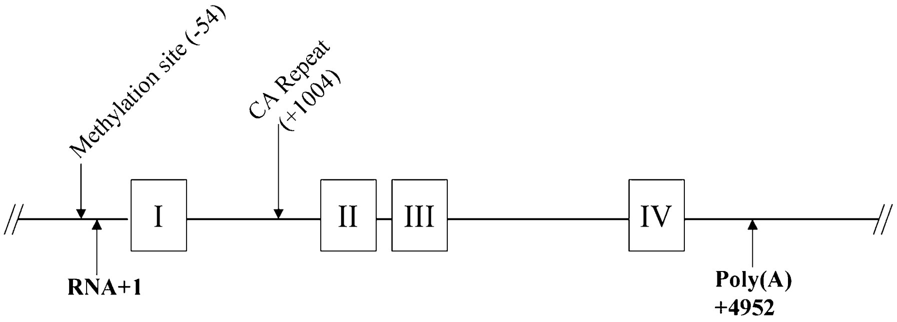

**FIGURE 1** Human IFN-γ: genomic structure. Boxes I–IV represent exons.

that interact with the nuclear factor κB (NF-κB) and signal transducers and activators of transcription (STAT) families of transcription factors. Interestingly, the STAT-binding region of the first intron is not conserved in the mouse gene and its role in regulating human IFN-γ gene transcription has not been defined.

The length of the CA repeat in the first intron of the human gene varies between individuals, ranging in size from 11 to 16 repeats. Two of the six alleles are present in 37% of the population. Correlations of the incidence of a particular CA repeat length and gene expression or the occurrence of diseases, such as multiple sclerosis and allograft fibrosis in lung transplant patients, have been reported. However, no functional role in regulating gene expression has been identified for the CA repeat, and the reported associations with disease incidence are thought to be due to linkage with other changes in the gene regulatory regions. Consistent with this hypothesis, a strong correlation between the repeat length, IFN-γ production, and a single-nucleotide polymorphism (SNP) in a NF-κB-binding site in the first intron of the gene has been reported. Other SNPs in the human promoter, introns, and 3′-untranslated region have been identified and although specific nucleotide alterations have been shown to result in changes in protein complexes binding to the DNA, no association of the specific SNPs with specific disease occurrence has been demonstrated. A polymorphism in the promoter of one strain of rat has been found and this polymorphism has been shown to alter DNA binding of a nuclear protein. Although C57/Bl6 mice are considered to be the prototype high IFN-γ producer strain and Balb/C mice are thought of as low IFN-γ producers, no direct nucleotide changes have been reported between these two strains. In contrast, a deletion in the promoter has been detected in SJL/J mice but the effect of this deletion on promoter function was not directly investigated. Although mice that lack the IFN-γ gene have been generated, there have been no reported cases of a human that has lost the IFN-γ gene due to mutation of the structural portion of the gene. In contrast, mutations in the IFN-γ receptor chains have been identified and result in a partial or complete loss of IFN-γ-induced signal transduction.

Early attempts to understand the regulation of IFN-γ expression in T cells mapped several DNase I hypersensitivity sites in the promoter and first intron regions of the IFN-γ gene. One of these DNase I hypersensitive sites falls close to the proximal promoter region that contains a number of putative *cis*-acting elements that may aid in tissue-specific expression of IFN-γ. Abbreviated lists of nuclear factors that interact with these elements include T-bet (T-box expressed in T cells), GATA-3, $Ca^{2+}$/cAMP-response element-binding protein (CREB)-ATF (activating transcription factor), NF-κB, NFAT (nuclear factor of activated T cells), and YY-1 (Yin–Yang 1). (Fig. 2). T-bet appears to play a major role in $CD4^+$ T helper 1 (Th1) IFN-γ gene expression as T helper 2 (Th2) cells can be induced to express IFN-γ by introduction of the T-bet protein. In addition, T-bet null mice demonstrate significantly decreased IFN-γ gene expression in $CD4^+$ T cells and NK cells. STAT4 is also essential for interleukin-12 (IL-12)-induced IFN-γ expression as demonstrated by the inability of IL-12 to induce IFN-γ in STAT4 null mice. Although the abundance of regulatory sites in the IFN-γ promoter as well the complexities associated with the number of DNA-binding proteins has blocked full understanding of tissue-specific transcriptional control of IFN-γ at this time, it is clear that multiple cell signaling pathways can activate IFN-γ transcription and these pathways appear to work through different regulatory elements within the IFN-γ genomic DNA.

The induction of IFN-γ mRNA expression by extracellular signals involves many different cell

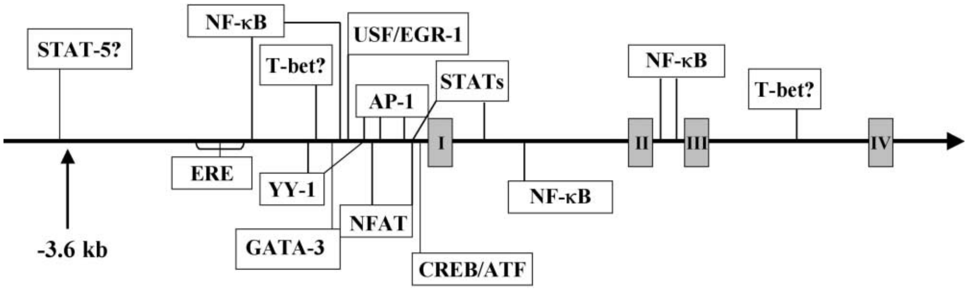

**FIGURE 2** Potential regulatory elements in the human IFN-γ gene. EGR-1, early growth response 1; ERE, estrogen-response element; USF, upstream stimulatory factor. Boxes I–IV represent exons.

signaling pathways. Major contributors to IFN-γ gene regulation are the extracellular signal-regulated kinase (ERK) signaling pathway and the p38 mitogen-activated protein (MAP) kinase pathway. These pathways are activated by distinct routes; TCR- and NK-activating receptors involve the ERK pathway and IL-12 and IL-18 signaling involves the p38 MAP kinase pathway. When both pathways are concurrently activated, strong synergy in IFN-γ mRNA and protein expression is observed. In addition to the ERK and MAP kinases, protein kinase C has been demonstrated to be involved in the signal transduction pathways leading to IFN-γ mRNA induction.

Methylation appears to be another important regulator of IFN-γ gene promoter expression. In all species, except chicken, for which the genomic DNA has been sequenced, the proximal promoter contains a highly conserved methylation site at position − 54. Methylation of this site correlates with an absence of interferon-γ expression in Th2 cells and adult naive T cells. Likewise, IFN-γ-producing cells such Th1 and CD4$^{+}$ memory T cells lack methylation at this site. Methylation-mediated control of IFN-γ expression is further supported by the induction of interferon-γ expression in Th2 cells treated with the demethylating agent 5-azacytidine.

Alterations in IFN-γ expression are not strictly due to changes in transcription. Additional regulation of the gene is due to posttranscriptional mechanisms. Stabilization of IFN-γ mRNA has been observed in both T and NK cells. The mechanism responsible for posttranscriptional control of IFN-γ has been studied in NK cells and appears to be mediated by events that result in nuclear retention of the IFN-γ mRNA. Retention of the mRNA is not permanent in that transcription-independent nucleo-cytoplasmic shuttling of the IFN-γ mRNA occurs very rapidly following stimulation of cells by an IFN-γ-inducing agent, such as IL-2.

## III. PROTEIN STRUCTURE AND PRODUCING CELLS

The human IFN-γ protein is a 166-amino-acid polypeptide. The first 23 amino acids represent a hydrophobic signal sequence that is removed by proteolysis, resulting in a 17 kDa protein of 143 aa. The murine protein, only 40% homologous to the human protein at the amino acid level, is 15.4 kDa and the mature protein is composed of 134 aa. The active protein is a noncovalent homodimer and is N-glycosylated at two sites. However, glycosylation does not have significant effects on protein activity as nonglycosylated protein produced in bacteria is biologically active. The structure of the molecule is mostly helical (62%) and is composed of six α-helices without any β-sheet structure. The N-terminal and C-terminal regions of the protein are important for receptor interaction and signal transduction based on studies with truncated proteins, peptides, and antibodies directed against these regions. The proteins are also highly species specific as human and mouse proteins signal only through their homologous receptor. Interestingly, a nuclear localization signal (NLS) is present in the molecule and accounts for evidence of an intracellular function and nuclear localization of the protein.

The cell types that account for most of the IFN-γ production *in vivo* are T cells, NK cells, and NKT cells. The primary stimulus for T-cell IFN-γ production is antigen in the context of either MHC class II (CD4$^{+}$ T cells) or MHC class I antigens (CD8$^{+}$ T cells). IFN-γ expression has been utilized to define T-cell populations as CD4$^{+}$ Th1 and CD8$^{+}$ T cytotoxic 1 (Tc1) cells are considered IFN-γ producers, whereas Th2 and Tc2 cells are defined by their production of IL-4 and lack of production of IFN-γ. In contrast, NKT cells produce both IFN-γ and IL-4. Cytokines, including IL-2, IL-7, IL-12, IL-18, IL-21, tumor necrosis factor, and IFN-α, chemokines, and triggering through other cell surface receptors, including CD28, can also play an important role in either directly inducing or enhancing IFN-γ expression from T cells. Expression of IFN-γ mRNA in T cells is transient, occurring as quickly as 1 h after induction and peaking at approximately 6–8 h. However, some signals (e.g., CD28 co-stimulation) result in a stabilization of the IFN-γ mRNA. NK cells are the major producers of IFN-γ in the innate arm of the immune system. The major inducing agents are the cytokines IL-2, IL-12, IL-15, and IL-18, and combinations of these cytokines (e.g., IL-2 + IL-12, IL-12 + IL-18) show dramatic synergy in their ability to induce IFN-γ mRNA. In addition to transcriptional activation, IL-2 + IL-12 results in the stabilization of IFN-γ mRNA. In the mouse, cross-linking of the LY49-activating receptors also potently induces IFN-γ expression. Triggering of other cell surface receptors, such as CD16, on IL-2-activated human NK cells further enhances IFN-γ expression. Other cell types, including macrophages and macrophage cell lines, B cells, mast cells, neutrophils, astrocytes, sensory neurons, trophoblasts, and even preimplantation human embryos, have been reported to express IFN-γ. As with T cells, B cells have been subdivided into IFN-γ producers and nonproducers. The major inducer of expression in B cells and macrophages is the combination of IL-12 + IL-18.

## IV. RECEPTOR STRUCTURE AND SIGNALING PATHWAYS

The IFN-γ receptor (IFNGR) is a heterodimeric receptor composed of the IFNGR-1 (α chain) and IFNGR-2 (β chain) chains. The IFNGR-1 subunit binds IFN-γ with the highest affinity. It is a 90 kDa glycoprotein that is encoded on human chromosome 6 and mouse chromosome 10. The IFNG-2 chain is a 60 to 67 kDa glycoprotein encoded on human chromosome 21 and mouse chromosome 16. The IFNGR-2 subunit does not actively bind IFN-γ; however, it is necessary for the IFN-γ-specific activation of the intracellular signal transduction pathway.

Both chains of the IFN-γ receptor belong to the class II family of cytokine receptors. This family of receptors lacks intrinsic kinase activity. Thus, signaling through the IFNGR requires both Janus kinase 1 (JAK1) and JAK2 tyrosine protein kinases. JAK1 and JAK2 tyrosine kinases are constitutively associated with IFNG-1 and IFNG-2 subunits, respectively (Fig. 3). Ligand binding initiates receptor oligomerization, with two IFNGR-1 chains binding one IFN-γ homodimer and subsequent recruitment of the two IFNGR-2 chains. The clustering of the receptor complex brings the inactive JAK1 and JAK2 kinases in close proximity with one another. The JAKs become activated by sequential auto- and transphosphorylation events. The activated JAKs phosphorylate a specific C-terminal tyrosine residue (Y440 in humans) in the IFNGR-1 chain. This allows for Src homology 2 (SH2)-mediated docking of two STAT family proteins (STAT1α) to the IFNGR-1 subunits. The receptor-associated JAKs phosphorylate the STAT1α proteins, which homodimerize to form a gamma-activated factor. The STAT1α homodimer translocates to the nucleus, where it interacts with a 9-nucleotide sequence known as a gamma-activated site (GAS) element to mediate IFN-γ-specific activation of gene transcription.

Nuclear translocation of the STAT1α homodimers is due to endocytosis of the IFNGR-1/IFN-γ complex. After ligand-mediated IFNGR oligomerization, JAK1 and JAK2 activation, and STAT1α binding and dimerization, the IFNGR-1 subunit and its bound cargo (JAK1, JAK2, STAT1α dimer, and IFN-γ) dissociate from the IFNGR-2 subunit and undergo receptor-mediated endocyotosis. Nuclear translocation of this complex is mediated by a NLS in the C-terminus of the IFN-γ protein. The NLS is retained on the cytoplasmic side of the endocytic vesicle and is recognized by the importin-α homologue Npi-1, which in turn facilitates binding to importin-β to initiate RAN (RAs related nuclear protein) GTPase import of the IFN-γ/IFNGR-1/STAT1α complex into the nucleus. Once in the nucleus, STAT1α dissociates from the ligand/receptor complex. The STAT1α homodimer binds concurrently to the IFN-γ-specific DNA GAS elements and the transcriptional co-activator CREB-binding protein to activate transcription of IFN-γ-specific genes.

Although IFN-γ requires the JAK/STAT pathway to mediate the majority of IFN-γ-dependent cellular events, a published study utilizing STAT1 null mice demonstrates the existence of a STAT1-independent signaling pathway. The alternative signaling pathway regulates *in vitro* proliferation and differentiation of myeloid cells as well as cellular migration. In this system, gene activation requires the presence of the IFNGR and JAK1. Although STAT1 is not required, when present it can influence the IFN-γ alternative signaling pathway. To this end, some genes are equally regulated in the presence or absence of

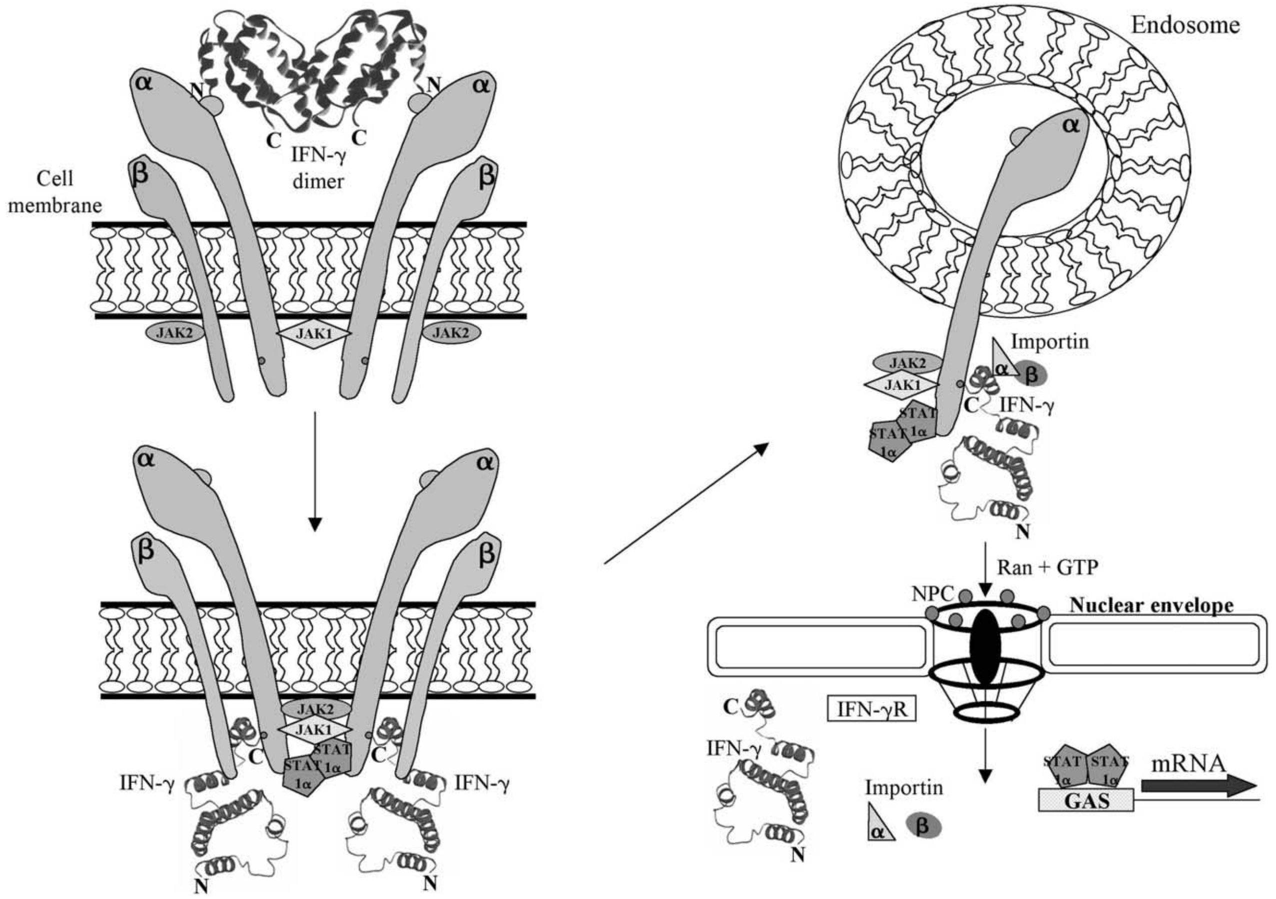

**FIGURE 3** IFN-γ signal transduction pathway. Binding of IFN-γ to the IFNGR activates JAK1 and JAK2 and induces binding of a STAT1α dimer to the α chain of the IFNGR. The IFN-γ/IFNGα receptor complex and the receptor-associated STAT1α/JAK1/JAK2 proteins are removed from the cytoplasmic membrane by endosome-mediated endocytosis and relocate to the cytoplasm. In the cytoplasm, the C-terminal portion of IFN-γ associates with the importin α/β complex. Nuclear import of the STAT/IFN-γ/IFNGα receptor complex is mediated by the Ran GTPase transport system. Figure by Mike Sanford based on Subramaniam *et al.* (2001).

STAT1, whereas other genes are regulated only in the absence of STAT1. The molecular mechanism for the STAT1-independent signal transduction pathway is unknown at this time. It is possible that another STAT family member may become activated and substitute for STAT1 to transduce a cellular response; however, further studies are required to support this hypothesis.

## V. ACTIVITIES AND FUNCTIONS

IFN-γ is a multipotent protein, acting on many cell types by inducing or inhibiting many cellular functions through direct effects on gene expression. IFN-γ has a clear role in host defense against viruses, including hepatitis B, herpes simplex, lymphocytic choriomeningitis virus, and mousepox, as has been demonstrated by experiments in IFN-γ and IFN-γ receptor null mice as well as studies utilizing IFN-γ neutralizing antibodies. The antiviral properties of IFN-γ are primarily due to the induction of double-stranded activated protein kinase and 2′-5′ oligoadenylate synthase. Interestingly, poxviruses have evolved mechanisms to defend against the biological activities of IFN-γ by encoding genes that act either as direct IFN-γ receptor decoys or as decoys for the IL-18 receptor. IFN-γ enhances immune function by promoting the growth of Th1 cells while inhibiting Th2 cell growth and enhancing NK cytotoxic activity. Furthermore, IFN-γ promotes switching to immunoglobulin G2a production by B cells and enhances antigen presentation by activating dendritic cells and inducing MHC class I and class II gene expression on the surface of target cells. IFN-γ is the major activator of macrophages, primarily through the induction of superoxide, nitric oxide production, and NRAMP (natural resistance-associated macrophage protein) gene expression. This function of IFN-γ is critical for host resistance to intracellular pathogens. In addition to these activities, IFN-γ has been shown to inhibit growth and induce apoptosis of many cells, including tumor cells. Indeed, IFN-γ has been found to be critical for enhanced host resistance to the development of chemically induced tumors. Curiously, IFN-γ has been described as both promoting and inhibiting angiogenesis, depending on the experimental system being tested. Part of these effects may be mediated by the direct induction in the tumor targets of the chemokines IP-10 (IFN-γ inducible protein 10) and MIG (monokine induced by IFN-γ). IFN-γ also has numerous negative effects, including suppression of erythropoiesis and hematopoietic progenitor precursor formation. In addition, high-level expression of IFN-γ is associated with the development of autoimmune disease and allograft rejection. The association of IFN-γ expression in autoimmune disease is consistent with findings of higher levels of IFN-γ expression in female mice and the report that estrogen acts as a co-factor for IFN-γ expression, through three estrogen-response elements identified in the IFN-γ promoter.

It should be noted that although studies in mice lacking various components of the IFN-γ signaling pathway have demonstrated increased susceptibility to numerous viral, microbial, and fungal pathogens, humans with defects in the signaling pathway show a predominant susceptibility to *Mycobacterium sp.* and *Salmonella sp.* infection.

## VI. SUMMARY

Interferon-γ is a single-copy gene that has pleiotropic effects on many cell types in the body. It functions as a homodimer and mediates its effects through specific cell surface receptors. Gene expression, although generally transient, is induced by a number of soluble mediators (e.g., interleukins) and via cross-linking of cell surface receptors. Importantly, strong synergy of IFN-γ gene expression is observed when multiple activating signals are combined. Although IFN-γ was initially identified as protein produced by the host in response to viral infection, subsequent research has provided strong evidence for its multifunctional role in modulating the host response to infection, inflammation, and cancer.

## Glossary

**cytokines** Soluble proteins that act as humoral regulators at nano- to picomolar concentrations and modulate the functional activities of individual cells and tissues.

**cytotoxic T cell** A white blood cell (lymphocyte) defined by expression of CD8 ($CD8^+$) on the cell surface; it expresses a specific T-cell receptor and has the ability to specifically recognize and kill target cells.

**interferons** Secreted proteins that elicit a non-virus-specific antiviral activity.

**interleukins** Proteins secreted by leukocytes or other cells that are involved in cell–cell communication.

**lymphokines** Soluble proteins (generally 12–30 kDa) produced by various lymphocyte populations following their stimulation by an antigen or another mode of cell activation.

**natural killer cell** A white blood cell (leukocyte) that can nonspecifically kill virally and bacterially infected cells and tumor cells.

**regulatory elements** Regions of genomic DNA that are bound by specific proteins and involved in the regulation of gene expression.

**single-nucleotide polymorphism** Single-nucleotide differences in the DNA sequence identified when sequences from multiple individuals are compared.

**T helper cell** A white blood cell (lymphocyte) defined by expression of CD4 ($CD4^+$) on the cell surface and that is capable of expressing a specific T-cell receptor. T helper 1 cells are defined by their ability to express IFN-γ, whereas T helper 2 cells are defined by their lack of expression of IFN-γ and expression of interleukin-4.

## See Also the Following Articles

**Interferons: α, β, ω, and τ • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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Encyclopedia of Hormones.

# Interferons: α, β, ω, and τ

Joseph Bekisz, Hana Schmeisser,
Carol Pontzer*, and Kathryn C. Zoon†

*Food and Drug Administration • *University of Maryland •
†National Cancer Institute*

I. INTRODUCTION
II. STRUCTURE AND FUNCTION
III. RECEPTORS
IV. SIGNAL TRANSDUCTION
V. CLINICAL APPLICATIONS OF HUMAN INTERFERON-α AND -β
VI. SUMMARY

**Interferons (IFNs) constitute a family of species-specific vertebrate proteins that display a number of biological activities. Type I IFNs include IFN-α, IFN-β, IFN-τ, and IFN-ω; they are classified according to their gene sequences. IFN-α and -β have been used as model systems to elucidate receptor interactions and signal transduction. Interferons confer nonspecific resistance to a broad range of viral infections, affect cell proliferation, and modulate immune responses.**

## I. INTRODUCTION

Interferons (IFNs) were originally discovered in 1957 by Isaac and Lindenmann. They were originally characterized as soluble proteins that induce antiviral activity in chicken cells. These molecules were subsequently identified in many vertebrates including humans. Numerous biological activities have subsequently been attributed to IFNs including anti-tumor, immunomodulatory, antiparasitic, and antiproliferative properties. Type I IFNs include IFN-α, IFN-β, IFN-τ, and IFN-ω. They were originally categorized based on their antigenicity; however, later they were described by their gene sequences.

In addition to viruses, a number of agents including bacteria, mycoplasma, fungal cell wall products, and protozoans induce the synthesis of IFN-α and IFN-β. It has also been shown that exposure of cells to synthetic double-stranded RNA molecules, growth factors such as colony-stimulating factor-1, and cytokines such as interleukin-1 (IL-1), IL-2, and tumor necrosis factor also induce the expression of type I IFNs. Interferon-α production is mediated by interferon regulatory factors (IRFs). These DNA-binding proteins, especially IRF-3 and IRF-7, modulate the transcription of IFN-α (see Fig. 1). Type I interferon proteins are secreted by the cells and then interact with specific cell surface receptors. It has been shown that IFN-α and IFN-β share the same receptor complex, which is composed of two proteins, IFNAR-1 and IFNAR-2. Following their interaction with their cell surface receptors, the signal is transduced to the nucleus by a series of steps that lead to transcription of messenger RNAs and ultimately translation of proteins that result in the many biological properties associated with these molecules.

Interferon-ω (IFN-ω) was first described in 1985 and the IFN-ω genes are believed to have diverged from the IFN-α genes over 100 million years ago. It appears to be as potent as IFN-α with respect to antiviral and antiproliferative activities, and no unusual biological activities have been attributed to it. IFN-ω has been shown to compete for binding to the common type I interferon receptor shared by IFN-α and IFN-β. Interferon-τ (IFN-τ), of which there are at least 12 species, constitutes a new class of type I interferons that is not virus inducible. It is expressed in the outer epithelial layer of the developing placenta

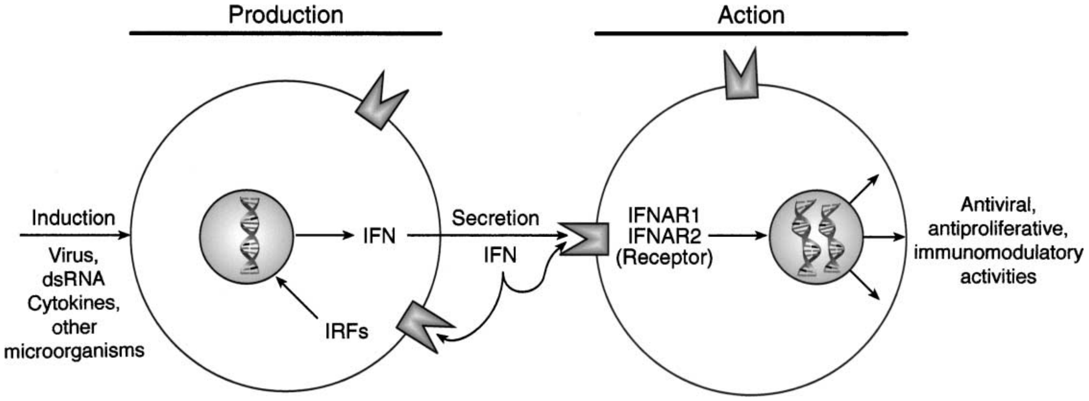
Production
Action
Induction
Virus, dsRNA Cytokines, other microorganisms
IFN
IRFs
Secretion
IFN
IFNAR1
IFNAR2
(Receptor)
Antiviral, antiproliferative, immunomodulatory activities

**FIGURE 1** Production and action of type I interferons. Interferon production can be induced by a number of agents (viruses, cytokines, etc.). Upon secretion, interferon binds to the type I receptor complex (IFNAR-1/IFNAR-2), resulting in antiviral, antiproliferative, and immunomodulatory effects.

in a number of ruminants and allows for the maintenance of the corpus luteum. It is glycosylated, with an apparent molecular weight of 20,000. Like IFNs-α and-ω, it has two disulfide bonds and, like IFN-ω, it consists of 172 amino acids. Like the other type I interferons, IFN-τ also binds to the type I receptor complex (IFNAR-1/IFNAR-2) (see Table 1).

## II. STRUCTURE AND FUNCTION

Distinct cellular genes encode various type I IFNs. In humans, there are at least 18 IFN-α nonallelic genes, 4 of which are pseudogenes found on chromosome 9. These genes encode a minimum of 14 different IFN-α proteins (165–166 amino acids). The IFN-α species are homologous in amino acid sequence structure (75–99%) and display a high level of species specificity in their biological properties. Human IFN-β is present as a single copy in the human genome (chromosome 9) and is expressed as a single protein (166 amino acids). IFN-ω consists of a family of 7 genes (only 1 of which is functional) also found on chromosome 9. Like IFN-α and IFN-ω, IFN-τ constitutes a multigene family. There is approximately 30% amino acid sequence homology between IFN-α and IFN-β. IFN-τ and IFN-ω (with approximately 50 and 75% sequence identity to IFN-α, respectively) contain 172 amino acids each compared to the 166 amino acids that constitute IFN-β and most IFN-αs (IFN-$\alpha_2$ contains 165 amino acids). These 6 additional amino acids are located at the C-terminal end of the molecule in both IFN-τ and IFN-ω (Table 1).

Twenty-two IFN-α subtypes derived from Sendai virus-induced Namalwa cells have been purified and characterized. Each subtype was found to consist of a single polypeptide chain of 165–166 amino acids with apparent molecular weights under reducing conditions ranging from 17,500 to 27,000 Da. Of the 22 subtypes examined, there are 3 major glycoforms. Removal of the carbohydrate moieties has no appreciable effect on antiviral or antiproliferative activities *in vitro*. However, it is well known that glycosylation can play a major role in the pharmacokinetics of proteins.

**TABLE 1 Human Interferons -α, -β, and -ω**

| | Class | | |
|---|---|---|---|
| | α | β | ω |
| Number of species | ≥22 | 1 | 1 |
| Glycosylation | ≥3 species | Yes | Yes |
| Apparent molecular weight (reducing) | 17,500–27,000 | 20,000 | 25,000 |
| Disulfide bonds | 2 | 1 | 2 |
| Number of amino acids | 165–166 | 166 | 172 |
| Chromosome coding for IFN | 9 | 9 | 9 |
| Chromosome coding for IFN receptor | 21 | 21 | 21 |

Type I IFNs are similar to members of the α-helical cytokine family, which have a common four-helix bundle topology, but they differ from the other α-helical cytokines in that they possess a fifth helix. A computer-built, three-dimensional, atomic-level model for human IFN-α has been constructed using the primary amino acid sequence of consensus IFN-α and the α-carbon Cartesian coordinates of murine IFN-β (see Fig. 2). The tertiary structure that is derived consists of five major α-helix segments: helix A (encompassing amino acids 12–24), helix B (amino acids 51–67), helix C (amino acids 80–99), helix D (amino acids 115–132), and helix E (amino acids 141–165). The interconnecting regions are designated loops AB (amino acids 25–50), BC (amino acids 68–79), CD (amino acids 100–114), and DE (amino acids 133–140). IFN-β and IFN-τ also have the same type of structure with five helices (A, B, C, D, and E) and four loops (AB, BC, CD, and DE) although the amino acids that make up those regions are not the same as those cited for IFN-α. X-ray crystallographic structures have been published for three type I IFNs, murine IFN-β, HuIFN-α2b, and HuIFN-β, and the structure of HuIFN-α2a was determined by nuclear magnetic resonance spectroscopy. Experiments using monoclonal antibodies against IFN-α/β as well as site-directed mutagenesis revealed the functional importance of the N-terminal half of loop AB. IFN-αs contain two disulfide bonds (Cys-1–Cys-99, Cys-29–Cys-139), whereas human IFN-β contains one disulfide bond (Cys-31–Cys-141). It has been reported that the two cysteine residues forming the disulfide bridge 29–139 (connecting loop AB with loop DE) in IFN-α are critical. Loss of activity results if this disulfide bridge is not present or even if it is displaced by one residue position. The disulfide bridge found in HuIFN-β (Cys-31–Cys-141) connects loop AB (Cys-31) to loop DE (Cys-141) and both of these loops are thought to be important for interaction of the IFN with its receptor. Three domains (amino acids 29–35, 78–95, and 123–140) within the IFN molecule appear to be important in receptor binding. Changes in the amino acid residues in the 78–95 domain may determine the differential specificity of action between IFN-αs and IFN-β. Mutations of only one of the residues at positions 22, 30, 33, 121, 125, 130, and 131 have been reported to increase or decrease the biological activity by more than 10-fold compared to the unmodified, parent protein. The residues at positions 33 and 121 are so critical that even replacement with an amino acid thought to be similar (Arg vs Lys) has a profound effect on activity of the molecule. Site-directed mutagenesis of IFN-α21a/α2c hybrids has shown that the amino acid region 81–95 is important in antiproliferative activity on Daudi (human, lymphoma) cells. More specifically, it was determined that tyrosine (Y) and/or isoleucine (I) at position 86 and tyrosine (Y) at position 90 play a very significant part in IFN-α's antiproliferative effects. There is considerable evidence, beyond that presented here, that even minute changes or differences in the primary sequences could be responsible for a host of biological activities, which could suggest that type I IFNs, rather than being the result of a redundant duplication, in fact, have distinct biological roles in living organisms.

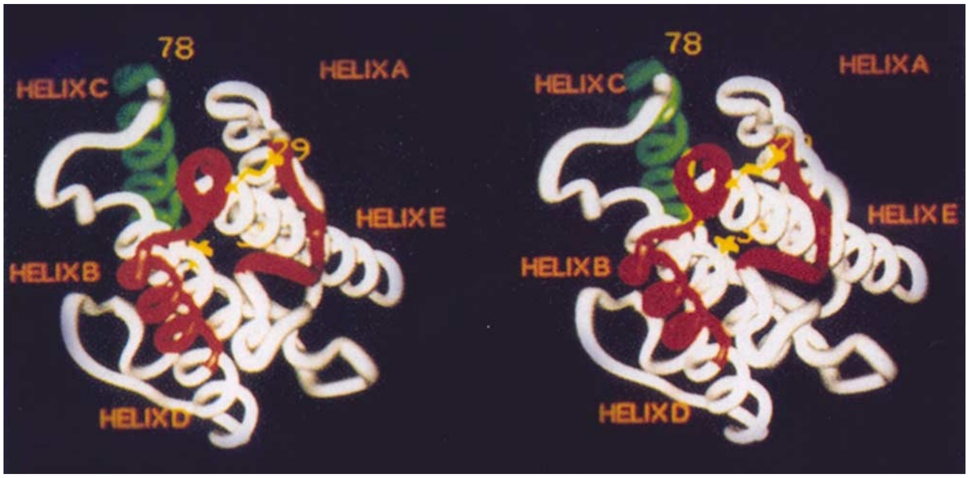

**FIGURE 2** Representation of a model of human interferon-α Con (Consensus IFN). Tertiary structure of human IFN-α constructed using the primary amino acid sequence of Consensus IFN and the α-carbon Cartesian coordinates of murine IFN-β. Reprinted from Korn *et al.* (1994) *J. Interferon Res.* **14**, 1–9, with permission.

## III. RECEPTORS

The type I interferons (IFN-α, IFN-β, IFN-τ, and IFN-ω) induce different cellular effects, but act through a common receptor complex, IFNAR, present at low levels on the surface of all cell types (100–5000 molecules/cell). The receptor complex consists of two subunits, IFNAR-1 and IFNAR-2. On the basis of modular organization and also sequence alignment with other cytokine receptor chains, IFNAR-1 and IFNAR-2 are members of the class II helical cytokine receptor family. All share conserved structural fibronectin type III (FNIII) "building blocks," forming the extracellular ligand-binding domain. The extracellular portion of IFNAR-2 has two FNIII domains (characteristically partitioned in two β-sheets), each containing 100 amino acids with seven β-strands and connecting loops. The extracellular portion of IFNAR-1 is composed of four fibronectin type III-like repeats, which are composed of two domains separated by a three-proline motif. There is 19% sequence identity and 50% sequence similarity (identity plus conserved amino acid changes) between these two domains. Each domain is composed of approximately 200 residues and can be further subdivided into two homologous subdomains of approximately 100 amino acids. Both IFNAR-1 and IFNAR-2 are glycosylated proteins.

IFNAR-1 and IFNAR-2 contribute to ligand binding to different extents, but both are necessary to form a productive binding site for IFNs. Human IFNAR-2 (HuIFNAR-2, 115 kDa) binds all type I IFNs but with lower affinity and different specificity than the IFNAR complex and has moderate intrinsic affinity for the range of human type I IFNs ($K_d \sim 10^{-9}$ M). Human IFNAR-1 (HuIFNAR-1, 135 kDa) has low intrinsic binding of human type I IFNs ($K_d \sim 10^{-7}$ M) but strongly affects the affinity and differential ligand specificity of the IFNAR complex. When HuIFNAR-1 is co-transfected with HuIFNAR-2, it increases the affinity of the receptor complex for binding most type I IFNs, including IFN-β by approximately 10-fold.

Mature HuIFNAR-1 (after removal of the peptide leader sequence) is a 530-amino-acid integral membrane protein that consists of an extracellular domain containing 409 residues, a transmembrane domain made up of 21 residues, and an intracellular domain composed of 100 residues. Mature HuIFNAR-2 has been isolated as three forms: the full-length receptor chain, composed of 487 amino acids (referred to as IFNAR-2c, 115 kDa) with 251 residues in the cytoplasmic portion, and two shorter forms, one composed of 303 amino acids (referred to as IFNAR-2b, 55 kDa) with only 67 residues in the cytoplasmic portion and one that is a soluble form lacking the transmembrane and cytoplasmic portions (referred to as IFNAR-2a, 40 kDa). Transmembrane domains of IFNAR-1 and IFNAR-2 do not have intrinsic enzyme activities, but their cytoplasmic domains associate noncovalently with tyrosine kinase 2 (Tyk2) and Janus kinase 1 (JAK1), respectively. The importance of both IFNAR-1 and IFNAR-2 for IFN-mediated signaling is highlighted by the observation that in the absence of either chain there is neither high-affinity binding of IFN nor activation of the requisite JAK1/Tyk2 or signal transducers and activators of transcription (STAT) proteins nor biological response.

One of the most intriguing aspects of the function of the type I IFN receptor is its ability to discriminate between the various types and subtypes of IFN and to elicit different biological responses depending upon which ligand is bound. IFN-α and IFN-β interact with HuIFNAR-1 and HuIFNAR-2 differently although they share a common receptor. For example, IFN-β mediates the association of tyrosine-phosphorylated IFNAR-1 and IFNAR-2 (as detected by immunoprecipitation with anti-IFNAR-1 antibodies), whereas IFN-α-1, -α-2, -α-6, -α-7, -α-8, and IFN-ω do not. Previously, the antiproliferative and competitive binding activities of 20 purified components of human lymphoblastoid interferon (IFN-α) were compared with that of recombinant human IFN-α2b on Daudi and AU937 cells. These data suggest that there may be more than one binding site for human IFN-α and/or there may be a multicomponent receptor involved in the biological actions of these molecules. Despite their sequence differences, various type I IFNs probably bind to extracellular portions of IFNAR-1 and IFNAR-2 in a similar manner.

The binding site for IFNAR-2 was mapped mainly on the AB loop and D helix of IFN-α2 and the binding site for IFNAR-1 on the C helix of IFN-α2. However, comparison with homologous cytokines suggests the involvement of the E helix of IFN-α2 in binding of IFNAR-2. Despite extensive studies, the interactions between IFNs and their receptor and the molecular mechanisms underlying the functional differences between IFNs are still unknown.

## IV. SIGNAL TRANSDUCTION

Studies on the induction of transcription by IFN-α have provided an understanding of the signal transduction pathways that connect the events from ligand binding to the type I IFN receptor at the cell's surface to gene activation. The primary signaling pathway initiated by IFN-α is the JAK/STAT pathway. It begins with dimerization of the two receptor subunits induced by IFN binding and the subsequent activation of protein tyrosine kinases. As can be seen from Fig. 3, the two kinases involved in type I IFN signal transduction are Tyk2, which is associated with IFNAR-1, and JAK1, which is associated with IFNAR-2. These kinases then rapidly phosphorylate receptor tyrosine residues, which act as docking sites for STAT SH2 domains. In addition, in the type I IFN system, STAT2 is preassociated with the IFNAR-2 chain of the receptor, and nonphosphorylated STAT1 is also constitutively associated with a more proximal region of IFNAR-2 via the adapter RACK1 (receptors for activated C kinase). This combination of constitutive and receptor phosphotyrosine-dependent binding of STATs leads to efficient phosphorylation of STAT2 and STAT1 by the kinases. STAT1 phosphorylation, however, is dependent on the prior activation of STAT2. The importance of STAT2 to IFN-α-induced signaling is demonstrated by the fact that STAT2 knockout mice are unresponsive to type I IFNs. Normally, IFN-α stimulation also leads to serine as well as tyrosine phosphorylation of STAT1. The phosphorylated forms of STAT1 and STAT2 associate with one another, and the heterodimer interacts with IFN regulatory factor-9 (IRF-9; p48) forming a complex called the IFN-stimulated gene factor 3 (ISGF3). STAT1 homodimers can also be formed. However, ISGF3 is the primary positive regulator of type I IFN-induced transcription and is quickly translocated to the nucleus after formation. This complex associates with a promoter, the interferon-stimulated response element (ISRE), that is located upstream of interferon-responsive genes, and transcription begins. The ISRE responsible for the IFN-α response is a highly conserved region of 12 to 15 bp, found within 200 bp of the transcription start site of most genes that respond to IFNα. In contrast, STAT1 homodimers target gamma-activated sites (GAS).

In addition to STAT1 and STAT2, other STAT proteins (STATs 3, 4, 5, and 6) have been shown to be phosphorylated in particular cell types in response to

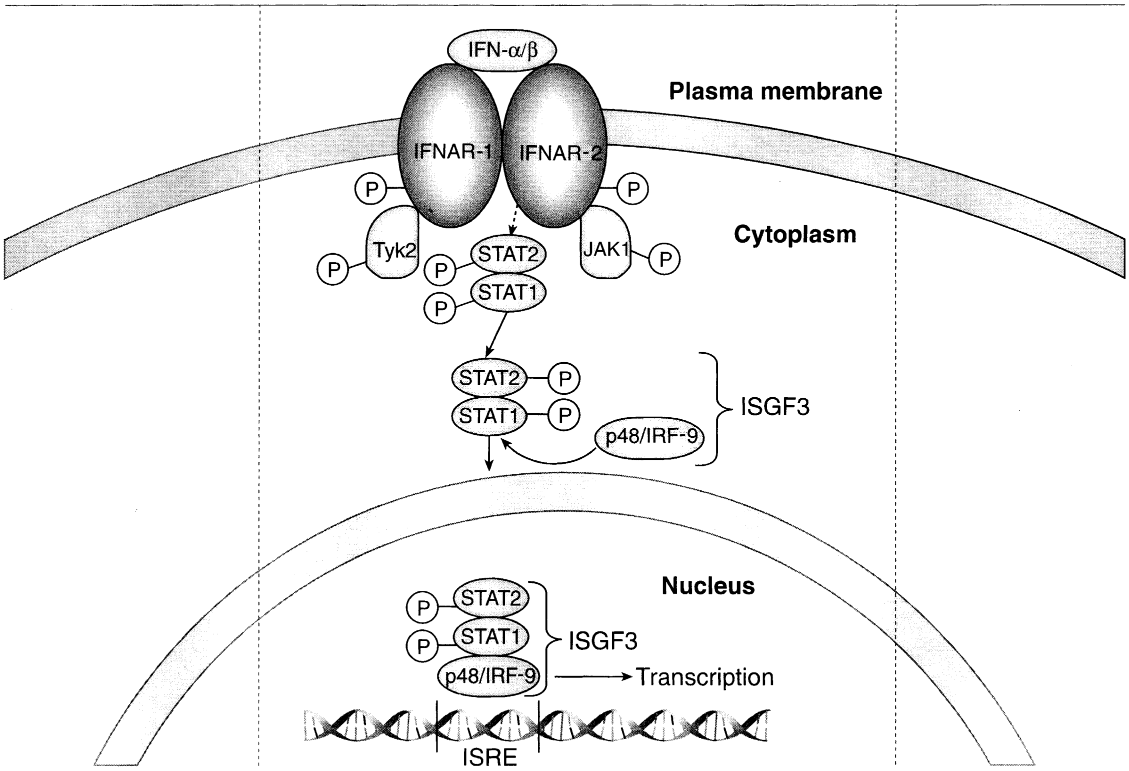

FIGURE 3 Signal transduction mechanism of Hu IFN-α/-β. Interferon binding causes the two subunits of the receptor (IFNAR-1/IFNAR-2) to aggregate, which leads to activation and phosphorylation of JAK1 and Tyk2. These then phosphorylate STAT1 and STAT2. The DNA-binding protein p48 (IRF-9) forms a complex with STAT1 and STAT2 that moves to the nucleus, where it stimulates transcription of genes bearing an interferon-response element (ISRE).

type I IFNs. STAT3 binds to tyrosine-phosphorylated IFNAR-1 and is itself phosphorylated at Tyr-656. The p85 regulatory subunit of phosphatidylinositol 3-kinase (PI-3K) has been shown to bind phosphorylated STAT3, which may be one way to couple activation of a serine kinase cascade to the IFN receptor. STAT4 activation is a convergence point of innate immunity and an adaptive IL-12-mediated response. STAT5 associates with JAK2 and becomes activated. IFNα treatment of Daudi cells leads to activation of both STAT5 and STAT6, and formation of a STAT2:STAT6 heterodimer that associates with IRF-9 has been reported. Although activation of these additional STAT proteins may be important in cell type-specific responses, their exact role in antiviral or even antiproliferative activity of the type I IFNs has yet to be determined.

In addition to the STAT proteins, another key player in type I IFN signaling appears to be PI-3K. PI-3K is a serine kinase consisting of a p85 adapter subunit and a p110 catalytic subunit. It has been shown to associate with IFNAR-1 in the absence of IFN binding. In addition, members of yet another signaling pathway, insulin receptor substrate proteins IRS-1 and IRS-2, regulate activation of PI-3K by IFN-α. There are many downstream targets of activated PI-3K, many of which have a role in cell growth and survival. Activation of the downstream target Akt has been shown to lead to serine phosphorylation and degradation of IκB and subsequent nuclear factor κB activation, which promotes cell survival. The extracellular regulated kinase (ERK) pathway is also downstream of PI-3K. ERK2 and STAT1 were found to co-immunoprecipitate following treatment of cells with type I IFNs. ERK is part of the Ras–Raf–MEK–mitogen-activated protein kinase (MAPK) pathway, which is also involved in promoting cell survival. It has been suggested that Akt or ERK pathways can promote cell survival in the presence of viral infection, and the choice is dependent on the virally induced apoptotic mechanism. The p38 MAPK is another important PI-3K effector pathway. The p38 MAPK appears to be regulated by the small GTPase Rac1, and the Rac 1/p38 pathway has been implicated in IFN-α-induced gene transcription via GAS elements and mediation of growth inhibition. IFN-α suppression of the bcr–abl fusion and inhibition of growth of chronic myelogenous leukemia cells also appears to be dependent on the p38 MAPK pathway. Thus, PI-3K and additional downstream signaling pathways may play a significant role in mediation of the antiproliferative and apoptotic activity of the type I IFNs.

Another family of transcription factors involved in IFN signaling is the IRF family. IRF-1 expression is driven by the GAS element and occurs subsequent to STAT activation. IRF-1 acts as a transcriptional activator at the ISRE, and its function is critical for expression of some IFN-inducible proteins such as guanylate-binding protein and inducible nitric oxide synthase. Another member of this family, IRF-2, is a constitutively expressed transcriptional repressor. Regulation of cell growth has been shown to depend in part on the ratio of IRF-1 to IRF-2, and any change that interferes with the balance of the two factors can result in a significant alteration in cell proliferation. Interestingly, IRF-2 has also been found to antagonize ISGF3, further attenuating type I IFN signaling.

There are additional mechanisms for regulation of JAK/STAT signaling. STAT1 is inactivated by tyrosine phosphatases and degraded by the ubiquitin–proteasome pathway. Regulatory proteins of the suppressor of cytokine signaling (SOCS) family and protein inhibitors of activated STAT (PIAS) also play a role in damping IFN-α-mediated signal transduction. SOCS1 and SOCS3 bind the JAK1 kinase domain and inhibit its activity, and PIAS1 inhibits STAT1-induced gene expression.

Examination of signal transduction from the type I IFN receptor provides insight into the mechanisms responsible for differences in function among IFN-α subtypes as well as other type I IFNs. Another point to consider is that the kinetics of activation of signal transduction molecules may affect observed activity, in that prolonged STAT1 activation in specific cell lines has been associated with enhanced IFN-α antiproliferative activity. Since a variety of signaling pathways are involved in IFN responsiveness, there is also potential for cross talk between pathways. For example, cross-phosphorylation of the IFN-γ receptor has been detected following IFN-α treatment. In T cells, the antiproliferative activity of type I IFNs is dependent on components of the TCR (T cell antigen receptor) signaling cascade, CD45, Lck (lymphocyte cell kinase) and Zap-70 (zeta chain associated protein 70) associating with IFNAR-1. Furthermore, STAT1 and NFAT (nuclear factor of activated T cells) have been found to be preassociated, and type I IFNs have been shown to induce nuclear translocation of NFAT. STAT proteins can also interact with transcription adapter molecules, and IFN-α leads to interaction of STAT2 with p300/CBP (CREB-binding protein, where CREB is cAMP-response element-binding protein).

Thus, the unique activities of particular type I IFNs may be the result of differences in activation or interaction of the various signal transduction cascades, JAK/STAT, IRF, IRS–PI-3K, ERK, or p38 MAPK, and others associated with IFN function.

## V. CLINICAL APPLICATIONS OF HUMAN INTERFERON-α AND -β

### A. Human Interferon-αs

The first human interferon-αs, human interferon alfa-2a, recombinant (Roferon A, Hoffmann-La Roche, Inc.) and human interferon alfa-2b, recombinant (Intron A, Schering Corp.), were licensed in the United States in 1986 for the treatment of hairy cell leukemia. Interferon alfa-2a is a recombinant DNA-derived protein prepared from genetically engineered *Escherichia coli* containing the human interferon α-2a gene. This protein is highly purified and has an antiviral specific activity of approximately $2 \times 10^8 \times$ IU/mg protein. It consists of 165 amino acids and has an apparent molecular weight of 19,000. Interferon alfa-2b is also produced from genetically engineered *E. coli* containing a plasmid encoding the human interferon-α-2b gene. It is also highly purified, has a molecular weight of 19,271, and has an antiviral specific activity of $2.6 \times 10^8$ IU/mg protein. Subsequently, both of these IFNs have been approved for a variety of clinical indications ranging from chronic myelogenous leukemia to chronic hepatitis C. See Table 2 for the complete list of approved indications in the United States as of March 2002. Some of the side effects of these two products are influenza-like symptoms (e.g., fever, fatigue, myalgia, and chills), and headache, nausea, vomiting, depression, alopecia, and anorexia. The higher the dose, the higher the incidence of severe psychiatric disorders (see their respective package inserts for the complete list of adverse effects).

Pegylated interferon alfa-2b is a covalent conjugate of human interferon alfa-2b with monomethoxy-polyethylene glycol (PEG). The molecular weight of the PEG is approximately 12,000. The average molecular weight of the PEG-INTRON is approximately 31,000. The specific antiviral activity of the product is approximately $0.7 \times 10^8$ IU/mg protein. PEG-INTRON (Schering Corp.) is approved for use either alone or in combination with REBETOL (ribavirin, USP) for the treatment of chronic hepatitis C in individuals with compensated liver disease. The dose of PEG-INTRON as a monotherapy is 1.0 μg/kg/week injected subcutaneously (sc). When administered in combination with REBETOL, the recommended dose of PEG-INTRON is 1.5 μg/kg/week. The recommended dose of REBETOL is 800 mg/day in two divided doses. Some major adverse effects include injection site inflammation/reaction, fatigue, headache, rigor, fever, weight decrease, dizziness, nausea, anorexia, neutropenia, myalgia, arthralgia, insomnia, depression, psychiatric disorders, anxiety, alopecia, dyspnea, and some cardiovascular effects (e.g., hypotension, arrhythmia) (see the package insert for the complete list of adverse effects).

PEGASYS, pegylated interferon alfa-2a, is a covalent conjugate of human interferon alfa-2a with a single branched bis-monomethoxy-polyethylene glycol (PEG) chain (approximate MW = 40,000). The apparent average molecular weight of the product is approximately 60,000. PEGASYS (Hoffman–La Roche Corp.) was FDA approved in 2002 as a monotherapy or in combination with COPEGUS (ribavirin) for the treatment of chronic hepatitis C in individuals with compensated liver disease who have not been previously treated with interferon alfa. The recommended dose of PEGASYS is 180 μg/week

TABLE 2 **Human Interferons -α, and -β Licensed in the United States**

| Product | Protein | Indications | Route of administration |
|---|---|---|---|
| Intron-A | Interferon alfa-2b | Hairy cell leukemia, chronic hepatitis B, condylomata acuminata, follicular lymphoma, AIDS-related Kaposi's sarcoma, chronic hepatitis C, malignant melanoma | im, sc, iv, il |
| Roferon | Interferon alfa-2a | Chronic myelogenous leukemia, hairy cell leukemia, AIDS-related Kaposi's sarcoma, chronic hepatitis C | im, sc |
| Infergen | Interferon alfacon-1 | Chronic hepatitis C | sc |
| Alferon | Interferon alfa-n3 | Condylomata acuminata | il |
| Betaseron | Interferon β-1b | Multiple sclerosis | sc |
| Avonex | Interferon β-1a | Multiple sclerosis | im |
| Rebif | Interferon β-1a | Multiple sclerosis | sc |

*Note.* il, intralesional; im, intramuscular; iv, intravenous; sc, subcutaneous.

for 48 weeks injected subcutaneously (sc) while the ribavirin dosage is 800–1200 mg, twice daily in a split dose. Some major adverse effects include injection site inflammation/reaction, fatigue, neutropenia, anorexia, myalgia, depression, and alopecia (see the package insert for the complete list of adverse events).

Interferon alfacon-1 (Infergen, Intermune) is a recombinant IFN-α that was developed based on a comparison of the sequences of the naturally derived human IFN-α's and targeting the most common sequence elements. It has a very high antiviral and antiproliferative specific activity, $10^9$ IU/mg protein. It consists of 166 amino acids and has an apparent molecular weight of 19,434. Interferon alfacon-1 exhibits 88% similarity to human interferon-α2 and 70% similarity to human interferon-β based on an amino acid sequence comparison. Infergen is approved in the United States for the treatment of chronic hepatitis C infection in individuals with compensated liver disease. Adverse effects are similar to those described for other recombinant interferon-αs (see package insert for the complete list of adverse effects).

Interferon alfa-n3 (ALFERON N Injection, Interferon Sciences, Inc.) is the only natural IFN-α licensed in the United States. It is manufactured from source leukocytes induced with Sendai virus and purified by immunoaffinity and size-exclusion chromatography. It consists of multiple interferon-αs, each with approximately 165–166 amino acids. The apparent molecular weights range from 16,000 to 27,000. Interferon alfa-n3 has an antiviral specific activity of $2 \times 10^8$ IU/mg protein or greater. ALFERON N Injection is approved in the United States for the intralesional treatment of refractory or recurring external condylomata acuminata in patients 18 years of age or older. Some of the major adverse effects are fever, chills, fatigue, headache, and myalgias.

## B. Human Interferon-β

At this time, three human interferon-βs are licensed in the United States: Betaseron (interferon-β-1b; Berlex Laboratories), Avonex (interferon-β1a; Biogen, Inc.), and Rebif (interferon-β-1a; Serono, Inc.). Betaseron, interferon-β-1b is a recombinant DNA-derived product made in *E. coli* that contains the human interferon β-ser17 gene. The antiviral specific activity of this interferon is $3.2 \times 10^7$ IU/mg protein. This a nonglycosylated protein with an apparent molecular weight of 18,500. Betaseron is indicated for use in ambulatory patients with relapsing–remitting multiple sclerosis to reduce the frequency of clinical exacerbations. The dosage and route of administration are 0.25 mg sc every other day. Some of the side effects of this product include injection site reactions including necrosis, headache, fever, flu-like symptoms, chills, malaise, decreased neutrophil and white blood cell counts, depression, and suicides/suicide attempts (see the package insert for the complete list of adverse effects). Avonex, interferon beta-1a is produced by recombinant DNA technology using Chinese hamster ovary cells into which the human interferon beta-1a gene was introduced. This interferon is a glycoprotein (166 amino acids) with an apparent molecular weight of 22,500. It has an antiviral specific activity of approximately $2 \times 10^8 \times$ IU/mg protein. Avonex is indicated for the treatment of relapsing forms of multiple sclerosis to delay the accumulation of clinical exacerbations. The dosage and route of administration are 30 μg injected intramuscularly once per week. The major adverse effects include headache, flu-like symptoms, fever, nausea, muscle aches, depression, and suicidal ideation (see the package insert for the complete list of adverse effects). Rebif is also a recombinant DNA-derived human interferon β-1a made in Chinese hamster ovary cells. It is a 166-amino-acid glycoprotein with a molecular weight of approximately 22,500. It has an antiviral specific activity of $2.7 \times 10^8$ IU/mg protein. The recommended dose is 44 μg injected 3 times per week sc. It is approved in the United States for patients with relapsing multiple sclerosis to decrease the frequency of exacerbations and delay the accumulation of physical disability. Again, the most common side effects include injection site disorders, influenza-like symptoms, abdominal pain, depression, an increase in liver enzymes, and hematologic disorders (for the complete list of adverse effects see the package insert).

## VI. SUMMARY

Interferon-αs and -β were the first cytokines to be discovered in 1957. Over the years, they have served as model systems to understanding receptor interactions and signal transduction. Interferon alfa-2a and Interferon alfa-2b were the first two cytokines licensed for marketing in the United States in 1986. Today these molecules and other interferon-αs are used to treat a wide variety of diseases ranging from hairy cell leukemia to malignant melanoma to hepatitis B and C. Interferon-β has also proven medically valuable, particularly for certain forms of multiple sclerosis. Interferon-τ and -ω continue to be explored to elucidate their biological roles.

## Glossary

**interferon** A family of species-specific vertebrate proteins that confer nonspecific resistance to a broad range of viral infections, affect cell proliferation, and modulate immune responses.

**interferon-α** A family of highly homologous species-specific interferons, the natural forms of which are derived primarily from either leukocytes or lymphoblastoid cells upon exposure to live or inactivated virus.

**interferon-β** An interferon produced primarily by fibroblasts in response to stimulation by live or inactivated viruses or by certain synthetic polynucleotides.

**interferon-ω** First described in 1985 and now recognized as sufficiently distinct from IFN-α to deserve the separate ω-subtype status. It appears to possess comparable biologic activity to many Hu-IFN-αs but its particular function is not understood.

**interferon-τ** A relatively new class of type I interferon that is not virus inducible but is constitutively produced by the trophectoderm of the ruminant conceptus during a very short period in early pregnancy. This interferon displays high antiviral and antiproliferative activities with a prominent lack of cytotoxicity *in vitro* and possibly *in vivo*.

**interferon receptor** All type I interferons (α, β, ω, and τ) act through a common receptor complex (IFNAR-1/IFNAR-2). Binding of interferon to its receptor mediates activation of numerous genes through different signal transduction pathways.

**licensed interferons** Licensure of interferons (or any biologic) in the United States is granted only after the Food and Drug Administration is ensured of its safety, purity, and potency as outlined by regulations set forth in the Code of Federal Regulations (21 CFR 601).

**signal transduction** The transmission of signals from the exterior of the cell to the interior. The type of signal transduction seen with IFN involves the coupling of ligand (IFN-α,β) and receptor (IFNAR-1/IFNAR-2), with the outcome being alterations in cellular activity and changes in the program of cellular gene expression.

## See Also the Following Articles

**Colony-Stimulating Factor-1 • Interferon-γ • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries) • Tumor Necrosis Factor (TNF)**

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Encyclopedia of Hormones.

# Interleukin-1 (IL-1)

Alberto Mantovani
*Istituto di Ricerche Farmacologiche Mario Negri and University of Milan*

I. INTRODUCTION
II. AGONISTS AND ANTAGONISTS
III. RECEPTORS
IV. CELLULAR SOURCES AND PRODUCTION
V. BIOLOGICAL ACTIVITIES
VI. CLINICAL IMPLICATIONS

**Interleukin-1 (IL-1) is the term for two polypeptide mediators, IL-1α and IL-1β, that are among the most potent and multifunctional cell activators thus far described in immunology and cell biology. The spectrum of action of IL-1 encompasses cells of hematopoietic origin, from immature precursors to differentiated leukocytes, vessel wall elements, and cells of mesenchymal, nervous, and epithelial origin. Occupancy of a few receptors, or perhaps only one receptor, per cell is sufficient to elicit cellular responses. The activity of IL-1 overlaps largely with that of tumor necrosis factor (TNF) and other cytokines.**

## I. INTRODUCTION

The production and action of interleukin-1 (IL-1) are regulated by multiple control pathways, including receptor antagonists and a decoy receptor. The complexity and uniqueness of IL-1 are best represented by the term "IL-1 system." The IL-1 system consists of the two agonists, IL-1α and IL-1β, a specific activation system (IL-1-converting enzyme, or ICE, caspase 1), a receptor antagonist (IL-1ra) of which there are different isoforms, and two high-affinity surface-binding molecules (Fig. 1). This article will focus largely on IL-1 and its receptors, with summaries of the properties of the other elements of the system (IL-1ra, caspase 1).

IL-1 is a member of a multigene family that includes 10 members to date. These molecules are denoted IL-1F1 through IL-1F10, where F stands for family. Receptors and functions have been identified for IL-1F4 (IL-18), IL-1F5, and IL-1F9; the last two molecules are the antagonist and agonist for the same receptor.

## II. AGONISTS AND ANTAGONISTS

The mature human IL-1α and IL-1β are polypeptides that share 26% amino acid (aa) identity. Comparison of mature IL-1β from different animal species indicates that the amino acid sequence is 75 to 78% conserved. IL-1α sequences are less conserved among species (60–70%). The primary translation products of IL-1α and IL-1β are 271 and 269 aa long, corresponding to a molecular weight of 30,606 and 30,749, respectively. The IL-1α propeptide is biologically active, whereas the IL-1β precursor is not. Although the sequence contains glycosylation sites, sugars are not important in the biological activity of IL-1. IL-1α may be glycosylated and mannose sites may be important for association with the cell membrane.

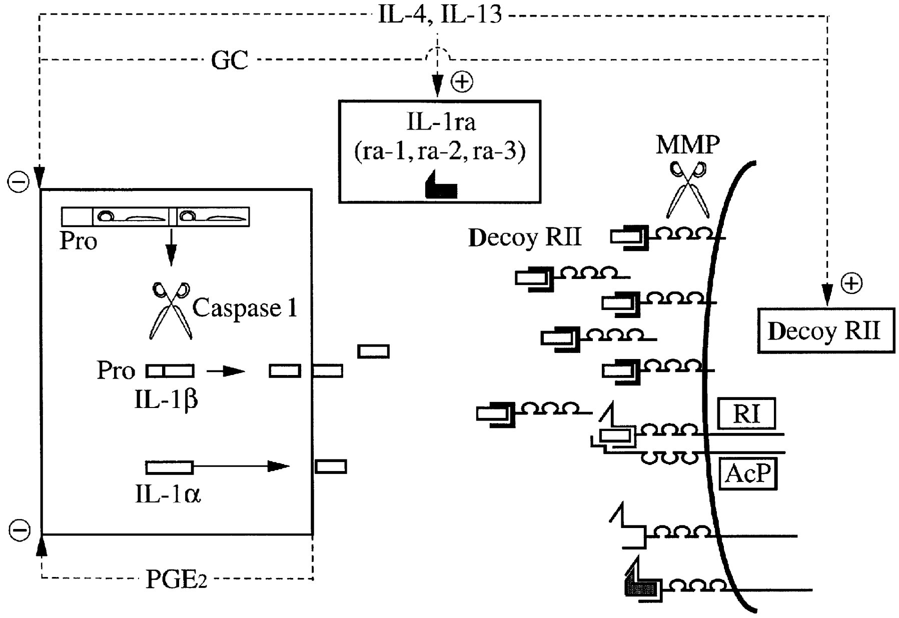

FIGURE 1 An overview of the IL-1 system. GC, glucocorticoid hormones; ra, receptor antagonist. The plus and minus signs indicate stimulation or inhibition of production, respectively. RI, receptor I; RII, receptor II; AcP, accessory protein. Reprinted with permission from "The Cytokine Network and Immune Functions" edited by Théze, J. (1999), copyright Oxford University Press.

IL-1α and IL-1β lack a signal peptide. IL-1α remains mostly in the cytosol and associated with the plasma membrane. The pathway of secretion of mature IL-1β (aa 117–269 of the precursor) is not completely defined and may be the same as that of other leaderless proteins. Cleavage of pro IL-1β to mature IL-1β is mediated by caspase-1, a cysteine protease representative of a novel class of proteolytic enzymes. IL-1α and IL-1β have been crystallized. Despite limited sequence similarity, IL-1α and IL-1β have a similar three-dimensional structure, consisting of a β barrel with four triangular faces that form a tetrahedron.

Multiple forms of IL-1ra have been generated by differential splicing: soluble IL-1ra (sIL-1ra) is a 152 aa protein (p*I* 5.2), secreted in both unglycosylated (18 kDa) and glycosylated (22 kDa) forms; intracellular IL-1ra type I (icIL-1ra I) has been cloned in keratinocytes and consists of 159 aa; it lacks a signal sequence and remains within cells; and icIL-1ra type II contains an extra 21 aa at the N-terminus.

IL-1ra is produced by different cell types, including monocyte–macrophages, polymorphonuclear leukocytes (PMN), and fibroblasts. Keratinocytes and other cells of epithelial origin produce icIL-1ra almost exclusively. The intracellular versions of IL-1ra are likely to represent a reservoir of anti-inflammatory molecules, to be released under conditions of extreme tissue damage.

## III. RECEPTORS

IL-1 receptors belong to a complex superfamily of molecules. They activate a signaling cascade starting from the adapter protein MyD88 leading to activation of nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (Fig. 2). Members of the IL-1/Toll-like receptor (TLR) superfamily share a Toll-IL-1 receptor (TIR) domain in the cytoplasmic region, which plays a key role in recruiting the adapter protein MyD88, the first step in a stereotyped signaling cascade leading to NF-κB and AP-1 activation (Fig. 1). The signaling IL-1 receptor complex includes the type I IL-1 receptor (IL-1RI) and the accessory protein (AcP), which have cytoplasmic TIR domains. The genes encoding IL-1RI, IL-1RII, and AcP are located on chromosome 2 (band q12–q22) in humans and in the centromeric region of chromosome 1 in mice. IL-1RI and IL-1RII have different affinities for the three ligands of the IL-1 family. Although results vary between studies, IL-1RI binds IL-1α with higher affinity than IL-1β ($K_d \simeq 10^{-10}$ and $10^{-9}$ M, respectively). By contrast, IL-1RII binds IL-1β more avidly than IL-1α ($K_d \simeq 10^{-9}$ to $10^{-10}$ and $10^{-8}$ M, respectively). IL-1ra binds to IL-1RI with an affinity similar to that of IL-1α, whereas IL-1RII binds IL-1ra at least 100 times less efficiently than IL-1RI. Plasmonic resonance analysis revealed that IL-1β has a slow off-rate from IL-1RII, whereas IL-1ra rapidly dissociates from IL-1RII but not from IL-1RI. Cell-associated IL-1RII did not bind pro-IL-1β, but sIL-1RII did bind it and blocked its processing by IL-1-converting enzyme. The kinetic data are consistent with a decoy function for IL-1RII. Moreover, they indicate that the two anti-IL-1 molecules in the system (IL-1ra and IL-1RII) minimally interfere with each other and actually synergize.

The IL-1RII (68 kDa) is structurally characterized by a short cytoplasmic tail (29 aa) with no TIR domain (Fig. 2). It is the predominant IL-1-binding protein found in monocytes, neutrophils, and B cells. Several lines of evidence suggest that IL-1RII is not required for signaling. Actually, cells that express only IL-1RI and AcP, such as endothelial cells, are exquisitely sensitive to IL-1. Moreover, under no experimental conditions has evidence been obtained that IL-1RII can trans-signal (i.e., in soluble form induce or amplify responsiveness to IL-1, in analogy to IL-6Rα).

Several lines of evidence are consistent with the view that the IL-1RII is a pure decoy for IL-1. The original proposal of the decoy paradigm was based on blocking antibodies to IL-1RII, which amplified responsiveness of myelomonocytic cells to IL-1, as well as on stimulation of expression by anti-inflammatory agents (see below). These observations have been confirmed and extended *in vivo*. Gene transfer experiments have shown that IL-1RII gene transfer blocks *in vitro* cellular responses to IL-1 in diverse cellular contexts. Moreover, as discussed below, transfer of IL-1RII-transfected cells ameliorates collagen-induced arthritis in mice. Finally, genetically modified mice yielded results consistent with the decoy paradigm. IL-1RII was expressed under the control of the human keratin promoter in transgenic mice; keratinocytes from these mice did not respond to IL-1. IL-1RII transgenic mice were resistant to phorbol myristate acetate (PMA)-induced vascular permeability changes and to chronic inflammation caused by repeated topical application of PMA. The effect of IL-1RII was restricted to the skin, with normal systemic inflammatory responses.

In agreement with the view that IL-1RII is a professional anti-IL-1 molecule, poxviruses encode an IL-1RII-like receptor that plays a key role in

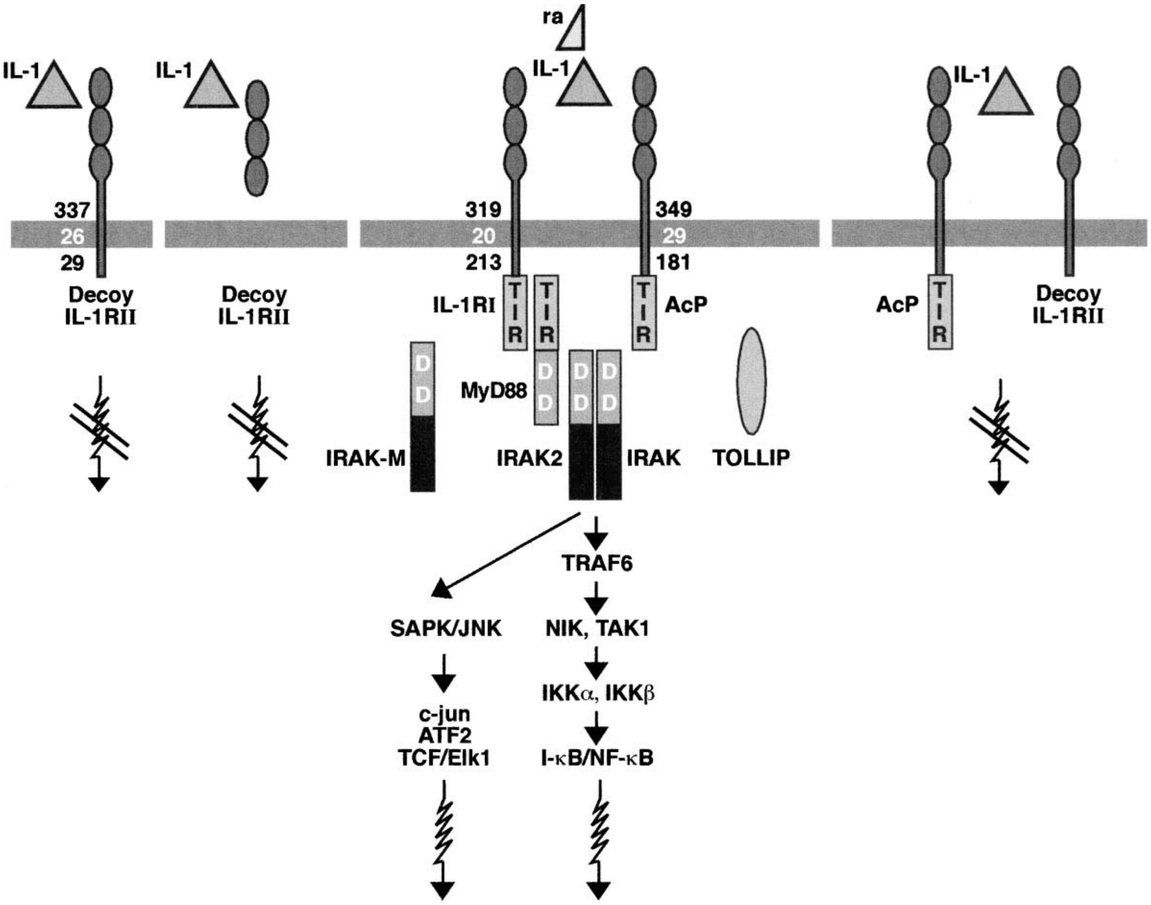

FIGURE 2 The type II interleukin-1 receptor (IL-1RII) as a decoy. The signaling IL-1R complex [IL-1RI and accessory protein (AcP)] activates the MyD88 cascade, leading to activation of NK-κB and AP-1. The cascade consists of adapter proteins (MyD88 and TRAF6), kinases (IRAKS, IKK, and JNK), and a shuttle molecule (Tollip for IRAK). In membrane-bound or soluble form, IL-1RII captures IL-1 and prevents it from forming a signaling receptor complex. In addition, it forms a dominant negative nonsignaling complex with the AcP. ATF-2, activated transcription factor 2; DD, death domain; IL-1ra, IL-1R antagonist; IKK, inhibitor of nuclear factor κB kinase; IRAK, IL-1R-associated kinase; JNK, Jun N-terminal kinase; NK, nuclear factor; NIK, nuclear factor κB-inducing kinase; SAPK, stress-activated protein kinase; TAK, transforming growth factor β-activated kinase; TCF, T-cell factor; TIR, Toll/IL-1R domain; TRAF, tumor necrosis factor receptor-associated factor. Circles represent immunoglobulin domains. Numbers refer to the number of amino acids per domain. Reproduced from Mantovani *et al.* (2001), with permission from Elsevier Science.

the regulation of pathogenicity. In addition to binding IL-1, the IL-1RII forms a complex with the agonist and the IL-1R AcP.

Therefore, IL-1RII acts as a dominant negative molecule for the formation of a signaling receptor complex by sequestering AcP, which is essential for signal transduction (Fig. 2). This dominant negative action probably explains early results obtained in studies in which, after IL-1RII gene transfer, under certain conditions, excess agonist did not completely revert inhibition. In summary, IL-1RII blocks IL-1 *in vitro* and *in vivo* in two complementary ways. First, in membrane-bound or soluble form it acts as a decoy, capturing IL-1 and preventing it from interacting with IL-1RI. Second, it acts as a dominant negative molecule for signaling receptor complexes.

IL-1RII is the main IL-1-binding protein in neutrophils, monocytes, macrophages, and B cells. Monocyte-to-macrophage differentiation results in increased expression of IL-1RII. Endothelial cells and fibroblasts generally have only IL-1RI and IL-1R AcP, whereas other cell types (e.g., T cells and epithelial cells) have a mixture of the two receptors. In addition to being regulated during lineage differentiation, IL-1RII expression is influenced by pro- and anti-inflammatory signals. Glucocorticoid hormones

(GCs) and the anti-inflammatory Th2 cytokines IL-4 and IL-13 induce augmented surface expression and release of the decoy RII (Fig. 1). For example, resting monocytes express $\simeq 1.3 \times 10^3$ molecules per cell. Exposure to IL-13 for 24 h increased the number of surface receptors to $3.5 \times 10^3$/cell and induced the release of $12 \times 10^3$ sIL-1R molecules/cell. A Stat-6-binding site in the IL-1R promoter could be involved in induction by IL-4 and IL-13. IL-10 does not induce or inhibit IL-1RII *in vitro* but *in vivo* increased circulating levels have been observed after IL-10 treatment in mice. Aspirin increases the levels of sIL-1RI released *in vitro* from mononuclear cell cultures. GCs, IL-4, and aspirin also increase IL-1RII in the circulation and/or tissues after *in vivo* administration. The stimulation of IL-1RII levels by anti-inflammatory signals is consistent with it being a negative regulator of IL-1 activity.

In contrast to anti-inflammatory signals, the two prototypic pro-inflammatory molecules bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ) inhibit IL-1RII expression in myelomonocytic cells. LPS causes a rapid (within 30 min) shedding of surface IL-1RII in monocytes, followed by down-regulation of transcript expression. Concomitantly, it stimulates expression of IL-1RI, IL-1R AcP, and the adapter protein MyD88. Stimulation with high concentrations of LPS causes the appearance of incompletely spliced IL-1RI transcripts, whose significance is unclear. PMA has similar effects, inducing also alternatively spliced soluble IL-1R AcP. IFN-γ suppresses IL-1RII mRNA levels and receptor release in monocytes but not in keratinocytes. The reason for the divergent effect of IFN-γ on myelomonocytic cells versus keratinocytes has not been established. It also blocks the IL-4-mediated stimulation of IL-1RII. Therefore, at least certain pro- and anti-inflammatory signals have divergent effects on IL-1RII expression and release in myelomonocytic cells, with inhibition, for instance, by IFN-γ and stimulation by IL-4 and GCs. The IL-1RII balance is a component in setting myelomonocytic cells in a pro- or anti-inflammatory mode.

sIL-1RII is found in culture supernatants and in biological fluids. In normal blood, relatively high concentrations are found, on the order of nanograms per milliliter, presumably reflecting constitutive release observed *in vitro* in cells such as monocytes and neutrophils. An alternatively spliced transcript encoding a soluble version of IL-1 RII has been identified in COS-1 and Raji cells. However, inhibitors of matrix metalloproteinases (MMPs) completely block the induction of IL-1RII release from monocytes, neutrophils, transfected fibroblasts, and hepatocytes exposed to diverse stimuli. Therefore, shedding mediated by an MMP closely related or identical to TNF-α-converting enzyme is the major mechanism of sIL-1RII release (Fig. 2). Shedding is to some extent selective in that PMA induces rapid shedding of IL-1RII but not of IL-1RI.

IL-1RII release is a regulated process (Fig. 2). Augmented surface expression induced by GCs or IL-4/IL-13 is associated with shedding and both are inhibited by IFN-γ. In addition to this default pathway, a defined and restricted set of stimuli cause rapid (in seconds to minutes) shedding mediated by activation of a preformed MMP. These include chemoattractants (e.g., fMLP), reactive oxygen intermediates, TNF, and LPS, but not other pro- and anti-inflammatory cytokines. Shedding of IL-1RII by circulating phagocytes induced by chemoattractants in the early steps of recruitment could prepare cells to respond to IL-1 once they enter tissues and the released decoy IL-1RII can buffer IL-1 leaking in the systemic circulation from sites of inflammation.

Alterations of tissue or body fluids levels of sIL-1RII have been detected in diverse pathological conditions. Increased blood levels of sIL-1RII have been detected in experimental endotoxemia, sepsis, acute meningococcal infection and in patients treated with IL-2, aspirin, or GCs. Overexpression of IL-1RII has been detected in the basal compartment of psoriatic skin, in the context of an imbalance of the IL-1 system. Glomerular overexpression of IL-1RII was associated with the therapeutic activity of IL-4 in experimental glomerulonephritis.

Joint inflammation has been extensively investigated and could represent a target for IL-1RII-based therapeutic strategies. sIL-1RII was present in inflammatory synovial fluids. Gene array analysis of osteoarthritis lesions showed a lack of expression of IL-1RII and IL-1ra. Overexpression of IL-1RII in chondrocytes protected them from IL-1 stimulation. Moreover, transfer of cells transfected with IL-1RII that overexpress and release this molecule resulted in inhibition of collagen arthritis. These results, as well as the binding properties of IL-1RII (high for IL-1, low for IL-1ra; see above), suggest that this molecule may be an ideal therapeutic tool to inhibit IL-1.

## IV. CELLULAR SOURCES AND PRODUCTION

Cells of the monocyte–macrophage lineage are the main cellular source of IL-1, though most cell types have the potential to express this cytokine. In the absence of *in vitro* or *in vivo* stimulation, the IL-1 genes are not expressed. Diverse inducers, including bacterial products (e.g., LPS), complement components, and cytokines (TNF, IFN-γ, granulocyte/macrophage colony-stimulating factor, and IL-1 itself), cause transcription (see above), which does not necessarily result in translation. For instance, adhesion causes an accumulation of IL-1 mRNA, which requires a triggering stimulus (minute amounts of LPS) for translation into protein. Similarly, gene expression profiling has recently revealed that chemokines induce IL-1β gene transcription, but that translation requires a second signal.

## V. BIOLOGICAL ACTIVITIES

### A. Spectrum of Action of IL-1

IL-1 affects a wide range of cells and organs. Its spectrum of action is similar to that of TNF and, to a lesser degree, of IL-6. Induction of secondary cytokines, including IL-6, colony-stimulating factors (CSFs), and chemokines, is involved in many of the *in vitro* and *in vivo* activities of IL-1. The vast phenomenological literature on the activities of IL-1 is concisely summarized here based on target organs/tissues, and the reader is referred to previous reviews for more detailed analyses.

### B. Hematopoietic Cells

IL-1 affects the hematopoietic system at various levels, from immature precursors to mature myelomonocytic and lymphoid elements. "Hemopoietin-1" activity was found to be mediated by IL-1α. IL-1 induces the production of CSFs in a variety of cell types including elements of the bone marrow stroma. It acts synergistically with hematopoietic growth factors at various stages of hematopoietic differentiation. IL-1 is active as an hematopoietic growth factor also *in vivo*: it stimulates production of CSFs, accelerates bone marrow recovery after cytotoxic chemotherapy or irradiation, and has radioprotective activity.

IL-1 acts on T and B lymphocytes. In particular, it co-stimulates T-cell proliferation in the classic co-stimulator assay. The AP-1 transcription complex in the promoter of IL-2 represents one molecular target for the co-stimulatory activity of IL-1. The LAF (lymphocyte activating factor) assay has been invaluable for the identification of IL-1, but the actual role of IL-1 co-stimulation in T-cell physiology has not been fully established. Recent data showing that IL-1ra favors the development of Th1-type responses may suggest a role for IL-1 in the generation of Th2-type responses.

IL-1 affects mature myelomonocytic elements. It induces cytokine production in monocytes, although it is a poor inducer in PMN (see above). It prolongs the *in vitro* survival of PMN by blocking apoptosis.

### C. Vascular Cells

IL-1 profoundly affects the function of vessel wall elements, in particular, that of endothelial cells. IL-1 activates endothelial cells in a pro-inflammatory, prothrombotic sense. IL-1 induces the production of tissue factor and platelet-activating factor, downmodulates the protein C-dependent anti-coagulation pathway, and induces the production of an inhibitor of thrombus dissolution (PAI-1). IL-1 induces gene expression-dependent production of vasodilatory mediators (nitric oxide, prostacyclin [PG]I2), expression of adhesion molecules, and production chemokines in cultures of endothelial cells. The concerted action of changes in rheology, adhesion, and chemotactic factors underlies leukocyte recruitment at sites of IL-1 production or injection.

### D. Neuroendocrine System

Infection and inflammation induce an elevation of blood corticosteroids (CSs) through activation of the so-called hypothalamus–pituitary–adrenal axis (HPAA), very similar to that observed with stress. This is the result of a central action whereby IL-1 stimulates the release of corticotropin-releasing hormone (CRH) by the hypothalamus, which induces ACTH production by the pituitary, ultimately causing a release of CSs in the bloodstream by the adrenals. This release in inhibited by anti-CRH antibodies. The increase in CSs resulting from HPAA activation by IL-1 may have several consequences in view of the wide range of immunosuppressive, anti-inflammatory, and metabolic actions of GC. It should be stressed here that CSs are (as mentioned in Section IV) potent inhibitors of the synthesis of IL-1 (and of other cytokines). They also prevent the hemodynamic shock associated with injection of IL-1, TNF, or LPS and protect against IL-1 or LPS toxicity. Therefore, activation of HPAA may be considered a feedback mechanism to control IL-1 production and toxicity. The fact that adrenalectomized animals are extremely susceptible to IL-1 toxicity strongly supports this hypothesis.

## E. The Acute-Phase Response

IL-1 is a key mediator of the series of host responses to infection and inflammation known as the acute-phase response. One of the early acronyms for IL-1 before it was named under the current terminology was LEM (leukocytic endogenous mediator). This was originally identified as a major mediator of the acute-phase response, particularly hypoferremia and induction of acute-phase proteins (APPs).

Hypoferremia seems to be mediated by an effect on neutrophils, which would be stimulated to release lactoferrin to sequester iron in the tissues. It could constitute a “nutritional immunity” against infection since iron is essential for the growth of many bacteria.

The synthesis of APPs is increased during inflammation. APPs include C-reactive protein, serum amyloid component, fibrinogen, hemopexin, and various proteinase inhibitors; these molecules may have protective, anti-toxic, and other functions yet to be defined. The synthesis of some of these proteins (e.g., serum amyloid A) can be directly induced in hepatocytes by IL-1. Other proteins, such as fibrinogen, are induced indirectly through IL-6. Therefore, both IL-1 and IL-6 (like other cytokines) behave like hepatocyte-stimulating factors (cytokines that stimulate liver APP synthesis).

The increased synthesis of APPs is part of a rearrangement of liver metabolism in which the synthesis of “normal liver proteins” is decreased; one such negative acute-phase reactant is albumin, whose gene expression is decreased by IL-1.

## F. Central Nervous System

IL-1 is the main endogenous pyrogen. In 1943, Menkins suggested that leukocytes release a pyrogenic substance, “pyrexin,” that was subsequently detected in the circulation of febrile rabbits. Human leukocytic pyrogen was purified in 1977 and an immunoassay to determine its presence was developed. It is now clear that the main endogenous pyrogen was IL-1 and that recombinant IL-1 induces fever in experimental animals, an activity shared with other cytokines including TNF and IL-6 (although these are much less potent than IL-1). The pyrogenic action of IL-1 is due to the increased production of prostaglandin (PG). In fact, IL-1 fever is abolished by inhibitors of PG synthesis, and IL-1 directly stimulates prostaglandin E2 release by hypothalamic tissue.

In addition to fever, IL-1 has other effects on the CNS. These include induction of slow-wave sleep and anorexia, typically associated with infections. IL-1 also activates the hypothalamus to produce CRH.

## G. Other Effects

It is almost impossible to list all the activities of IL-1. Since it became available as a recombinant protein, a large number of papers have been published reporting very disparate effects of this cytokine. IL-1 has a number of local effects that have been termed “catabolic” and it plays a role in destructive joint and bone diseases. In particular, IL-1 induces the production of collagenase by synovial cells and of metalloproteinases by chondrocytes.

IL-1 also stimulates fibroblast proliferation and collagen synthesis and thus plays a role in fibrosis. It induces a profound hypotension, an effect that is inhibited by a cyclooxygenase inhibitor and in which IL-1 and TNF act synergistically.

Another important action of IL-1 is its toxicity for insulin-producing beta cells in Langerhans islets, supporting a role for IL-1 in the pathogenesis of insulin-dependent type 1 diabetes.

## H. Gene-Modified Mice

The development of transgenic or knockout mice for components of the IL-1 system has provided new evidence as to the *in vivo* function of these molecules. IL-1β −/− mice show a reduced acute-phase response to local irritants, whereas the responses to systemic LPS are normal. Gene targeting and overproduction have revealed a critical role for endogenous IL-1ra in animal growth, responses to infection and inflammation, and cytokine regulation. Selective overexpression of IL-1β and the type IR and type II decoy R in skin resulted in pathology consistent with the concept that IL-1 is important in skin pathophysiology.

# VI. CLINICAL IMPLICATIONS

IL-1 is the central mediator of local and systemic inflammatory reactions. IL-1 levels in the blood reach relatively modest levels in response to septic conditions, but IL-1ra increases to levels that are orders of magnitude higher. This may represent a feedback control mechanism. Most of the interest generated for IL-1 is for its pathogenic role in septic shock syndrome and rheumatoid arthritis. However, *in vitro* studies, animal models, and results of studies reporting levels of IL-1 in human diseases have indicated other pathologies in which the blockade of IL-1 might be beneficial. These include vasculitis, disseminated intravascular coagulation, osteoporosis, neurodegenerative disorders such as Alzheimer's disease, diabetes, lupus nephritis, immune complex glomerulonephritis, and autoimmune diseases in general. Inhibition of IL-1 by IL-1Ra, anti-IL-1, or anti-IL-1-R antibodies is protective in various animal models including endotoxin-induced hemodynamic shock and lethality, arthritis, inflammatory bowel disease, spontaneous diabetes in BB strain rats, graft-versus-host disease in mice, heart allograft rejection, and experimental autoimmune encephalomyelitis.

Since TNF is produced concomitantly and IL-1 synergizes with TNF (but not with IL-6) in many systems, it is likely that these two cytokines act in concert in the pathogenesis of these disorders.

IL-1 is a autocrine/paracrine growth factor for acute myeloid leukemia (AML) cells, chronic myeloid leukemia cells, plasmacytoma (via IL-6), and possibly some solid tumors such as ovarian carcinoma. Interestingly, AML blasts usually express IL-1β, but not IL-1ra. Blocking IL-1 using IL-1ra inhibits AML proliferation *in vitro*.

The exploration of the therapeutic potential of IL-1 has provided an opportunity to examine the *in vivo* activity of systemic IL-1 administration in humans. Phase I trials have been conducted with IL-1α and IL-1β in studies ultimately aimed at exploiting the hematopoietic/radioprotective action of these molecules. By and large, the results obtained confirm data from experiments in animals. Systemic (intravenous) IL-1 (1–10 ng/ml) causes fever, sleepiness, anorexia, myalgia, arthralgia, and headache. At doses of 100 ng/ml or higher, a rapid fall in blood pressure occurs.

Therapeutic strategies aimed at blocking IL-1 have received considerable attention in experimental models and in humans. Results obtained with IL-1ra in graft-versus-host disease and in rheumatoid arthritis are encouraging. The IL-1 decoy RII is a logical candidate for blocking the action of IL-1. Preclinical results (e.g., in rheumatoid arthritis in the mouse) are encouraging and this compound is currently undergoing clinical evaluation.

## Glossary

**chemokines** Chemotactic cytokines, with the typical chemokine structure, that interact with seven-transmembrane-domain, G-protein-coupled receptors.

**cytokines** Soluble non-antigen-specific polypeptides that act as communication signals among diverse cell types.

**decoy receptor** A receptor that interacts with the ligand with high affinity and specificity but that is unable to signal or to be part of signaling receptor complexes. Decoy receptors act as a sink for the ligand and, in some cases, as dominant negatives for signaling receptors.

**interleukins** A group of cytokines, with no features that distinguish them from other cytokines.

## See Also the Following Articles

**Decidualization • Erythropoietin, Biochemistry of • Interleukin-2, Interleukin-4, etc. (multiple Interleukin entries) • Tumor Necrosis Factor (TNF)**

## Further Reading

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# Interleukin-2

Kendall A. Smith
*Weill Medical College of Cornell University*

I. INTRODUCTION
II. STRUCTURES
III. FUNCTIONS
IV. IL-2 REGULATION OF THE IMMUNE RESPONSE
V. IL-2 THERAPY
VI. IMMUNOSUPPRESSIVE THERAPY
VII. SUMMARY

**Interleukin-2, originally termed T-cell growth factor, was the first cytokine to be isolated, purified, and characterized at the molecular level. Interleukin-2 is a small, globular glycoprotein composed of four amphipathic antiparallel α-helices; it is prototypic of the interleukins as well as the hematopoietic cytokines and**

**TABLE 1 IL-10 Signaling in Different Cell Types**

| Cell type | Stats | Interaction with other pathways | Regulation | Effect on the cell |
|---|---|---|---|---|
| Monocyte | Stat1, Stat3 | Inhibits IKK activity and NFkB DNA binding; activates PI3K and p70 S6K that may be involved in proliferation; inhibits p38 MAPK to modulate ARE dependent TNF mRNA translation | Inhibited by IL-1 TNFα, LPS signaling, SOCS1, and SOCS3 | Inhibits proliferation, production of inflammatory cytokines, IFN-induced gene expression by inhibiting Stat1 phosphorylation, p19$^{INK4D}$ expression, CD40-mediated activation of ERK1/2, induction of IL-IR, and COX-2 expression; up-regulates expression of CD95 and CD95L, thus promoting apoptosis; up-regulates FcγRI and SOCS-3 |
| Dendritic cell | Stat1, Stat3 | Inhibits activation of SAPK/JNK, p38$^{MAPK}$, and ERK2 | Antagonized by TNFα and CD40 ligand | Represses TNF-induced changes and modulates CCR expression |
| B cell | Stat1, Stat3, Stat5 | Not described | Not described | Induces differentiation and proliferation; expression of CD32/16, CD2, LECAM-1, heat-stable antigen, and c-fos; and Bcl-2 expression; prevents apoptosis |
| T cell | Stat1, Stat3 | Inhibits Cd28 tyrosine phosphorylation and PI3K recruitment | Not described | Enhances TGF-βR expression on activated T cells, prevents release of T$_H$1 cytokines, blocks proliferation, enhances Bcl-2 expression, and prevents apoptosis |
| Neutrophil | Stat1, Stat3 | Not described | SOCS proteins | Inhibits mobilization of specific granules to membrane and suppresses production of superoxide |
| NK cell | Stat1, Stat3 | Not described | Not described | Enhances cytotoxicity and proliferation |
| Mast cell | Stat1, Stat3 | Not described | Not described | Inhibits kit expression and reduces TNF induction |
| Melanoma cell | — | Not described | Not described | Induces proliferation and down-regulates MHC class I and II complex expression |
| Oligodendrocyte | — | Not described | Not described | Inhibits iNOS expression and promotes survival |

TABLE 2 Cellular Function of IL-10

| Cell type | Function: Immunosuppressive, anti-inflammatory | Function: Stimulation, proliferation |
|---|---|---|
| T helper ($T_H$) | Decreased cytokine synthesis (IFNγ); anergy, immune deviation, generation of suppressor-type cells; inhibits $T_H1$ | Stimulates $T_H2$ |
| T cell | — | Stimulates maturation and proliferation |
| Thymocyte | — | Proliferation |
| Natural killer | Decreased cytokine synthesis (IFNγ) | Increased proliferation, increased cytotoxicity |
| Macrophage, monocyte | Decreased antigen-presenting cell function, cytokine synthesis (IL-1α, IL-1β, IL-6, IL-8, IL-12, VEGF, MMP-9, TNFα, GM-CSF, NO, $H_2O_2$), and receptor expression (CD80, CD86, MHC II, IL-1RI, IL-1RII) | — |
| Dendritic cell | Decreased cytokine synthesis, receptor expression (CD80, CD86, MHC II, MHC I), and APC function | — |
| Vascular endothelial cell | Decreased receptor expression (ICAM-1) | Increased receptor expression (CD62E) |
| B cell | T-Dependent responses | T-Independent responses, isotype switching, growth and differentiation, and increased MHC II |
| Mast cell | — | Stimulates proliferation |
| Neutrophil | Decreased cytokine secretion | — |
| Oligodendrocyte | Decreased cytokine synthesis | — |

## II. GENE AND PROTEIN STRUCTURE

IL-10 is produced by activated T cells, B cells, monocytes/macrophages, mast cells, and keratinocytes. Human IL-10 (hIL-10) is encoded by a single 3.5-kb exon; the mouse IL-10 (mIL-10) gene contains five exons that span 5.1 kb of the genomic DNA, and both genes are located on chromosome 1. Detailed analysis of the mIL-10 gene has revealed that there are possible transcriptional control sequences present in noncoding regions of the mIL-10 genomic sequence. The hIL-10 and mIL-10 cDNA sequences have 80% homology and encode peptides of 178 amino acids, including hydrophobic leader

TABLE 3 Pathophysiologic Function of IL-10

| Disease state | Function[a]: Anti-inflammatory immunosuppression | Function[a]: Proinflammatory immunostimulation |
|---|---|---|
| Cytokine syndrome | + | |
| Endotoxemia | + | |
| Rheumatoid arthritis | + | |
| Thyroiditis | + | |
| Collagen arthritis | + | |
| Herpetic keratitis | + | |
| Insulin-dependent diabetes mellitus | + | + |
| Experimental autoimmune encephalomyelitis | + | + |
| Tumor immunity | + (vIL-10) | + (cIL-10) |
| Allograft immunity | + (vIL-10) | + (cIL-10) |
| Graft-versus-host disease | | + |
| Colitis | + | |
| Systemic lupus erythematosus | | + |
| Angiogenesis | + | |

[a] vIL-10, Viral interleukin-10; cIL-10, cellular interleukin-10.

sequences. Both proteins exist in solution as noncovalent homodimers. hIL-10 is an 18-kDa nonglycosylated polypeptide; mIL-10 is heterogeneously N-glycosylated at a site near the N-terminus, resulting in a mixture of 17-, 19-, and 21-kDa species. A mIL-10 mutant lacking the N-linked site is active and recombinant mIL-10 expressed in *Escherichia coli* retains all known biological activities, thus glycosylation of mIL-10 is not required for biological activity.

The crystal structure reveals that human IL-10 is a tight, noncovalent homodimer and that each monomer contains two intrachain disulfide bonds, between residues 12 and 108 and between residues 62 and 114 (hIL-10 numbering). Each monomer consists of six α-helices containing specific residues (hIL-10 numbering): A (residues 18–41), B (residues 48–58), C (residues 60–82), D (residues 87–108), E (residues 118–131), and F (residues 133–159). Residues A–F tightly associate with the other monomer (A′, B′, C′, D′, E′, and F′) to form two interpenetrating domains. Each domain is composed of six α-helices (A, B, C, D, E′, and F′), four originating from one monomer (A, B, C, and D) and two from the other (E′and F′). The overall topology of the helices bears close resemblance to IFNγ. The two most notable differences between IL-10 and IFNγ dimers are the size and orientation of the domains. In hIL-10, the helix bundles are perpendicular to one another, whereas in IFNγ the domains are oriented at an angle of about 60°. Studies on the crystal structure of IL-10 bound to a soluble receptor (R) complex (IL-10R1) reveal that there are two primary sites for IL-10 and IL-10R interaction.

## III. VIRAL IL-10

Several viruses, including Epstein–Barr Virus (EBV), equine herpes virus type 2, orf virus (OV), and human cytomegalovirus, encode IL-10 homologues that have functions similar to those of cellular IL-10 (cIL-10). It is postulated that these viruses might take advantage of the IL-10-like products to suppress production of early immunoregulatory cytokines, leading to ineffective immune responses.

BCRF1 is the EBV homologue of IL-10. This viral IL-10 (vIL-10) exhibits a high degree of DNA and amino acid sequence homology to both mIL-10 and hIL-10. Viral IL-10 is expressed in the late phase of the lytic cycle of EBV and has 85% amino acid identity with hIL-10 and 71% homology with hIL-10 at the DNA sequence level. vIL-10 and cIL-10 have identical immunosuppressive properties, inhibiting IFNγ production, major histocompatability complex (MHC) class II expression, T-cell proliferation, and B-cell IgE production, further reinforcing the notion that IL-10 is an immunosuppressive cytokine.

Viral IL-10 lacks several of the immunostimulatory activities of cellular IL-10 on certain cells. vIL-10 neither effectively enhances class II MHC expression on mouse B cells nor costimulates mouse thymocyte or mast cell proliferation. *In vivo*, it has been shown that cIL-10 can be immunosuppressive or immunostimulatory, whereas vIL-10 is mostly immunosuppressive. Thus, experiments in which mouse tumor cells were infected with recombinant retroviruses expressing mIL-10 or vIL-10 cDNAs show that mIL-10 accelerates and vIL-10 impedes tumor rejection. Experiments also show that rejection of mouse heart allografts is substantially inhibited when the grafts are induced to express vIL-10 but not mIL-10. These observations suggest that vIL-10 has conserved only a subset of the activities of cIL-10, perhaps as a pathogenetic mechanism for successful viral infection. These data also suggest the existence at least two functional domains, only one of which has been conserved by EBV.

The most significant difference in the amino acid sequence of viral and cellular IL-10 is found at the N-terminus. The crystal structure of vIL-10 shows that its structure is similar to that of human IL-10, forming an intercalated dimer of two 17-kDa polypeptides. Viral IL-10 exhibits novel conformations of the N-terminal coil and of the loop between helices A and B compared to hIL-10. It has been suggested that these differences might account for the different activities of vIL-10 and cellular IL-10. Viral IL-10 binds to the IL-10R at least 1000-fold less avidly than does cIL-10, but, like cIL-10, requires the presence of both IL-10R1 and IL-10R2 to induce signaling and immune responses. Mutagenesis of cIL-10 and vIL-10 reveals that the single amino acid isoleucine at position 87 of cIL-10 is required for its immunostimulatory function. Substitution in cIL-10 of isoleucine with alanine, which corresponds to the vIL-10 residue, abrogates immunostimulatory activity for thymocytes, mast cells, and alloantigenic responses but preserves immunosuppressive activity for inhibition of IFNγ production and prolongation of cardiac allograft survival. Conversely, substitution in vIL-10 of alanine with isoleucine converts vIL-10 to a cIL-10-like molecule with immunostimulatory activity. The mechanism of this switch is mostly due to changes in receptor binding affinity.

Human cytomegalovirus encodes a viral IL-10 homologue (cmvIL-10) that has only 27% identity with hIL-10 at the protein level. cmvIL-10 is able to bind to the human IL-10 receptor and to compete with human IL-10 for binding sites, and requires both subunits of the IL-10 receptor complex to induce signaling and biological function. A new cellular homologue of interleukin-10, AK155, has been identified by transformation of T lymphocytes with *Herpesvirus saimiri*. AK155 protein shows 24.7% amino acid identity and 47% amino acid similarity to hIL-10 and has been mapped to the human chromosome 12q15. The biological function of this homologue is not known. There is also a new molecule, IL-22, that is structurally related to IL-10 and it shares the same IL-10R2 with IL-10. Little is known about the biological activities of IL-22, and it has been described to up-regulate acute-phase reactant production by hepatocytes.

A monomeric form of human IL-10 has been engineered by inserting six amino acids (GGGSGG; G is glycine and S is serine, using the one-letter code) into the D–E loop, connecting the swapped secondary structural elements between Asn-116 and Lys-117. The monomeric form (IL-10M1) is stable under native conditions and is structurally similar to one domain of IL-10. IL-10M1 binds to soluble IL-10R1 (sIL-10R1) with a 1:1 stoichiometry and is biologically active.

## IV. IL-10 EXPRESSION

IL-10 is expressed in a variety of cells, including T cells, B cells, macrophages/monocytes, NK cells, keratinocytes, eosinophils, mesangial cells, epithelial cells, and tumor cells. Activated $T_H0$-, $T_H1$-, and $T_H2$-like CD4 + T cells, as well as CD8 + T cells, all express IL-10. CD4 + CD45RA + cells produce low levels of IL-10, whereas CD4 + CD45RA − "memory" cells produce higher levels of IL-10. IL-10 has been implicated in the induction of regulatory or suppressor $T_H$ cell subsets and it also appears to contribute to their effector function.

IL-10 production is induced by many pathogens, which can directly activate monocytes/macrophages [bacterial cell wall components, parasites, fungi, human immunodeficiency virus (HIV)], B cells (EBV), and T cells (human T cell leukemia virus type 1). In the course of severe infections and stress conditions, cytokines, hormones, and arachidonic acid derivatives are released and up-regulate IL-10 synthesis in monocytes, macrophages, and T cells.

Elevated IL-10 expression is associated with human diseases such as septicemia, bacterial meningitis, malaria, rheumatoid arthritis, lepromatous leprosy, visceral leishmaniasis, and lymphatic filariasis. IL-10 protein is also detected in many tumors, such as those in Burkitt's lymphoma, AIDS lymphoma, non-Hodgkin lymphoma, multiple myeloma, melanoma, ovarian cancer, and other intra-abdominal cancers. IL-10 production is regulated by other cytokines. IL-4, IL-13, and IFNγ inhibit IL-10 production in monocytes activated by lipopolysaccharide (LPS). IL-10 also strongly inhibits IL-10 mRNA synthesis, suggesting that IL-10 has autoregulatory activities. In contrast, IL-1, IL-2, IL-3, IL-6, IL-7, IL-12, and IL-15 induce IL-10 production in monocytes, T cells, NK cells, B cells, and mast cells. Tumor necrosis factor α (TNFα) induces IL-10 production in monocytes and mouse liver. IFNα induces IL-10 production in human T cells and monocytes. Transforming growth factor-β (TGF-β) induces IL-10 production in macrophages, mesangial cells, and liver cell lines.

## V. IL-10 IN DISEASE STATES

### A. Inflammation

IL-10 has protective effects in experimental endotoxemia and rescues mice from LPS-induced toxic shock, which is correlated with reduced levels of serum TNFα. IL-10 inhibits the production of TNFα and macrophage inflammatory protein-2 (MIP-2); regulates hemodynamic parameters, leukocyte–endothelial cell interactions, and microvascular permeability; and reduces mortality in experimental endotoxemia. Mice treated with anti-IL-10 from birth or IL-10-deficient mice are more susceptible to endotoxin-induced shock than are normal mice. Human volunteers receiving IL-10 after endotoxin challenge suffer fewer systemic symptoms and less cytokine production.

### B. Inflammatory Bowel Disease

IL-10-deficient and IL-10R2-deficient mice develop chronic intestinal inflammatory disorders and are successfully treated by administration of IL-10, indicating the role of IL-10 in the development of inflammatory bowel disease (IBD) and the effect of IL-10 in the treatment of IBD. Transfer of CD4 + $CD45RB^{high}$ T cells (which produce IFNγ and TNFα) into severe combined immune-deficient (SCID) mice causes IBD, and administration of CD4 + $CD45RB^{low}$ T cells (which produce IL-4 and IL-10) or mIL-10 prevents the development of IBD. CD4 + $CD45RB^{high}$ T cells isolated from IL-10 transgenic mice do not induce IBD but inhibit IBD induced by wild-type CD4 + CD45RB$^{high}$ T cells. Furthermore, intraperitoneal injection of adenovirus, producing IL-10, successfully prevents experimental colitis in rats, and oral treatment with mIL-10 reduces colitis in mice and prevents colitis in IL-10-deficient mice.

A double-blind, randomized, multicenter trial in humans has been conducted to evaluate the safety, tolerance, and pharmacokinetics of IL-10 in the treatment of Crohn's disease. The results indicate that intravenous bolus injection of IL-10 over 7 days is safe, well tolerated, and results in clinical improvement. Another trial has shown that subcutaneous administration of recombinant human IL-10 (rhIL-10) for 28 days to patients with mild to moderately active Crohn's disease is safe, well tolerated, and clinically efficacious; 23.5% of patients receiving 5 μg/kg of rhIL-10 achieve clinical remission and endoscopic improvement at the end of treatment.

## C. Experimental Autoimmune Encephalomyelitis

The effects of IL-10 in the treatment of experimental autoimmune encephalomyelitis (EAE) are controversial, and various reports have yielded conflicting results. Initial studies show that IL-10 mRNA in the spinal cord dramatically rises with the recovery from EAE, and intracerebral synthesis of IL-10 is also increased during the recovery phase of EAE. IL-10-deficient mice develop more severe EAE and do not achieve spontaneous recovery, suggesting an important role of IL-10 in the disease. IL-10 prevents the induction of EAE in Lewis rats and SJL mice on systemic administration during the initial phase of the disease. IL-10 decreases the relapse rate when given with TNFα, and neutralization of IL-10 increases the incidence and the severity of the relapse. Adenovirus-mediated IL-10 gene transfer inhibits development and prevents subsequent relapse of EAE. Murine IL-10 transgenic mice are protected from EAE induced by proteolipid protein (PLP) immunization. Human IL-10 transgenic mice are highly resistant to EAE; this is mediated by suppression of autoreactive T-cell function. In contrast, intravenous injection of IL-10 exacerbates the disease in an adoptive transfer model of EAE. Intracranial injection of IL-10 or IL-10 plasmid does not inhibit EAE. Adoptive transfer of a myelin basic protein (MBP)-specific T-cell hybridoma transduced with IL-10 also does not suppress EAE. Hence, the role of IL-10 in EAE may be related to the route and timing of cytokine administration.

## D. Insulin-Dependent Diabetes Mellitus

Systemic administration of IL-10 to adult nonobese diabetic (NOD) mice delays the onset of diabetes, indicating the potential therapeutic effect of IL-10 in autoimmune diabetes. Daily subcutaneous administration of IL-10 to 9- and 10-week-old NOD mice delays the onset and decreases the incidence of diabetes. Combined therapy with IL-4 and IL-10 suppresses $T_H1$ cytokine production in the islet grafts and inhibits insulin-dependent diabetes mellitus (IDDM) recurrence in syngeneic islet-transplanted NOD mice. IL-10/Fc protein, a long-lived noncytolytic fusion protein, completely prevents the occurrence of diabetes in 5- to 25-week-old NOD mice and the mice remain disease free for a long time after cessation of IL-10/Fc therapy. Intramuscular plasmid injection provides long-term systemic delivery of cytokines. When an IL-10 expression plasmid is injected into the skeletal muscles of NOD mice, the incidence of diabetes is significantly reduced. Intravenous injection of IL-10 plasmid also prevents autoimmune insulitis in NOD mice.

In contrast, transgenic expression of IL-10 on beta cells accelerates the development of diabetes in NOD mice, suggesting a proinflammatory effect of IL-10 in the pathogenesis of autoimmune diabetes. In IL-10 transgenic NOD mice, the incidence of diabetes in the first and second generations is greatly increased, the onset of diabetes is much earlier than in the nontransgenic NOD mice, and histological analysis shows severe insulitis and prominent ductal proliferation. IL-10 transgenic NOD mice back-crossed with NOD.B6 Idd3 Idd10 mice, which have diabetes-resistance alleles at *Idd3* and *Idd10* on chromosome 3 and have a very low frequency of diabetes, results in IL-10 transgenic back-cross mice that develop diabetes. As for EAE, the role of IL-10 in the pathogenesis of autoimmune diabetes is not clear at this time. Its effects may depend on timing, location, and dose of cytokine, along with contributions from other regulatory events.

## E. Angiogenesis

IL-10 down-regulates the production of macrophage-derived angiogenic factors such as vascular endothelial growth factor (VEGF), IL-1β, TNFα, IL-6, and matrix metalloproteinase-9 (MMP-9), inhibits angiogenesis, and hence inhibits tumor growth and metastasis. IL-10 stimulates tissue inhibitor of metalloproteinase-1 (TIMP-1) production, inhibits MMP-2 and MMP-9 production, inhibits angiogenesis in human prostate cancer cells *in vitro*, and significantly increases mouse survival after implantation with IL-10- and TIMP-1-expressing tumors *in vivo*. Transferring the IL-10 gene into Burkitt's lymphoma cells significantly reduces tumor formation in SCID mice, with IL-10 inhibiting VEGF-induced neovascularization and proliferation of microvascular endothelial cells. Local expression of vIL-10 in thrombosed veins decreases thrombosis-associated inflammation and inhibits the expression of cell adhesion molecules such as P- and E-selectin.

The role of IL-10 in angiogenesis has also been studied in a hindlimb ischemia model in IL-$10^{-/-}$ and IL-$10^{+/+}$ mice. In IL-$10^{-/-}$ mice, angiogenesis in the ischemic hindlimb is greatly increased and VEGF expression is also increased. After transferring IL-10 cDNA into IL-$10^{-/-}$ mice, angiogenesis and VEGF expression are significantly inhibited, suggesting an effect of IL-10 on angiogenesis via down-regulation of VEGF expression. Further experiments show that MMP-2 and MMP-9 are increased in the ischemic hindlimb in IL-$10^{-/-}$ mice, and the MMP inhibitor BB-94 abolishes the increase in vessel density and the blood perfusion index, suggesting a role for MMP in angiogenesis.

## F. Transplant Rejection

IL-10 prolongs allograft survival in nonvascularized and vascularized transplantation in mice and rats. Adenovirus-mediated IL-10 gene transfer reduces allograft rejection in sheep corneal transplants. Cationic lipid-mediated hIL-10 gene transfer prolongs survival of allogeneic hepatocytes in rats. Intracoronary infusion of an adenoviral vector expressing IL-10 efficiently prolongs graft survival in rabbits. Injection of rhIL-10 down-regulates cytokine-induced neutrophil chemoattractant and prolongs liver allograft survival in rat orthotopic liver transplantation. Intraportal injection of an adenoviral vector expressing IL-10 significantly prolongs survival in orthotopic liver transplantation. IL-10/Fc fusion protein inhibits macrophage-mediated immune responses and prolongs pancreatic islet xenograft survival.

In contrast, infusion of IL-10 in murine recipients of allogeneic bone marrow grafts accelerates graft rejection and increases graft-versus-host disease (GVHD)-induced mortality. mIL-10 inhibits the proliferation of donor spleen cells in the mixed leukocyte reaction (MLR) assay, but cannot reduce morbidity and mortality from GVHD. Postoperative intraperitoneal administration of mIL-10 simulates B-cell and T-cell alloimmune responses and exacerbates allograft rejection in heterotopic vascularized heart transplantation in mice. Subconjunctival or intraperitoneal administration of mIL-10 does not prolong corneal allograft survival and even accelerates rejection.

Viral IL-10 encoded by Epstein–Barr virus BCRF1 open reading frame has the immunosuppressive activity of cellular IL-10 but lacks the immunostimulatory activity and is a more potent immunosuppressant. Plasmid-mediated or adenoviral-mediated vIL-10 gene transfer prolongs allograft survival in nonvascularized cardiac transplantation. Retroviral-mediated vIL-10 gene transfer also prolongs allograft survival, whereas murine IL-10 gene transfer does not prolong graft survival. Similar results are also observed in tumor rejection: vIL-10 inhibits the process of immune rejection in tumors and vIL-10-transduced tumors still grow, whereas mIL-10 causes tumor rejection and inhibits tumor growth. Hence, vIL-10 may act as a more effective therapeutic agent in transplant rejection.

## G. Regulatory T Cells

It is currently believed that various types of T regulatory cells (Tr cells) are critical for the inhibition of T-cell responses to antigens and play an important role in immunological tolerance. Several types of Tr cells have been characterized in different experimental models, including Tr1 cells, CD4 + CD25 + cells, $T_H3$ cells, and others. IL-10 has been related to the differentiation and function of some Tr cells, especially Tr1 cells and CD4 + CD25 + cells.

Tr1 cells result from T-cell priming in the presence of IL-10 and IFNγ and produce large amounts of IL-10 and TGF-β, moderate levels of IFNγ and IL-5, and little or no IL-2 and IL-4. Tr1 cells exert regulatory effects via the production of IL-10 and TGF-β and down-regulate both $T_H1$ and $T_H2$ immune responses. The suppressive effects of Tr1 cells are reversed by the addition of anti-IL-10 and/or anti-TGF-β mAb.

CD4 + CD25 + cells comprise about 5–10% of peripheral T cells in naive mice and humans, constitutively express the α-chain of IL-2-receptor, and exert immune regulatory functions in a variety of immune assays. CD4 + CD25 + cells express IL-10 mRNA and secrete IL-10 protein, whereas CD4 + CD25 − cells do not express IL-10 mRNA, even after CD3 stimulation. Some reports have shown that the role of CD4 + CD25 + cells in the control of autoimmune diseases and inflammatory responses is dependent on IL-10. For example, the production of IL-10 is essential for CD4+ CD25+ cells to prevent colitis in immunodeficient mice; IL-$10^{-/-}$ mice develop colitis in a conventional environment and Tr cells from IL-$10^{-/-}$ mice are not effective in preventing inflammation. However, the role of IL-10 in the inhibitory effect of CD4+ CD25+ cells remains controversial; other reports show that neutralization of IL-10 or IL-10R has no effect on the suppressive effect of CD4+ CD25+ cells.

## VI. IL-10 SIGNALING

### A. IL-10 Receptor Structure

The IL-10 receptor is a heterodimer composed of subunits known as IL-10 receptor 1 and IL-10 receptor 2); IL-10R2 was formerly known as orphan receptor CRF2-4. The human IL-10R1 gene has been mapped to chromosome 11q23.3. Human IL-10R1 messenger RNA is around 3.6 kb long and encodes a polypeptide of approximately 110 kDa. The predicted amino acid sequences of murine and human IL-10R1s are 60% identical and 73% similar. Although the human IL-10 receptor is species specific, murine IL-10 receptor binds both human and murine IL-10 with comparable affinity. The $K_d$ of binding is in the range of 50–200 p*M*.

Sequence analysis and predicted structure revealed that IL-10 receptor is related to interferon receptors. The polypeptide was shown to be 578 amino acids long, with a putative signal peptide sequence of 21 amino acids, a 215-amino-acid extracellular domain, a transmembrane segment of 25 amino acids, and a cytoplasmic domain of 317 amino acids. Although the calculated molecular mass is approximately 61 kDa, the observed size is around 110 kDa, suggesting glycosylation on at least one extracellular site of the six identified.

Structurally, the extracellular portion of mIL-10R consists of two homologous segments of approximately 110 amino acids. A soluble form of the extracellular domain of hIL-10R1, sIL-10R1, has been cloned and expressed. The soluble receptor is capable of binding hIL-10 and inhibiting its biological activity. From the analysis of this binding, the stoichiometry of the ligand–IL-10R1 interaction has been determined. Crystallographic analysis of the sIL-10R1/IL-10 interaction reveals a complex consisting of two IL-10 dimers and four soluble IL-10R1s. The stoichiometry of the vIL-10:hIL-10R complex has been determined to be the same. Although the domains of sIL-10R1 are structurally similar to IFNγR1, the interdomain angle in sIL-10R1 is around 90°, which is more similar to cytokine class I receptors, rather than the 120° observed for cytokine class II receptors. The topology of each domain is closely related to FBN-III modules that form a sandwich from two antiparallel β-sheets.

### B. Receptor Signaling

Functionally important regions of the intracellular domain of the murine IL-10R have been identified using deletion and substitution mutagenesis approaches. Two tyrosine residues (Tyr-427 and Tyr-477) have been found to be important in recruitment and activation of the signal transducer and activator of transcription protein Stat3, but not Stat1 or Stat5. Tyr-427 and Tyr-477 in the murine receptor correspond to Tyr-446 and Tyr-496 in their human counterpart. The sequences surrounding each of the conserved tyrosines have also been found to be highly conserved between murine and human receptors.

The first chain of the IL-10R is capable of binding the ligand on its own and possesses Stat recruitment sites, but it also requires a second chain for initiating a signaling pathway. The second chain of the receptor was originally identified as an orphan cytokine receptor (CRF2-4). The gene was mapped to chromosome 16 in mice and chromosome 21 in humans. The human gene spans more than 30 kb and encodes a 325-amino-acid protein that is 69% identical with its 349-amino-acid murine counterpart. The encoded protein is a transmembrane protein with a 19-amino-acid leader, an extracellular portion of 201 amino acids, a 29-amino-acid transmembrane domain, and a 76-amino-acid intracellular portion. The relatively close location of its gene to the IFNαR gene (less than 35 kb away) and its homology in structure to the interferon receptors suggest that the receptor protein belongs to the interferon class II cytokine receptor family and possibly plays a role in interferon signaling. Unlike IL-10R1, CRF2-4 is constitutively expressed in most cells and tissues and has not been found to be regulated by any stimuli. Interestingly, it has been shown that CRF2-4 also functions as a second chain of the receptor for IL-22, an IL-10 homologue. The importance of CRF2-4 receptor has been further demonstrated in CRF2-4 knockout mice. These mice are developmentally normal but they do not respond to IL-10 and develop chronic colitis and splenomegaly, similar to the IL-10 knockout models. The mice showed normal responses to interferon, suggesting that CRF2-4 is not obligatory for interferon signaling.

## C. Jak–Stat Activation and Transcriptional Regulation

Stimulation of T cells and monocytes with IL-10 leads to assembly of the IL-10 receptor complex consisting of the ligand dimer, two IL-10R1 chains, and two IL-10R2 chains, which results in induced proximity between their respective associated Janus kinase (Jak) family members. IL-10R1 has been shown to associate with Jak1, whereas IL-10R2 is associated with Tyk2. Receptor assembly leads to tyrosine phosphorylation of Janus kinase family members Jak1 and Tyk2, but not Jak2 or Jak3. On assembly of the receptor complex, Janus kinases transphosphorylate each other and the intracellular domain of the IL-10R1, leading to recruitment of Stats, predominantly Stat3. Electrophoretic mobility-shift assays revealed that in all cell populations expressing IL-10 receptor, IL-10 stimulation results in activation of Stat1 and Stat3, whereas in pro-B cells expressing IL-10 receptor, Stat5 is also activated.

## D. SOCS Regulation

IL-10 is an efficient stimulus for suppressor of cytokine signaling (SOCS-3)/CIS3 induction in neutrophils and monocytes. Incubation of monocytes with IL-10 results in inhibition of gene expression for several IFN-induced gene products, such as IP-10, ISG54, and ICAM-1. This correlates with suppression of IFNα-induced assembly of Stat factors to specific promoter motifs (ISRE and GAS elements) on IFNα- and γ-inducible genes and inhibition of tyrosine phosphorylation of Stat1. Because vanadate, a protein tyrosine phosphatase (PTP) inhibitor, had no effect on inhibition of Stat1 phosphorylation, the effect induced by IL-10 is probably not through SHP1 or other PTPs. Induction of SOCS proteins may therefore be involved in inhibition of interferon signaling. IL-10 has been found to suppress IFNα-activated Stat1 in the liver, possibly through induction of SOCS-2, SOCS-3, and CIS expression.

The IL-10 pathway may also be regulated by other pathways. For example, in addition to Stat3 tyrosine phosphorylation, phosphorylation of Stat3 at Ser-727 plays a role in regulation of transcription. Active MEK kinase 1 (MEKK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates a c-Jun $NH_2$-terminal kinase signaling pathway and is induced by growth factors, cytokines, and environmental stress. MEKK1 carries out phosphorylation of Stat3 at Ser-727 *in vitro*, whereas *in vivo*, MEKK1 activates Src and Jak kinases, which phosphorylate Stat3 at Tyr-705. MEKK1 thus may be involved in modulation of IL-10 signaling by growth factors and environmental stress.

## E. Downstream Genes

Signaling through the IL-10 receptor activates a number of genes that mediate both anti-inflammatory and proliferative effects induced by IL-10. Activated/memory T cells become refractory to TGF-β-mediated inhibition of proliferation through the down-regulation of the TGF-βRII. TGF-βR expression and inhibitory function can be restored on activated/memory T cells by addition of IL-10. IL-10 has been shown to down-regulate the expression of MHC class I and II complexes and ICAM-1, thus inhibiting human leukocyte antigen (HLA)-dependent antigen presentation and T-cell activation. In monocytes, IL-10 has also been shown to inhibit proliferation directly by up-regulating expression of $p19^{ink4D}$ and $p21^{cip}$. The $p19^{ink4D}$ promoter has been determined to have two Stat3 binding sites. Blocking Stat3 signaling with dominant negative Stat3 or mutant IL-10 receptor prevents induction of $p19^{ink4D}$, but it has no effect on $p21^{cip}$ expression, suggesting that $p21^{cip}$ is regulated through other signaling mechanisms. The antiproliferative effect of IL-10 on macrophages is also exerted through up-regulation of the CD95 receptor and ligand on the monocyte cell surface, leading to apoptosis, and through down-regulation of c-kit protooncogene expression, a protein tyrosine kinase that plays a role in hematopoiesis. In T cells, IL-10 has been shown to induce anergy through inhibition of tyrosine phosphorylation of CD28, thus preventing recruitment of phosphatidylinositol 3-kinase (PI3K) to the receptor.

The anti-inflammatory effects of IL-10 are mediated by down-regulation of production of pro-inflammatory cytokines, and inhibition of cellular responses to mediators of inflammation. In monocytes, IL-10 inhibits the production of IL-1 and TNFα and down-regulates the expression of IL-1RI and IL-1RII. In addition, IL-10 inhibits cyclooxygenase 2 (COX-2) expression in LPS-stimulated monocytes and accelerates degradation of COX-2 mRNA, thus controlling the inflammatory response at yet another level.

## Glossary

**IL-10R** Interleukin-10 receptor complex, composed of the ligand-binding IL-10R1 and the signal-transducing IL-10R2 chains.

**Jak–Janus kinase** Signaling molecules that bind to the cytoplasmic region and are involved in IL-10R signal transduction.

**SOCS** Suppressor of cytokine synthesis; inhibitory molecules that bind to receptor docking sites, preventing Stat activation and inhibiting signal transduction.

**Stat** Kinase signaling molecules downstream of Jak; involved in IL-10R signal transduction and activation of transcription.

## See Also the Following Articles

**Angiogenesis • Diabetes Type 1 • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

## Further Reading

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# Interleukin-12 Family Members

Michael T. Lotze and Pawel Kalinski
*University of Pittsburgh*

I. INTRODUCTION
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**Interleukin-12 was originally identified as a T-cell-stimulating factor promoting T-cell activation, as a natural killer-stimulating factor enhancing cytolytic activity and interferon-γ production, and as a cytotoxic lymphocyte maturation factor promoting the acquisition of T-cell effector function with interleukin-2.**

## I. INTRODUCTION

Cloning of both of the subunits of interleukin-12 (IL-12), the p35 and p40 chains in both mouse and human, revealed the close homology of IL-12 to the IL-6 family of cytokines and receptors as well as the extended hematopoietin family. Recent identification

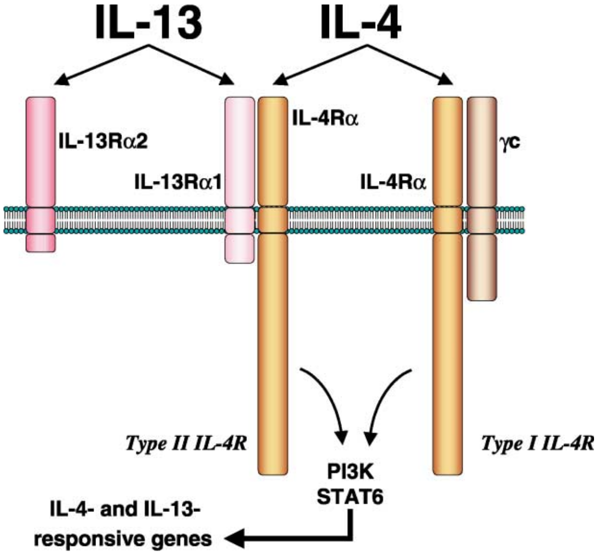

FIGURE 1 IL-13 and IL-4 receptor complexes. IL-13 binds to two different proteins, IL-13Rα1 and IL-13Rα2. IL-13 signaling requires interaction with a heterodimeric complex consisting of IL-4Rα and IL-13Rα1 chains, which is called type II IL-4R since it is also used by IL-4. IL-4 also signals through the type I IL-4R consisting of IL-4Rα and γc chains. The IL-13Rα2, despite having high affinity for IL-13, does not induce the activation of IL-13-responsive genes and acts as a decoy receptor.

and has potent anti-inflammatory effects *in vitro* and *in vivo*. Finally, IL-13 can promote the growth of certain tumors *in vitro* and *in vivo* either directly or indirectly by down-regulating tumor immunosurveillance mechanisms.

## II. IL-13 PROTEIN AND IL-13 GENE

Human IL-13 is a cytokine with a molecular mass of ~12 kDa. Two functionally similar IL-13 isoforms consisting of 131 or 132 amino acids have been identified. Mouse IL-13 contains 131 amino acids and has 58% identity with its human homologue. IL-13 contains four potential N-glycosylation sites on four cysteine residues, which pair to form two intramolecular disulfide bonds. The genes encoding human or mouse IL-13 are located on the long arm of chromosome 5q31–q1 or the syntenic region of mouse chromosome 11, respectively, in the same 160 kb cluster of genes encoding IL-4 and IL-5. The IL-13 gene is only 12 kb upstream of the IL-4 gene and both genes together with the IL-5 gene are coordinately regulated in T helper 2 ($T_H2$) cells.

Polymorphisms in the coding and promoter regions of the IL-13 gene have been identified and are associated with asthma and atopic dermatitis in families belonging to different ethnic groups in Asia, Europe, and the United States. IL-13 protein has only approximately 25% homology with IL-4, but has many structural and functional properties in common with IL-4, which are determined by shared receptor components expressed on various cell types.

## III. IL-13 RECEPTOR COMPLEXES

There are two human IL-13-binding proteins: IL-13Rα1 and IL-13Rα2 (Fig. 1). The IL-13Rα1 protein binds IL-13 with low affinity ($K_d \sim 4$ n*M*) but does not bind IL-4. IL-13Rα1 associates with the IL-4Rα chain (CD124) to form a high-affinity receptor that binds IL-13 with a $K_d$ of 30 p*M*. The IL-4Rα chain alone binds IL-4 with relatively high affinity ($K_d \sim 100$ p*M*) but does not bind IL-13. These IL-4Rα/IL-13Rα1 heterodimeric complexes are expressed on many cell types, with a high level of expression in heart, liver, ovary, and skeletal muscle. IL-4Rα/IL-13Rα1 complexes are also expressed on human B cells, human and mouse monocytes/macrophages, basophils, eosinophils, mast cells, endothelial cells, fibroblasts, smooth muscle cells, and airway epithelial cells. In contrast, mouse and human T lymphocytes and mouse B lymphocytes do not express functional IL-13R. Furthermore, IL-4Rα/IL-13Rα1 complexes are also expressed on keratinocytes, ciliated respiratory epithelial cells, heart muscle cells, faveola cells, hepatocytes, gastric glands, sebaceous glands, and sweat glands.

The human IL-13Rα1 chain has 76% homology with mouse IL-13Rα1 at the protein level. The IL-4Rα/IL-13Rα1 complex can also serve as a functional IL-4R (type II IL-4R). This has been demonstrated with T and B cells from X-linked severe combined immunodeficiency patients who have defective common γ chains (γc). The γc is shared with IL-2, IL-4, IL-7, IL-9, and IL-15R and is required for signaling through these receptors. As a consequence, the lymphocytes of these patients do not respond to IL-2, IL-7, IL-9, or IL-15, which causes the severe defects in T- and B-cell functions observed in these patients. However, their B cells respond to IL-4 using the type II IL-4R, indicating that both IL-4 and IL-13 can signal through the IL-4Rα/IL-13Rα1 complex in the absence of a functional γc. This is consistent with the observation that IL-4 and IL-13 signal transduction in the absence of γc occurs in macrophages from γc-deficient mice.

The second IL-13R, IL-13Rα2, has 27% homology with the IL-13Rα1 chain and binds IL-13 with high affinity ($K_d \sim 50$ p$M$) in the absence of IL-4Rα. The genes encoding IL-13Rα1 and IL-13Rα2 are both located on the X chromosome. Human and mouse IL-13Rα2 proteins have 59% homology. The IL-13Rα2 has a very short intracytoplasmic tail, which lacks the domains required for signaling. This is probably the reason that binding of IL-13 to the IL-13Rα2 chain does not result in cell signaling. IL-13Rα2 is thought to function as a decoy receptor for IL-13. The IL-13Rα2 is expressed on various carcinomas including glioblastomas, ovarian carcinoma, renal carcinoma, pancreatic carcinoma, and acquired immunodeficiency syndrome-associated Kaposi's sarcoma and on fibroblast cell lines. In addition, soluble IL-13Rα2 can be detected in mouse serum. The IL-13Rα2 chain is not expressed at detectable levels on cells of the immune system and normal endothelium.

## IV. IL-13 SIGNAL TRANSDUCTION

In hematopoietic cells, binding of IL-13 to the IL-13Rα1 chain results in its dimerization with the IL-4Rα chain and the activation of the Janus kinases JAK1 and tyrosine kinase 2. Subsequent tyrosine phosphorylation of the IL-4Rα chain and the 170 kDa insulin receptor substrate-2 (IL-4-induced phosphotyrosine substrate) provides a docking site for the Src homology 2 domain-containing 85 kDa subunit of phosphatidylinositol 3-kinase in lymphoid cells. This results in the recruitment, phosphorylation, dimerization, and nuclear translocation of signal transducer and activator of transcription 6 (STAT6) and the activation of IL-4-/IL-13-responsive genes in various cells expressing IL-4Rα/IL-13Rα1 chain complexes. IL-13-induced phosphorylation of the IL-4Rα chain and activation of STAT6 are also observed in nonhematopoietic cells such as endothelial cells, fibroblasts, and colon and ovarian carcinoma cells. The requirement for STAT6 in IL-13-mediated signaling was confirmed in STAT6-deficient mice that failed to respond to IL-13. In keratinocytes and lung fibroblasts, IL-13 can also induce the phosphorylation of STAT3.

## V. IL-13 PRODUCTION

IL-13 is produced by many different cell types, including T cells, natural killer (NK) cells, NK-T cells, basophils, mast cells, eosinophils, and dendritic cells following activation. In mouse T cells, IL-13 production is restricted to the CD4$^+$ T$_H$2 and CD8$^+$ T cytotoxic 2 subset. In contrast, IL-13 is produced at relatively high levels by human T$_H$2 cells and also by T$_H$0 cells, T$_H$1 cells, CD8$^+$ T cells, and naive CD45RA$^+$ T cells, albeit at lower levels. Since IL-13 is produced by all single IL-4-producing cells, IL-13 is considered primarily a T$_H$2 cytokine.

IL-13 production by T cells is further enhanced by IL-4, IL-18, and IL-25. However, IL-4 is not absolutely required for IL-13 production, because IL-4-deficient mice are able to produce IL-13. Production of IL-13 by activated mast cells and basophils following cross-linking of the high-affinity receptor for Immunoglobulin E (FcϵRI) likely contributes to the initiation and maintenance of allergic inflammatory responses. IL-13 is also produced by Epstein-Barr virus-transformed B cells, B-cell lymphomas, keratinocytes, Hodgkin/Reed-Sternberg cells, and pancreatic carcinoma cells.

## VI. IMMUNOSTIMULATORY AND PRO-INFLAMMATORY EFFECTS OF IL-13 *IN VITRO*

Like all other cytokines, IL-13 has pleiotropic effects on many different cell types including B cells, monocytes/macrophages, dendritic cells, eosinophils, endothelial cells, synoviocytes, epithelial cells, fibroblasts, smooth muscle cells, and various tumor cells. However, IL-13 does not act on mouse or human T cells. In contrast to IL-4, IL-13 does not support T-cell proliferation and fails to drive the differentiation of naive human CD4$^+$ T cells toward a T$_H$2 phenotype, which is consistent with the absence of functional IL-13R on these cells.

IL-13 modulates the phenotype of human B cells by up-regulating CD23, CD71, CD72, CD80, CD86, major histocompatibility complex (MHC) class II molecules, and surface immunoglobulin M (IgM). IL-13 has also growth-promoting effects on human B cells stimulated by anti-IgM or anti-D40 monoclonal antibodies (mAbs). IL-13 enhances the production of IgM, IgG, and IgA by human B cells stimulated with anti-CD40 mAbs or CD40 ligand and induces these B cells to switch to IgG4 and IgE synthesis independently of IL-4. Allergen-specific IgE binds to FcϵRI on mast cells and basophils. Binding of allergen to receptor-bound IgE results in FcϵRI triggering and activation of these cells, leading to the release of mediators of allergic inflammation such as histamines, prostaglandins, leukotrienes, and pro-inflammatory cytokines. These data indicate that IL-13 may play a role in IgE-mediated allergic diseases (Fig. 2).

IL-13 modulates the phenotype of both human and murine monocytes/macrophages. It up-regulates the expression of various adhesion molecules on

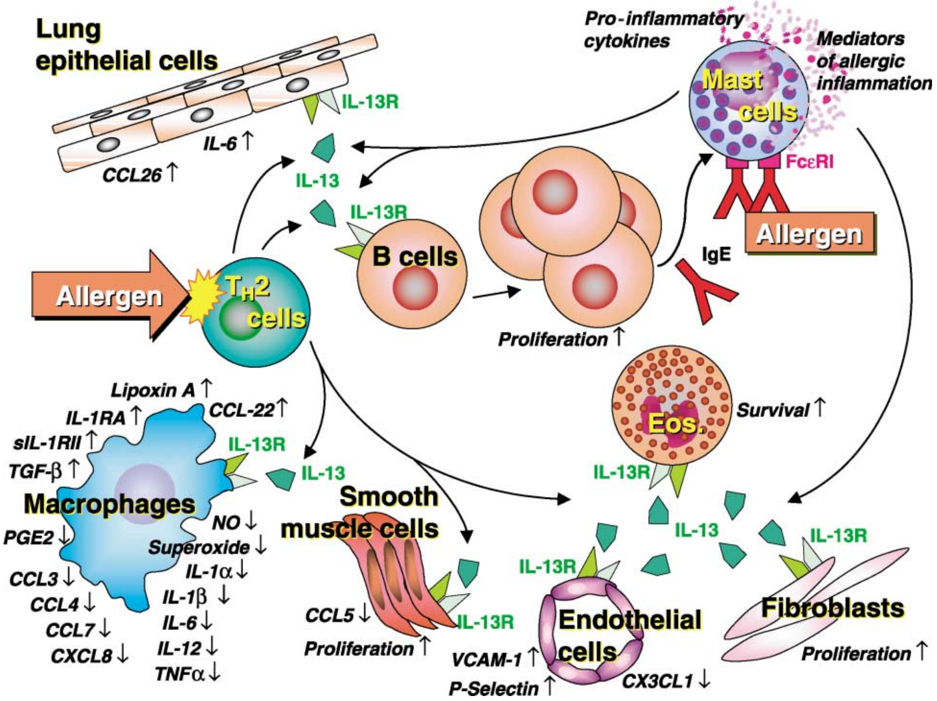
Lung epithelial cells
IL-13R
IL-6 ↑
CCL26 ↑
Pro-inflammatory cytokines
Mediators of allergic inflammation
Mast cells
FcεRI
Allergen
IL-13
IL-13R
B cells
IgE
Allergen
TH2 cells
Proliferation ↑
Lipoxin A ↑
IL-1RA ↑
sIL-1RII ↑
TGF-β ↑
CCL-22 ↑
IL-13R
IL-13
Macrophages
PGE2 ↓
NO ↓
Superoxide ↓
IL-1α ↓
IL-1β ↓
IL-6 ↓
IL-12 ↓
TNFα ↓
CCL3 ↓
CCL4 ↓
CCL7 ↓
CXCL8 ↓
Eos.
Survival ↑
IL-13R
Smooth muscle cells
IL-13
IL-13R
IL-13R
CCL5 ↓
IL-13R
Proliferation ↑
Endothelial cells
Fibroblasts
VCAM-1 ↑
P-Selectin ↑
CX3CL1 ↓
Proliferation ↑

FIGURE 2 Effects of $T_H2$ cell-secreted IL-13 on various target cells. IL-13, produced by $T_H2$ cells following specific stimulation (i.e., with allergens), has stimulatory/pro-inflammatory effects on B cells, eosinophils, lung fibroblasts, lung epithelial cells, vascular endothelial cells, lung smooth muscle cells, mast cells, and basophils (not shown). Allergen-activated mast cells (and basophils) release mediators of allergic inflammation, including IL-13, which further exacerbate $T_H2$ responses (i.e., allergic asthma). On the other hand, IL-13 has anti-inflammatory effects on activated monocyte/macrophages. IL-13-induced changes are indicated in the figure by arrows: up-regulation (↑); down-regulation (↓).

monocytes including CD11b, CD11c, CD18, CD29, and CD49e, suggesting that IL-13 contributes to enhanced extravasation and migration of these cells to sites of inflammation. In addition, IL-13 enhanced the expression of MHC class II molecules and the expression of CD80 and CD86, the ligands for CD28 on T cells, resulting in an enhanced T-cell-stimulating capacity. IL-13 induces the expression of the low-affinity receptor for IgE (CD23) on monocytes, which directs allergen complexes to allergen-specific T cells, thereby enhancing allergic responses *in vitro*. IL-13 enhances the production of the chemokine CCL22 (macrophage-derived chemokine) by monocytes and dendritic cells. Since CCL22 preferentially attracts skin homing $T_H$ cells, this effect of IL-13 may exacerbate cutaneous inflammatory $T_H2$ processes such as those found in atopic dermatitis. IL-13 directly acts as a chemoattractant for human monocytes. It also enhances the chemotaxis of these cells through up-regulation of the chemokine receptors CXCR1 and CXCR2. Up-regulation of the leukotriene D4 (LTD4) receptor by IL-13 results in enhanced chemotactic responses toward LTD4, which is implicated in bronchoconstriction, mucus production, and chronic airway inflammation in asthma.

IL-13 induces vascular cellular adhesion molecule-1 (VCAM-1) and P-selectin expression on human umbilical vein endothelial cells, which induces adhesion and the subsequent extravasation of eosinophils, T lymphocytes, neutrophils, and monocytes. IL-13 also induces lung epithelial cells and nasal and dermal fibroblasts to produce CCL26 (eotaxin-3), which is a potent chemoattractant for eosinophils in the airways and skin. IL-13 also prolongs the survival of eosinophils directly or through up-regulation of granulocyte/monocyte colony-stimulating factor production by lung epithelial cells. Accumulation of eosinophils at sites of allergic inflammation plays an important role in the pathogenesis of asthma, emphasizing the role of IL-13 in this process. This notion is further supported by the observation that IL-13 inhibits the production of the chemokine CX3CL1 (Fractalkine) by activated vascular endothelial cells, which reduces the recruitment of NK cells and $T_H1$ cells and the amplification of polarized $T_H1$ responses, thereby favoring the development of $T_H2$-dominated allergic inflammatory responses.

IL-13 interacts directly with primary epithelial cells, fibroblasts, and smooth muscle cells isolated from human lung and induces profound changes of virtually nonoverlapping gene expression profiles in these three airway cell types. In addition, IL-13 promotes the proliferation of lung fibroblasts and lung smooth muscle cells. IL-13 also acts on human mast cells by inducing c-fos and up-regulating intercellular adhesion molecule-1 (ICAM-1) expression, contributing to the activation, enhanced adhesion, and extravasation of these cells. The production of the pro-inflammatory cytokine IL-6 by human microglial and epithelial cells is enhanced by IL-13 (Fig. 2).

## VII. IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY EFFECTS OF IL-13 *IN VITRO*

In addition to the immunostimulatory and pro-inflammatory activities associated with allergic inflammatory disorders, IL-13 has important immunosuppressive and anti-inflammatory activities. IL-13 inhibits the production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and tumor necrosis factor α (TNFα), the pro-inflammatory chemokines CXCL8 (IL-8), CCL3 (macrophage inflammatory protein 1α, MIP-1α), CCL4 (MIP-1β), and CCL7 (monocyte chemoattractant protein 3), and the chemokine receptor CXCR4 by activated monocytes, alveolar macrophages, and synovial macrophages from patients with rheumatoid arthritis. IL-13 also inhibits IL-12 production by activated monocytes, but priming of these monocytes prior to activation resulted in increased IL-12 production. In contrast, IL-13 enhances the production of the IL-1 receptor antagonist (IL-1RA) and the release of soluble type II IL-1R (sIL-1RII) from activated monocytes and neutrophils. Both IL-1RA and sIL-1RII have anti-inflammatory activities by functioning as IL-1 antagonists (Fig. 2).

The anti-inflammatory effects of IL-13 are further exemplified by its capacity to down-regulate either directly or indirectly various mediators of inflammation. For example, IL-13 induces the expression of 15-lipoxygenase, which catalyzes 15-(*S*)-hydroxyeicosatetraenoic acid and lipoxin A, both of which inhibit the production of pro-inflammatory leukotrienes. In addition, IL-13 inhibits the function of prostaglandin E2 from arachidonic acid through inhibition of cyclooxygenase induction by activated monocytes. IL-13 also inhibits nitric oxide and superoxide anion production by murine macrophages and activated human monocytes, respectively.

IL-13 down-regulates the expression of the Fcγ receptors CD16, CD32, and CD64 on human monocytes. Down-regulation of CD64 expression correlated with decreased antibody-dependent cellular cytotoxicity. IL-13 may also inhibit the production of CCL5 (regulated on activation of normal T cell expressed and secreted, RANTES) by airway smooth muscle cells, airway epithelia, and endothelial cells. CCL5 is a ligand for CCR3 and CCR5 expressed on $T_H2$ cells, eosinophils, and monocytes and is a potent chemoattractant for these cells.

## VIII. BIOLOGICAL EFFECTS OF IL-13 *IN VIVO*

Both immunostimulatory/pro-inflammatory and immunoprotective/anti-inflammatory effects of IL-13 have been observed *in vivo*.

### A. The Role of IL-13 in Asthma

It is well established that IL-13 levels are up-regulated in the lungs, blood, and bronchial alveolar lavage fluid of asthmatic patients. In addition, IL-13 mRNA and IL-13 protein expression levels are enhanced in bronchial biopsies from asthmatic patients. IL-13 levels are also up-regulated in allergic asthma patients following allergen challenge. These observations suggest that IL-13 is associated with human asthma. This notion is supported by several lines of evidence indicating that IL-13 plays a central role in experimental asthma models in mice.

Delivery of IL-13 into the airways causes airway hyperresponsiveness, mucus overproduction, eosinophilia, and airway inflammation, phenomena that reflect the hallmarks of asthma. IL-13 failed to induce this asthma phenotype in IL-4Rα- and STAT6-deficient mice, indicating that IL-13 signaling through the IL-4Rα/IL-13Rα1 complex and subsequently through STAT6 is necessary for the induction of allergic inflammation.

Transgenic mice in which IL-13 is selectively expressed in the lungs developed a comparable asthma phenotype with pulmonary inflammation, infiltrating $T_H2$ lymphocytes, eosinophils, and macrophages, airway epithelial cell hypertrophy, goblet cell hyperplasia, and enhanced mucus production. In addition, airway fibrosis, airway hyperresponsiveness, and high levels of eotaxin production are observed in these mice. Enhanced eotaxin production together with IL-5 produced by the infiltrating $T_H2$ cells is responsible for the airway eosinophilia.

The IL-13-induced airway and peribronchial fibrosis (which is comparable to fibrotic responses observed in remodeled asthmatic airways in human) is in large part mediated through transforming growth factor-β1 (TGF-β1). IL-13 overexpressed in the lungs induces TGF-β1 production predominantly by macrophages. In addition, IL-13 converts latent TGF-β1 to its active form *in vivo*. The pro-fibrotic effects of TGF-β1 were considerably reduced by treatment with soluble TGF-βR, demonstrating that IL-13-induced lung fibrosis is for the most part indirectly mediated by TGF-β1.

IL-13 overexpression in the lungs of STAT6-deficient mice did not result in any pulmonary pathology, confirming that IL-13 signaling through STAT6 is required for the development of the asthma phenotype. Interestingly, reconstitution of STAT6 only in lung epithelial cells is sufficient to restore IL-13-induced airway hyperresponsiveness and mucus overproduction, indicating that the direct effects of IL-13 on lung epithelial cells are sufficient to induce these two hallmarks of asthma. However, restoration of alveolar inflammation and fibrosis was not observed in these mice. Furthermore, these data indicate that IL-13 induces airway hyperresponsiveness independent of inflammation, eosinophilia, subepithelial fibrosis, and airway smooth muscle layer thickening and that the effects of IL-13 on cells other than epithelial cells are required for the development of these pathological processes associated with experimental asthma.

Mice with allergen- or fungus-induced experimental asthma have enhanced levels of IL-13. Blockade of these enhanced endogenous levels of IL-13 by administration of soluble IL-13Rα2, which neutralizes specifically IL-13, and not IL-4, inhibits airway hyperreactivity, goblet cell hyperplasia, and mucus overproduction. IL-13 blockade also inhibits asthma in guinea pigs and dogs. Thus, IL-13 plays a critical role in inducing the asthma phenotype, and compared to IL-4, IL-13 is the dominant cytokine in inducing mucus hypersecretion and airway hyperresponsiveness through direct effects on lung epithelial cells.

In addition to its critical role in asthma, IL-13 expressed in an inducible way in adult murine lungs can cause emphysema, which mirrors human chronic obstructive pulmonary disease. This targeted expression of IL-13 in lung epithelial cells results in enhanced lung volumes and pulmonary compliance, mucus metaplasia, and macrophage-, lymphocyte-, and eosinophil-rich inflammation. Local overproduction of IL-13 induced the expression of matrix metalloproteases (MMPs)-2, -9, -13, and -14 and cathepsins B, S, L, H, and K in lung tissues. Treatment of these mice with a broad-spectrum MMP antagonist or cysteine protease inhibitors strongly reduced lung volume and inflammatory cell influx but had no effect on mucus metaplasia. These data indicate that MMP- and cathepsin-dependent pathways play an important role in IL-13-induced emphysema, but that IL-13, in this setting, utilizes different pathways for induction of airway mucus metaplasia.

## B. The Role of IL-13 in Intestinal Nematode Infections

IL-13 has protective effects on mice infected with the gastrointestinal nematodes *Nippostrongylus brasiliensis*, *Ticheris muris*, and *Trichinella spiralis* and induces the expulsion of these worms. IL-13 fails to expel nematodes in IL-4Rα chain- and STAT6-deficient mice, indicating that signaling through IL-4Rα/IL-13Rα1 and STAT6 is required for worm clearance. This signaling pathway is utilized by both IL-4 and IL-13, but studies using an IL-13 antagonist or IL-4- and IL-13-deficient mice have shown that IL-13 is the dominant cytokine for the expulsion of *N. brasiliensis*, whereas either IL-4 or IL-13 suffices to expel *T. spiralis*. Both IL-13 and IL-4 are necessary to clear *T. muris*. The mechanisms underlying nematode expulsion are complex and not fully understood and differ for various nematodes. They include IL-13-induced increased mucus production, enhanced intestinal smooth muscle cell contractility, and activation of mast cell-mediated effector mechanisms.

Although IL-13 provides protection from nematode infections, this protective immunity also causes inflammatory responses, which can be detrimental to the host and are particularly of clinical importance when they develop in the lungs or in the liver. The damaging effect of IL-13 in chronic infections has been illustrated in a mouse model for *Schistosoma mansoni* infection. Mice infected with this helminth develop lung and liver granulomas, which contain large numbers of infiltrating $T_H2$ cells and eosinophils. Treatment with an IL-13 antagonist results in a reduction in granuloma size and eosinophilia, whereas nearly complete inhibition is observed in IL-4-deficient mice, indicating that both IL-4 and IL-13 contribute to granuloma formation and eosinophilia. However, hepatic fibrosis developing in *S. mansoni*-infected mice is strongly blocked by an IL-13 antagonist, indicating that compared to IL-4, IL-13 is the dominant cytokine responsible for induction of fibrosis associated with chronic infectious diseases.

### C. Protective Effects of IL-13 in Autoimmune Diseases

IL-13 down-regulates inflammatory responses by activated monocytes and macrophages. IL-13 also inhibits the production of the pro-inflammatory cytokines IL-1β and TNFα by synoviocytes from patients with rheumatoid arthritis. IL-13 is also effective in the treatment of experimental arthritis *in vivo*. Injection of cells genetically engineered to produce IL-13 reduces the severity and the incidence of collagen-induced arthritis in mice. The beneficial effects of IL-13 in this disease model correlated with reduced TNFα production in the spleen. A similar approach had positive effects on experimental autoimmune encephalomyelitis (EAE). IL-13 reduced the mean duration, severity, and incidence of the disease. These protective effects were mediated by the anti-inflammatory activity of IL-13 on macrophages, which resulted in down-regulation of the disease causing $T_H1$ responses. IL-13 also inhibits TNFα-induced lung inflammation in guinea pigs and reduces acute lung inflammatory injury induced by intrapulmonary deposition of IgG immune complexes in rats by reduction of TNFα and CXCL2 (MIP-2α) production, ICAM-1 expression, and neutrophil recruitment in the lung. In addition, IL-13 administration reduced the intravascular leak of albumin in this model. Exogeneously administered recombinant IL-13 also increases survival in murine models of endotoxic shock by reducing excessive inflammation and circulating TNFα levels.

## IX. EFFECTS OF IL-13 ON TUMOR CELLS

IL-13 is an autocrine growth factor for cutaneous T-cell lymphomas and Hodgkin's lymphoma/Reed-Sternberg cells. Blocking IL-13 activity inhibits the proliferation of these cells *in vitro*. On the other hand, the growth of other types of tumor cells such as gliomas and renal cell carcinomas is inhibited both by IL-13 and IL-4 and requires signaling through the IL-4Rα/IL-13Rα1 chain. These *in vitro* growth inhibitory effects of IL-13 are overcome by transfection of the IL-13Rα2 chain in these cells, which acts as a nonsignaling decoy receptor. In contrast, growth of human breast or pancreas tumor cells expressing high levels of IL-13Rα2 following transfection in immune-deficient mice was strongly inhibited by neutrophil-mediated anti-tumor activity. Restoration of tumor growth was observed on loss of IL-13Rα2 expression. IL-13 has also indirect effects on tumor growth by modulating the immune response. IL-13 produced by NK-T cells inhibited immunosurveillance mechanisms that provided protection from tumor recurrence. Since certain tumors such as gliomas, renal cell carcinomas, and ovarian carcinomas express high levels of the IL-13Rα2 chain, which binds IL-13 with high affinity, the use of IL-13 as a vehicle to deliver cytotoxic compounds into tumors is being explored as a therapeutic modality for these types of cancers.

## X. SUMMARY

IL-13 plays an important role in the induction and maintenance of allergic inflammatory processes associated with experimental asthma. IL-13 induces airway hyperreponsiveness, goblet cell hyperplasia, enhanced mucus production, pulmonary inflammation with infiltrating $T_H2$ lymphocytes, macrophages, and eosinophils, enhanced IgE production, and, on a more chronic basis, airway fibrosis via signaling through the IL-4Rα/IL-3Rα1 complex (type II IL-4R) and the STAT6 pathway. Because IL-13 utilizes the type II IL-4R, it shares many (but not all) of its biological activities with IL-4. However, IL-13 also has clearly distinctive properties. IL-13 is, compared to IL-4, the dominant cytokine inducing airway hyperresponsiveness and mucus overproduction in allergen- or fungus-induced experimental asthma in mice. These effects of IL-13, which represent two of the hallmarks of asthma, are mediated by direct effects on lung epithelial cells. IL-13 is also the dominant cytokine in inducing lung fibrosis, which is largely mediated through the *in vivo* induction and activation of TGF-β. Another major difference between IL-13 and IL-4 is that IL-13, in contrast to IL-4, fails to induce naive human T cells to differentiate into $T_H2$ cells, which is related to the fact that human and mouse T cells do not express functional IL-13R. Since $T_H2$ responses are critical for the induction of allergic inflammation and asthma, IL-4 must be considered the dominant cytokine for the induction phase of asthma, whereas IL-13 plays a major role in the effector phase. Both IL-4 and IL-13 are probably required for the maintenance of allergic asthma, but the relative contribution of each of these cytokines in allergic disease and asthma in human remains to be established.

IL-13 also has anti-inflammatory effects *in vitro* and *in vivo*. It down-regulates pro-inflammatory cytokine and chemokine production by activated monocytes and macrophages *in vitro*. In addition, IL-13 has protective effects in models of rheumatoid arthritis, EAE, and acute lung inflammatory injury. Exogeneously administered IL-13 also increases survival in murine models of endotoxin-induced shock. The beneficial effects of IL-13 in these disease models are mediated through down-regulation of excessive inflammation and inhibition of *in vivo* TNF$\alpha$ production. In addition, IL-13 protects against gastrointestinal nematode infections; in particular, the expulsion of *N. brasiliensis* is solely dependent on IL-13. This protective immunity is associated with excessive inflammatory responses, which in a *S. mansoni* infection model in mice result in lung and liver granulomas and fibrosis, which are predominantly mediated by IL-13. Generally, the most significant function of IL-13 (which distinguishes it from IL-4) is its capacity to regulate the functions of epithelial cells, smooth muscle cells, and fibroblasts on mucosal surfaces. Finally, IL-13 can directly or indirectly affect tumor cell growth. It functions as an autocrine growth factor for T-cell lymphomas and Hodgkin/Reed-Sternberg cells *in vitro*. In addition, endogenously produced IL-13 down-regulates immunosurveillance, resulting in enhanced tumor growth and tumor recurrences in a mouse tumor model.

Based on the current knowledge, it is clear that inhibition of IL-13 would provide a novel way to intervene in allergic asthma, granuloma formation, fibrosis associated with chronic inflammations or nematode infections, and fibrotic diseases in general. In addition, anti-IL-13 therapy could have potential benefits in the treatment of T-cell and Hodgkin's lymphomas and in enhancing tumor immunosurveillance. The large-scale availability of specific IL-13 antagonists such as anti-IL-13 or anti-IL-4R$\alpha$ chain mAbs or soluble IL-13R$\alpha$2 for the treatment of human disease will in the near future allow final conclusions on the role of IL-13 in health and disease to be reached.

## Glossary

**airway hyperresponsiveness** Exaggerated bronchoconstrictor response to a wide variety of endogenous and exogenous stimuli. Airway hyperreactivity is generally assessed by inhalation challenge with metacholine or histamine.

**asthma** Chronic inflammatory disorder of the airways characterized by recurrent episodes of wheezing, breathlessness, chest tightness, and coughing in response to physical, chemical, or immunological stimuli. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness. Asthma is frequently associated with atopy.

**chemokines** Small proteins characterized by the presence of a four-cysteine motif in their primary structure and by their ability to promote chemotactic responses in leukocytes. Chemokines are divided into subclasses (CCL, CXCL, and CX3CL) on the basis of the spacing between their two conserved N-terminal cysteine residues (adjacent cysteines; cysteines separated by a single amino acid; and cysteines separated by three amino acids, respectively). XCL1 is an exceptional chemokine since it lacks two cysteines. Chemokines exert their functions by binding G-protein-coupled seven-transmembrane receptors.

**T helper 2 response** Reaction of the immune system to certain antigens such as allergens and infectious helminths that results in the generation of memory $CD4^+$ T-cell populations secreting high levels of the cytokines IL-4, IL-5, IL-10, and IL-13 but little or no IL-2 and interferon-$\gamma$. $T_H2$ cells are excellent helpers for B-cell antibody synthesis and promote allergic reactions.

## See Also the Following Articles

**CC, C, and $CX_3C$ Chemokines • CXC Chemokines • Glucocorticoids and Asthma • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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# Interleukin-15

Thomas A. Waldmann

*National Cancer Institute, National Institute of Health, Maryland*

I. INTRODUCTION
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**Interleukin 15 (IL-15) is a 14 to 15 kDa glycoprotein that is a member of the four-α-helix-bundle family of cytokines. There is widespread expression of IL-15 mRNA in a number of tissues including placenta, skeletal muscle, heart, lung, kidney, fibroblasts, and activated monocytes. IL-15 plays a role in natural killer cell differentiation, T-cell functions, and the host response to intracellular pathogens. Abnormalities of IL-15 expression have been reported in a variety of inflammatory, autoimmune, and neoplastic diseases.**

## I. INTRODUCTION

Interleukin 15 (IL-15) is a 14 to 15 kDa member of the four-α-helix bundle family of cytokines that is involved in natural killer (NK) cell differentiation, T-cell functions, and the host response to intracellular pathogens. In May 1994, two groups simultaneously described the cytokine, now known as IL-15, based on the ability of culture supernatants from two cell lines, CV-1/EBNA and the human T-cell lymphotropic virus I (HTLV-I)-associated HuT-102 cell line, to stimulate the proliferation of the cytokine-dependent murine T-cell line CTLL-2. As a part of an analysis of the cytokines produced by the retrovirus HTLV-I-associated adult T-cell leukemia cell line, Burton and co-workers reported purifying a NK and T-cell stimulatory cytokine. Simultaneously, the group of Grabstein and co-workers, by analysis of the cell line CV-1/EBNA, identified the same cytokine, IL-15. IL-15 expression is controlled at the levels of transcription, translation, and intracellular trafficking. In particular, in contrast to most cytokines, IL-15 is posttranscriptionally regulated by multiple controlling elements that impede its translation including 11–13 upstream AUGs of a long, >500-nucleotide $5'$-untranslated region ($5'$-UTR), two unusual signal peptides, and the C-terminus of the mature protein. IL-15 uses two distinct receptor and signaling pathways. In T and NK cells, the receptor for IL-15 includes IL-2Rβ and γc subunits, which are shared with IL-2, as well as an IL-15-specific receptor subunit, IL-15Rα. Mast cells respond to IL-15 with a distinct receptor system that does not share elements with the IL-2 receptor. IL-15 plays a pivotal role in the development, survival, and function of NK cells. Although IL-2 and IL-15 share two receptor subunits and many functions, especially in innate immunity, at times they provide distinct and contrasting contributions to T-cell-mediated immune responses. IL-2, through its pivotal role in activation-induced cell death (AICD), is involved in peripheral tolerance through the elimination of self-reactive T cells. In contrast, in general, IL-15 manifests anti-apoptotic actions and inhibits AICD and stimulates the persistence of memory phenotype CD8$^+$ T cells. Abnormalities of IL-15 expression have been reported in inflammatory, autoimmune, and neoplastic diseases. In particular, abnormally high levels of IL-15 transcription and translation are observed in HTLV-I-associated diseases such as the neurological disorder tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). Furthermore, abnormalities of IL-15 expression have been noted in patients with autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease. Therapeutic agents are being developed to target the receptor and signaling elements shared by IL-2 and IL-15 to provide effective treatment for such autoimmune disorders as well as the leukemia/lymphoma associated with the retrovirus HTLV-I.

## II. THE STRUCTURE AND GENOMIC ARCHITECTURE OF IL-15

IL-15 is a 14 to 15 kDa glycoprotein that is a member of the four-α-helix bundle cytokine family. The cDNA defining IL-15 encodes a 162 aa peptide with a 48 aa leader sequence yielding a 114 aa mature protein. Two disulfide cross-links are present at Cys-42–Cys-88 and Cys-35–Cys-85. The predicted folding topology of IL-15 suggests three loops connecting the four helices in an up-up-down-down configuration. The IL-15 gene maps to chromosome 4q31 (human) and to the central region of chromosome 8 (mouse). The IL-15 gene consists of nine exons (exons 1–8 and a newly discovered exon 4a). This exon–intron organization contrasts with the four-exon–three-intron architectural pattern observed with IL-2, IL-4, IL-5, and IL-13.

Human IL-15 mRNA contains a 5′-UTR of at least 500 nt, a mature protein coding sequence of 486 nt, and a 3′-UTR of at least 400 nt. There are two alternative leader peptides, one containing 48 aa and one with 21 aa. The classical long (48 aa) signal peptide that is associated with all secreted IL-15 proteins is encoded by exons 3, 4, and 5 of the human IL-15 gene. The short 21 aa signal peptide is encoded by exon 5 and by an additional 119 nt sequence inserted between exons 4 and 5 (new exon 4a). The two signal peptides share 11 amino acids encoded by exon 5. The introduction of the 119 nt of exon 4a disrupts the 48 aa signal sequence by inserting a premature termination codon and then provides an alternative initiation codon. IL-15 associated with the short 21 aa signal peptide is not secreted but rather is stored intracellularly, appearing in the cytoplasm and nucleus.

## III. THE REGULATION OF IL-15 EXPRESSION

### A. Regulation of Transcription

IL-2 and IL-15 exhibit major differences in terms of the levels of control of their synthesis and secretion and in terms of their sites of synthesis. IL-2 is produced by activated T cells and its expression is regulated predominantly at the levels of mRNA transcription and message stabilization. In contrast, there is widespread constitutive expression of IL-15 mRNA in a variety of tissues including placenta, skeletal muscle, kidney, lung, heart, fibroblasts, and activated monocytes.

The regulation of IL-15 expression is multifaceted. Modest control occurs at the level of transcription, whereas a dominant control occurs posttranscriptionally at the levels of translation and intercellular trafficking. In terms of transcriptional control, freshly isolated monocytes express a very low level of IL-15 mRNA that is up-regulated when the monocytes are activated with IFN-γ. In addition, infection of monocytes with herpes virus 6 or 7, *Bacillus Calmette-Guerin* (BCG), *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Mycobacterium leprae*, or *Candida albicans* is associated with an up-regulation of IL-15 mRNA expression. A series of conserved motifs between mouse and human IL-15 5′ regulatory regions, including the transcriptional motifs, nuclear factor κB (NF-κB), interferon regulatory factor-element (IRFE), myb, NF-IL-6, and interferon-α2 (IFN-α2), has been identified. It was demonstrated through the use of a series of reporter assays involving IL-15 promoter deletion mutants that the IRF-E motif is critical for the activation of the IL-15 promoter. Another transcription factor that appears to play an important role in IL-15 transcription is NF-κB acting on an NF-κB motif adjacent to the IRF-E motif.

### B. Posttranscriptional Regulation of IL-15 Synthesis

IL-15 is predominantly regulated posttranscriptionally at the levels of translation and intracellular trafficking. Although IL-15 mRNA is widely expressed constitutively, it has been difficult to demonstrate IL-15 within the cells or the supernatants of cells that express such IL-15 mRNA. There are multiple controlling elements that impede IL-15 mRNA translation. Initial studies focused on the 5′-UTR of IL-15 mRNA. In general, the 5′-UTRs of effectively translated messages are short, simple, and unencumbered by AUGs upstream of the initiation AUG. In contrast to this pattern, the 5′-UTR of the message encoding IL-15 is long and quite complex and includes multiple upstream AUGs (11 or 13 in different human isoforms of the UTR). Kozak emphasized that the presence of such AUGs in the 5′-UTR of mRNAs may drastically reduce the efficiency of their translation. Cells transfected with IL-15 constructs with the 5′-UTR deleted produced approximately 12- to 15-fold more IL-15 than cells transfected with the wild-type construct. Furthermore, using expression constructs that exchanged the signal peptide coding sequences and initiation ATGs of IL-2 with those of IL-15 linked to the alternative mature protein coding sequence, it was shown that the IL-15 long signal peptide was an important factor in the negative regulation of IL-15 expression. Finally, a third negative element was shown to exist in the C-terminus of the IL-15 mature coding sequence or protein. These multiple negative regulatory features controlling IL-15 expression may be required in light of the potency of IL-15 as an inflammatory cytokine that stimulates the expression of tumor necrosis factor α (TNFα), IL-1β, and inflammatory chemokines, which, if indiscriminately expressed, could lead to inflammatory autoimmune diseases. In terms of a more positive role for IL-15, by maintaining a pool of translationally inactive mRNA, cells may respond rapidly to an intracellular infection by transforming the IL-15 mRNA into a form that can be translated effectively, yielding secreted IL-15 that may activate T and NK cells, which could then aid in the host response to the invading pathogen.

### C. Intracellular Trafficking of IL-15

The two isoforms of IL-15 generated by usage of alternative signal peptides have different intracellular trafficking patterns. The isoform with the short (21 aa) putative signal peptide is not secreted but appears in nuclear and cytoplasmic compartments, suggesting a biological function for this isoform of IL-15 as an intracellular molecule. The IL-15 associated with the long 48 aa signal peptide is translocated into the endoplasmic reticulum (ER). On translocation, the 48 aa signal peptide may be completely processed or alternatively processed, leaving a residual 3′ element of the signal peptide linked to the mature coding sequence. This latter signal element contains a conserved retention sequence that impedes the trafficking of IL-15 from the ER to the Golgi. Thus, there are impediments to intracellular trafficking that in addition to the impediments to translation are involved in the negative regulation of the synthesis and secretion of IL-15.

## IV. IL-15 RECEPTOR AND SIGNAL TRANSDUCTION PATHWAYS

IL-15 uses two receptor and signaling pathways. The type I receptor expressed in T and NK cells is made up of three membrane components. Two of these components, IL-2β and γc, are shared with IL-2; the γc is also shared with IL-4, IL-7, IL-9, and IL-21. In addition, the two cytokines have their own unique cytokine-specific α chains, IL-15Rα for IL-15 and IL-2Rα for IL-2. The IL-15Rα chain, the unique chain of IL-15, is a type 1 membrane protein with a signal peptide of 32 aa, a 173 aa extracellular domain, a single membrane-spanning region of 21 aa, as well as alternative 37 to 41 aa and 21 aa cytoplasmic domains. In contrast to the IL-2Rβ and γc chains, IL-15Rα is not a member of the cytokine receptor superfamily. However, IL-2Rα and IL-15Rα may constitute their own family. IL-15Rα and IL-2Rα have a similar intron–exon organization. Moreover, they are closely linked in both the human (10p15–p14) and the murine genomes (chromosome 2). IL-15 combines with IL-15Rα with a $K_d$ (dissociation constant) of $10^{-11}$ M, a level 3 logs higher than that of IL-2 for IL-Rα. IL-2Rβ in association with γc is able to bind IL-15 at a lower affinity ($K_d$ of $10^{-9}$ M) and can induce an IL-15 signal in the absence of IL-15Rα. IL-15Rα has a wide cellular distribution and is expressed in T cells, B cells, monocytes, and thymic and bone marrow stromal cells, as well as in liver, heart, spleen, lungs, skeletal muscle, and activated endothelial cells. The heterotrimeric IL-15 receptor/-signaling pathway in activated T cells utilizes JAK3 and JAK1, as well as STAT-3 and STAT-5. IL-15 uses a distinct type 2 pathway in mast cells. Mast cells respond to IL-15 with a receptor system that does not share elements with the IL-2R but uses a novel 60 to 65 kDa IL-15 RX subunit. In mast cells, IL-15 signaling through this type 2 receptor involves JAK-2/STAT-5 activation rather than the JAK1/JAK3 and STATS-5/STAT-3 system used by IL-15 in activated T cells.

## V. BIOLOGICAL ACTIONS OF IL-15

### A. IL-15 Actions in the Development, Survival, and Function of NK Cells

IL-15 is important in the development, survival, and activation of NK cells. NK cells are virtually absent in knockout mice deficient in IL-15 or IL-15Rα. A similar defect in NK development is observed in mice deficient in interferon regulatory factor I (IRF-I), which is required for the interferon-induced transcription of IL-15 mRNA. Similarly, NK cells are deficient in mice lacking IL-2Rβ, γc, and JAK3, a series of elements that are required for IL-15 action. The role of IL-15 in NK development has been studied directly using *in vitro* stromal-independent cultures of hematopoietic precursors. IL-15 is very effective at inducing such bone marrow progenitors to differentiate into NK cells. A similar IL-15-mediated propagation of NK cells from their progenitors was observed in NK cell-deficient IRF$^{-/-}$ mice treated with IL-15. This latter observation supports the view that the defect in such mice is not in the NK cell progenitors themselves but in the induction of bone marrow stromal cell IL-15 synthesis.

IL-15 facilitates the survival of NK cells *ex vivo*, a phenomenon that is associated with the prevention of programmed cell death. This anti-apoptotic action requires B-cell CLL/lymphoma-2 (BCL-2) expression. In addition, IL-15 synergizes with IL-12 to stimulate the production by NK cells of IFN-γ, TNF-α, and granulocyte/macrophage colony-stimulating factor (GM-CSF). Furthermore, IL-15 stimulates the expression of a stimulatory receptor, NKG2D, involved in effective cytotoxic action that is expressed by activated NK cells.

### B. Distinct Functions of IL-15 and IL-2 in the Life and Death of Lymphocytes

As might be anticipated from their sharing of the IL-2Rβ and γc subunits in T and NK cells, IL-15 and IL-2 share a number of biological activities including the stimulation of the proliferation of activated CD4$^+$, CD8$^+$, and γδ subsets of T cells. IL-2 and IL-15 also facilitate the induction of cytolytic effector cells including cytotoxic T lymphocytes and lymphokine-activated killer cells. In addition, both IL-2 and IL-15 act as chemokinetic and chemoattractants for T cells. Finally, both cytokines induce the proliferation and synthesis of immunoglobulins (Ig) by human B cells co-stimulated with anti-IgM or the CD40 ligand.

Although IL-2 and IL-15 share many functions, at times they provide distinct and contrasting contributions to the life and death of lymphocytes. IL-2 through its pivotal role in activation-induced cell death (AICD) is active in peripheral tolerance involved in the elimination of self-reactive T cells. IL-15, in contrast, inhibits IL-2-mediated AICD. In addition to their distinct actions in AICD, IL-2 and IL-15 play opposing roles in the control of the homeostasis of CD8$^+$ memory phenotype T cells. IL-15 stimulates the development and persistence of such CD8$^+$ memory phenotype T cells, whereas IL-2 inhibits their survival. Taken together, the reported studies support the view that in terms of their special adaptive immune functions, IL-2 and IL-15 favor opposing actions that tend to emphasize one or the other of two competing goals of the immune response. IL-2, through its contribution to AICD for CD4$^+$ cells and its interference with the persistence of CD8$^+$ memory phenotype T cells, favors the elimination of lymphocytes that are directed toward self, thereby playing a crucial role in the maintenance of peripheral tolerance. In contrast, IL-15, through its inhibition of IL-2-mediated AICD and its positive role in the persistence of CD8$^+$ memory cells, favors the maintenance and survival of CD4$^+$ and CD8$^+$ T cells. Thus, IL-15 contributes to the maintenance of a specific immune response to foreign pathogens.

### C. Phenotype of IL-2, IL-2R, IL-15, and IL-15R Knockout Mice

An analysis of IL-2 and IL-2Rα as well as IL-15 and IL-15Rα knockout mice supports these contrasting roles for IL-2 and IL-15 in AICD and in the expression of memory CD8$^+$ cells. IL-2 and IL-2Rα gene-targeted mice manifest the massive enlargement of peripheral lymphoid organs, high levels of IgG1 and IgE, as well as autoimmune disorders, such as hemolytic anemia, inflammatory bowel disease, and infiltrative granulopoiesis, that are associated with impaired AICD. In contrast, IL-15 gene-targeted mice manifest no lymphoid organ enlargement, autoimmune disease, or impaired AICD. Rather, they manifest a marked reduction in the number of NK cells and memory phenotype CD8$^+$ cells. Such mice have a reduced capacity to mount a protective immune response to challenge with infectious pathogens.

## VI. ABNORMALITIES OF IL-15 EXPRESSION IN DISEASE

### A. Disorders of IL-15 in Autoimmune Diseases

Feldmann and co-workers proposed that TNF-α is at the apex of a cytokine cascade that includes IL-1β, IL-6, and GM-CSF, as well as a series of inflammatory chemokines that are ultimately involved in the development and progression of rheumatoid arthritis (RA). McInnes and co-workers reported abnormalities of IL-15 in this disease and suggested that IL-15 may precede TNF-α in this cytokine cascade. In murine models, the intra-articular injection of IL-15 induced a local tissue inflammatory infiltrate consisting predominantly of T lymphocytes. In support of the view that IL-15 plays a role in inflammatory arthritis, the injection of an IL-15 antagonist, the soluble form of IL-15Rα, led to the suppression of development of collagen-induced arthritis. Abnormalities of IL-15 were also reported in other inflammatory autoimmune disorders including ulcerative colitis, Crohn's disease, type C chronic liver disease, and sarcoidosis, as well as multiple sclerosis.

### B. Aberrant IL-15 Expression in Retroviral Diseases

Increased IL-15 expression has been observed in retroviral diseases and neoplasia. HTLV-I-infected T cells of patients with the neurological disorder TSP/HAM express the HTLV-1-encoded transactivator $p40^{tax}$. The expression of tax leads to the induction of IL-15 and IL-15Rα as well as IL-2 and IL-2Rα expression. The induction of IL-15 and IL-15R expression involves NF-κB and IRF-1 or IRF-4. The *ex vivo* proliferation of HTLV-1-infected T cells in TSP/HAM can be inhibited in part by an antibody to IL-15 or to IL-2 and can be virtually abrogated by the simultaneous administration of antibodies to both cytokines or to both cytokine receptors, suggesting that these two cytokines mediate autocrine/paracrine stimulatory systems as a consequence of HTLV-I infection. IL-15 also appears to play a role in the expression of antigen-specific major histocompatibility complex (MHC) class I-restricted memory phenotype $CD8^+$ cells that participate in the pathogenesis of TSP/HAM. Patients with TSP/HAM were shown using tetramer technology to have from 3 to over 20% of their $CD8^+$ cells as MHC class I-restricted, antigen-specific cells (directed to amino acids 11–19 of the tax protein transactivator). The number of such cells that persisted in *ex vivo* cultures of patient peripheral blood mononuclear cells for 6 days was decreased in the presence of antibodies to IL-15 or to its receptor. This observation is in accord with the view presented above that IL-15 plays a major role in the generation and persistence of antigen-specific $CD8^+$ memory and effector cells. An increased production of IL-15 by HTLV-I-associated T cells is also observed in adult T-cell leukemia (ATL), an aggressive leukemia of mature $CD4^+$ cells that is associated with HTLV-I. Taken together, the evidence supports the view that the retrovirus-induced IL-15 and its private receptor play meaningful roles in the pathogenesis and persistence of both autoimmune and leukemic disorders associated with HTLV-I infection.

## VII. THERAPY USING IL-15 OR DIRECTED TOWARD THE IL-15 RECEPTOR OR ITS SIGNAL TRANSDUCTION SYSTEM

The opposing effects of IL-2 and IL-15 on the life and death of lymphocytes have implications for their use in cancer therapy and as a component of vaccines. IL-2 has been approved for use in the treatment of metastatic renal cell carcinoma and malignant melanoma. However, in the presence of IL-2 due to IL-2-mediated AICD, the tumor-specific T cells generated may interpret the tumor cells as self and may not survive. Furthermore, the inhibition mediated by IL-2 on the survival of memory $CD8^+$ T cells directed toward the tumor is not desirable. In contrast, IL-15 with its activation of T cells, its inhibitory action on AICD, and its facilitation of the persistence of memory phenotype $CD8^+$ T cells may be superior to IL-2 in the treatment of cancer and as a component of vaccines.

The majority of therapeutic trials directed toward the IL-2/IL-2R or IL-15/IL-15R systems have focused on the IL-2 receptor α-subunit. Such IL-2Rα-directed therapeutic efforts have met with considerable success in the treatment of leukemia and select autoimmune disorders and in the prevention of allograft rejection. However, efforts targeting IL-2Rα have limitations. In particular, antibodies to IL-2Rα do not inhibit the actions of IL-15, a cytokine that does not bind to this subunit. Another limitation of IL-2Rα-directed therapy is that antibodies directed toward this receptor do not act on resting NK or NK-T cells that express IL-2Rβ and γc but not IL-2Rα. An additional limitation is suggested by our discussion above on the role of IL-2R in AICD where antibody-mediated inhibition of AICD may prevent the generation of peripheral tolerance to the transplantation antigens expressed on the allografts. Due to these limitations in IL-2Rα-directed therapy, IL-15 receptor-directed therapy is being developed for use in organ transplantation protocols and for application in the treatment of autoimmune disorders as well as diseases caused by the retrovirus HTLV-I. The administration of an IL-15 inhibitor, the soluble high-affinity IL-15Rα receptor chain linked to the immunoglobulin Fc element, prevented the development of murine collagen-induced arthritis and inhibited allograft rejection. Furthermore, an IL-15 receptor antagonist produced by mutation of a glutamine residue within the C-terminus of IL-15 to aspartic acid competitively inhibited IL-15-triggered cellular proliferation. The administration of this IL-15 mutant markedly attenuated antigen-specific delayed hypersensitivity responses in mice and enhanced the acceptance of pancreatic islet cell allografts. Additional IL-15-directed therapeutic approaches have focused on the IL-2Rβ receptor subunit shared by IL-2 and IL-15. A humanized version of HuMikβ1, an antibody directed toward IL-2Rβ that inhibits IL-15 action on T and NK cells, prolonged renal allograft survival in cynomolgus monkeys. In an initial clinical trial, the antibody Mikβ1 is being evaluated in the therapy of patients with T-cell-type large granular lymphocytic leukemia associated with hematocytopenia. The monoclonal large granular lymphocytes involved in this disease respond to IL-15 and express IL-2Rβ and γc but not IL-2Rα. In addition, this monoclonal antibody will soon be evaluated in the treatment of autoimmune diseases in which abnormalities of IL-15 have been demonstrated including rheumatoid arthritis, multiple sclerosis, and TSP/HAM. In conclusion, our emerging understanding of the IL-15/IL-15R system including the definition of the actions that this cytokine manifests that are shared with IL-2 as well as those that are distinct is opening new possibilities for the development of more rational IL-15/IL-15 receptor-directed immune interventions that may be of value in the prevention of allograft rejection, in the treatment of cancerand autoimmune diseases, as well as in the therapy of diseases associated with the retrovirus HTLV-I.

## Glossary

**activation induced cell death** A multistep process involved in peripheral tolerance that is initiated by TCR/CD3 stimulation; there is a subsequent interaction of the induced IL-2 and IL-2R that when followed by TCR/CD3 restimulation leads to the induction of the cell death effector Fas ligand that interacts with the Fas receptor, which culminates in the death of the self-reactive T cell.

**adult T-cell leukemia** An aggressive malignancy of mature CD3-, CD4-, and CD25- (IL-2Rα) expressing lymphocytes found predominantly in Japan, the Caribbean Islands, and sub-Saharan Africa caused by the retrovirus human T-cell lymphotropic virus I.

**human T-cell lymphotropic virus I (HTLV-I)** A retrovirus that is found predominantly in Japan, the Caribbean Islands, and sub-Saharan Africa that induces the expression of IL-2 and IL-15 and their receptors and that is the etiological agent of a number of human diseases including adult T-cell leukemia and autoimmune disorders including tropical spastic paraparesis/HTLV-I-associated myelopathy.

**tropical spastic paraparesis/human T-cell lymphotropic virus I (HTLV-I)-associated myelopathy** A demyelinating neurological disease that is caused by the retrovirus HTLV-I and that is associated with progressive weakness and bowel and bladder dysfunction.

## See Also the Following Articles

**Glucocorticoids and Autoimmune Diseses • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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Encyclopedia of Hormones.

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# Interleukin-16

Kevin Wilson, Yujun Zhang, Hardy Kornfeld, David Center, and William Cruikshank
*Boston University*

I. GENE STRUCTURE AND SYNTHESIS
II. CELLULAR SOURCES
III. RECEPTOR INTERACTION AND SIGNALING
IV. BIOACTIVITY
V. IL-16 AND HIV INFECTION AND INFLAMMATION

**Interleukin-16 (IL-16) was initially reported in 1982 as the first T-cell chemoattractant. IL-16 was isolated from supernatants of mitogen- or antigen-stimulated human peripheral blood mononuclear cells with chemoattractant activity for both human and rat T cells. Based on this activity, IL-16 was originally designated lymphocyte chemoattractant factor; however, its ability to attract immune cells *in vivo* may be secondary to its newly described role as an immunoregulatory molecule for cell motility and activation. This article will discuss many of the current concepts regarding IL-16 gene structure, synthesis, and processing, its biochemical characteristics, and its interaction with CD4. Its potential roles in inflammation and human immunodeficiency virus infection are also discussed.**

## I. GENE STRUCTURE AND SYNTHESIS

### A. Gene Structure

Interleukin-16 (IL-16) is located on chromosome 15q26.1–q26.3 in humans and on chromosome 7 D2–D3 in mice. The IL-16 gene contains seven exons and six introns and encodes a large precursor protein, pro-IL-16, which is enzymatically processed to generate mature (secreted) IL-16 from the C-terminal of pro-IL-16. There is high gene sequence homology for both pro-IL-16 and mature IL-16 when the human is compared with mouse, rat, feline, or simian, greater than 84% for all species. The IL-16 promoter contains two CAAT-box-like motifs and multiple binding sites for GA-binding protein (GABP) and GATA-1, -2, and -3 transcription factors. Two of the motifs constitute a dyad symmetry element that complexes with GABPα and GABPβ. Activation then occurs when the co-activator, CREB-binding protein/p300 (where CREB is $Ca^{2+}$/cAMP-response element-binding protein), binds to GABPα and induces the IL-16 gene promoter. The promoter lacks a TATA-box.

### B. Transcription

The IL-16 message is constitutively expressed at high levels in >95% of human T lymphocytes and does not appear to be inducible following antigenic or mitogenic activation, suggesting that posttranscriptional modifications are required for translational regulation. In T lymphocytes, the gene is transcribed into mRNA of approximately 2.6 kb, which is capable of undergoing alternate splicing. The message degrades relatively quickly with a half-life of 2 h. Under nondisease conditions, IL-16 mRNA is limited almost exclusively to lymphatic tissue and circulating immune cells. Synthesis of IL-16 is not restricted to T cells because during an inflammatory response *in situ* IL-16 message is also detected in other cell types, such as epithelial cells obtained from the airways of asthmatics or the footpads of mice undergoing a delayed-type hypersensitivity reaction. Airway epithelial cells do not have constitutive IL-16 message; however, the message is inducible by a variety of stimulants [tumor necrosis factor( (TNFα), transforming growth factor-β (TGF-β), and histamine]. A similar profile exists for mast cells where the message is induced following C5a or phorbol myristate acetate (PMA) stimulation and fibroblasts where the message is induced following IL-1β, TNFα, and leukoregulin stimulation. B cells appear to have a constitutive message that cannot be induced further. It is not clear at present why many of the cell types express high constitutive levels of IL-16 mRNA. A possible explanation is that IL-16 is part of a rapid response mechanism as IL-16 secretion occurs rapidly following stimulation of T cells, mast cells, and eosinophils.

## C. Translation and Posttranslational Modifications

Translation of the 2.6 kb mRNA results in a 631-amino-acid precursor protein, pro-IL-16 (Fig. 1). Using immunofluorescence and confocal analysis, pro-IL-16 was localized to the perinuclear region of the cytoplasm. Following cleavage by caspase 3, the N-terminal domain is found primarily in the nucleus, indicating cleavage-dependent translocation. Sequence analysis of pro-IL-16 indicates the presence of a casein kinase II (CKII) substrate site; a cdc2 enzymatic substrate site; a bipartite nuclear translocation signal (NLS) domain; two PDZ (PSD-95/Dlg/ZO-1) domains; and a SH3-binding domain. The casein kinase II substrate site, cdc2 kinase substrate site, and nuclear localization signal function together as a CcN motif: a nuclear localization signal regulated by dual phosphorylation. In light of its nuclear localization and the identification of several potential cell cycle-related kinase substrate sites, the N-terminal domain may be important in contributing to the regulation of the lymphocyte cell cycle. Along those lines, Zhang *et al.* have demonstrated that the presence of pro-IL-16 in the nucleus is associated with increased $G_0/G_1$ cell cycle arrest. This effect is not detected when the CKII substrate site or NLS is mutated. The physiologic importance of these findings has yet to be determined.

## D. Neuronal IL-16

A unique form of IL-16, neuronal IL-16 (NIL-16), is found in the cerebellum and hippocampus. Neuronal IL-16 differs from lymphocyte-derived IL-16 in the size and structure of the N-terminal domain. The C-terminal domains of NIL-16 and pro-IL-16 are identical in protein sequence and in association with CD4. Stimulation of granule neurons, which express CD4, with secreted NIL-16 results in the expression of the immediate-early gene, c-*fos*, via a signaling pathway involving protein phosphorylation. The N-terminus of NIL-16, however, is unique as it is approximately twice the size of lymphocyte pro-IL-16 and contains four PDZ domains. It has not been determined whether NIL-16 translocates to the nucleus following cleavage and processing. It has been determined that NIL-16 does interact selectively with a variety of neuronal ion channels. The presence of PDZ domains and the interaction with ion channels are typical of several neuronal intracellular scaffolding proteins. It is hypothesized that NIL-16 may have two roles in the nervous system; extracellularly, mature IL-16 acts to activate $CD4^+$ neuronal cells, and intracellularly, pro-IL-16 may function to anchor ion channels to the membrane.

## E. Biochemical Characteristics

The mechanism for IL-16 secretion has not been clearly defined. IL-16 is similar to another

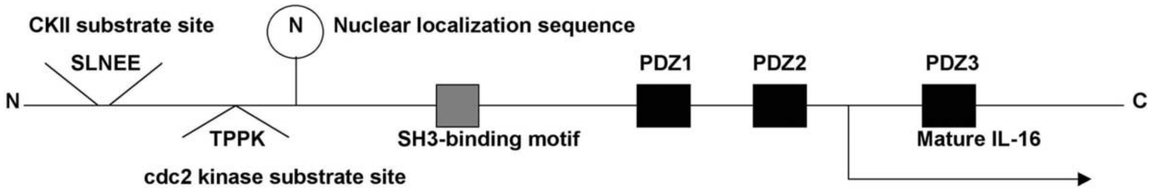

**FIGURE 1** Schematic diagram of pro-IL-16. Human pro-IL-16 is a 631-amino-acid protein that contains multiple consensus motifs. There exists a functional CcN motif consisting of a casein kinase II substrate site, a cdc2 kinase substrate site, and a classical bipartite nuclear localization sequence. There are three PDZ domains and an SH3-binding motif. Pro-IL-16 is cleaved by caspase 3. The C-terminal fragment of pro-IL-16 is secreted as mature IL-16. Following cleavage, pro-IL-16 translocates to the nucleus, where it can potentially regulate cell cycle progression.

interleukin, IL-1, in that they both are processed by caspase enzymes and they both lack a consensus secretory leader sequence. IL-1 is secreted by virtue of its interaction with caspase-1, resulting in the formation of a membrane pore. In addition, pro-IL-1 has been shown to translocate into the nucleus following caspase cleavage and affect cell cycle progression. A parallel system for IL-16 secretion has not been identified as yet. It has been determined, however, that the N-terminal may play a critical role in secretion. When the N-terminal 20 amino acids of IL-16 are deleted, there is reduced capacity for secretion. Conversely, when a secretory leader sequence is added, secretion is enhanced.

Biologically active IL-16 is composed of homo-aggregates of monomeric peptides. Maximal activity appears with tetrameric formation; however, dimers may also contain some activity. IL-16 monomers lack all IL-16 bioactivity described thus far; however, they are capable of functioning as competitive inhibitors. As both monomeric and aggregated forms of IL-16 are detected in cell supernatants or biological fluids, the presence of the monomer may have some physiological relevance in regulation of IL-16 bioactivity. Each monomeric IL-16 peptide contains a PDZ domain. Typically, PDZ proteins are intracellular proteins that associate with other intracellular proteins and are identified by a conserved G-L-G-F sequence. Although not definitively proven, the PDZ domains likely facilitate autoaggregation. As PDZ proteins are typically intracellular, IL-16 may be the first example of a secreted PDZ protein. Of the 121 amino acids that constitute the IL-16 monomer, the N-terminal 22 amino acids and C-terminal 16 amino acids are not contained within the core PDZ structure and appear to be hinged to facilitate a large degree of flexibility. All described function associated with secreted IL-16 is transmitted by a 4-amino-acid sequence contained in the C-terminal. Synthetic peptides corresponding to the C-terminal sequence, Arg-106 to Ser-109, inhibit all activity of IL-16. When alanine is substituted for Arg-107 in the synthetic oligopeptide, inhibition is lost, suggesting that Arg-107 is essential for IL-16-induced chemotactic activity. These data suggest that the bioactive site on IL-16 is located in the C-terminal end, distinct from the PDZ domain. There is preliminary data to suggest that the N-terminal also contributes to the function of IL-16 through interaction with CD4. Although the N-terminus is not essential for bioactivity, activity is greater when the sequence is present.

### F. Species Homology

IL-16 is a highly conserved protein with functional conservation as well in all species examined thus far. Simian pro-IL-16 and human pro-IL-16 are approximately 96% homologous, and the secreted forms of IL-16 for each species are $>98\%$ homologous. There is also intra- as well as interspecies conservation of function. Simian IL-16 has similar function and magnitude of effect whether stimulating simian or human $CD4^+$ T cells. Similarly, murine pro-IL-16 and mature IL-16 demonstrate a high level of homology and functional cross-reactivity with the human protein. In fact, there is functional cross-reactivity and cross-antibody neutralization for all species investigated to date, including rat, sheep, bovine, and feline IL-16. Interestingly, IL-16 was originally identified functionally as lymphocyte chemoattractant factor in 1982, based on its ability to induce migration in both human and rat T cells. These findings suggest that IL-16, along with its interaction with CD4 (see below), represents an evolutionarily conserved molecule with rudimentary roots in the immune system.

## II. CELLULAR SOURCES

Initially identified as the major chemoattractant factor produced by $CD8^+$ T cells, IL-16 has now been shown to be a product of a variety of cell types including $CD4^+$ T cells, mast cells, eosinophils, dendritic cells, bronchial epithelial cells, B cells, and most recently, fibroblasts. $CD8^+$ T cells contain constitutively bioactive IL-16 as stimulation with vasoactive amines, such as histamine or serotonin, results in a rapid release of IL-16 that is not altered in the presence of either translation or transcription inhibitors. In addition, $CD8^+$ cell lysates from unstimulated cells contain bioactive IL-16. This suggests that $CD8^+$ T cells have not only constitutive expression of activated caspase 3 but also a mechanism for storage of processed mature IL-16. Antigenic activation of $CD8^+$ cells results in IL-16 production that is sensitive to translation inhibitors, indicating *de novo* synthesis. These data suggest that in $CD8^+$ T cells, two pathways may exist for IL-16 expression. Preformed bioactive IL-16 is stored in vesicles responsive to secretagogues such as vasoactive amine stimulation, and new IL-16 is synthesized and released following T-cell receptor activation. In contrast, $CD4^+$ T cells contain constitutive message and pro-IL-16 but no preformed bioactive IL-16. These cells must be stimulated for 18–24 h with antigenic or mitogenic stimuli before bioactive IL-16 is detected in the supernatant as a result of caspase 3 processing of pro-IL-16. As IL-16mRNA is constitutively present, the 18 to 24 h period required to produce IL-16 likely also reflects the time required for generation and activation of caspase 3.

The initial observation that airway epithelial cells can produce IL-16 was reported by Bellini *et al*. In their studies, bronchial epithelial cells were obtained by bronchoscopic biopsy from normal, atopic asthmatic, and atopic nonasthmatic volunteers and cultured for 48 h with or without histamine. Only the supernatants from the cell cultures obtained from asthmatics were chemotactic for T lymphocytes and contained IL-16. The induced production of IL-16 by asthmatic epithelial cells was confirmed later using immunohistochemistry and *in situ* hybridization on biopsy lung tissue. In addition to histamine, epithelial cell lines have been reported to produce IL-16 in response to TNFα and TGF-β, as well as following stimulation by Th2 cytokines IL-9 and IL-13.

The most recently identified cell source is the fibroblast. Cultured fibroblasts obtained from all types of tissues sites are capable of generating IL-16 in response to IL-1β, TNFα, and leukoregulin. Cultured fibroblasts appear to have constitutive message but no preformed IL-16 protein. The lack of preformed IL-16 is confirmed by histological studies demonstrating that normal tissue fibroblasts lack IL-16; however, fibroblasts obtained from diseased tissue such as the synovium of patients with rheumatoid arthritis (RA), but not in patients with osteoarthritis (OA), contain high levels of IL-16. In those studies, the synovial lining contained IL-16 message with increased IL-16 protein detected in the synovial fluid.

## III. RECEPTOR INTERACTION AND SIGNALING

IL-16 requires the expression of CD4 on T cells to induce a cellular response. A direct interaction between IL-16 and CD4 was first demonstrated in studies in which murine L3T4–T cell hybridoma cells were transfected with the cDNA encoding human CD4. Originally unresponsive to IL-16 stimulation, hybridoma cells expressing CD4 demonstrated IL-16 responsiveness as measured by induction of second messengers and cell migration. The physical interaction between IL-16 and CD4 has been demonstrated by CD4 affinity chromatography and by immunoprecipitation.

The IL-16-binding site on CD4 has recently been determined. High species cross-reactivity suggested a high degree of protein sequence conservation between the active site on IL-16 from different species with its binding site on CD4 from different species. Comparison of the human CD4 amino acid sequence with several different species revealed the D4 domain to be the most conserved. Initially, using peptide inhibition studies, the active site was mapped to the Trp-345QCLLSer-350 sequence within the D4 domain. This was confirmed and further analyzed using regional and point mutational analyses that suggested that both Leu-347 and Leu-348 are essential for induced signaling but that a larger region is required for binding. The binding site on CD4, the proximal region of the D4 domain, is the identical site where CD4 autoaggregates during antigenic activation. Therefore, the presence of IL-16, either in aggregated form or as a monomer, in high enough concentrations prevents CD4 aggregation. Therefore, as a consequence of this interaction, CD4 is sequestered from the T-cell receptor (TCR) complex, resulting in significant alteration of TCR signaling and lymphocyte activation (Fig. 2) (see also bioactivity section). It is this function of IL-16 that appears to predominate in a number of inflammatory conditions.

It does not appear that a co-receptor is necessary to elicit IL-16-induced cellular signaling in T cells and neuronal cells; however, this may not be the case for all cell types. Recent reports have suggested that alternate or co-receptor signaling may be present for both murine monocytes and human dendritic cells; however, identification of an alternative receptor has not been reported. A possible relationship exists between IL-16 signaling and several of the chemokine receptors. IL-16 stimulation of monocyte-derived macrophages results in loss of membrane-expressed CCR5 and CXCR4. In T cells, IL-16 binding to CD4 has been shown to decrease the chemotaxis induced by macrophage inflammatory protein-1β (MIP-1β) binding to CCR5 and stromal cell-derived factor-1α, which binds to CXCR4. As both of these chemokine receptors can physically associate with CD4, the potential exists for a co-receptor relationship with IL-16; however, more studies are required to assess this further.

IL-16 bioactivity is induced only when aggregated into multimeric form, indicating that cross-linking of CD4 is necessary for IL-16-induced intracellular signaling. Further supporting this finding are the observations that oligomerization of CD4 is required for optimization of major histocompatibility complex class (MHC) class II-dependent cell activation and that cross-linking of CD4 by multimeric IL-16 results in the generation of several second messengers not detected by monovalent binding. In lymphocytes,

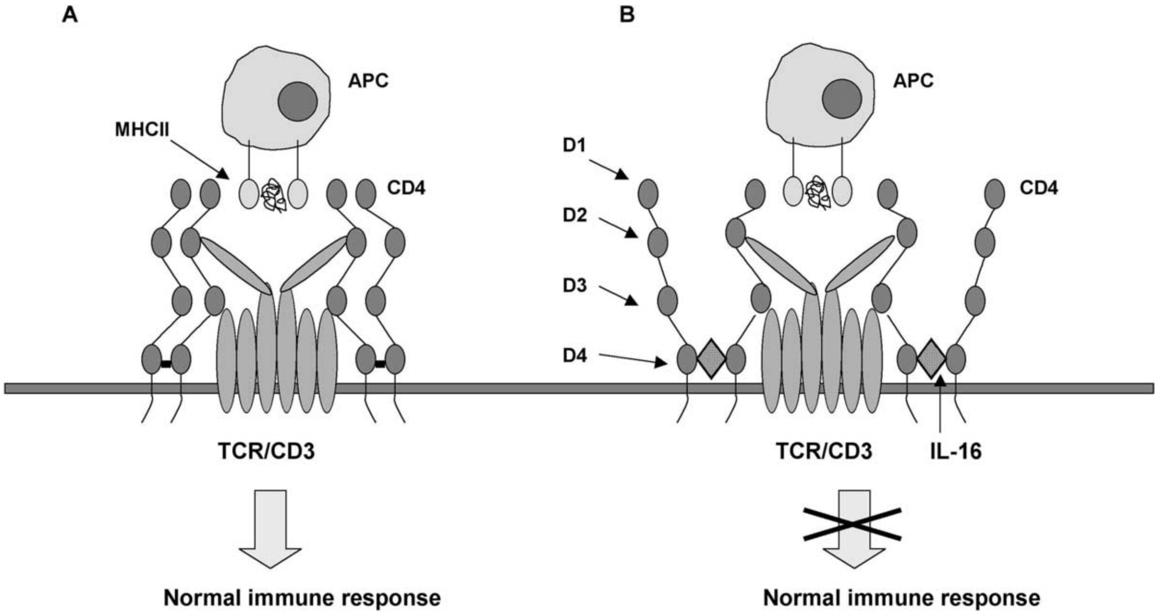

FIGURE 2 Inhibition of TCR-mediated signaling by IL-16. (A) During normal immune responses, an antigen is presented by an antigen-presenting cell (APC) on the major histocompatibility complex class II site (MHCII). The MHCII associates with the D1 domain of CD4. The D3 domain of CD4 interacts with T-cell receptors (TCRs) and CD4 autoaggregates via interaction of the D4 domains, initiating a normal immune response. (B) Interleukin-16 (IL-16) binds to D4 and oligimerizes CD4, changing the steric conformation of CD4. As a result, a normal immune response is inhibited. It is not known whether the mechanism of inhibition is interruption of normal signaling and/or generation of an inhibitory signal.

detectable increases in intracellular calcium, inositol 1,4,5-triphosphate, and phosphorylation of CD4 and p56$^{lck}$ are observed within minutes after stimulation with aggregated IL-16. IL-16 stimulation of monocytes is likely mediated through a Src tyrosine kinase family member, p59$^{lyn}$, and results in activation of the stress-activated protein kinase (SAPK) pathway. Following IL-16 stimulation, SEK-1 (mitogen-activated protein kinase kinase-4) is phosphorylated, resulting in the activation of SAPKs p46 and p54. In addition, c-Jun and p38 MAPK (mitogen-activated protein kinase) are phosphorylated without inducing phosphorylation of MAPK-family members extracellular signal-related kinase-1 (ERK-1) and ERK-2. However, distinct from other signaling pathways involving activation of SAPKs and p38 MAPK in macrophages, there is no apoptosis associated with activation of this apoptotic pathway component. In T cells, IL-16-induced signals are dependent on the interaction of CD4 with another Src tyrosine kinase family member, p56$^{lck}$. This effect was identified by transfecting L3T4 murine hybridoma T cells with CD4 constructs that prevented or allowed interaction with p56lck. IL-16-induced signals were detected only when p56$^{lck}$ physically associated with CD4. Interestingly, p56$^{lck}$ constructs lacking the catalytic SH1 kinase domain functioned normally, indicating that kinase activity was not required for induction of a migratory signal. All signaling was lost, however, when the adapter SH3 domain was deleted. This suggests that a migratory signal induced by an IL-16/CD4 interaction is not transmitted by the enzymatic activity of the SH1 domain, but rather by the SH3 domain.

## IV. BIOACTIVITY

IL-16 induces a variety of cellular responses on a number of different CD4$^{+}$ cell types (Table 1). The bioactivities include induction of cell motility, cell proliferation, and cellular differentiation. In addition, as mentioned above, IL-16 can regulate antigen-induced TCR signaling. Although initially characterized as a chemoattractant selective for CD4$^{+}$ T cells, it was later determined that IL-16 is a potent chemoattractant for all peripheral immune cells expressing CD4$^{+}$ including eosinophils, monocytes, and dendritic cells. As a distinction from most of the chemokines, IL-16 can induce migration in naive (inactivated) T cells. In addition to chemoattractant

**TABLE I Bioactivities of Interleukin-16 in CD4$^+$ Lymphocytes**

Mature IL-16
- Induces migration/chemotaxis
- Regulates immune and inflammatory processes mediated through the TCR
- Primes cells for IL-2 or IL-15-induced proliferation
- Regulates the responsiveness of chemokine receptors CCR5 and CXCR4
- Suppresses HIV-1 and SIV-1 replication

Pro-IL-16
- Translocates to the nucleus where it regulates T-cell growth

Neuronal IL-16
- NIL-16 activates immediate-early gene, c-*fos*, in CD4$^+$ cerebellar granule neurons
- Pro-NIL-16 interacts with the mouse brain calcium channel, mbC$\alpha$1

activity, IL-16 is also a competence growth factor, capable of stimulating cell cycle progression in CD4$^+$ T cells. Stimulation of CD4$^+$ T cells with IL-16 results in up-regulation of surface-expressed IL-2R$\alpha$ and $\beta$, which is associated with progression from a $G_0$ to a $G_1$ phase shift in the cell cycle. Cell proliferation does not occur until the cells are stimulated with either IL-2 or IL-15. Stimulation of peripheral blood mononuclear cells with IL-16 followed by IL-2 results in a 1000-fold increase in cell numbers with the expanded cell population being homogenously composed of CD4$^+$CD25$^+$CD29$^+$CD45RO T cells. A similar cell phenotype but with less dramatic expansion is observed when IL-16 and IL-2 are used to stimulate T cells from human immunodeficiency virus (HIV)-infected individuals. In cells that have lost regulated cell growth such as T-cell lines, IL-16 can act as a complete growth factor. CD4$^+$ T-cell lines such as Jurkat and HUT 78 increase their growth rate when stimulated by IL-16. In addition, cells obtained from patients with mycosis fungoides, a common cutaneous T-cell lymphoma, have been shown to have high levels of IL-16 expression. IL-16 stimulation also has an effect on CD4$^+$ pro-B-cell differentiation. Murine bone marrow cells, obtained from aged mice with low levels of circulating mature B cells, stimulated with IL-16 resulted in the differentiation of CD4$^+$ pro-B cells into pre-B cells, the increased expression of RAG-1 and RAG-2, and ultimately, the expansion of the number of pre-B cells in the bone marrow.

Interaction of CD4 with the ligands HIV-1 gp120 or anti-CD4 antibody can induce second messengers and cellular responses such as chemotaxis. The earliest known function for CD4, however, was as a regulatory receptor for TCR-induced activation. Anti-CD4 antibodies were thought to stimulate a "negative signal," resulting in inhibition of TCR signaling and cell proliferation. As a ligand for CD4, IL-16 similarly inhibits antigen-induced TCR signaling. The addition of IL-16 to cell cultures containing a mixed lymphocyte reaction or anti-CD3 stimulation results in inhibition of T cell proliferation. Point-mutated IL-16 is incapable of signaling through CD4 but can still inhibit TCR signaling. This suggests that the interaction with CD4 alone and not signaling through CD4 facilitates the inhibitory activity.

The mechanism by which IL-16/CD4 binding stimulates many cellular responses while inhibiting antigen-dependent stimulation via TCR is becoming more clear. IL-16's interaction with the D4 domain on different CD4 molecules serves to cross-link CD4 and induce cellular signals via activation of phosphatidylinositol 3-kinase, resulting in both a migratory signal and cell cycle progression. If IL-16 interacts with CD4 prior to antigenic stimulation, CD4 is unable to autoaggregate and is unlikely to participate in the formation of a TCR/MHC class II complex. Without CD4's contribution, TCR signaling is significantly less effective and cell activation is reduced or completely inhibited. Binding of IL-16 to a previously activated cell has little effect on the activation state of that cell. Therefore, IL-16's effect to restrict CD4$^+$ T-cell activation is selective for unstimulated T cells. As a consequence of decreased cell activation, cytokine production is also reduced. This is particularly true of the Th2 cytokines, IL-4, IL-5, and IL-13, where IL-16 stimulation results in almost a complete reduction in synthesis (Fig. 3). Th1 cytokines, such as interferon-$\gamma$, are less affected. The overall hypothesis for the role of IL-16 in inflamed tissue is less clear. One possibility is that IL-16 contributes to antigen-independent, nonclonal recruitment and priming of CD4$^+$ cells in an inflammatory process. The ability of IL-16 to selectively inhibit chemokine-induced migration would serve to further define the recruited cell population. Although the recruited cells would be responsive to cytokine stimulation (IL-2, IL-15), they would be transiently refractory to antigen-specific activation. The effect would be to increase selectively the number of cells recruited to an inflammatory focus and to further increase the number of viable cells by simultaneously reducing their susceptibility to antigen-specific induced cell death.

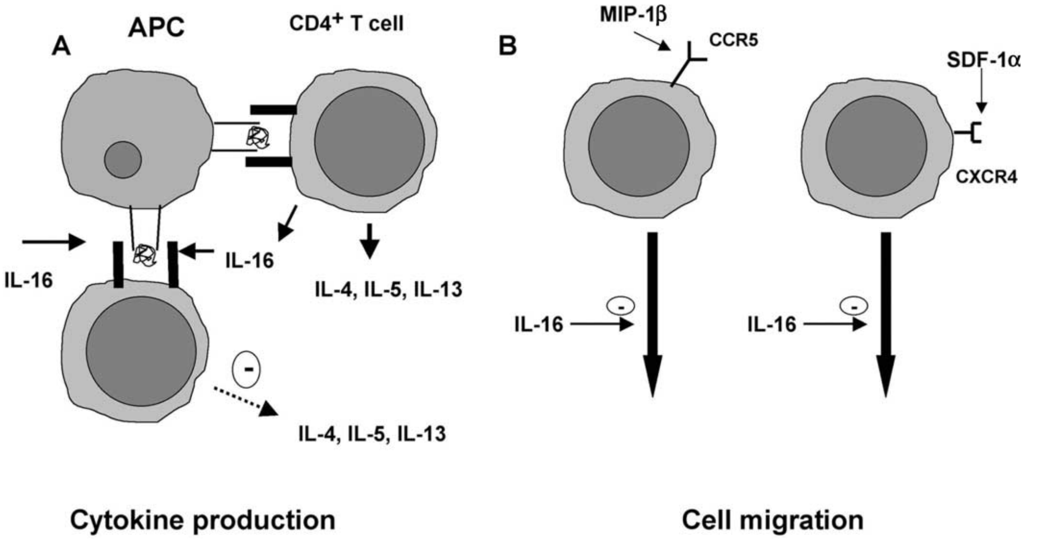

FIGURE 3 Regulatory effects of IL-16 on T-cell recruitment and cytokine production. (A) Following antigen activation, T lymphocytes secrete proinflammatory cytokines such as IL-4, IL-5, and IL-13. IL-16, which can associate with CD4 on unstimulated cells, is also produced, resulting in a reduction or complete inhibition of antigen-induced activation and subsequent cytokine production. There are many other cell sources for IL-16 such as airway epithelium, fibroblasts, and other immune cell types. (B) Binding of IL-16 to its receptor, CD4, decreases chemotaxis induced by several chemokines, in particular, the chemotactic signal generated by macrophage inflammatory protein-1β (MIP-1β) binding to CCR5 and stromal derived factor-1α (SDF-1α) binding to CXCR4.

## V. IL-16 AND HIV INFECTION AND INFLAMMATION

### A. IL-16 in HIV-1 Infection

As a product of CD8$^+$ T cells, IL-16 was investigated as a potential contributing factor to the anti-HIV-1 viral activity identified by Levy *et al*. Based on this, several studies have identified that binding of IL-16 to CD4$^+$ cells results in suppression of both HIV-1 and simian immunodeficiency virus infection and replication. As IL-16 binds to the D4 domain, distinct from the HIV-1-binding site located in D1, there is no inhibitory effect of IL-16 on HIV-1 binding or internalization. The mechanism for the antiviral activity of IL-16 therefore is different from the mechanism used by RANTES, MIP-1α, and MIP-1β, which bind to CCR5 co-receptor and prevent HIV-1 binding. The inhibitory effect of IL-16 appears to be at the level of transcriptional regulation. Maciaszek *et al*. reported that IL-16 pretreatment of CD4$^+$ lymphoid cells suppressed HIV-1 promoter activity 60-fold. This effect required sequences within the core enhancer, but was not due to the down-regulation of the binding activity of transcription factors such as nuclear factor κB. Therefore, it appears that IL-16 stimulates the activation of a transcriptional repressor that functions through sequences within or immediately adjacent to the core enhancer. Confirmation of this concept was generated when studies revealed that cells transfected with the bioactive portion of IL-16 are resistant to HIV-1 infection. The effect was a result of CD4 engagement by IL-16 on the plasma membrane rather than by intracellular interaction with CD4 prior to surface expression. Several studies have reported that IL-16 is capable of inhibiting HIV even when it is added postinfection. The effects of IL-16 are not limited to T cells. Truong*et al*. reported that IL-16 not only inhibits viral replication but also prevents viral entry in dendritic cells.

The magnitude of viral inhibition for IL-16 is variable depending on the strain of virus; however, for most strains tested there was an average of approximately 50–60% reduction in viral replication. Although the antiviral activity of IL-16 is not as effective as that of many agents currently used in clinical trials, its real benefit may be its ability to facilitate CD4$^+$ T-cell expansion. As discussed earlier, IL-16 stimulation results in up-regulation of IL-2Rα, CD25. This suggests a potential use for HIV therapeutics as an adjunct to IL-2 for immune reconstitution. IL-16 stimulation results in up-regulation of IL-2Rα and imparts IL-2 responsiveness to CD4$^+$ lymphocytes. Thus, IL-16 treatment would be expected to increase the IL-2R$^+$ population, potentially increasing the effectiveness of exogenous IL-2 and decreasing the required amount of IL-2, thereby reducing the risk of IL-2 toxicity. Expansion of IL-2R$^+$ cells would theoretically also increase TCR diversity. *In vitro* studies have indicated that peripheral blood mononuclear cells obtained from HIV-1$^+$ individuals, cultured with both IL-16 and IL-2, result in an increase in a homogenous CD4$^+$ T-cell population. In addition, preliminary studies have demonstrated that cells from some patients demonstrated renewed antigen responsiveness. Clinically, a pattern has developed correlating HIV disease progression with systemic levels of IL-16. IL-16 levels are low in patients with acquired immune deficiency syndrome, but rise dramatically after treatment with indinavir, concomitant with a rise in CD4 counts. Interestingly, IL-16 levels are higher than the normal range in long-term nonprogressors of disease.

## B. IL-16 in Inflammation

Initial characterization of IL-16 bioactivity, induction of cell migration, and priming T cells for proliferation would suggest a pro-inflammatory cytokine. As such, IL-16 has been detected at sites of inflammation in association with a variety of diseases, such as rheumatoid arthritis (RA), asthma, sarcoidosis, systemic lupus erythematosus, inflammatory bowel disease (IBD), delayed-type hypersensitivity (DTH), and Graves' disease. Consistent with the *in vitro* assays indicating pro-inflammatory activity, animal models of IBD, RA, and DTH, where the animals were treated intraperitoneally with IL-16 neutralizing antibodies, there was a significant reduction in parameters of inflammation. However, as with a number of cytokines, its presence in different environments results in the induction of different cellular responses. Although under certain conditions, IL-16 may function as a pro-inflammatory cytokine, recent data have suggested that in inflammation associated with other diseases, such as asthma or RA, IL-16 acts in an immunomodulatory fashion.

Identifying a role for IL-16 in inflammation has focused on diseases characterized by CD4$^+$ infiltrates. Asthma was the first disease to be directly associated with IL-16 production. Bellini *et al.* identified IL-16 in cultures of histamine-stimulated primary epithelial cells obtained from asthmatics, but not from normals. Cruikshank *et al.* challenged asthmatic individuals with antigen, resulting in detection of IL-16 in the bronchoalveolar lavage fluid 4 h postchallenge. IL-16 could not be detected in normal or atopic nonasthmatic individuals, indicating selective production of IL-16 in association with asthma rather than as a result of general inflammation. Subsegmental histamine challenge of asthmatics, but not nonasthmatics, also resulted in the elaboration of IL-16. When biopsies obtained from asthmatics were assessed for immunohistochemical staining and *in situ* hybridization, IL-16 message and protein were detected in the airway epithelium and infiltrating CD4$^+$ cells. There was a significant correlation between the amount of protein and message and the number of infiltrating CD4$^+$ cells. Nonasthmatics had little detectable IL-16 protein or message. The role of IL-16 in asthmatic inflammation is now thought to predominate as an immunoregulatory factor. Intratracheal instillation of IL-16 in a murine model of asthma has indicated a significant reduction in airway hyperreactivity (AHR) as well as a reduction in lung inflammation. Conversely, preliminary studies using an IL-16 − /− knockout mouse have indicated that AHR is significantly increased when these mice are subjected to an OVA model of allergic AHR. These mice also demonstrate an increase in lung inflammation characterized by a cellular influx of T cells and eosinophils. In another animal model, Klimiuk *et al.* demonstrated that the transfer of IL-16-producing CD8$^+$ T cells in a murine model of RA reduces IFN-γ, IL-1β, and TNFα levels by more than 90% and significantly reduces CD4$^+$ T-cell infiltrates, effects that were blocked by co-administration of anti-IL-16. The mechanism for the anti-inflammatory effect of IL-16 in these models has not been clearly defined. The current hypothesis is that IL-16, via its direct interaction with CD4 (see above), limits or completely inhibits CD4$^+$ T-cell activation. Since much of the inflammatory process for these diseases is orchestrated by the CD4$^+$ T cell, alteration in their function would markedly effect downstream events.

IL-16 has been detected in several other diseases; however, its role has not been identified. Immunohistochemical staining of sarcoidosis-associated granulomas from the lymph node and lung reveals high levels of IL-16 staining, with the greatest prevalence in the perivascular areas of lymphocyte accumulation. IL-16 is abundant in the bronchoalveolar lavage fluid of patients with sarcoidosis and tuberculosis. In patients with Crohn's disease, colonic tissue sections show increased IL-16 protein and message compared to uninvolved colonic tissues from the same patients or normal controls. In an animal model of Crohn's disease, anti-IL-16 antibody treatment significantly reduced all parameters of inflammation.

Similarly, using an animal model of DTH, Yoshimoto *et al.* demonstrated that anti-IL-16 antibody treatment significantly reduced granuloma formation in murine footpads.

In summary, IL-16 is a multifunctional cytokine generated by a variety of cell types. In T cells, bioactivities of IL-16 are restricted to surface expression of CD4; however, in monocytes and macrophages there may be an alternative receptor. Expression of IL-16 is primarily limited to T cells during homeostasis conditions, but is dramatically up-regulated at sites of inflammation where it appears to have the capacity to function as either a proinflammatory or an immunomodulatory cytokine depending on the surrounding environment. A better understanding of how IL-16 fits into the pathogenesis of inflammation is required as the specificity of IL-16, through CD4, is directed to those cell types that orchestrate much of the inflammatory process.

IL-16 stimulation of T cells, macrophages, and dendritic cells results in a cellular state that retards HIV-1 viral replication. This effect appears to occur through the generation of a transcriptional repressor molecule. The antiviral activity of IL-16 in conjunction with its ability to induce IL-2R in $CD4^+$ T cells suggests that IL-16 may be beneficial in HIV-1 immune reconstitution as an adjunct to IL-2 therapy.

## Glossary

**casein kinase II (CKII)** A ubiquitous protein kinase that phosphorylates over 160 known substrates. Among its many actions, CKII changes the activity of transcription factors, proteins involved in DNA replication and DNA repair (topoisomerase II, DNA ligase), and growth factors (p53, p21).

**cdc2 protein kinase** A protein kinase that associates with cyclin A or B to form a complex resembling the invertebrate mitosis-promoting factor (MPF). MPF phosphorylates histone-1 protein, disrupting its association with chromatin. It is hypothesized that this provides DNA topoisomerase II access to chromatin, thereby promoting chromatin condensation and advancement to the mitotic phase of the cell cycle. Also, it phosphorylates lamins, impairing their ability to polymerize and leading to nuclear membrane breakdown.

**nuclear localization sequence** A consensus sequence that is short, is single or bipartite, and contains clusters of basic amino acids. It functions as a nuclear-targeting motif.

**$p56^{lck}$** An intracellular tyrosine kinase, from the Src family of tyrosine kinases, that associates with the intracellular portion of CD4 via its unique $NH_2$-terminal domain. Its presence appears to be essential for CD4 co-receptor activity. However, its tyrosine kinase function is dispensable for certain CD4 functions.

**PDZ domain** An approximately 90-amino-acid segment with a distinctive three-dimensional shape that contains a binding cleft for protein interaction. The PDZ domain typically allows homologous and heterologous recruitment and assembly into larger protein complexes.

**SH3-binding motif** A consensus motif, PXXP, where P denotes proline and X denotes an unspecified amino acid. The motif is recognized by Src homology (SH) domains of *Src* and other nonreceptor tyrosine kinases, permitting the SH domain to bind to SH-binding motifs.

## See Also the Following Articles

**Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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# Interleukin-18

HIROKO TSUTSUI, TOMOHIRO YOSHIMOTO,
HARUKI OKAMURA, AND KENJI NAKANISHI

*Hyogo College of Medicine, Japan and Japan Science and Technology Corporation, Tokyo*

I. INTRODUCTION
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IV. RECEPTOR AND SIGNAL TRANSDUCTION
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VI. PATHOPHYSIOLOGICAL ROLES
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**Interleukin-18 (IL-18) is produced by various types of cells as a biologically inactive precursor and becomes active after cleavage with appropriate enzymes. Playing roles in both the innate and the adaptive immune systems, IL-18 acts not only on NK cells, macrophages, dendritic cells, basophils, and mast cells, but also on T cells and B cells. IL-18 activates both Th1 and Th2 responses. In addition, IL-18 has a wide spectrum of biological actions, a characteristic that has not been shown by other cytokines.**

## I. INTRODUCTION

Interleukin-18 (IL-18) was discovered as a potent interferon-γ (IFN-γ)-inducing factor in 1995. Its structural properties resemble those of IL-1 and there is some amino acid sequence homology between them. IL-18, like IL-1β, is produced as a biologically inactive precursor and becomes active after cleavage with appropriate processing enzymes, such as caspase-1. The receptor for IL-18 (IL-18R) is composed of two chains, a ligand-binding component, the α chain, and a signaling component, the β chain, both of which belong to the IL-1R family. Furthermore, IL-1 and IL-18 share a common signal transduction pathway. In contrast to its structural homology, IL-18 has biological actions distinct from those of IL-1. IL-1 exerts its actions primarily in the innate immune system, whereas IL-18 plays roles equally in both the innate and the adaptive immune systems. IL-18 acts not only on NK cells, macrophages, dendritic cells, basophils, and mast cells, but also on T cells and B cells. In addition, IL-18 has a wide range of biological functions, a characteristic that has not been shown by other cytokines. IL-18 not only activates Th1 responses but also induces Th2 responses. Because Th1 and Th2 immune responses have been believed to be dichotomous outcomes of adaptive immunity, the finding that IL-18 up-regulates both of them appears to be contradictory and would cause immunological chaos. This may reflect the wide-ranging pathophysiological roles of IL-18 in various immune responses and diseases. IL-18 is profoundly involved in Th1-dependent host defenses against various pathological microbes, such as *Listeria monocytogenes*, a gram-positive intracellular facultative bacterium, *Leishmania major*, an intracellular protozoan, and *Cryptococcus neoformans*, an intracellular fungus. IL-18 plays major roles in the induction and/or activation of autoimmune diseases or inflammatory diseases, including multiple sclerosis and inflammatory bowel disease. Here, we summarize the immunological aspects of IL-18 based on previous reports.

## II. MOLECULAR STRUCTURE AND GENE EXPRESSION

Human and mouse IL-18 genes are located on chromosome 11 and chromosome 9, respectively. Human and mouse IL-18 cDNAs encoding precursor IL-18 are composed of 193 and 192 amino acids, respectively, and lack the usual leader sequence that is required for secretion from the cells. Instead, the IL-18 gene contains an unusual leader sequence of 35 amino acids at the N-terminal. The IL-18 gene consists of seven exons, and the first two exons are noncoding. Two promoter regions are located upstream: exon I and exon II. One of them has a PU.1-binding site, suggesting constitutive activation of the IL-18 gene and inducible activation by lipopolysaccharide (LPS). The other exon, containing an interferon consensus site-binding protein-binding site, is activated by IFN-α, IFN-β, and IFN-γ. Based on these characteristics, it is conceivable that macrophages and keratinocytes constitutively express IL-18. The IL-18 gene has a second unique feature.

Cytokine genes usually contain many copies of an RNA-destabilizing element that result in a short half-life of the mRNA for the cytokine. In contrast, the IL-18 gene has at most one copy, presumably resulting in long-lived IL-18 mRNA (described below).

## III. PRODUCTION AND PROCESSING

Macrophages, particularly Kupffer cells, store large amounts of proIL-18 under normal conditions. Many cell types, including keratinocytes, dendritic cells, and immune competent B cells, also constitutively express IL-18. This constitutive and continuous production of IL-18 might be caused by the unique gene expression of IL-18 as described above. Therefore, posttranscriptional regulation seems to be quite important for the release of biologically active IL-18.

Two distinct processing pathways have been identified, such as the pathogen-associated molecular pattern (PAMP)-mediated caspase-1-dependent pathway and the Fas/Fas ligand (FasL)-mediated caspase-1-independent pathway (Fig. 1). Both processing machineries are utilized for processing of IL-1β.

On stimulation with LPS, preformed inactive precursor caspase-1 is cleaved into biologically active caspase-1, which cleaves proIL-18 into mature IL-18, eventually leading to the release of IL-18 from macrophages. This process does not always require transcriptional and translational signals, although most cytokines, including IL-1β, require them. In fact, Kupffer cells that constitutively express both precursor IL-18 and precursor caspase-1 can secrete IL-18 but not IL-12, tumor necrosis factor α (TNFα), or IL-1β in the presence of transcriptional or translational inhibitors after being stimulated with LPS. Interestingly, IL-18 secretion from LPS-stimulated Kupffer cells does not require MyD88, although these cells use MyD88 for transduction of cytoplasmic signals required for the production of many other cytokines. Indeed, MyD88-deficient Kupffer cells secrete IL-18 but not IL-12 although Toll-like receptor 4 (TLR4) equally transduces signals for IL-12 and IL-18 secretion. The same scenario seems to be employed by natural infection for this dissociated release of IL-18 and IL-12. *Li. monocytogenes* infection induces elevation of both IL-12 and IL-18 serum levels in wild-type mice. MyD88-deficient mice show elevation of IL-18 but not IL-12. This is also the case for the infection with *Plasmodium berghei*, a pathogenic protozoan in mice.

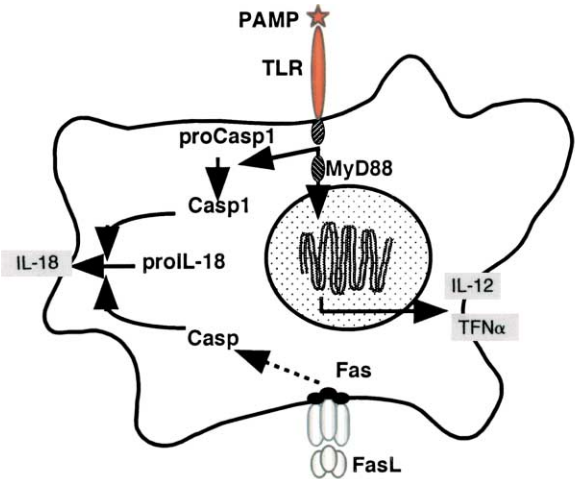

**FIGURE 1** Processing of IL-18. Kupffer cells store both IL-18 and caspase-1 (Casp1) as the biologically inactive precursor forms (proIL-18 and proCasp1) under normal conditions. After stimulation with PAMPs, such as LPS, Casp1 is activated independent of MyD88 and then cleaves prepared proIL-18 into biologically inactive mature IL-18, resulting in the secretion of mature IL-18. In contrast, LPS-activated Kupffer cells produce IL-12 or TNFα in a MyD88-dependent manner. On stimulation with Fas ligand (FasL), Kupffer cells release IL-18 independent of caspase-1. However, FasL-stimulated IL-18 secretion is inhibited by caspase inhibitor, indicating that this processing requires a caspase (Casp) other than caspase-1.

A caspase other than caspase-1 is also involved in the processing of IL-18. Kupffer cells isolated from mice that had been treated with heat-killed *Propionibacterium acnes*, a gram-positive bacterium, can secrete IL-18 in response to FasL, whereas those from untreated mice do not. This is because *P. acnes* treatment induces Fas expression on Kupffer cells. *P. acnes*-elicited caspase-1-deficient Kupffer cells can secrete IL-18 after stimulation with FasL, but this is completely inhibited by caspase inhibitors that inhibit caspase-1 or broad caspase activity. This Fas/FasL-mediated IL-18 secretion is observed in mice with acute graft-versus-host disease (GVHD). IL-18 increases in F1 mice ($H2^{bXd}$) that had been transplanted with wild-type splenic cells ($H2^{b}$). In contrast, F1 mice transplanted with *gld/gld* splenocytes ($H2^{b}$) that lack functional FasL do not show elevated serum levels of IL-18. Therefore, at least in acute GVHD mice, IL-18 is secreted on stimulation with FasL.

## IV. RECEPTOR AND SIGNAL TRANSDUCTION

Like IL-1R, IL-18R is composed of a ligand-binding α chain and a signaling β chain (Fig. 2). IL-18Rα-deficient lymphocytes do not bind or respond to IL-18. Anti-IL-18Rβ antibodies inhibit lymphocytes to respond to IL-18 both *in vivo* and *in vitro*.

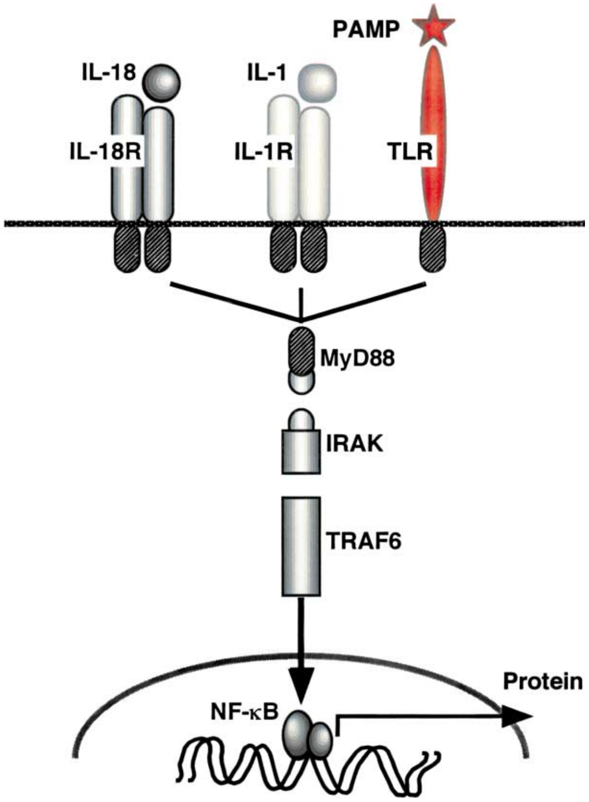

**FIGURE 2** IL-18 signaling pathway. IL-18R shares a signal transduction pathway with IL-1R and TLRs. On stimulation with the corresponding ligand, the intracellular domain of each receptor recruits MyD88 to relay a signal, leading to activation of NF-κB.

Freshly isolated murine $CD4^+$ or $CD8^+$ T cells bind with low affinity to IL-18, whereas T cells treated with IL-12 and/or type 1 IFN (IFN-α and IFN-β) bind with high affinity to IL-18. Indeed, the former express a trace amount of IL-18Rα and/or IL-18Rβ, whereas the latter have much larger amounts of both components. Th1 cells, but not Th2 cells, express high levels of IL-18Rα, strongly suggesting that IL-18Rα is a surface marker of Th1 cells. In contrast to T cells, NK cells constitutively express large amounts of IL-18R.

IL-18R shares a signal transduction pathway with IL-1R as well as TLR (Fig. 2). After activation of IL-18R with IL-18, intracellular portions of both chains recruit MyD88 to relay a signal to activate NF-κB, through the sequential activation of IL-1R-associated kinase (IRAK) and TNFR-associated factor (TRAF) 6. Deletion of any gene encoding MyD88, IRAK, or TRAF6 results in failed responses to all the ligands, such as IL-18, IL-1, and PAMPs.

IL-18-binding protein (IL-18BP) is believed to be a natural inhibitor that inhibits binding of mature IL-18 to IL-18R. IL-18BP shows very high binding affinity for IL-18 but has no homology with IL-18Rα or IL-18Rβ. IL-18BP lacks a transmembrane portion, indicating that it is a soluble decoy receptor like the soluble IL-1R type II, which efficiently inhibits binding of IL-1 to IL-1R. IL-18BP is present in serum or urine under normal conditions and is up-regulated by IFN-γ, suggesting a negative circuit between IL-18BP and the IFN-γ, IL-18 product. Although it is not known whether IL-18BP physiologically regulates IL-18 biology, IL-18BP seems to have the potential to be utilized as a therapeutic against IL-18-associated diseases, such as inflammatory diseases, autoimmune diseases, and atopic diseases (described below).

## V. BIOLOGICAL FUNCTIONS

One of the prominent characteristics of IL-18 is a wide range biological action. In the immune system, IL-18 has the capacity to activate both Th1 and Th2 responses depending on its cytokine environment, as well as innate immune responses (Fig. 3). In principle,

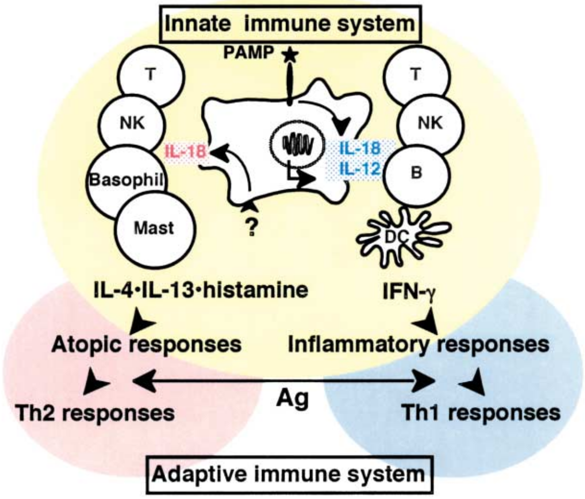

**FIGURE 3** Biological functions of IL-18 in the immune system. On stimulation with microbe or microbe products via TLRs, macrophages and/or dendritic cells release IL-18 together with IL-12 (Fig. 1), which activates various types of cells to produce a large amount of IFN-γ, which induces inflammation. Then IL-18 promotes IL-12-committed Th1 responses. If IL-18 producers release only IL-18, Th2-related cytokines are produced, which evokes atopic responses. IL-18 is then involved in Th2-cell development when appropriate antigenic stimulation is present. Thus, IL-18 produces the opposite immunological results depending on whether or not it acts cooperatively with IL-12.

IL-18 stimulates not only conventional constituents of the innate immune system, such as NK cells, macrophages, dendritic cells, mast cells, and basophils, but also adaptive immunity members, such as T cells and B cells, but is dispensable for their T-cell receptor (TCR) and B-cell receptor (BCR) engagement, respectively. These IL-18-responsive cells show immunologically opposite responses depending on whether they encounter IL-18 with or without IL-12. If only IL-18 is released, these cells produce Th2-related cytokines. Such conditions are referred to as innate atopic responses, which would drive adaptive immunity toward Th2 responses if antigenic stimulation works. If IL-18 acts on them together with IL-12, they produce Th1-related cytokines. This is referred to as the innate inflammatory response, which would drive and promote Th1 responses. In natural infection, intracellular microbes induce Th1 responses by two means. One is their PAMPs, which are capable of inducing IL-12 and IL-18 production, and the other is the protein that is presented to naive T cells as a peptide/major histocompatibility complex combination. Under other conditions, IL-18 that is secreted without IL-12 and that is stimulated by unknown molecules directly induces Th2 responses.

## A. Roles of IL-18 in Innate Immunity

IL-18 stimulates various constituents of innate immunity to release IFN-γ, particularly in collaboration with IL-12 (Fig. 3). IL-18 together with IL-12 induces very high levels of IFN-γ production by NK cells, dendritic cells, and macrophages. Naive CD4$^+$ T cells and even Th1 cells, although important constituents of the adaptive immune system, also produce a large amount of IFN-γ in response to IL-12 and IL-18 without TCR engagement, indicating that CD4$^+$ T cells also have the capacity to play a role in innate immunity. Although the molecular mechanisms of the synergistic action of IL-12 and IL-18 on IFN-γ production are unclear, IL-12-induced up-regulation of their IL-18R expression might be partially involved in this synergism. Recently, it was demonstrated that Tyk2, an essential JAK kinase for IL-12 signaling, is also required for IL-18-induced IFN-γ production by NK cells. Tyk2-deficient NK cells produce no IFN-γ in response to IL-18, suggesting the possible involvement of Tyk2-mediated signaling in IL-18-induced IFN-γ production. The molecular basis for Tyk2-mediated IL-18 signaling is unknown. As expected, the synergistic action of IL-18 and IL-12 for IFN-γ production is essential for clearance of some types of pathogenic microbes, particularly intracellular pathogens (described below).

IL-18 solely induces production of hematopoietic cytokines, such as IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) by CD4$^+$ T cells. Interestingly, IFN-γ-deficient CD4$^+$ T cells produce much more IL-5 in response to IL-18 than wild-type cells, indicating a down-regulatory action of endogenous IFN-γ for IL-18-induced IL-5 production. Daily administration of IL-18 increases circulatory eosinophil numbers in mice, followed by neutrophilia. IL-18-transgenic mice or caspase-1-transgenic mice that spontaneously release mature IL-18 display extramedullar hematopoiesis, particularly in myeloid lineages. However, we do not know whether IL-18 is involved in homeostasis and/or pathogenesis of the hematopoietic system.

IL-18 has the capacity to induce Th2-related cytokine production by CD4$^+$ T cells, NK cells, basophils, and mast cells (Fig. 3). CD4$^+$ T cells produce IL-4 and IL-13 in the presence of IL-2 but without TCR engagement, whereas NK cells produce only IL-13 on stimulation with IL-18. Bone marrow-derived mast cells and basophils express IL-18R and release IL-4, IL-13, and histamine in response to IL-18 in collaboration with IL-3. These cytokines and chemical mediators are major relevant and/or effector molecules that induce atopic phenotypes, such as broncho-constriction and mucous formation in asthma and itching in atopic dermatitis. It is well established that cross-linkage of FcϵR I (high affinity receptor for Fc portion of IgE) on these cells by allergen-specific immunoglobulin E (IgE)/allergen is essential for the release of these effector molecules. In contrast, IL-18 can exert these actions without help from allergen-specific IgE/allergen, suggesting abnormal accumulation of IL-18 as a causative factor for atopic diseases.

IL-18, like IL-12, enhances the cytolytic activity of NK cells. In contrast to the synergism of IL-18 and IL-12 in IFN-γ production, there is no synergy in enhancement of their cytolytic activity. IL-18 does not increase production of their cytolytic effector molecules, such as perforin and granzyme B, a serine protease, whereas IL-12 does up-regulate their perforin and granzyme B production, suggesting an up-regulating mechanism of IL-18 distinct from that of IL-12. Unlike IL-12, IL-18 can induce FasL expression on NK cells and Th1 cells. This is also the case for hepatic NKT cells, a subpopulation expressing both NK-cell marker and the TCR.

Like IL-12, IL-18 plays a role in the functional development of NK cells. Both IL-12-deficient mice and IL-18-deficient mice have comparable numbers of splenic NK cells as wild-type mice. However, the cytolytic activity of IL-12-deficient or IL-18-deficient NK cells against NK-cell targets is decreased compared with wild-type NK cells. NK-cell activity in IL-12 and IL-18 double-knockout mice is most strongly impaired. Interestingly, NK cells from any mutant or wild-type mice equally increase their cytolytic activity in response to IL-12 and/or IL-18, indicating that NK cells do not require endogenous IL-12 or IL-18 for acquisition of responsiveness to IL-18 and/or IL-12.

### B. Roles of IL-18 in Th1 Immune Responses

Although IL-18, like IL-12, is a potent factor to induce IFN-γ production, IL-18, unlike IL-12, fails to induce Th1-cell development. CD4$^+$ T cells from IL-18-deficient mice that had been administered heat-killed *P. acnes*, a prototype *in vivo* Th1-cell inducer, show only slight impairment in Th1 development compared with *P. acnes*-primed wild-type mice, whereas CD4$^+$ T cells from *P. acnes*-primed IL-12-deficient mice lack Th1 development, indicating that IL-12 but not IL-18 is a Th1-cell-inducing factor. This is also the case for *Le. major* infection, clearance of which is completely dependent on the intensity of *Le. major*-specific Th1 cells. Because they normally develop into Th1 cells, IL-18-deficient mice with a resistant background show weak susceptibility to *Le. major* compared with wild-type mice with the same background. Indeed, due to their failure in Th1-cell development, IL-12-deficient mice display profound susceptibility to *Le. major.*

### C. Roles of IL-18 in Th2 Immune Responses

Although IL-18 was initially recognized as a Th1-activating factor, recent studies show that IL-18 is a potent Th2-cell-differentiation factor. Exogenous IL-18 stimulates CD4$^+$ T cells to develop into Th2 cells on TCR engagement via induction of endogenous IL-4. However, Th2 cells lose responsiveness to IL-18 but retain responsiveness to IL-4, indicating that IL-4 is a Th2-initiating and -sustaining factor but that IL-18 only initiates Th-cell development.

It is noted that CD4$^+$ T cells that have been treated solely with IL-18 are not Th2 cells, indicating that Th2 as well as Th1-cell development definitely requires simultaneous signaling through their TCRs in addition to signals of Th2- and Th1-driving factors, respectively. Interestingly, exogenous IL-18 can up-regulate their CD40 ligand expression, which is essential for inducing IgE production by B cells. Daily administration of IL-18 increases serum levels of IgE in wild-type mice, but not in IL-4-deficient mice, in 2 weeks without any antigen stimulation. IL-18 or caspase-1-transgenic mice that overexpress mature IL-18 spontaneously show high serum levels of IgE, whereas depletion of the Stat6 gene inhibits their high levels of IgE, indicating that accumulation of IgE is dependent on Stat6 in these transgenic mice. This IL-18 induction of IgE is inhibited by anti-CD40 ligand but not by a soluble decoy receptor for IL-13, which inhibits selective biological actions of IL-13 but not IL-4. All the results indicate a requirement of both CD40/CD40 ligand and IL-4/Stat6 but not IL-13 for IL-18-induced IgE induction in mice.

### D. Others

IL-18 acts not only on the immune system but also on other systems and physiological processes, such as the endocrine system, the nervous system, and bone metabolism. Under conditions of stress, IL-18 is released from the adrenal gland, suggesting the possible involvement of IL-18 in stress-induced systemic alterations. Osteoblast-derived IL-18 suppresses osteoclast development via induction of GM-CSF production, indicating that IL-18 is an important factor for bone metabolism. Recently, it was shown that intracerebroventricular administration of IL-18 prolongs nonrapid eye movement sleep, suggesting the involvement of IL-18 in sleep control.

## VI. PATHOPHYSIOLOGICAL ROLES

Genetic deletion and transgenic techniques have revealed diverse actions of IL-18 in homeostasis of the immune system and various immune system diseases. IL-18 plays important protective roles in host defense against microbial infection or tumor. However, an overabundance of IL-18 is often harmful enough to induce inflammatory or even atopic diseases.

### A. IL-18 in Host Defense

As briefly described above, IL-18 is critical for host defense against some types of pathogenic microbes. In particular, IL-18 plays a major role in the clearance of intracellular microbes, such as *Li. monocytogenes*, *Mycobacterium* spp., *C. neoformans*, a pathogenic fungus, *Toxoplasma gondii*, a pathogenic protozoan, and *Le. major.* In the defense against these microbes, endogenous IL-18 as well as IL-12 evokes local and/or systemic inflammatory reactions, eventually leading to the induction of effector molecules, such as reactive oxygen intermediates and nitric oxide derivatives (Fig. 4).

IL-18 and IL-12 are also essential in expelling some types of viruses. Previously, type I IFN produced by various types of cells was believed to be only the first line against viral infection. However, recent studies have shown that double-stranded RNA activates TLR3 to release various proinflammatory cytokines, which together result in the production of virus-killing substances and/or directly inhibit viral replication (Fig. 4). Depletion of IL-18 renders mice susceptible to some types of viral infection.

## B. IL-18 Against Tumors

Like IL-12, exogenous IL-18 induces regression of tumor in mice. However, molecular mechanisms are distinct. IL-12 treatment enhances perforin/granzyme-mediated anti-tumor actions of host cytotoxic cells, and IL-18 elevates FasL expression on effector cells that kill Fas-expressing tumor cells *in vivo*. IL-18 gene therapy is useful to reduce the growth of some types of tumors.

## C. IL-18 in Immune Diseases

IL-18 plays critical roles in the development of various types of diseases from Th1-cell-associated autoimmune diseases, inflammatory diseases, and Th2-cell-related atopic diseases (Table 1).

IL-18 promotes various autoimmune diseases. Autoimmune diseases are caused by a disturbance of self-tolerance through unknown mechanisms. IL-18 appears to be involved in local autoimmune diseases associated with Th1 cells. Here, we show several examples.

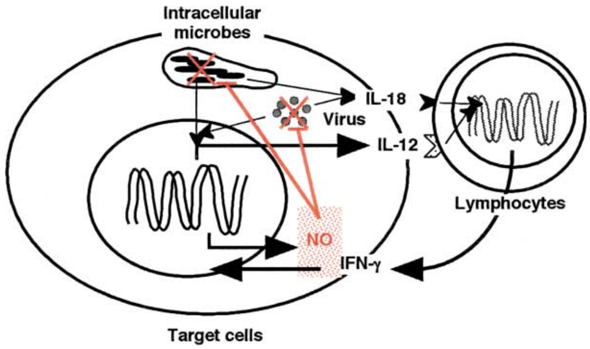

**FIGURE 4** Involvement of IL-18 in host defense. Intracellular microbes or viruses stimulate host macrophages presumably through the appropriate TLR to release proinflammatory cytokines, including IL-18 and IL-12. IL-18, in combination with IL-12, activates lymphocytes to release a large amount of IFN-γ, which in turn fully activates macrophages to produce microbicidal molecules such as nitric oxide (NO).

In patients with insulin-dependent diabetes mellitus (IDDM) or nonobese diabetes (NOD) mice, which is a mouse model of spontaneous IDDM, pancreatic beta cells are selectively destroyed, presumably by insulin or insulin-related enzyme-specific T cells that would be depleted in healthy or wild-type mice. Administration of neutralizing anti-IL-18 reduces pathological changes in NOD mice, suggesting that IL-18 is an exacerbating factor for development of IDDM.

Patients with multiple sclerosis, an autoimmune disease of the central nervous system, and mice with experimental autoimmune encepharitis (EAE), which is induced by immunization with neural myelin-derived products, display high serum levels of IL-18 and express activated caspase-1 in their neural lesions. The severity of the disease correlates with the levels of IL-18 in the lesion in the mouse model. Administration of neutralizing anti-IL-18 or caspase inhibitors reduces the disease score in EAE mice.

Many investigators have reported a possible contribution of IL-18 in rheumatoid arthritis, which is a Th1-cell-driven autoimmune disease of the joints. High levels of IL-18 are expressed in the synovia of the patients. This is also the case for a mouse model that is induced by collagen administration.

IBD, especially Crohn's disease, is also associated with IL-18. Lesions in patients with Crohn's disease express the active form of IL-18 and caspase-1. Mice having received trinitorobenzene sulfate (TNBS), a mouse model of IBD, accumulate IL-18 in the colitis lesion. Their disease score parallels IL-18 concentration in the lesion. Furthermore, administration of IL-18BP protects against colitis induced by TNBS, indicating that IL-18 is an endogenous causative molecule for IBD.

IL-18 is profoundly involved in inflammatory diseases, such as septic syndrome and acute GVHD (Table 1). Septic syndrome is initiated by the activation of host innate immunity by the causative bacteria or bacterial products. It is plausible that IL-18 is released via activation of TLRs in septic syndrome. In addition, treatment with neutralizing anti-IL-18 inhibits lethality and/or tissue damage associated with sepsis. In particular, liver injury is prevented by neutralization of IL-18 or depletion of the gene encoding IL-18 or caspase-1. Acute GVHD, a potentially fatal side effect of allogeneic cell transplantation, is tightly associated with the elevation of serum levels of IL-18. The disease severity is also correlated with the level of IL-18.

TABLE 1 Roles of IL-18 in Various Immune Diseases

| Types | Examples | Pathological roles | |
|---|---|---|---|
| | | Without IL-12 | With IL-12 |
| Autoimmune diseases | Insulin-dependent diabetes mellitus | + | ++ |
| | Multiple sclerosis | + | ++ |
| | Rheumatoid arthritis | + | ++ |
| | Inflammatory bowel diseases | + | ++ |
| Inflammatory diseases | Septic syndrome | + | ++ |
| | Acute graft-versus-host disease | + | ++ |
| Atopic diseases | Atopic dermatitis | + | − |
| | Bronchial asthma | +[a] | − |

*Note*. Endogenous IL-18 (without IL-12) exerts its biological actions to up-regulate (+) the severity of individual immune diseases. Exogenous IL-12 (with IL-12) up-regulates (++) or down-regulates (−) each immune disease.

[a] IL-18 shows reciprocal effects on the disease severity depending on the time points when IL-18 acts.

Atopic diseases are also targeted by IL-18. Transgenic mice that express an excess amount of active IL-18 spontaneously develop atopic dermatitis-like cutaneous changes associated with high serum levels of IgE. In fact, patients with atopic dermatitis have a higher concentration of IL-18 in their sera than do healthy volunteers.

## VII. SUMMARY

Various types of cells produce IL-18 as a biologically inactive precursor. On appropriate stimulation, bioactive IL-18 is secreted after cleavage with the appropriate enzymes. IL-18 has a wide spectrum of biological actions. IL-18 with IL-12 potentially forces inflammatory responses by inducing IFN-$\gamma$ production by various types of cells, whereas IL-18 solely drives atopic responses via IL-4 production by the restricted cell types T cells and basophils. IL-18, whether acting as an inflammation-driving factor or an atopy-inducing element, plays a critical role in host defense but is sometimes involved in various immunological diseases. Furthermore, IL-18 has the potential to modulate nonimmune systems. Future studies, particularly studies on the roles of IL-18 in nonimmune systems, will establish a comprehensive characterization of IL-18, only a part of which we now know.

## Glossary

**adaptive immune system** Consists mainly of T cells and B cells that show hyperdiversity based on their DNA rearrangement and somatic mutation.

**atopic disorder** Condition resulting from the immediate hypersensitivity based on the abnormal production of immunoglobulin E (IgE) against environmental antigens. Atopic disorders have different clinical manifestations, such as hay fever, asthma, urticaria, or chronic eczema. Antigen, usually called allergen, cross-links allergen-specific IgE bound to Fc$\epsilon$RI on mast cells and activates them to release various chemical mediators and cytokines, leading to development of pathological alterations.

**autoimmune diseases** Diseases that are caused by defects of self-tolerance and are divided into two groups based on their effector mechanisms. Abnormal production of autoantibodies against autoantigen results in immune complex-induced systemic or autoantigen-specific disorders, depending on the properties of autoantibodies. Self-reactive T cells also cause tissue-specific disorders.

**caspases** Cysteine proteases that cleave corresponding substrates following the aspartate site and are involved in apoptosis and/or cytokine processing. More than 10 members have been identified. Caspase-1, originally termed interleukin-1$\beta$ (IL-1$\beta$)-converting enzyme, has the capacity to cleave biologically inactive precursors IL-1$\beta$ and IL-18 into the corresponding biologically active forms.

**innate immune system** The primitive defensive system, including barriers between the inner and the outer environments, defensive proteins in the circulation, and immune competent cells except for T cells and B cells. Recently, it was shown that innate immunity can strictly discriminate microbe products from self-molecules by the corresponding receptors.

**Th1/Th2 immune responses** On stimulation with antigens, T cells develop into Th1 and Th2 cells in the presence of interleukin-12 (IL-12) or IL-4, respectively. Th1 cells selectively secrete interferon-$\gamma$ and IL-2 but not IL-4, IL-5, or IL-13, whereas Th2 cells express the inverse cytokine profile. Imbalance or dysregulation of

Th1/Th2 immune responses occasionally determines successful host defense or disease severity.

**Toll-like receptor (TLR)** The signaling receptor for various microbe products in innate immunity; it is required for discrimination of microbes from self-molecules. TLR is composed of more than 10 members. Each TLR recognizes distinct microbe products called pathogen-associated molecular patterns (PAMPs). All the TLRs examined share a common signaling pathway. On stimulation with PAMPs, the intracellular domain of the corresponding TLR binds to an intracellular adapter molecule, myeloid differentiation factor 88 (MyD88), to relay a signal to activate nuclear factor κB, a transcriptional factor.

## See Also the Following Articles

**Glucocorticoids and Autoimmune Diseases • Diabetes Type 1 • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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# Interleukin-22

Sidney Pestka[*], Sergei V. Kotenko[†], and
Paul B. Fisher[‡]

[*]*University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School* • [†]*University of Medicine and Dentistry—New Jersey Medical School* • [‡]*Columbia University College of Physicians and Surgeons*

I. DISCOVERY OF IL-22
II. THE IL-22 GENE
III. THE IL-22 RECEPTOR COMPLEX
IV. EXPRESSION OF THE IL-22R1 mRNA
V. NATURALLY OCCURRING IL-22 ANTAGONIST
VI. EXPRESSION OF THE IL-22BP mRNA
VII. IL-22R1, IL-10R2, AND IL-22BP GENES

**Interleukin-22 is an interleukin-9-induced cytokine that is active on B and T lymphocytes, mast cells, and eosinophils. The roles of this**

**hormones such as growth hormone and prolactin.**

## I. INTRODUCTION

Interleukin-2 (IL-2), a 15.5-kDa glycoprotein, is functionally confined to the immune system: only antigen-activated T cells produce it and lymphocytes are the only cells that express IL-2 receptors. IL-2 promotes the proliferation, differentiation, and survival of mature lymphocytes, especially T cells. Consequently, IL-2 is obligatory for a physiologic antigen-specific acquired cellular immune response, and it also can promote innate host defenses by activating natural killer (NK) cells. *In vivo*, IL-2 regulates the tempo, magnitude, and duration of the T-cell immune response. As well, because the size of the memory cell pool is dependent on IL-2, the degree of protective immunity is determined in large part by the availability of IL-2. Accordingly, IL-2 is presently in use therapeutically for the augmentation of the immune system in the treatment of cancer and infectious diseases.

The interleukin-2 receptor (IL-2R) was the first of the cytokine receptors to be identified and characterized. The IL-2R is composed of three distinct chains ($\alpha$, $\beta$, and $\gamma$), each of which participates in the formation of the binding site for IL-2. The $\alpha$-chain contributes to IL-2 binding a very rapid association rate; the $\beta\gamma$ dimer contributes a slow dissociation rate, making for a very high-affinity binding site ($K_d = 10$ p$M$).

If a deficiency of IL-2R triggering occurs, either as a result of genetic defects, or pharmacologically, profound immunodeficiencies can be produced. For example, genetic deficiencies of IL-2 or IL-2R $\alpha$ or $\beta$ chains result in immunodeficiency early after birth, with a later accumulation of activated T cells that do not function properly. It is significant that these phenotypic changes cannot be compensated by other cytokines. Mutations of the IL-2R $\gamma$ chain results in severe combined immunodeficiency. Given these findings, it is not surprising that pharmacological agents, and therapeutic monoclonal antibodies reactive with the IL-2R that block the IL-2-IL-2R system, are potent immunosuppressives and are the main agents employed to prevent organ transplant rejection.

## II. STRUCTURES

Human IL-2 is a small globular glycoprotein of 133 amino acids. There are four antiparallel amphipathic $\alpha$-helices with one intrachain disulfide bond that is essential for molecular integrity and activity. The high-affinity IL-2R is composed of three noncovalently linked type I transmembrane chains of 55 ($\alpha$-chain), 75 ($\beta$-chain), and 65 ($\gamma$-chain) kDa. The $\alpha$-chain has only 13 intracellular amino acid residues and does not participate in signaling, whereas the $\beta$- and $\gamma$-chains have large intracellular portions and are responsible for signaling the interior of the cell. The IL-2R binds IL-2 with an extremely high affinity ($K_d = 10$ p$M$), owing to a rapid association rate contributed by the $\alpha$-chain ($k = 10^7\ M^{-1}\ s^{-1}$) and a slow dissociation rate ($k' = 10^{-4}\ s^{-1}$) contributed by the $\beta\gamma$ chain heterodimer.

## III. FUNCTIONS

The concentrations of IL-2 that bind to IL-2Rs are identical to the concentrations that promote a T-cell proliferative response, i.e., the $K_d = EC_{50}$. Thus, the IL-2 binding and biological response curves are coincident, and there are no “spare” receptors. Moreover, there are a finite number of IL-2–IL-2R interactions that must occur before a cell is triggered to undergo cell cycle progression. Accordingly, the concentration of IL-2 available, the density of IL-2Rs, and the duration of their interaction determine the biological effects of IL-2, and each individual cell responds in a quantal (all-or-none) fashion. The number and duration of intermolecular interactions determine the “strength” of the IL-2–IL-2R signal delivered to the cell. The signaling molecules triggered by the IL2–IL2R interaction include the Janus tyrosine-specific kinases, JAK1 and JAK3, which are activated by the stabilized IL-2–IL-2R complex. Subsequently, the JAKs catalyze the phosphorylation of themselves as well as the $\beta$- and $\gamma$-chains of the IL-2R, which then serve as docking sites for the signal transducers and activators of transcription (Stat5a and Stat5b). In addition to the JAK/Stat pathway, the IL-2–IL-2R interaction activates the Ras/Raf/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase/Akt pathways, which then are involved in activating transcription factors that are responsible for activating the expression of specific genes that promote cell survival, proliferation, and differentiation. Some of the important genes expressed as a consequence of IL-2 signaling are known; Bcl-2 and Bcl-X, for example, are important in promoting cell survival, and cyclin-D2 is important in promoting progression through the cell cycle. However, many other genes are yet to be discovered.

## IV. IL-2 REGULATION OF THE IMMUNE RESPONSE

IL-2 is the principle cytokine responsible for activating the proliferation, differentiation, and survival of peripheral mature T cells once they have become activated by antigen. In particular, IL-2 stimulates a marked proliferative expansion of CD8 + T cells, which are responsible for recognizing and reacting with intracellular infections, such as those caused by viruses. As well, this subset of cytolytic T cells (CTLs), which acquire the capacity to lyse infected target cells, is also active in the destruction of transplanted organs. By comparison, the other major subset of T cells, which express the CD4 surface molecule, differentiate into “helper” cells ($T_H$ cells), by virtue of their capacity to secrete large amounts of cytokines and also to express surface helper molecules. Thus, CD4 + T cells help CD8 + T cells by secreting IL-2. Although CD8 + T cells can also produce IL-2, CD4 + T cells produce approximately 80% of the IL-2 released during an immune reaction. In experimental viral infections, a rapid release of IL-2 promotes a massive expansion of CD8 + T cells, as much as 100,000-fold, within just a few days. However, in mice that have had the IL-2 genes deleted, the proliferative expansion is only ~10% of that observed in wild-type mice. The degree to which antigen-specific T cells expand after a primary antigenic stimulation determines the eventual size of the “memory” T-cell population. Therefore, IL-2 is responsible for the size of this population and for the efficacy of the immune response to reinfection.

IL2 is also responsible for the differentiation that produces CTLs and $T_H$ cells. IL-2 augments the expression of genes that encode cytolytic molecules, such as perforin and the granular proteases (granzymes), and the cytokine molecules that define the so-called $T_H1$ and the $T_H2$ cell subsets. Additionally, IL-2 appears to maintain T-cell homeostasis by generating feedback down-regulatory effects, thereby limiting the proliferation and differentiation initiated on the encounter with antigen.

## V. IL-2 THERAPY

### A. Pharmacodynamics

The pharmacodynamics of IL-2, or how IL-2 affects the cells and tissues of the body, are based on the type and distribution of the IL-2Rs. High-affinity, trimeric (αβγ) IL-2Rs are expressed only transiently on antigen-activated T cells and B cells, whereas they are constitutively expressed by ~10% of NK cells. Because the affinity of the heterotrimeric IL-2Rs is very high (10 p*M*), only very low IL-2 concentrations (<100 p*M*) are necessary to saturate them. By comparison, 90% of NK cells express the β- and γ-chains of the IL-2R, but not the α-chain, so that the $K_d$ is 100-fold higher. This 100-fold difference in affinity, combined with the small number of recently antigen-activated T cells and B cells (<1%), vs NK cells ($\sim 10^9$ cells/μl), accounts for the main difference in the effects of high doses of IL-2, as have been used in cancer therapy, vs low doses, as used in infectious diseases such as human immunodeficiency virus (HIV) infection. High IL-2 doses result in systemic IL-2 concentrations that saturate most of the IL-2Rs expressed by NK cells, which leads to their activation and their secretion of proinflammatory cytokines, resulting in severe systemic toxicity. In contrast, if the IL-2 doses are lowered, and the systemic IL-2 concentration remains <100 p*M*, only the high-affinity IL-2Rs are saturated, thereby avoiding the activation of most of the NK cells and the attendant toxicity.

### B. Pharmacokinetics

The pharmacokinetics of IL-2, or what the body does with the glycoprotein, are dependent on the characteristics of the IL-2 molecule. Because IL-2 is a small (15.5 kDa) globular glycoprotein, it passes freely between capillary and lymphatic endothelial cells; after intravenous administration, it distributes into the total extracellular space, which in a normal adult is about 15 liters. This α-decay phase, due to distribution, occurs with a half-time of ~10 min. Therefore, within 40 min, >94% of the initial peak concentration is dissipated. Subsequently, there is a β-decay due to renal excretion, which has a half-time of ~3 h. After subcutaneous administration, there is a half-time of ~1 h for absorption, followed by renal excretion, so that peak plasma concentrations occur at ~2–3 h and detectable plasma concentrations are still present after 12–16 h. Therefore, low plasma IL-2 concentrations can be maintained by once or twice daily subcutaneous injections. After a subcutaneous or intradermal injection of IL-2, there is a characteristic and classic delayed-type hypersensitivity (DTH) reaction that develops within 24 h. The cardinal signs of inflammation (i.e., rubor, calor, dolor, tumor) are due to the IL-2-activation of NK cells and T cells, which then produce proinflammatory cytokines, leading to the extravasation of plasma and cells at the site of IL-2 injection.

### C. Clinical Results

In cancer treatment, IL-2 is used in very high doses (i.e., 150 million units (U)/day) for short treatment intervals of 3–5 days. At these doses, IL-2 causes acute systemic inflammation, with high fever, malaise, myalgia, fatigue, and hypotension. This therapy eventually results in an antitumor response in ~15% of subjects, and ~5% of patients with renal cell carcinoma and malignant melanoma achieve a long-term, complete response.

The treatment of HIV infection is the other major disease category for which IL-2 has been used. In patients with chronic infection and low levels of circulating CD4+ T cells, doses that are ~10-fold lower than those used in cancer therapy have been found to be effective in increasing the concentration of CD4+ T cells. However, these doses are still quite toxic and can be tolerated only for ~5 days. Low-dose daily IL-2 administration is now being tested in conjunction with therapeutic HIV vaccines with coadministration of antivirals, to augment HIV-specific immunity. This has become an important new area for immune-based therapies because the available antiviral drugs are effective in suppressing viral replication, but they do not cure the infection, so that when discontinued the virus begins replicating immediately and a viral relapse occurs almost universally. Consequently, the new therapeutic approaches are designed to augment HIV immune reactivity by boosting the number and function of HIV-specific T cells, especially CD8+ CTLs. A similar approach is planned to test IL-2 for the treatment of chronic hepatitis C virus (HCV) infection.

## VI. IMMUNOSUPPRESSIVE THERAPY

The mechanisms of action of the most commonly used immunosuppressive drugs are focused on the prevention of either production or action of IL-2. Glucocorticoid hormones, which have been widely used as immunosuppressives and anti-inflammatory agents for the past 50 years, inhibit the transcription of IL-2 and other cytokine genes. Similarly, the drugs cyclosporin A and FK506 also inhibit IL-2 gene expression, although by blocking slightly different transcriptional activating pathways.

A monoclonal antibody reactive with the IL-2R α-chain has recently been found to be very effective in blocking the IL-2–IL-2R interaction and attenuating the rejection response to renal and cardiac allografts. Also, in combination with rapamycin, which blocks IL-2R signaling, these antibodies have recently been shown to prevent the rejection of pancreatic islet cell grafts used for the treatment of type 1 diabetes mellitus.

## VII. SUMMARY

IL-2 is the major mature T-cell growth, differentiation, and survival factor (cytokine) operative in the immune system. It promotes the proliferative expansion and function of antigen-selected T-cell clones and is responsible for the generation of protective immunity and immunologic memory. As a small globular glycoprotein that interacts with high-affinity stereospecific cell surface receptors, it mediates its effects by activating tyrosine-specific kinases and the transcriptional activation of specific genes. In the clinic, it is being used to augment immune responses for the treatment of cancer and infectious diseases, and drugs that block the production or action of IL-2 are effective immunosuppressives.

## Glossary

**cytokines** Cell products that act as hormones, serving to convey messages from one cell to another.

**interleukin** Term meaning "between leukocytes"; originally coined to describe the first cytokines known to be involved in the regulation of the immune and inflammatory systems.

**Janus kinases** Class of tyrosine-specific enzymes that function as second messengers; act intracellularly to signal molecules in the cytoplasm and nucleus by catalyzing the phosphorylation of tyrosine residues on target molecules.

**signal transducers and activators of transcription (STATs)** Molecules that act as second messengers by receiving signals from the cytoplasmic domains of cell surface receptors; on becoming activated, they transform into activators of transcription.

## See Also the Following Articles

**Interleukin-1, Interleukin-4, etc. (multiple Interleukin entries)**

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# Interleukin-4

WILLIAM E. PAUL
*National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda*

I. INTRODUCTION
II. INTERLEUKIN-4 STRUCTURE
III. THE INTERLEUKIN-4 RECEPTOR
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**Interleukin-4 is a type I cytokine that expresses a wide range of biologic functions. Among its most important actions is control of immunoglobulin class switching to IgE and of differentiation of naive CD4 T cells into $T_H2$ cells. It is the principal regulator of allergic and antihelminthic responses.**

## I. INTRODUCTION

Interleukin-4 (IL-4) is produced by a subset of CD4 T helper cells ($T_H2$), by natural killer (NK) T cells, by basophils and mast cells, and by eosinophils. IL-4 mediates its function by interacting with two types of receptors (Rs), both of which utilize the IL-4Rα chain. The type I receptor also utilizes the common γ-chain (γc) whereas the type II receptor utilizes IL-13Rα1. In response to IL-4, IL-4Rα becomes tyrosine phosphorylated and mediates its signaling function by the recruitment of a series of signaling intermediates, including a signal transduction and activation transcription protein (Stat6) and insulin receptor substrate-2 (IRS-2). Regulation of IL-4 expression has potential importance in determining the severity of allergic diseases and, possibly, in controlling destructive autoimmunity.

## II. INTERLEUKIN-4 STRUCTURE

IL-4, a typical "short hematopoietin," is a ~20,000-Da glycoprotein consisting of 153 amino acids (in humans) or 140 amino acids (in mice). Its three-dimensional structure is typical of short hematopoietins. IL-4 is a globular protein consisting of four short α-helices, in which the first (A) and second (B) are parallel to one another and connected by a long, overhand loop. The third (C) and fourth (D) helices are parallel to each other but are antiparallel to helices A and B. The α chain of the IL-4 receptor interacts with regions in helices A and C, whereas contact residues for the γc chain of the receptor have been identified on helix D.

## III. THE INTERLEUKIN-4 RECEPTOR

The IL-4 receptor uses a typical short hematopoietin receptor chain, IL-4Rα, as a high-affinity binding element and a second polypeptide, either γc (the type I receptor) or IL-13Rα1 (the type II receptor). The type II receptor is also the principal receptor for IL-13. This implies that IL-4 and IL-13 mediate many functions in common. However, the type II receptor is not found on T cells, thus IL-4 is unique in its capacity to induce $T_H2$ differentiation.

For IL-4 to mediate signaling, it must heterodimerize IL-4Rα with either γc or 13Rα1. A mutation in human IL-4, Y124D (where Y is tyrosine and D is aspartic acid, using the one-letter amino acid code), results in inability to bind to the human γc chain. Because the mutant retains its capacity to bind to IL-4Rα, it is capable of binding but not signaling through the type I receptor and thus is a powerful competitive inhibitor of many IL-4 functions.

The extracellular segment of IL-4Rα consists of two type III fibronectin domains. The membrane distal extracellular domain contains conserved paired cysteines and the membrane-proximal extracellular domain contains a conserved WSXWS motif

**cytokine have yet to be fully elucidated, but it appears to be involved in mechanisms controlling immunoregulatory and inflammatory responses, including allergies and asthma.**

## I. DISCOVERY OF IL-22

Interleukin-22 (IL-22) is one of several recently discovered cytokines with limited primary homology to IL-10. Using a complementary DNA subtraction technique in mouse T cells, the cDNA for mouse IL-22 has been identified as a gene specifically induced by IL-9. A protein encoded by the cDNA that was originally designated IL-10-related T-cell-derived inducible factor (IL-TIF) is now designated IL-22. The protein demonstrates 22% identity to IL-10 and consists of 280 amino acids, including a potential signal peptide. The expression of IL-22 is induced by IL-9 in several cell lines, including BW5147 thymic lymphoma cells, T helper cell clone TS2, and MC9 mast cells, and is also induced by IL-9 or concanavalin A (Con A) in freshly isolated splenocytes. Constitutive expression of the IL-22 gene is detected in thymus and brain. Recombinant IL-22 produced by HEK293 cells transiently transfected with the IL-22 cDNA represents a heterogeneous protein that migrates on gel in the region of 25–30 kDa, indicating glycosylation of the protein. Recombinant IL-22 activates signal transducers and activators of transcription (Stat) factors, predominantly Stat3 and Stat5, in MES13 murine kidney mesangial cells and PC12 rat pheochromocytoma cells.

Human IL-22 has been cloned based on its homology to the mouse orthologue and also as a novel secreted protein. Recombinant human IL-22 expressed in COS cells appears to be a glycosylated secreted protein migrating on gel as several bands in the region of about 25 to 40 kDa. IL-22 mRNA expression is induced in T cells by anti-CD3-stimulation and is further up-regulated by the addition of Con A. It has been reported that activated human and mouse $T_H1$ rather than $T_H2$ CD4 + T helper cells produce IL-22. It has also been demonstrated that lipopolysaccharide (LPS) injection *in vivo* stimulates IL-22 mRNA expression in various organs. As a first step in determining biological activities and the receptor for IL-22, several IL-22-responsive cell lines, as measured by Stat activation, have been identified. Rapid Stat activation following treatment with IL-22 is observed in TK-10 renal carcinoma, SW480 colon adenocarcinoma, A549 lung carcinoma, HepG2 and HepG3 hepatoma, HT29 intestinal epithelial cell line, and several melanoma cell lines. Stat3, to a lesser extent Stat1, and in certain cells Stat5 are the transcriptional factors of the Stat family activated in cells after IL-22 treatment. Biological activities of IL-22 are not well defined yet. It has been shown that stimulation of HepG2 human hepatoma cells with IL-22 up-regulates the production of acute-phase reactants such as serum amyloid A, 1-antichymotrypsin, and haptoglobin. Similar effects are observed *in vivo*: IL-22 injection induces production of serum amyloid A in mouse liver. These observations suggest involvement of IL-22 in the inflammatory response. IL-22, in contrast to IL-10, has no effect on LPS-induced production of tumor necrosis factor α (TNFα), IL-1, and IL-6 from freshly isolated human monocytes. Furthermore, IL-22 does not inhibit the action of IL-10 in these assays. IL-22 has no effect on interferon γ (IFNγ) production from *in vitro* polarized $T_H1$ cells; however, modest inhibitory effects of IL-22 on IL-4 production from $T_H2$ cells is observed. Administration of murine IL-22 either by intravenous injection or by adenovirus results in systemic effects, i.e., decreased red cell count and serum albumin levels and increased platelet count, serum amyloid A levels, fibrinogen levels, and body weight. Basophilia in the proximal renal tubules is also observed. Further experiments are necessary to elucidate the role of IL-22 in inflammation and in immunoregulation.

## II. THE IL-22 GENE

A search for the IL-22 gene led to a surprising discovery. Whereas there is a single copy of the IL-22 gene in the human genome and in genomes of BALB/c and DBA/2 mice, genomes of other mouse strains, such as C57BL/6, FVB, and 129, possess two IL-22 genes, which were originally designated IL-TIFα and IL-TIFβ. Apparently, the gene has been duplicated and two copies are well conserved, demonstrating 98% nucleotide identity in the coding region. The murine genes map to chromosome 10 in the region of the IFNγ gene. The human IL-22 gene is located on chromosome 12 about 90 kb from the IFNγ gene (Fig. 1). The human IL-22 gene and the murine IL-TIFα gene consist of six exons; exon 1 contains a part of the 5′ untranslated region (UTR) and exons 2–5 cover the coding region of the IL-22 mRNA. The murine IL-TIFβ gene, in addition to several single nucleotide variations, contains a 658-nucleotide deletion covering the first noncoding exon and

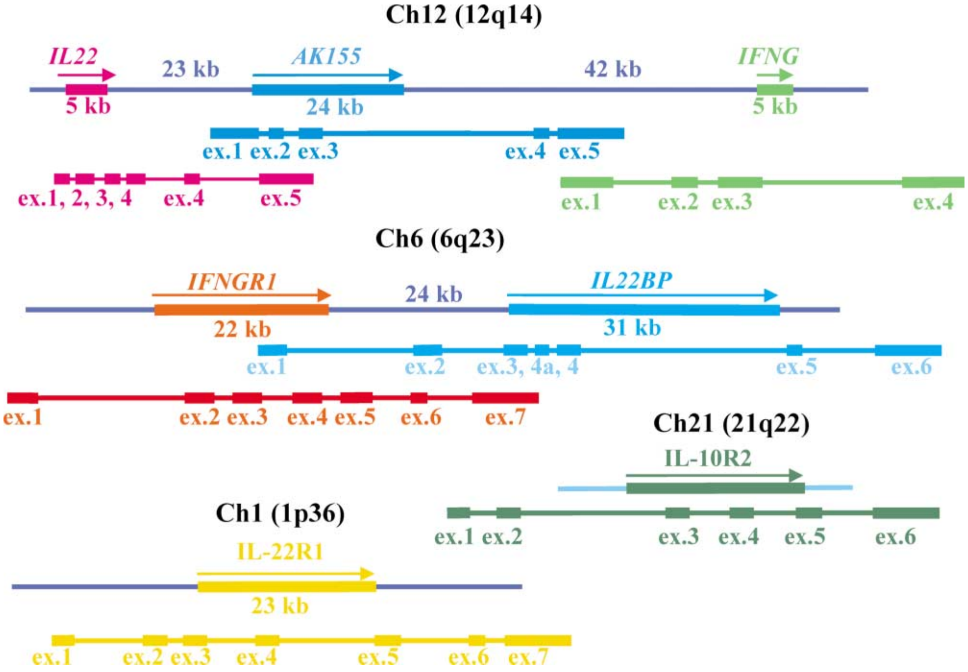

**FIGURE 1** Chromosomal localization of the IL-22 gene and genes encoding subunits of the IL-22 receptor complex and the IL-22 binding protein. The IL-22 gene is positioned near the interferon $\gamma$ (*IFNG*) and *AK155* genes on human chromosome 12 (Ch12). The functional IL-22 receptor complex consists of IL-22R1 and IL-10R2 subunits. Both subunits are common shared receptors for other receptor complexes. IL-22R1 is also a receptor subunit for the IL-20/IL-24 receptor complex, whereas IL-10R2 is also a chain for the IL-10 receptor complex. The IL-22R1 and IL-10R2 genes are mapped to human chromosomes 1 and 21, respectively. IL-22 binding protein is a soluble receptor that acts as an IL-22 antagonist. Its gene (*IL22BP*) is on human chromosome 6 in the vicinity of the *IFNGR1* gene.

a segment of a putative promoter, suggesting that the IL-TIFβ gene is either differentially regulated or not expressed at all.

There are data linking IL-22 to allergy and asthma. IL-22 is induced by IL-9, a $T_H2$ cytokine active on T and B lymphocytes, mast cells, and eosinophils and thus potentially involved in allergy and asthma. The IL-22 gene and another IL-10-related gene, *AK155*, are located on human chromosome 12q15, where several loci potentially linked to asthma and atopy have been identified by genetic studies, particularly in the 12q13–q23.3 region. The strongest evidence for linkage is in a region near the gene encoding IFNγ. However, the gene for IFNγ appears to be highly conserved (no sequence variations were detected in 265 individuals), suggesting that mutations of the IFNγ gene are unlikely to be a significant cause of inherited asthma. The IL-22 and *AK155* genes are positioned next to the IFNγ gene (Fig. 1) and, thus, are possible candidates for linkage to asthma.

## III. THE IL-22 RECEPTOR COMPLEX

Cytokines exert their actions by binding to specific cell-surface receptors, which leads to the activation of cytokine-specific signal transduction pathways. Based on the fact that IL-22 demonstrates homology to IL-10, it is logical to propose that their receptor complexes may share at least structural homology and perhaps receptor subunits. The functional IL-10 receptor complex consists of two chains, the ligand-binding IL-10R1 subunit and a second IL-10R2 subunit that supports signaling through the IL-10R1 chain. Both chains belong to the type II cytokine receptor family. In addition to members with defined functions, i.e., two receptor chains for type I and type II interferons, two receptor chains for IL-10, and the tissue factor that binds coagulation factor VIIa, several orphan receptors belong to this family.

The first step in identifying the IL-22 receptor complex was to rule out the possibility that IL-22 may utilize the canonical IL-10 receptor complex. This was demonstrated by inability of IL-22 to induce Stat activation in IL-10-responsive cells, such as the RAW264 murine macrophage-like cell line and the MOLT-4 human lymphoblast cell line, and also in freshly isolated human peripheral blood mononuclear cells (PBMCs). In addition, IL-22 failed to induce MHC class I antigen expression and Stat1 activation in hamster cells expressing the chimeric human IL-10R1/γR1 chain and the intact human IL-10R2 chain, whereas human IL-10 did. Thus, it became clear that IL-22 requires its own specific receptor complex for signaling, but might share a subunit with the IL-10 receptor complex, particularly the IL-10R2 chain, which is ubiquitously expressed.

Orphan receptors from the type II cytokine receptor family have been examined for being likely subunits of the IL-22 receptor complex. Out of several candidates, only one receptor, a cytokine receptor family type II member (CRF2-9), was capable of binding IL-22 when expressed in monkey COS cells or in Chinese hamster cells, as demonstrated by flow cytometry with an IL-22-Fc fusion protein or by cross-linking, respectively. However, only in COS cells was CRF2-9 able to sustain IL-22 signaling as measured by Stat activation, whereas CRF2-9 expressed in hamster cells did not render them responsive to IL-22. CRF2-9 was designated IL-22R1, or IL-22R. These results suggest that an additional receptor subunit is required to support signaling through IL-22R1 in hamster cells. This subunit might not be expressed in hamster cells or may be species specific, whereas it is endogenously expressed in COS cells. A similar scenario was observed for the IL-10 receptor complex. It was demonstrated that the IL-10R2 chain has limited species specificity: the IL-10R2 chain of mouse, human, or monkey origin can support signaling through the IL-10R1 chain of these species. However, the hamster IL-10R2 chain can support signaling only through mouse IL-10R1, but not through human IL-10R1. Thus, it was hypothesized that IL-10R2 could be a common second chain for both IL-10 and IL-22 receptor complexes. Indeed, when the human IL-10R2 chain is coexpressed with IL-22R1 in hamster cells, the cells become sensitive to IL-22, demonstrating involvement of IL-10R2 in the IL-22 receptor complex. In addition, anti-IL-10R2 antibody completely blocks IL-22-mediated expression of serum amyloid A and luciferase reporter activity in HepG2 cells. Thus, IL-22 requires expression of both chains, IL-22R1 and IL-10R2, for signaling and induction of biological activities. The pattern of IL-22-induced Stat activation in COS cells expressing the IL-22 receptor is characteristic of IL-22 signaling in intact IL-22-responsive cells. The analysis of the IL-22R1 intracellular domain demonstrates that Stat proteins are likely to be recruited through this receptor. There are several tyrosine residues in the IL-22R1 intracellular domain that are potential sites for phosphorylation. Analysis of amino acids surrounding tyrosine residues within the IL-22R1 intracellular domain reveals the presence of four potential Stat3 recruitment sites, the phospho-Tyr-XX-Gln sequence (YXXQ motif). In addition, when the chimeric IL-22R1/γR1 chain (in which the IL-22R1 intracellular domain was replaced by the intracellular domains of the human IFNγR1 chain) was expressed in COS cells, the pattern of IL-22-induced Stat activation changed to that characteristic of IFNγ signaling—only Stat1 DNA-binding complexes were observed. Thus, the activation of Stats is indeed mediated by the IL-22R1 intracellular domain, because substitution of the IL-22R1 intracellular domain with the IFN-γR1 intracellular domain causes a change in the pattern of Stat activation. The use of chimeric receptors also allowed evaluation of responsiveness of cells to IL-22 treatment without knowing IL-22-specific biological activities. In cells expressing the chimeric IL-22R1/γR1 and intact IL-10R2 chains, IL-22 induced IFNγ-like biological responses. Thus, in these cells, in addition to measuring IL-22-induced signaling events (Stat activation), it was also possible to measure IL-22-induced IFNγ-like biological activities, such as major histocompatibility complex (MHC) class I antigen expression.

The IL-22 receptor (Fig. 2) is likely to be structurally homologous to the IFNγ, IL-10, and other IL-10 family cytokine receptor complexes. IL-22 binding is likely to induce oligomerization of two IL-22R1 chains and two IL-10R2 chains. However, the IL-22 receptor complex has a distinct feature: the IL-22R1 chain and the IL-10R2 chain are both capable of binding IL-22 independently. The ability of IL-10R2 to bind IL-22 has been demonstrated by flow cytometry using an IL-22-Fc fusion protein and COS cells overexpressing IL-10R2 alone, and also by cross-linking radiolabeled IL-22 to hamster cells expressing only the human IL-10R2 chain, without the IL-22R1 chain. Thus, both chains of the IL-22 receptor complex can independently bind ligand, whereas in the IL-10, IFNα, and IFNγ receptor complexes only one chain (the R1 chain, which also recruits Stat proteins) can bind ligand in the absence of the other. Nevertheless, in all of these receptors,

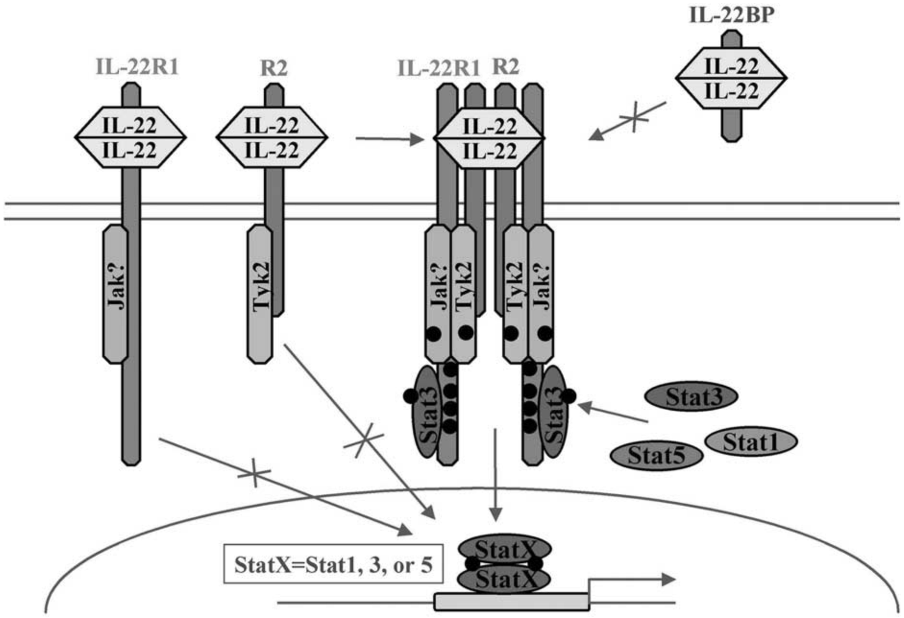

FIGURE 2 Model of the IL-22 receptor complex and signal transduction mechanism. The functional IL-22 receptor complex consists of two receptor chains, IL-22R1 and IL-10R2 (R2), which is likely to be structurally homologous to the functional IL-10 or interferon γ receptor complexes. Both chains can independently bind IL-22, but both chains must be present in the receptor complex to induce signal transduction events. The IL-22 activity can be negatively regulated by the expression of the R2 chain alone because IL-22 binding to the R2 chain does not lead to signaling, preventing shedding of IL-22 into the circulation (local suppression). In addition, secretion of the soluble IL-22 binding protein (IL-22BP) into the circulation can provide systemic inhibition of IL-22 action. Both chains are necessary to assemble the functional receptor complex, which is able to induce signaling after binding IL-22. The IL-10R2 chain is associated with Tyk2. IL-22 activates a set of Stat proteins (Stat1, Stat3, and Stat5).

the second (R2) chains are necessary for signaling. The ability of the IL-10R2 alone to bind IL-22 was particularly surprising because this chain is unable to bind IL-10. Whether this unusual binding is of functional significance remains to be determined. Because IL-10R2 is ubiquitously expressed but is unable to transduce a signal without an additional chain (IL-10R1 or IL-22R1), it is possible that secreted IL-22 will be retained at the site of secretion by being bound to the IL-10R2 chain, providing local action but preventing its action at remote sites. However, this "exception" could become a rule for receptor complexes for other IL-10-related cytokines, for which only the combination of both chains provides receptor complexes with high affinity for a ligand, and each chain alone provides very low binding affinity for a ligand.

In conclusion, it is clear that both IL-22R1 and IL-10R2 chains are required for reconstitution of the functional IL-22 receptor complex; and that the IL-10R2 chain also functions as the IL-22R2 chain. However, sharing of receptor chains between cytokines of the IL-10 family goes beyond the use of IL-10R2 as a second chain in both IL-10 and IL-22 receptor complexes. The IL-22R1 chain combined with IL-20R2, the second chain of the IL-20 receptor complex, could form a functional receptor complex for both IL-20 and IL-24.

## IV. EXPRESSION OF THE IL-22R1 mRNA

Expression of IL-22R1 mRNA has been detected by Northern blotting in several normal tissues, including kidney, liver, and pancreas, in which the level of expression was the highest. The fact that the IL-22R1 gene is expressed in normal liver tissue correlates with its functions as a hepatocyte-stimulating factor. The transcript size is approximately 3.0 kb, which is comparable with the size of IL-22R1 cDNA (2.8 kb). Several solid tumor cell lines constitutively express the IL-22R1 mRNA. Among them are colorectal adenocarcinoma SW480, lung carcinoma A549, melanoma G361, hepatoma HepG2, and renal carcinoma Caki-1 and TK-10 cell lines, the

HT29 intestinal epithelial cell line, and the HaCaT keratinocyte cell line also constitutively express IL-22R1 mRNA. It is interesting to note that all cell lines expressing IL-22R1 mRNA are nonhematopoietic tumor cell lines. Because IL-22 induces Stat3 activation in IL-22-responsive cells, and expression of constitutively active Stat3 has been shown to cause cellular transformation, it is possible that in certain cells IL-22 could act as an autocrine or paracrine factor, driving cells to malignancy.

## V. NATURALLY OCCURRING IL-22 ANTAGONIST

The fact that IL-22 is capable of binding to the singly expressed IL-10R2 suggests that the activity of IL-22 can be regulated on a local level by sequestering locally expressed IL-22, because IL-10R2 is ubiquitously expressed. However, regulation of IL-22 action is not limited to the local level. Systemic regulation is provided by the soluble receptor, i.e., IL-22 binding protein (IL-22BP).

The use of decoy or soluble receptors for cytokines utilizing members of the type II cytokine receptor family for signaling is rather uncommon. It has been demonstrated only for interferon α (IFNα), in which the human IFNαR2c, one of the functional chains of the IFNα receptor complex, has two splice variants, the membrane-bound IFNαR2b (with a short intracellular domain that appears unable to transduce the signal) and the soluble IFNαR2a form. These various receptor chains are the result of alternative splicing events. The gene encoding the only soluble receptor from the type II family was recently, identified. This novel receptor was originally designated cytokine receptor family type II member 10/X (CRF2-10/CRF2-X). When its function as an IL-22 antagonist was discovered, the protein was renamed IL-22 binding protein (other names include IL-22RA2 and CRF2-s1).

IL-22BP was discovered by searching the sequence of the human genome for genes encoding novel receptors from the type II cytokine receptor family. IL-22BP demonstrates 34% identity to the IL-22R1 extracellular domain. The purified protein migrates on gel as a broad band in the region of about 35–45 kDa, suggesting possible glycosylation of the protein. Indeed, there are five potential sites for N-linked glycosylation (Asn-X-Thr/Ser) in CRF2-10. IL-22BP binds IL-22, as demonstrated by cross-linking of IL-22BP to radiolabeled IL-22 in solution and by binding IL-22BP-Fc fusion protein on IL-22-coated plates. Also, IL-22 is unable to interact with the membrane-bound IL-22 receptor complex in the presence of IL-22BP, as demonstrated by cross-linking. In addition, IL-22BP neutralizes IL-22 activity. IL-22BP inhibits the ability of IL-22 to induce Stat activation in intact A549 human lung carcinoma cells and the ability of IL-22 to induce the expression of the SOCS-3 gene in HepG2 human hepatoma cells; it also blocks luciferase reporter activity induced by IL-22 in HT29 intestinal epithelial cells and neutralizes IL-22 activity in a proliferation assay with BAF3 cells expressing the IL-22 receptor complex. IL-22BP inhibits IL-22-induced activities and signaling in specifically designed IL-22-responsive hamster cells expressing the chimeric human IL-22 receptor complex. The affinity of the IL-22 receptor complex when both IL-22R1 and IL-10R2 chains are present is higher than the affinity of each chain alone. It seems that IL-22BP has even higher affinity for IL-22 binding than the membrane-bound IL-22 receptor complex, because incubation of cells expressing the functional IL-22 receptor complex with IL-22 in the presence of IL-22BP inhibits binding of IL-22 to the cellular receptors. It also appears that the IL-22–IL-22BP complexes are stable and that little or no dissociation of the complexes occurs, because cells incubated for 3 days with IL-22–IL-22BP complexes in conditioned media do not demonstrate IL-22-induced activities. The binding appears to be very specific, because IL-22BP-Fc fusion protein fails to bind to plates covered with bound cytokines from the IL-10 family, including IL-19, IL-20, and IL-24; IL-22BP also fails to neutralize IL-10 activity. Thus, by binding IL-22, IL-22BP blocks the activities of IL-22.

## VI. EXPRESSION OF THE IL-22BP mRNA

Because each chain of the IL-22 receptor complex alone is able to bind IL-22, it is likely that IL-22 activity can be negatively regulated by expression on the cell surface of the R2 chain, unpaired by the IL-22R1 chain. Because IL-22 binding to the R2 chain expressed alone does not lead to signaling, it may prevent shedding of IL-22 into the circulation (local suppression). In addition, secretion of the soluble IL-22BP into the circulation can provide systemic inhibition of IL-22 action. Thus, the production of IL-22BP may be one of the mechanisms to regulate IL-22 function precisely (Fig. 2). Because IL-22 has been implicated in inflammation, the expression of IL-22BP in certain tissues can, perhaps, modulate local inflammation. In this light, it is of interest to note that IL-22BP expression has been detected by *in situ* hybridization in the mononuclear cells of inflammatory infiltration sites, plasma cells, and a subset of epithelial cells. Cells expressing IL-22BP as detected by *in situ* hybridization are observed in several tissues, including placenta, skin, inflamed appendix, lung, gastrointestinal tract, lymph node, thymus, and spleen. Also, strong signals are detected in epithelial cells and some interstitial cells (most likely mononuclear cells) in ovarian carcinoma; however, no signal is observed in normal ovarian tissue. The regulation mechanism of IL-22BP expression is not known. Induction of IL-22BP expression in response to LPS or anti-CD3 antibodies in peripheral mononuclear cells has not been detected. IL-22 stimulation does not induce IL-22BP production. The IL-22BP expression profile generated by Northern blot and polymerase chain reaction (PCR) analysis indicates that IL-22BP is highly expressed in placenta, mammary gland, breast, spleen, skin, and lung, with lower expression in a variety of other tissues such as heart, pancreas, testis, thymus, and prostate. IL-22BP expression is also detected in the digestive system (stomach, small intestine, esophagus, gastro-esophageal, pancreas, duodenum, ileum, colon, and small bowel), female reproductive system (mammary gland, endometrium, and breast), and other systems (lymph nodes, lung, skin, parotid, bladder, bronchus, heart ventricles, and kidney). It is noteworthy, that IL-22BP is expressed in certain tissue-specific tumors such as ovarian, uterine, and rectal cancers, but not in the corresponding normal tissues.

## VII. IL-22R1, IL-10R2, AND IL-22BP GENES

Genes encoding receptors from the type II cytokine receptor family are clustered on several human chromosomes. The IL-10R2 gene maps to chromosome 21 along with IFNαR1, IFNαR2, and IFNγR2 genes. The IL-22R1 gene is on chromosome 1. The IL-22BP gene is positioned on chromosome 6 in the vicinity of the IFNγR1 gene in a head-to-tail orientation and is in close proximity to the IL-20R1 gene. IL-10R2, IL-22R1, and IL-22BP genes have similar structural architecture, with two exceptions for the IL-22BP gene: the presence of an additional exon (exon 1) encoding the 5′ UTR and the lack of the two last exons encoding transmembrane and intracellular domains of the IL-22R1 and IL-10R2 chains. Thus, IL-10R2 and IL-22R1 genes contain seven exons. The first exon encodes the 5′ UTR and the signal peptide of the proteins. Exons 2, 3, 4, and 5 encode the extracellular domain of the receptors. Exon 6 encodes the entire transmembrane domain and the beginning of the intracellular domain of the receptors. Exon 7 encodes the rest of the intracellular domain and the 3′ UTRs of the proteins. Numbering of exons is shifted by one in the IL-22BP gene because of the presence of an additional exon, exon 1, encoding the 5′ UTR. Thus, the IL-22BP gene is composed of six exons. The entire first exon and a part of the second exon encode the 5′ UTR. The rest of the second exon encodes the signal peptide of the protein. Exons 3, 4, and 5 and a portion of exon 6 encode the secreted IL-22BP receptor. The rest of exon 6 encodes the long 3′ UTR with the presence of multiple polyadenylation signals. In addition, alternative splicing events either insert exon 4a between exons 4 and 5, creating a longer CRF2-10 splice variant (CRF2-$10_L$), or eliminate exon 5, creating prematurely terminated short protein (CRF2-$10_S$). It remains to be determined whether alternative slicing events are regulated and whether splice variants are functional.

## Glossary

**cytokine receptor family type II** Cell membrane protein group that includes interferon receptors, interleukin-10 receptor, coagulation factor VIIa receptor, and several orphan receptors.

**interferon** Member of a family of proteins, some of which are glycoproteins; when secreted by virus-infected cells, can protect noninfected cells from viral replication.

**interleukin** Generic term for a group of protein factors that affect primary cells; derived from macrophages and T cells that have been activated.

**major histocompatibility complex** Genetic regions and encoded antigenic proteins that confer tissue compatibility/incompatibility between donor and recipient cells and tissues.

## See Also the Following Articles

**Interferons: α, β, ω, and τ • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

## Further Reading

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# Interleukin-24

Sidney Pestka[*], Sergei V. Kotenko[†], and
Paul B. Fisher[‡]

[*]*University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School* • [†]*University of Medicine and Dentistry—New Jersey Medical School* • [‡]*Columbia University College of Physicians and Surgeons*

I. OVERVIEW OF *mda-7*
II. DISCOVERY
III. ACTIVITIES
IV. HUMAN *mda-7* (IL-24) GENE
V. HUMAN IL-24 RECEPTOR COMPLEX
VI. IL-24-RELATED MOLECULES

**Interleukin-24 (MDA-7) is a member of an interleukin-10 cytokine subfamily. It is encoded by a gene (*mda-7*) that resides in a cluster of genes associated with the interleukin-10 family of cytokines. The gene cluster contains four genes that encode IL-10, IL-19, IL-20, and MDA-7. Because of its growth-suppressive effects, interleukin-24 has antitumor properties in a broad array of tissues; potential therapeutic applications, particularly in combination with other suppressive agents, are under investigation.**

## I. OVERVIEW OF *mda-7*

Subtraction hybridization has identified a melanoma differentiation-associated gene-7 (*mda-7*) as a novel cDNA displaying inducible expression in the context of irreversible growth arrest and terminal differentiation of human melanoma cells. The *mda-7* gene is expressed in normal melanocytes; expression decreases progressively during the process of melanoma development and evolution, suggesting a potential involvement of this gene in human melanoma progression. When ectopically expressed in a wide variety of cancer cells with diverse genetic defects, *mda-7* induces growth suppression and apoptosis (programmed cell death). In contrast, *mda-7* does not elicit any overt harmful effects in normal human cells. Preclinical human tumor animal models have documented that *mda-7* administered by means of a replication-incompetent adenovirus, Ad.*mda-7*, suppresses tumor formation and cancer progression. Based on these preferential cancer specific growth-suppressive and apoptosis-inducing properties, phase I clinical trials with Ad.*mda-7* in patients with cancer are now in progress. Human *mda-7* encompasses seven exons and six introns and encodes a protein of 206 amino acids. The *mda-7*-encoded protein contains a predicted secretory motif of 49 amino acids, an interleukin-10 (IL-10) signature motif, and belongs to the four-helix bundle family of cytokine molecules most closely related to the IL-10 subfamily (although IL-10 contains six α-helices). The *mda-7* gene maps to human chromosome 1q, at 1q32.2–q41, a genomic area containing a cluster of genes associated with the IL-10 family of cytokines, including IL-10, IL-19, and IL-20. Members of the expanded IL-10 family have distinct functional properties and tissue expression profiles. Experimental evidence documents that the MDA-7 protein is secreted from both normal and tumor cells, and purified MDA-7 protein can bind to IL-20R1 and IL-20R2 receptor complexes, resulting in the activation of signal transducers and activators of transcription (Stat) signaling pathways. Based on its structure, cytokine-like properties, and proposed mode of action, MDA-7 has now been classified as IL-24. Still unanswered and important questions relate to the mechanism by which IL-24 (MDA-7) selectively induces its cancer growth suppressive and apoptosis-inducing effects and the programs of gene expression changes underlying these effects.

## II. DISCOVERY

Aberrant programs of differentiation occur frequently in many forms of cancer. A therapeutic approach that offers potential for exploiting this trait in tumor cells involves the use of agents that induce reprogramming of neoplastic cells to a more differentiated state, resulting in a reduction or loss in proliferative ability and tumorigenic potential. In human melanoma cells, treatment with a combination of fibroblast interferon β(IFNβ) and the protein kinase C activator mezerein (MEZ) results in an irreversible loss in growth potential, suppression of tumorigenic potential, and terminal differentiation. Combining this "differentiation therapy" model system with subtraction hybridization allows definition of the spectrum of gene changes that are associated with induction of melanoma terminal differentiation. Initial screening of 73 cDNA clones isolated from an HO-1 IFNβ + MEZ temporally spaced subtracted cDNA library has led to identification of the novel *mda*-7 cDNA.

## III. ACTIVITIES

Based on the context of its isolation following induction of terminal differentiation in human melanoma cells, it was initially hypothesized that *mda*-7 might play a role in human melanoma growth, differentiation, and progression. To test this possibility, HO-1 human melanoma cells were transformed with an expression vector containing the *mda*-7 gene under the transcriptional control of a mouse mammary tumor virus promoter (pMAMneo) that responds to dexamethasone (DEX). When appropriately engineered HO-1 clones were grown in a medium containing DEX, *mda*-7 was expressed and growth was suppressed. Analysis of *mda*-7 mRNA expression in cell lines and in patient-derived tissue demonstrated an inverse relationship between melanoma development and progression and *mda*-7 expression. Highest levels of *mda*-7 expression were observed in melanocytes and nevi and diminishing expression was found as the primary early radial growth phase (RGP) melanoma progressed to vertical growth phase (VGP) melanoma and then to metastatic melanoma, in which the majority of patient-derived cell lines and tumor samples displayed negligible or no *mda*-7 expression. This inverse relationship between melanoma progression and *mda*-7 expression, as originally proposed by Jiang *et al.* based on reverse transcriptase and polymerase chain reaction (RT–PCR) studies, has recently been confirmed by Western blotting with MDA-7 antibodies and by immunohistochemistry with patient tissue samples and cells in culture.

Considering the growth suppressive effects of *mda*-7 in metastatic human melanoma cells, experiments were performed to determine if this effect is restricted to melanoma or represents a more general property of this gene. Transfection of a wide spectrum of human tumor cell lines with an *mda*-7 expression vector, including glioblastoma multiforme, osteosarcoma, and carcinomas of the breast, cervix, colon, nasopharynx, and prostate, reduced colony formation. In contrast, no deleterious effects were observed when *mda*-7 was ectopically expressed in normal human cells, including epithelial cells and fibroblasts. To deliver *mda*-7 to cancer cells more efficiently, a replication-incompetent adenovirus (Ad.*mda*-7) expressing *mda*-7 was constructed. As observed with transfection, Ad.*mda*-7 selectively induced a growth inhibitory effect in diverse human cell types but spared a wide spectrum of normal cells, including endothelial and epithelial cells, fibroblasts, and melanocytes. Moreover, when expressed at high levels by adenovirus delivery, *mda*-7 selectively induced apoptosis in cancer cells. Initial experiments using breast carcinoma cells indicated that Ad.*mda*-7 induced apoptosis in a p53-independent manner and was associated with an increase in the pro-apoptotic protein BAX and a decrease in the ratio of the anti-apoptotic protein Bcl-2 to the BAX protein. Moreover, when breast carcinoma cells were cotransfected with a pMAMneo–*mda*-7 vector (permitting controlled *mda*-7 expression by dexamethasone and selection for resistance to G418) and a Bcl-2 or Ad.E1B expression vector, *mda*-7-induced growth inhibition was prevented. These findings document that the increase in BAX protein and the decrease in the ratio of BAX to Bcl-2 protein following Ad.*mda*-7 infection of breast cancer cells functionally contribute to the process of apoptosis. However, because DU-145 prostate cancer cells, which do not produce BAX protein, and NSCLC H1299 lung carcinoma cells, which do not show up-regulation of BAX expression following infection with Ad.*mda*-7, are also sensitive to the programmed cell death-inducing effects of *mda*-7, it is clear that this cancer-growth-suppressing gene can induce apoptosis in a BAX-dependent or BAX-independent manner. To date, Ad.*mda*-7 has been shown to induce apoptosis in multiple cancer subtypes, including melanoma, glioblastoma multiforme, osteosarcoma, and carcinomas of the breast, cervix, colon/rectum, lung, nasopharynx, and prostate, without affecting normal cells. These experiments confirm that Ad.*mda*-7 can induce antitumor effects in specific cancer cells independent of the genomic status of p53, retinoblastoma, p16, Ras, BAX, and caspase 3 genes. In specific cancer cell types, Ad.*mda*-7 also induces an alteration in the cell cycle, resulting in an accumulation of tumor cells in the $G_2$/M phase of the cell cycle, and this alteration in cell cycle regulation may contribute to the antitumor effects of *mda*-7. Based on the broad-spectrum antitumor activity of *mda*-7 and its apparent selectivity toward cancer cells without adversely affecting normal cellular physiology, phase I clinical trials have been initiated with Ad.*mda*-7 to determine its safety in patients. Initial results with a limited number of patients suggest that *mda*-7 is safe and has no unanticipated side effects following Ad.*mda*-7 intratumoral administration, and that direct injection into tumors can elicit tumor cell death by apoptosis. In view of these promising initial findings, further studies to define directly the potential cancer therapeutic effects of Ad.*mda*-7 in additional phase I and phase II clinical trials are warranted.

An apparent exception to the wide-spectrum antitumor properties of *mda*-7 is pancreatic cancer. Infection of human pancreatic cancer cells with Ad.*mda*-7 does not alter growth or induce apoptosis. In contrast, the combination of Ad.*mda*-7 with antisense phosphorothioate oligonucleotides (PS ODNs), thus targeting the K-*ras* oncogene (which is mutated in 85–95% of pancreatic carcinomas), induces growth arrest and decreased survival by inducing apoptosis uniquely in mutant K-*ras* pancreatic carcinoma cells. Similarly, by blocking K-*ras* expression using an antisense K-*ras* expression vector, thus targeting the AUG region of the K-*ras* gene, the growth and *in vivo* tumorigenic potential of mutant K-*ras* pancreatic carcinoma cells are inhibited. This study provides compelling evidence that a combinatorial approach, consisting of the *mda*-7 cancer-specific apoptosis-inducing gene and an oncogenic inactivation strategy, offers potential for developing an effective and rational therapy for pancreatic and perhaps other resistant cancers. Furthermore, this study emphasizes how molecular oncology, based on an understanding of the molecular defects in cancer cells, can be used for defining methods for early diagnosis, prevention, and new and effective therapeutic strategies for treating cancer. These experiments further highlight that, in specific cancers, such as pancreatic cancer, it may be necessary to correct or modify two disparate molecular changes to elicit a biological response that results in growth suppression and apoptosis. By defining the gene changes associated with cancer development and evolution, it may be possible to exploit these defects by correcting and blocking specific cancer-related changes, thereby short-circuiting the cancer cell and inducing its demise.

## IV. HUMAN *mda*-7 (IL-24) GENE

The human *mda*-7 cDNA is 1718 nucleotides long; there is an open reading frame (ORF) encoding a 23.8-kDa protein of 206 amino acids. The major ORF starts at position 275 and terminates at position 895. The predicted open reading frame is flanked by 5′ and 3′ untranslated sequences of 274 and 823 bp, respectively. The 3′ untranslated region (UTR) contains three consensus elements (AUUUA) involved in mRNA stability and three polyadenylation signals, AAUAAA, at −413, −204, and −20, from the first A of the poly(A) tail. The *mda*-7 promoter has been isolated and found to be constitutively active in human melanoma cells, and treatment with IFNβ + MEZ does not significantly alter promoter function. In contrast, *mda*-7 mRNA levels during terminal differentiation are partly regulated by differential posttranscriptional message stabilization regulated by the AU-rich (ARE) sequences present in the 3′ untranslated region of the *mda*-7 cDNA. *In vitro* translation of *mda*-7 in a rabbit reticulocyte system produces a protein with an apparent molecular mass of ~24 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Hydrophobicity plot analysis of the MDA-7 amino acid sequence predicts the presence of a hydrophobic stretch encompassing amino acids 25 to 45 of the protein. Analysis of cell lysates from Ad.*mda*-7-infected tumor and normal cells indicates the presence of an approximate 23/17-kDa doublet, suggesting that MDA-7 is processed intracellularly in a similar manner in cancer and normal cells. The first 48 amino acids of MDA-7 contain a potential signal sequence with a consensus proteolytic cleavage site at amino acids 48/49. Considering this arrangement of the primary MDA-7 amino acid sequence, it is predicted that a 23/17-kDa doublet would be generated, and indeed this is the case. Analysis of supernatants from Ad.*mda*-7-infected tumor and normal cells indicates the presence of higher molecular weight MDA-7 proteins, including multiple-sized proteins, presumably resulting from differential glycosylation.

Analysis of DNAs derived from different species by Southern blotting indicates homologous sequences in the genomic DNAs of yeasts, monkeys, bovines, dogs, and cats. The intensity of hybridization in Southern blots reveals that the highest sequence homology occurs between human, Rhesus monkey, and yeast genomic DNAs. Only weak hybridization signals are detected by probing Southern blots containing bovine, dog, and cat genomic DNA with *mda-7*. These findings confirm that *mda-7* is an evolutionarily conserved gene. However, there does not appear to be a yeast homologue of *mda-7*.

Southern blotting demonstrates that *mda-7* is a single-copy gene. Considering its unique presence in the human genome, a PCR-based method employing *mda-7*-specific primers with diploid human fibroblast genomic DNA was used to identify and isolate *mda-7* genomic DNA. This approach resulted in the identification of a 5.5 kbp amplification product containing the *mda-7* open reading frame. This approach plus subsequent screening of a human placental genomic library resulted in identification of a contiguous *mda-7* genomic sequence containing the *mda-7* transcription unit (6.33 kbp). By genomic walking, it was possible to clone an additional 2.2 kbp of the 5′ flanking region, which contains the *mda-7* promoter. The *mda-7* gene comprises seven exons and six introns that conform to the intron–exon consensus splicing signals (GT...AG). The exons range in size from 64 to 889 bp and the introns range from 115 to 1143 bp. Moreover, the *mda-7* cDNA deduced from the human exon sequence information corresponds exactly with the previously reported human *mda-7* cDNA. Sequence analysis of the seven exons from 15 different genomic sources, including non-small-cell lung carcinoma, colorectal, prostate, cervical, and normal peripheral blood leukocytes, identified an exon 4 polymorphism at residue 125 that causes a change in codon usage, resulting in either a histidine or tyrosine residue. The *mda-7* cDNA from human melanoma cells contained histidine at amino acid 125. A single amino acid insertion (Ser-15) in the signal peptide of the published *mda-7* sequence has been reported in colorectal carcinomas. However, this genetic alteration was not observed after sequencing all *mda-7* exons from more than 15 different genomic sources, including the DLD-1 colorectal cancer cell line. Nevertheless, this finding does not rule out the possibility of mutations in other regions of the gene locus.

A panel of rodent–human hybrid DNAs was employed in combination with a PCR amplification-based method to map the chromosomal location of *mda-7*. This approach generated a unique 129-bp human *mda-7*-specific product in positive clones. This approach documented that *mda-7* resides on chromosome region 1q. With the use of the gene-bridge radiation hybrids and the Whitehead Institute/MIT Center for Genome Research (WICGR) mapping server, *mda-7* displayed close linkage to markers WI-9461 (D1S06) and D1S491, which map to the region 1q32.3–q41. In this context, the genomic locus encoding *mda-7* appears to be within an IL-10-related gene cluster containing four genes, including those for IL-10, IL-19, IL-20, and MDA-7, in linear order, spanning 195 kb of genomic DNA. At the present time, the functional significance of this genomic organization is unclear.

Analysis of a large array of normal and cancer cell types demonstrates a lack of *de novo* expression of *mda-7*. An exception is melanocytes, which express both *mda-7* mRNA and protein. To define which normal tissue(s) express *mda-7*, multiple tissue Northern blots containing human tissue poly(A)$^+$ RNAs were probed with an *mda-7*-specific cDNA probe. This analysis confirmed restricted *mda-7* expression in tissues of the immune system, including spleen, thymus, and peripheral blood leukocytes. In addition, treatment of the human K562 erythroleukemic cell line with 12-O-tetradecanoylphorbol 13-acetate (TPA), which results in megakaryocyte differentiation, results in the induction of *mda-7* mRNA expression. Experiments with a rat model indicated elevated (9- to 12-fold) levels of *c49a* (a rat gene that encodes a protein with 58.7% overall identity with the human MDA-7 protein) mRNA during wound healing, specifically in areas surrounding the edge of the wound. Representational difference analysis (RDA) resulted in cloning of a mouse gene called *FISP* (IL-4-induced secreted protein), with sequence homology to *mda-7* and the rodent gene *c49a* (identical to a later described *mob-5* Ha-*ras*-induced rat gene). *FISP* is induced specifically as a consequence of induction of differentiation of T cells to T helper ($T_H2$) cells. Based on functional differences between human *mda-7*, rat *c49a*/*mob-5*, and mouse *FISP*, it appears that these genes are not true homologues but represent closely related members of a gene family with distinct functions; however, they do appear to have evolved from the same gene.

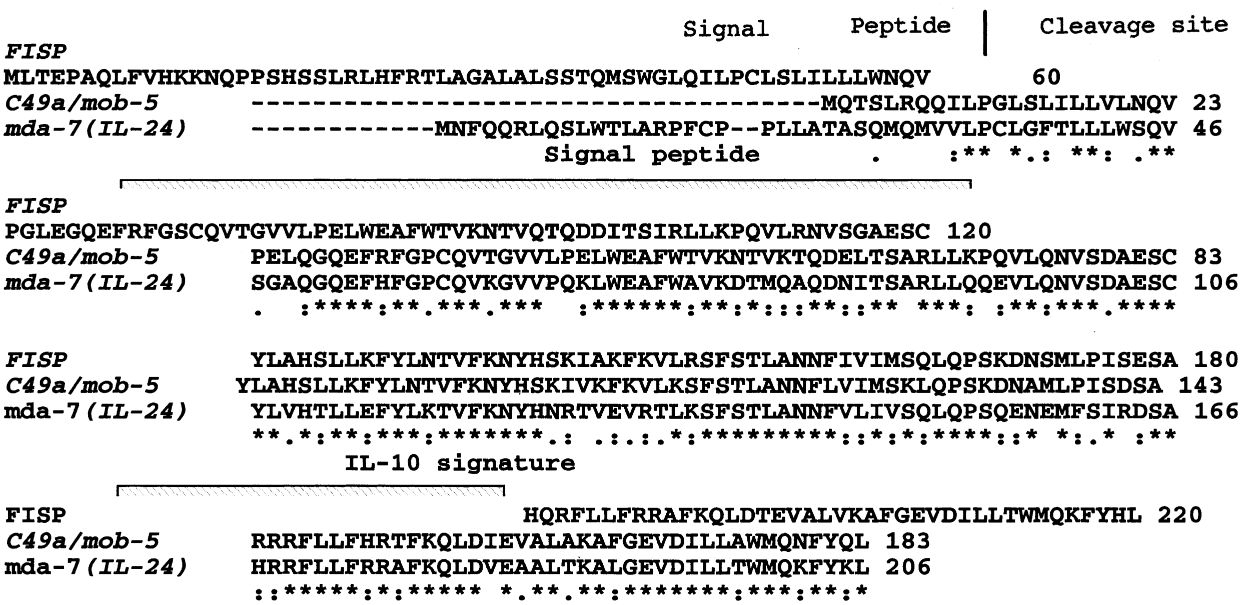
Although normally restricted to specific cell lineages, *mda*-7 mRNA can be induced under appropriate conditions in human cells that are not of melanocytic or hematopoietic lineage. A 24-h treatment of DU-145 (prostate carcinoma), HBL-100 (normal breast epithelial), MDA-MB-157 and MDA-MB-231 (breast carcinoma), NC (normal cerebellum astrocytes), GBM-18 (glioblastoma multiforme), Saos-2 (osteosarcoma), HeLa (cervical carcinoma), and HONE-1 (nasopharyngeal carcinoma) cells with IFNβ and mezerein induces *mda*-7 mRNA expression. In contrast, *de novo* expression and induction of *mda*-7 are not apparent in additional normal and tumor-derived human cells, including HuPEC (normal prostate epithelial), PC-3 and LNCaP (prostate carcinoma), MCF-7, T47D and MDA-MB-453 (breast carcinoma), T98G (glioblastoma multiforme), and SW613 (colon carcinoma). These results document that *mda*-7 is not expressed *de novo* in most normal and cancer cell types, but expression, at least at the level of mRNA, can be induced by IFNβ and MEZ in a spectrum of normal and tumor cell types independent of alterations in classic tumor suppressor genes such as *Rb* and/or *p53*. Further studies are required to understand the significance of the induction of *mda*-7 in regulating cellular physiology in normal and cancer cell types.

## V. HUMAN IL-24 RECEPTOR COMPLEX

IL-24 (MDA-7) has been shown to have many activities that might be explained by its interaction with two separate receptor heterodimeric complexes. IL-24, IL-19, and IL-20 bind to the IL-20 receptor complex that consists of two chains, IL-20R1 and IL-20R2. The IL-20R1 (IL-20Rα) chain is the cytokine receptor family 2-8 (CRF2-8) chain; whereas the IL-20R2 (IL-20Rβ) chain is the protein also designated DIRS1. Furthermore, IL-24 and IL-20 bind to a second receptor complex with which IL-19 does not interact. This second receptor complex consists of the IL-22R1 and IL-20R2 complex. When activated by the ligands, both receptors signal through Stat3. Because cytokines IL-19, IL-20, IL-22, and IL-24 signal through Stat3, the differences in their activities represent a mystery still to be understood. This paradox is reminiscent of the IFNα/β receptor that consists of the two chains, IFNαR1 and IFNαR2c, thus serving as the receptor for many human ligands (12 human IFNα species, plus one IFNβ, one IFNω, and one IFNκ). Nevertheless, these various interferons demonstrate remarkable differences in activities and signal transduction despite the fact that they function through the same receptor.

## VI. IL-24-RELATED MOLECULES

Since the original isolation of the *mda*-7 cDNA in a human melanoma differentiation screen, three independently isolated rodent sequences showing significant sequence identity have been reported. The rat c49a sequence was isolated by differential RNA display (DD-PCR) to identify new genes for which expression was correlated with wound healing. Elevated expression was observed in fibroblast-like cells at the wound edge or base, showing a 9- to 12-fold elevated level of expression from 12 h up to 5 days, followed by a gradual decrease to 1.5- to 3-fold over a 6- to 14-day period, compared to control samples. In contrast to the observed growth-suppressive properties of the human sequence, rat c49a appears to be associated with cellular proliferation of fibroblast populations involved in the wound-healing process. No further data with this model have been forthcoming since the initial report. A later study reported the isolation of a sequence identical to c49a, utilizing DD-PCR as a screen to identify oncogenic *ras* target genes. Two types of screens were used, one involving a Rat-1 fibroblast line containing an inducible Ha-*ras* gene and the other utilizing DD-PCR on a Rat-1 line constitutively expressing Ha-*ras* treated or not with the mitogen-activated protein kinase kinase (MAPKK) inhibitor PD98059. A sequence identified in the screen and designated mob-5 was confirmed to be rapidly induced concomitant with induction of the Ha-*ras* gene, identifying it as an early oncogenic *ras* target. This sequence was shown to be rapidly inducible downstream of oncogenic *ras* signaling (but not as a consequence of serum stimulation of starved normal fibroblasts) or to be otherwise expressed in any normal rat tissues examined in the study. The normal physiological role of c49a/mob-5 is presently unclear, though both screens and subsequent analyses indicated an association of expression with proliferative responses, as opposed to observed growth-inhibitory effects induced by ectopic expression of human IL-24. It is possible that these sequences, despite possessing regions of significant amino acid identity with the human



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                                                 Signal      Peptide  |  Cleavage site
FISP
MLTEPAQLFVHKKNQPPSHSSLRLHFRTLAGALALSSTQMSWGLQILPCLSLILLLWNQV      60
C49a/mob-5       -------------------------------------MQTSLRQQILPGLSLILLVLNQV 23
mda-7(IL-24)     ------------MNFQQRLQSLWTLARPFCP--PLLATASQMQMVVLPCLGFTLLLWSQV 46
                                     Signal peptide      .     :** *.: **: .**
         ____________________________________________________________

FISP
PGLEGQEFRFGSCQVTGVVLPELWEAFWTVKNTVQTQDDITSIRLLKPQVLRNVSGAESC 120
C49a/mob-5       PELQGQEFRFGPCQVTGVVLPELWEAFWTVKNTVKTQDELTSARLLKPQVLQNVSDAESC 83
mda-7(IL-24)     SGAQGQEFHFGPCQVKGVVPQKLWEAFWAVKDTMQAQDNITSARLLQQEVLQNVSDAESC 106
                 .   :****:**.***.***   :******:**:*:::**::** ***: :**:***.****

FISP             YLAHSLLKFYLNTVFKNYHSKIAKFKVLRSFSTLANNFIVIMSQLQPSKDNSMLPISESA 180
C49a/mob-5      YLAHSLLKFYLNTVFKNYHSKIVKFKVLKSFSTLANNFLVIMSKLQPSKDNAMLPISDSA 143
mda-7(IL-24)     YLVHTLLEFYLKTVFKNYHNRTVEVRTLKSFSTLANNFVLIVSQLQPSQENEMFSIRDSA 166
                 **.*:**:***:*******.: .:.:.*:*********::*:*:****::* *:.* :**
                       IL-10 signature
         __________________________

FISP                                      HQRFLLFRRAFKQLDTEVALVKAFGEVDILLTWMQKFYHL 220
C49a/mob-5       RRRFLLFHRTFKQLDIEVALAKAFGEVDILLAWMQNFYQL 183
mda-7(IL-24)     HRRFLLFRRAFKQLDVEAALTKALGEVDILLTWMQKFYKL 206
                 ::*****:*:***** *.**.**:*******:***:**:*
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FIGURE 1 Sequence comparison of mouse IL-4-induced secreted protein (FISP), rat c49a/mob-5, and human MDA-7 (IL-24). Multiple sequence alignments using CLUSTAL W (http://www.ebi.ac.uk/clustalw) with amino acid sequences of mouse (FISP), rat (*c49a*/mob-5), and human MDA-7 (IL-24) proteins. The specific regions of note, the secretory signal peptide and IL-10 signature sequences, are indicated under each relevant sequence by a hashed line; the signal peptide cleavage site is indicated by the vertical line. The markings under each multiple alignment have the following correspondence: *, identical amino acid; ●, weakly conserved residue; :, conservative change in amino acid residue; and no marking for an unrelated residue at the respective alignment position.

protein, are related moieties but are functional rodent homologues. This is supported by the observation that the genes appear to have an oppositely acting phenotypic effect. A screen designed to identify genes involved in T helper lymphocyte type 2 ($T_H2$) cell development resulted in the isolation, by representational difference analysis, of mouse IL-4-induced secreted protein, which showed significant homology to c49a/mob-5 and IL-24 (MDA-7). FISP is expressed selectively in $T_H2$ cells following culturing of splenocytes, resulting in $T_H2$ differentiation and not $T_H1$ lineage determination. FISP is not expressed in normal spleen, thymus, liver, brain, heart, lung, testicle, kidney, or embryo-derived tissues. It appears that, on a functional level, FISP more closely resembles IL-24 than c49a/mob-5 due to its close association with differentiation. It is presently unclear if ectopic expression of FISP results in induction of apoptosis similar to activity displayed by IL-24. In general, it appears that IL-24, c49a/mob-5, and FISP represent closely related sequences comprising a gene family, but remain functionally distinct. Sequence alignment of the proteins shows significant amino acid identity over significant stretches but also several specific residues that are not functionally conserved (Fig. 1). Further structure–function studies are needed to clarify the basis of the differential activity of these molecules.

## Glossary

**interferon** Member of a family of proteins, some of which are glycoproteins; when secreted by virus-infected cells, can protect noninfected cells from viral replication.

**interleukin** Generic term for a group of protein factors that affect primary cells; derived from macrophages and T cells that have been activated.

***mda*-7** Melanoma differentiation-associated gene.

## See Also the Following Articles

**Interferons: α, β, ω, and τ • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

## Further Reading

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# *In Vitro* Fertilization

Roger G. Gosden and William E. Gibbons
*Eastern Virginia Medical School*

I. INTRODUCTION
II. ROLE OF GONADOTROPINS IN THE MENSTRUAL CYCLE
III. CONTROLLED OVARIAN STIMULATION
IV. HYPOTHALAMIC DOWN-REGULATION
V. COMPLICATIONS OF OVULATION INDUCTION
VI. OOCYTE AND EMBRYO QUALITY
VII. THE FUTURE

***In vitro* fertilization (IVF) is a technology with wide-ranging applications for treating female and male infertility. To enhance its chances of success, the ovaries are stimulated with gonadotropic hormones to increase the numbers of mature oocytes that are available. Pharmacological suppression of endogenous gonadotropins enables greater control of the cycle and improves success rates. Normally, several embryos are created, but their quality is variable and cytogenetic anomalies are common. To compensate for the difficulty of identifying embryos of high implantation quality, two or more are transferred to the uterus. Nevertheless, only a quarter of treatment cycles are fully successful in young women and fewer in those over 35 years of age. Technology is evolving toward less intervention in the natural cycle and more screening of embryos by preimplantation genetic diagnosis.**

## I. INTRODUCTION

Infertility is arguably the most common medical condition worldwide, with at least 1 in 10 couples experiencing involuntary childlessness at some stage in their lives. *In vitro* fertilization (IVF) was pioneered by Robert Edwards and Patrick Steptoe in the 1970s. Although their primary objective was to use IVF to bypass blocked fallopian tubes, it has become a generic reproductive technology for treating many conditions, including male infertility using intracytoplasmic sperm injection. In essence, IVF involves aspirating oocytes from follicles immediately before ovulation, when they should be at the metaphase II stage of meiosis. A few days after fertilization *in vitro* with spermatozoa from ejaculated semen or surgically extracted in severely oligospermic men, the embryos reach the morula and blastocyst stages, when they can be transferred to the uterus.

The original breakthrough in IVF was achieved in natural cycles (Fig. 1a). Nowadays, treatment usually involves ovarian stimulation to increase the harvest of oocytes and improves the chances of producing some embryos with high implantation potential. Many oocytes either fail to fertilize or produce poor-quality embryos, and cells with abnormal numbers of chromosomes are common. Spare embryos of good quality can be cryopreserved for replacement in a subsequent undisturbed cycle or for donation to other patients.

## II. ROLE OF GONADOTROPINS IN THE MENSTRUAL CYCLE

At the beginning of each menstrual cycle, a cohort of 10–20 small antral follicles is present. Only one follicle becomes estrogenic and grows toward ovulation and luteinization, and the rest undergo atresia. Emergence of this dominant follicle depends on stimulation by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and intrafollicular (where W is tryptophan, S is serine, and X is an unknown amino acid).

The cytosolic domain of mouse IL-4Rα is 785 amino acids in length. The membrane-proximal cytosolic region interacts with the Janus kinase JAK1 (or, in some cases, JAK2); γc has a docking site for JAK3. It is generally believed that the interaction of JAK1 and JAK3 as a result of heterodimerization of IL-4Rα and γc leads to the activation of the Janus kinases and the phosphorylation of key tyrosines in IL-4Rα.

More distally in IL-4Rα, there is a docking site for phosphotyrosine-binding (PTB) domain proteins. In particular, IRS-1 and IRS-2, Shc, $p62^{dok}$, and $p56^{dok2}$ (FRIP) bind to this site when its central tyrosine (Tyr-497) is phosphorylated. The sequence in IL-4Rα that constitutes this site is designated the I4R motif. It is highly homologous to docking sites for PTB domain proteins found in the insulin receptor and the insulin-like growth factor-I (IGF-I) receptor, and many of the same PTB domain proteins dock to these receptors.

Further distally, there are three sites with the motif GYK/QXF (G, glycine; Y, tyrosine; K, lysine; Q, glutamine; F, phenylalanine) that are docking sites for the latent transcription factor Stat6. The central tyrosines of these sites are at positions 575, 603, and 631. There is an immunoregulatory tyrosine-based inhibitory motif (ITIM) in the cytosolic portion of IL-4Rα, centered on a tyrosine at position 713. Phosphorylated ITIMs serve as docking sites for SH2-containing phosphatases (SHP1, SHP2, and SHIP). It has been shown that in the presence of IL-4 SHP1 becomes associated with IL-4Rα, suggesting that this negatively regulates the receptor by dephosphorylating key tyrosines in IL-4Rα or in signaling intermediates.

## IV. IL-4 RECEPTOR SIGNALING MECHANISMS

Addition of IL-4 to resting T cells leads to rapid phosphorylation of Stat6; each of the three Stat6 sites in IL-4Rα appears competent to mediate the docking of Stat6 and its subsequent phosphorylation, because abolition of Stat6 phosphorylation is observed only if the central tyrosines of each of the sites have been replaced by phenylalanines.

Phosphorylated Stat6 dissociates from the receptor and dimerizes. The dimer enters the nucleus, where it binds to DNA; a motif to which dimeric Stat6 binds is $TTCN_4GAA$. Transcription mediated as a result of this binding tends to require interaction with other well-characterized transcription factors. These may include c-Jun and Sp-1. Among the genes known to be targets of Stat6 action are the germ-line *IgCϵ* gene and the gene for IL-4Rα. Indeed, in Stat6 knockout cells, neither gene is induced in response to IL-4.

Phosphorylation of PTB domain proteins, such as IRS-2, has been implicated in the anti-apoptotic effects of IL-4 in myeloid cells. This appears to be mediated by phosphoinositide 3-kinase (PI3K). Indeed, the regulatory subunit of PI3K binds to phosphorylated IRS-2.

The role of IL-4 in mediating cell growth is less well understood. Although phosphorylated IRS-2 can interact with Grb2 and Sos and thus should be able to induce the Ras pathway, IL-4 is inefficient in activating mitogen-activated protein kinases (MAPKs) in lymphoid cells. Furthermore, IL-4 has recently been shown to enhance the expression Gfi-1, a SNAG domain transcriptional repressor. Gfi-1-overexpressing cells show repression of $p27^{Kip1}$, which is also known to be repressed by IL-4, in a Stat6-dependent manner. Repression of $p27^{Kip1}$ allows cells to enter cycle. Thus, much of the IL-4-dependent growth promotion in lymphocytes may depend on the Stat6/Gfi-1/$p27^{Kip1}$ pathway rather than on signaling through IRS-2.

IL-4 generally mediates its functions in lymphocytes as a costimulant. In T cells, most IL-4 functions, such as $T_H2$ differentiation, depend on joint stimulation through the T-cell receptor (TCR) and the IL-4 receptor. It is striking that TCR engagement profoundly, although transiently, desensitizes the IL-4 receptor. Within 1 h of stimulation through the TCR, phosphorylation of IL-4Rα, JAK1, Stat6, and IRS-2 is strikingly attenuated. The failure of such cells to respond to IL-4 lasts for ~8 h, after which the cells regain their capacity to respond. The inhibition is not dependent on new protein synthesis and is reversed by MAPK kinase kinase (MEKK) inhibitors, suggesting that extracellular signal-related kinases (ERK1/2) play a major role in the inhibition process.

JAK/Stat signaling is inhibited by a set of suppressor of cytokine signaling (SOCS) proteins. It has been reported that interferon γ (IFNγ) induction of SOCS-1 results in inhibition of IL-4-mediated functions in macrophages and lymphocytes.

## V. REGULATION OF IL-4 PRODUCTION

IL-4 is produced by a series of cell types, including $T_H2$ cells, NK T cells, basophils, mast cells, and eosinophils. $T_H2$ cells are a polarized set of memory/effector CD4 T cells that have the capacity to produce IL-4 and a series of other cytokines, including IL-13, IL-5, IL-6, IL-9, and IL-10. Although there are no absolute markers of the $T_H2$ phenotype, these cells fail to express IL-12Rβ2 and IL-18 receptors and often express the IL-1 receptor analogue T1-ST2. They are more likely to express the chemokine receptor CCR4 and less likely to express CCR5 and CXCR3.

Naive CD4 T cells are capable of producing small amounts of IL-4 in response to TCR/CD28-mediated signaling, but they are not "high-rate" IL-4 producers. Differentiation of naive CD4 T cells into $T_H2$ cells is optimally achieved by culturing these cells with their cognate antigen and antigen-presenting cells (or with TCR stimulants such as anti-CD3, in association with anti-CD28) together with IL-4 and IL-2. In general, such differentiation is inhibited by IFNγ and possibly by IL-12.

IL-4 plays its role in $T_H2$ differentiation by acting through Stat6. This leads to the induction of Gata3. Retroviral transduction of TCR-stimulated cells with Gata3 leads to the development of IL-4-producing cells, strongly indicating that Gata3 plays an important role in $T_H2$ differentiation. Indeed, many of the cytokines that are produced by $T_H2$ cells have Gata-binding sites in their promoters and elsewhere in the gene. Several such sites have been identified in the *Il4*gene. On the other hand, transduced Gata3 is much less effective than transduced, constitutively active Stat6 in inducing IL-4-producing cells, suggesting that optimal induction of the $T_H2$ phenotype requires an additional Stat6-inducible factor.

Although IL-4 is very important in $T_H2$ induction *in vitro*, there are instances in which $T_H2$ cells develop in the absence of IL-4 or in the absence of IL-4Rα or Stat6. However, in one instance, it was shown that even Stat6 − /− cells that develop into IL-4 producers have up-regulated Gata3, arguing for the importance of Gata3 in IL-4-production.

Physiologically, the source of IL-4 that participates in $T_H2$ differentiation has been enigmatic. Other IL-4-producing cell types, such as NK T cells and basophils, have been proposed as sources of the IL-4 that plays a role in $T_H2$ differentiation. There is no convincing evidence implicating basophils or mast cells in this process. It has been suggested that NK T cells may play a role, particularly because of reports that both in models of type 1 diabetes in mice and in human type 1 diabetes, there is a deficiency of NK T cells. In humans in the prodromal stages of type 1 diabetes, a deficiency in serum IL-4 levels has also been suggested.

However, data continue to indicate that IL-4 production by naive T cells may play a role in IL-4-dependent $T_H2$ development, at least in some settings. There is evidence that naive T cells can transcribe IL-4 and that such IL-4 production is not blocked by anti-IL-4. Further, mice lacking IL-4Rα also produce "early" IL-4, further arguing that such production is a property of naive CD4 T cells rather than previously differentiated $T_H2$ cells. Finally, cloning experiments indicate that early IL-4 production is a property of the majority of CD4 T cells and does not represent production of large amounts of IL-4 by rare cells.

Intensity of T cell stimulation plays a role in the differentiation of naive CD4 T cells into $T_H2$ cells. In some well-characterized systems, naive CD4 T cells cultured without added polarizing cytokines tend to develop IL-4-producing capacity when stimulated with low concentrations of their cognate antigens. High concentrations favor the differentiation of these cells into IFNγ-producing $T_H1$ cells. These dose effects appear to be due, at least in part, to the endogenous induction of an appropriate cytokine environment, because the $T_H2$ differentiation that occurs in response to low concentrations of antigen is largely inhibited by anti-IL-4 antibody, whereas the $T_H1$ differentiation that occurs in response to high antigen concentration is blocked by anti-IFNγ.

Dendritic cells may play a substantial role in determining the degree of differentiation of naive cells into $T_H2$ cells. Highly activated dendritic cells, particularly those that have been stimulated with lipopolysaccharide or through CD40L, produce substantial amounts of IL-12p70, which aids in driving IFNγ production. These two cytokines, acting together or separately, favor the differentiation of naive cells into $T_H1$ cells and inhibit $T_H2$ differentiation. Forms of dendritic cell activation that fail to induce IL-12p70, or the loss of IL-12p70-producing capacity by dendritic cells, may render those cells more likely to induce a $T_H2$ response, because they would be less effective in inhibiting the activity of endogenously produced IL-4 in driving $T_H2$ differentiation.

## VI. IL-4 GENE STRUCTURE AND EXPRESSION

The *Il4* gene consists of four exons distributed over 6.2 kB located on human chromosome 5 at q23–q31 and in the syntenic region on mouse chromosome 11. The gene is in a cytokine complex. Genes *Il13, Il5, GM-CSF*, and *Il3* are linked to the *Il4* gene; *Il13* is located ~11 kB from *Il4*, and a highly conserved element in the *Il4–Il13* intergenic region, i.e., *CNS-1*, plays a role in regulating the level of expression of both IL-4 and IL-13.

The *Il4* gene appears to be in a closed conformation in naive T cells. The gene displays little evidence of DNase 1 hypersensitivity and most CpGs are methylated. As cells undergo $T_H2$ differentiation, they show progressive evidence of developing an open conformation in the *Il4* genetic region. The *Il4* gene in $T_H2$ clones has a series of DNase 1 hypersensitivity sites, most notably in the second intron and downstream of the fourth exon, in a site designated V. The *CNS-1* element also acquires DNase I hypersensitivity. Similarly, $T_H2$ clones display CpG demethylation and histone acetylation in regions of DNase 1 hypersensitivity, further indicating the acquisition of an accessible conformation. Newly differentiated $T_H2$ cells show some of the chromatin changes observed in $T_H2$ clones.

*Il4* has been reported to be expressed monoallelically. Using knock-in mice, in which one IL-4 allelic product can be distinguished from the other at the level of individual cells, it has been observed that, in many $T_H2$ cells, IL-4 encoded by only one of the two alleles is produced. Furthermore, $T_H2$ clones from heterozygous mice show distinctive ratios of expression of the two allelic products. This translates into distinctive proportions of cells that express the two alleles on stimulation. The ratio of expression of the two alleles is heritable, strongly suggesting that it represents an epigenetic trait, presumably due to distinctive patterns of accessibility of the two alleles in individual $T_H2$ cells and in the clones derived from them. The biological significance of monoallelism at the *Il4* locus is still not understood. It has been suggested that it relates to the potential of the *Il4* gene to exist in several different conformations, each associated with a distinctive probability of expression. This may represent a particularly effective way to control the proportion of antigen-specific CD4 T cells that can produce IL-4 and thus can engage in productive interactions with B cells, dendritic cells, macrophages, and other cell types.

## VII. IL-4 FUNCTIONS

IL-4 receptors are broadly expressed, indicating a wide range of cell types that are potential targets of IL-4 action. The earliest recognized activities of IL-4 were in regulating growth and survival of naive B cells stimulated by cross-linkage of membrane IgM. Indeed, IL-4 was first designated B cell growth factor (BCGF) because of this activity. By itself, IL-4 does not cause the growth of either resting T or B cells, although it is a good survival factor, particularly for resting T cells.

### A. Immunoglobulin Class Switching

IL-4 has a major role in controlling the class of immunoglobulin produced in the course of an antibody response. *In vitro*, IL-4, acting with anti-CD40 or with lipopolysaccharide (LPS), causes B cells to differentiate into IgE-secreting cells; when mouse cells are used, IL-4 also promotes the production of IgG1, but when human cells are used, IgG4 production is enhanced. This effect appears to be highly significant at the physiologic level. Mice in which the *Il4* gene, the *Il4R*$\alpha$ gene, or the *Stat6* gene has been inactivated by gene knockout technology have a profound diminution in levels of serum IgE and in IgE antibody responses. They also show defects in IgG1 production, but these are somewhat more variable. IL-4 transgenic mice have heightened serum levels of IgE.

IL-4 acts as a switch factor by regulating the transcription of the germ-line *IgC*$\epsilon$ and *IgC*$\gamma$1 H-chain genes. The induction of germ-line transcription targets these genes for switch recombination and leads to class switching to the $\epsilon$ and $\gamma$1 isotypes. Interestingly, these events appear to occur principally in germinal centers. It has recently been shown that the RNA editing enzyme, activation-induced cytidine deaminase, is critical for class switching to occur.

An indication of the relative balance of $T_H1/T_H2$ differentiation *in vivo* is provided by the ratio of IgG1 to IgG2a in mouse serum. Whereas IL-4 is the principal switch factor regulating IgG1 production, IFN$\gamma$ regulates IgG2a switching, so that in situations in which IFN$\gamma$ predominates, a mouse will tend to have a high IgG2a:IgG1 ratio, whereas when IL-4 dominates, there will be a low IgG2a:IgG1 ratio.

### B. $T_H2$ Differentiation

As already discussed in some detail, the most efficient way to induce IL-4-producing $T_H2$ cells is by priming naive CD4 cells in the presence of IL-4. IL-4 acts through Stat6. Gata3 is induced and plays an important role in the acquisition of the $T_H2$ phenotype. A high rate of IL-4 production requires c-maf as well as Gata3. IL-4-mediated $T_H2$ differentiation results in the capacity of cells to produce other $T_H2$ cytokines, notably IL-13, IL-5, IL-6, IL-9, and IL-10.

In various *in vivo* models, IL-4-mediated $T_H2$ differentiation appears to play an important role in the pathogenesis of infectious and allergic diseases.

For example, in BALB/c mice, infection with *Leishmania major* leads to progressive disease with increasing lesion size and increasing parasite burden. In general, T cells from the draining lymph nodes of these mice produce substantial amounts of IL-4 and relatively little IFNγ. By contrast, in mouse strains such as C57BL/6, in which infection with *L. major* results in a CD4 T-cell response dominated by IFNγ-producing cells, the infection is resolved. In susceptible strains, neutralization of IL-4 by administration of anti-IL-4 antibody allows recipients to resolve infections with *L. major.* Such mice develop CD4 cell responses dominated by IFNγ production. It is generally believed that anti-IL-4 neutralizes the "early" IL-4 that plays a role in $T_H2$ differentiation in naive cells.

However, the process may be substantially more complex. For example, when certain strains of *L. major* (e.g., LV39) are used for infection studies, it is seen that both IL-4 and IL-4 receptor knockout mice are susceptible to infection and that anti-IL-4 treatment renders wild-type mice resistant to infection with LV39. This suggests that the presence of IL-4 at low concentrations or possibly at specific times during infection may be important in the development of resistance, independent of the striking role of IL-4 in the acquisition of a susceptible state (see discussion on IL-4 effects on dendritic cells, Section VII,D).

In a model of immune-mediated airway hyperreactivity in mice, IL-4 plays a major role in the induction of the hypersensitive state. Immunization of mice with ovalbumin (Ova) followed by airway challenge with Ova results in bronchiolar constriction, excessive mucus production, and an inflammatory response dominated by eosinophils. Neutralization of IL-4 at the time of initial immunization strikingly diminishes the magnitude of the hyperreactivity to challenge, suggesting that a $T_H2$ response plays a major role in the immunopathogenesis of this process. By contrast, neutralization of IL-4 at the time of challenge has little or no effect on the magnitude of the resistance to flow in the airway or on the other manifestations of the response. However, neutralization of IL-13 will block airway hyperreactivity and mucus production, although eosinophilia still occurs.

This indicates that IL-4 is critical to induction of the hyperreactive state but that it is not the principal mediator of the effector phase of the airway response. By contrast, anti-IL-13 does inhibit airway hyperreactivity, implying that it is the major effector molecule. Because IL-4 and IL-13 largely use the same receptor on bronchiolar endothelial cells and because direct insufflation of either IL-4 or IL-13 causes airway hyperreactivity, the distinctive actions of IL-4 and IL-13 as effector molecules may be determined by the relative amounts of the cytokines made during the effector phase of the allergic response, rather than by intrinsic differences in their functionalities.

## C. IL-4 Inhibits Macrophage Activation

IL-4 exerts potent inhibitory activity on macrophages, where it opposes many of the effector functions mediated by IFNγ. IL-4 inhibits the production of pro-inflammatory molecules that are normally induced as a result of the action of IFNγ alone or in synergy with other stimulants of inflammation. Among the molecules for which synthesis is inhibited by IL-4 are TNFα, IL-1β, IL-12, and IP-10. Furthermore, IFNγ is a potent stimulator of inducible nitric oxide synthase, a key enzyme in the induction of reactive nitrogen species, which are potent antimicrobial agents. IL-4 inhibits this induction as well as the induction of reactive oxygen intermediates and thus opposes macrophage activation. In many instances, IL-4 acts on macrophages with a function similar to that of IL-10. It can be speculated that the role of IL-4 in opposing much of the IFNγ-mediated induction of enhanced microbicidal activity of macrophages may actually represent the function of IL-4 as a regulator of levels of inflammation, which reflects a physiologic effort to control levels of tissue damage during immune responses.

## D. IL-4 Plays a Role in Dendritic Cell Differentiation and Maturation

*In vitro* production of dendritic cells from bone marrow precursors is usually achieved by growth of the cells in a mixture of granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4, implying an important role for IL-4 in this process. In general, immature dendritic cells, which are the cells emerging from such differentiation, are excellent in uptake of antigen but require activation both to mediate their optimal antigen-presentation function and to acquire the capacity to produce IL-12p70. Such a combination of functions allows dendritic cells to prime naive CD4 T cells optimally to develop into $T_H1$ cells. Somewhat surprisingly, Shortman and colleagues have presented evidence that the presence of IL-4 during this maturation phase strikingly enhances the capacity of dendritic cells to produce IL-12p70 and thus favors $T_H1$ differentiation. Other have reported that the presence of IL-4 enhances the maturation of monocyte-derived dendritic cells, which preferentially induce $T_H1$ responses, while exerting a pro-apoptotic effect on plasmacytoid dendritic cells, which have been proposed to enhance $T_H2$ responses. Indeed, it has been shown in a variety of models, including contact hypersensitivity, *L. major* infection, and cytotoxic T cell destruction of tumors, that IL-4 present at specific times is important in the acquisition of effector function. Analysis of these examples has suggested that the target of IL-4 is an antigen-presenting cell, most likely a dendritic cell. Thus, the *in vitro* data showing a role of IL-4 in acquisition of optimal IL-12p70-secreting activity may be paralleled with a physiologic effect of IL-4 on dendritic cell function.

### E. IL-4 Regulation of Cell Migration

IL-4 plays a role in the migration of eosinophils into tissues, which is of central importance in allergic inflammation. It does so in part through the induction of eotaxin-3 production by human vascular endothelial cells; eosinophils home to eotaxin produced by vascular endothelial cells through the action of CCR3 receptors expressed by the eosinophils. IL-4 has also been reported to up-regulate expression of vascular cellular adhesion molecule 1 (VCAM-1) on vascular endothelium, which would further aid in the accumulation of eosinophils in areas of allergic inflammation.

IL-4 also controls migration of B cells; stimulation with lipopolysaccharide or with anti-CD40 plus IL-4 results in marked enhancement of the migration response to BCA-1, SLC, ELC, and SDF-1.

## VIII. TARGETING IL-4 FOR THERAPY

Because of the central role IL-4 plays in the induction of allergic diseases and in the production of IgE antibody, it has been regarded as an important target for drug development for these diseases. Thus far, it has been difficult to develop small-molecule inhibitors of the binding of IL-4 to the type I IL-4 receptors, although new approaches in this area should be considered. Alternative approaches have been the use of soluble IL-4 receptors to inhibit IL-4 binding to cell-associated receptors or the use of an IL-4 mutant (Y124D) that acts as a competitive antagonist. Thus far, although there have been preliminary reports of the use of these approaches, there have not yet been any dramatic successes flowing from this effort. Similarly, animal studies have indicated that IL-4 can, in some instances, ameliorate disease-causing autoimmunity. Perhaps the most dramatic effects have been in administration of IL-4 to nonobese diabetic (NOD) mice that express a spontaneous type 1 diabetes. NOD mice that receive IL-4 have a striking amelioration of diabetes. There have also been reports that IL-4 can diminish the incidence and severity of experimental allergic encephalomyelitis, which is often used as a model for multiple sclerosis.

## Glossary

**IgE** Immunoglobulin class that mediates many allergic-type responses; functions by binding to high-affinity Fc$\epsilon$RI receptors on mast cells and basophils. When IgE molecules are cross-linked, by binding their cognate antigen, they signal the release of preformed mediators and the synthesis and secretion of cytokines.

**interferon $\gamma$** Cytokine that is a member of the interferon/interleukin-10 family. Mediates a wide variety of cellular functions, particularly the enhancement of the microbicidal activity of macrophages.

**Stat6** Latent transcription factor that docks to phosphorylated IL-4 receptor $\alpha$-chains, becomes phosphorylated, then dissociates from the receptor, dimerizes, enters the nucleus, and binds to specific elements in DNA, where it cooperates with other transcription factors in regulating the expression of IL-4-inducible genes.

**$T_H2$ cells** Differentiated form of CD4 T helper cells that have acquired the capacity to produce a set of cytokines that orchestrate humoral immune responses, particularly allergic-type responses. These cytokines include IL-4, IL-13, IL-5, IL-6, and IL-10.

**type I cytokines** Also known as hematopoietins; four-$\alpha$-helix-bundle proteins generally secreted by T cells, in response to stimuli mediated through the T-cell receptor. These molecules are recognized by a family of receptors designated hematopoietin receptors.

## See Also the Following Articles

**Interferons: $\alpha$, $\beta$, $\omega$, and $\tau$ • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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Encyclopedia of Hormones.

# Interleukin-5

ANGUS J. LAUDER[*], ANDREW N. J. MCKENZIE[*], AND COLIN J. SANDERSON[†]

[*]*MRC Laboratory of Molecular Biology, Cambridge, England* • [†]*Western Australia Institute of Medical Research*

I. INTRODUCTION
II. GENE AND PROTEIN STRUCTURE
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IX. IL-5 IN DISEASE

**Interleukin-5 is a cytokine produced predominantly by T cells. It is important in inflammatory responses due to its control of eosinophil production and its ability to activate basophils and to modify mast cell secretions. Inhibition of interleukin-5 may be key to therapeutic interventions in certain malignancies and hyperimmune conditions.**

## I. INTRODUCTION

Interleukin-5 (IL-5) was originally isolated by several groups independently on the basis of two different biological activities. Several years of work identified a B-cell differentiation factor (BCDF) and an eosinophil differentiation factor (EDF) that were subsequently shown to arise from the same protein. Cloning of the human and murine genes led to this factor being named interleukin-5. IL-5 is expressed predominantly by T lymphocytes and encodes a 40- to 45-kDa protein. Although it has been clear for some time that IL-5 is crucial in the development of eosinophils, IL-5 has also been implicated in a range of conditions, such as asthma, cancer, and parasitic infections.

## II. GENE AND PROTEIN STRUCTURE

The human IL-5 gene is located in the cytokine gene cluster on chromosome 5q31; the murine homologue is located in the syntenic region of mouse chromosome 11. These regions also contain the respective human and murine IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-4, and IL-13 genes. The IL-5 gene consists of four exons and three introns, which encode precursor proteins of 134 and 133 amino acids in the murine and human systems, respectively. The four-exon and three-intron gene structure of IL-5 is also found in the cytokine gene cluster genes encoding IL-4, IL-13, and GM-CSF. There is little sequence conservation between these genes but the conserved exon/intron structure and their chromosomal proximity to each other suggest a common evolutionary origin whereby gene duplication and subsequent sequence divergence led to the evolution of new cytokine genes. The human and mouse IL-5 coding sequences are highly conserved at the nucleotide (77%) and the amino acid (70%) levels, although there are differences in the structures of the two genes. The mouse gene produces a transcript of 1.6 kb, compared to the shorter 0.9-kb human mRNA. This is due to the presence of a number of *Alu*-like repeats and a 738-bp insertion flanked by inverted repeats in the murine sequence.

In both humans and mice, the IL-5 protein precursors are cleaved to generate the 115-amino-acid mature peptide. The IL-5 protein is subject to both O-linked (Thr-3) and N-linked (Asn-28) glycosylation, with an additional N-linked glycosylation site at Asn-55 in the murine IL-5. Glycosylation does not appear to be required for the biological activity of IL-5 because deglycosylated IL-5 retains comparable receptor binding and activity. However, N-linked glycosylation does appear to increase the thermostability of IL-5 and may therefore be important in the function of IL-5. Unusually for cytokine molecules, the active IL-5 protein exists as a dimer joined by two disulfide bridges between conserved cysteine residues. Dimer formation is crucial for the activity of IL-5 because mutation of the conserved cysteine residues blocks not only dimer formation but biological activity as well. X-Ray diffraction studies reveal that each monomer consists of four $\alpha$-helices arranged in an up/up–down/down topology, forming a characteristic cytokine fold. This structural arrangement is found in a number of other cytokine molecules, including IL-2, IL-4, GM-CSF, and the human growth hormone molecule. These cytokines exist as monomers, with their four α-helices forming the up/up–down/down cytokine fold. In the case of the IL-5 dimer, rather than each monomer forming a cytokine fold independently, the fourth helix from each monomer supplies the D′ helix, to complete the fold of the other monomer. Even though the D′ helix is supplied by the other monomer, the resulting cytokine fold is homologous to the cytokine folds formed by individual monomers of IL-2, IL-4, and GM-CSF.

The IL-5 protein has also been shown to contain a functional nuclear localization sequence (NLS). Fluorescence labeling of the IL-5 receptor has revealed that in the presence of IL-5 ligand the IL-5 receptor can translocate to the nucleus. Unlike nuclear hormone receptors, for which a clear nuclear signaling role has been defined, the functional significance of IL-5 receptor nuclear targeting is currently unclear.

## III. CONTROL OF IL-5 EXPRESSION

IL-5 is expressed predominantly by T lymphocytes and is associated with a $T_H2$ cytokine expression profile. In addition to T cells, IL-5 expression has also been detected from mast cells, eosinophils, and Epstein–Barr virus-infected B cells. IL-5 gene expression can be activated by a wide range of factors, including parasitic infection, the cytokines IL-2, IL-4, and IL-25, and chemical reagents such as phorbol esters in conjunction with phorbol myristate acetate (PMA). The control of gene expression can be analyzed at a number of different levels. In addition to numerous IL-5 promoter elements that have been reported, regulatory elements exist that co-ordinate expression of multiple genes within the cytokine gene cluster. Located between the IL-4 and IL-13 genes, the conserved noncoding sequence-1 (CNS-1) region was identified by comparison of the mouse and human cytokine gene cluster sequences. Deletion of this 401-bp sequence in mice reduces expression of IL-4, IL-5, and IL-13, resulting in impaired $T_H2$ responses. IL-4 production in mast cells was unaffected in these mice, suggesting that this element may regulate $T_H2$ cytokine expression specifically in the T-cell lineage. Although the CNS-1 element appears to regulate these cytokines coordinately, there are many circumstances in which these genes are differentially expressed. For example anti-CD3 stimulation of murine $T_H2$ clones results in induction of IL-4 and IL-5. However, stimulation of these cells with recombinant IL-2 induces only IL-5 and not IL-4, indicating that different signals can activate subsets of genes from the cytokine gene cluster.

The complex range of signals that affect IL-5 gene expression is reflected at the level of the IL-5 promoter. Numerous promoter elements interact with a range of transcription factors, including members of the GATA, Oct, activator protein 1 (AP-1), NFAT, and Ets families. One of the first regulatory sequences to be identified was the conserved lymphokine element 0 (CLE0), which is not only present in the IL-5 promoter but is also found in the IL-3, IL-4, and GM-CSF promoters. The CLE0 sequence (nucleotides −56 to −42 in humans) is required for IL-5 promoter activity and binds members of the AP-1 and Oct transcription factor families. Upstream of the CLE0 element lie several GATA binding sites, which play a positive role in IL-5 transcription (−70, −152), and a further GATA site (−400), which negatively regulates IL-5 expression. The Ets1 and Ets2 transcription factors have been shown to transactivate a human IL-5 reporter construct through a GGAA motif adjacent to the AP-1 site within the CLE0 element. Ets-mediated transactivation acts with GATA-3 in a cooperative manner to further increase transcription levels. In addition to 5′ control elements, additional positive sequences have been identified 3′ to the IL-5 gene. The mouse downstream regulatory element-1 (mDRE1) is located in the region +4539 to +4585 in relation to the translational start site of the IL-5 gene and has been shown to bind Oct-1 and Oct-2 transcription factors.

Higher order gene regulation at the level of chromatin structure also plays a key role in gene expression. On T-cell differentiation to a $T_H2$ phenotype, $T_H2$ cytokine genes become located to transcriptionally active regions of chromatin whereas silenced $T_H1$ cytokine genes generally become localized to inactive heterochromatin regions. GATA-3 expression has been shown to promote $T_H2$ differentiation, and, remarkably, ectopic expression of GATA-3 can induce $T_H2$ cytokine expression in committed $T_H1$ cells. GATA-3 appears to act as a master switch to induce $T_H2$ cytokines. The IL-5 promoter contains numerous GATA binding sites that help to mediate its induction under $T_H2$-promoting conditions. Even though different genes may utilize the same transcription factors, differences in their mode of action may exist. For example, GATA-3 induces both IL-4 and IL-5, consistent with its role as an inducer of $T_H2$ differentiation. In the case of IL-5, GATA-3 appears to act directly on the IL-5 promoter. In contrast, GATA-3-mediated IL-4 induction may act through a global $T_H2$ enhancement and not directly through binding at the IL-4 promoter. Interestingly, activation of IL-4 and IL-5 by GATA-3 requires different regions of the GATA-3 protein. Transactivation of IL-4 requires only the C-terminal zinc finger domain, whereas induction of IL-5 by GATA-3 requires both the N- and C-terminal zinc finger domains, which may reflect a different mode of action.

Many of the transcription factors detailed above are involved in regulating multiple genes, including those of other cytokines. This raises the question as to how gene-specific transcriptional activation is achieved. The use of similar transcription factors may help to coordinate regulation of groups of genes such as $T_H2$ cytokines. Differences in the number of binding sites, cooperation with other sites, and the different sets of factors activated by different cellular stimuli allow specific regulation of genes within such a group. The potential of gene-specific regulatory sequences/proteins is clearly evident in this respect. Recent evidence has uncovered a pair of interacting palindromic sequences nearly 400 bp apart that positively regulate IL-5 expression. Mutation of the positive regulatory motifs within either of these sequences results in a 60 to 70% reduction in IL-5 expression. These elements form protein/DNA complexes with nuclear extracts from the EL4-23 T-cell line, and although the binding proteins have not yet been identified, elements such as these would help to achieve specific IL-5 expression under conditions in which other $T_H2$ cytokines may not be induced.

## IV. IL-5 RECEPTOR STRUCTURE/ISOFORMS/DISTRIBUTION

The IL-5 receptor consists of α and β chains that, when bound to IL-5 ligand, activate a series of signal transduction cascades. The β chain of the IL-5 receptor is also utilized by the IL-3 and GM-CSF receptors and is therefore known as the βc, or common, β chain. The shared usage of the βc chain explains the partially overlapping functions of these cytokines, such as their ability to promote eosinophil survival. IL-3, IL-5, and GM-CSF compete for receptor binding, presumably due to limiting amounts of the shared βc chain. Each cytokine receptor has a unique α chain that provides specificity, and the distribution of these chains defines the differing biological roles of these cytokines. In the human system, IL-5 receptors appear to be found only on eosinophils and basophils. In mice, in addition to eosinophils and basophils, the IL-5 receptor is also found on activated B cells and on B1 cells from the peritoneal cavity. The α and β chains are both required to generate a successful IL-5 signal. The βc chain contains a large cytoplasmic domain and is essential for signal transduction. Transfection of the α and β chains into the CTLL-2 T-cell line generates a functional IL-5 receptor and enables these cells to proliferate in response to IL-5. However, transfection of the wild-type βc chain with an α chain lacking its cytoplasmic tail generates a signaling-deficient receptor. It is not clear at this time if the small cytoplasmic domain of the α chain interacts directly with the intracellular signaling machinery or if it acts through interaction with the βc chain. Direct interaction of the cytokine-specific α chain with the signaling machinery is an attractive model because it provides a simple explanation for producing IL-3-, IL-5-, and GM-CSF-specific signals. A recent report describes the identification of the first signaling molecule shown to interact with the IL-5Rα. The protein syntenin was shown to interact with the IL-5R α chain but, significantly, not with the IL-3R or GM-CSFR α chains, thereby enabling an IL-5 receptor-specific signal to be generated.

The hIL-5Rα gene encodes a protein of 420 amino acids containing a 20-amino-acid N-terminal signal peptide, a 324-amino-acid extracellular domain, a 21-amino-acid transmembrane span, and a short 55-amino-acid cytoplasmic domain. The common β chain is much larger, at 897 amino acids, and contains a 16-amino-acid signal sequence, a 424-amino-acid extracellular domain, a 27-amino-acid transmembrane span, and a large 430-amino-acid cytoplasmic domain. These receptor proteins belong to the hemopoietin superfamily in which the extracellular domain is a modular structure and each module consists of a seven-β-sheet scaffold. The extracellular domain of the α chain contains three modules. The membrane-proximal module contains a canonical Trp-Ser-Xxx-Trp-Ser (WS-WS) motif that is characteristic of this receptor superfamily. The second module contains conserved cysteine residues that form disulfide bridges within the protein, and the third module contains a WS-WS-like motif. The extracellular portion of the βc chain contains four modules; two contain WS-WS motifs and the other two contain conserved cysteine residues that form disulfide bridges. The cytoplasmic domains of the IL-5 receptor lack intrinsic kinase activity but promote signaling through the association of numerous signaling molecules and kinases (see Section V).

The IL-5Rα chain in isolation binds IL-5 with low affinity, whereas the βc chain alone has no affinity for IL-5. Interaction of the α and β chains forms the high-affinity IL-5 receptor. Although the IL-5Rα chain is the major partner in ligand binding, interaction of IL-5 with the β chain is also required for signaling to proceed. Mutation of a glutamic acid to a glutamine residue at position 13 of hIL-5 disrupts the normal interaction with the βc unit. This mutant acts in an antagonistic fashion inasmuch that it binds functional receptors but cannot trigger signaling due to disruption of the IL-5 ligand/βc chain interaction. The IL-5R βc chain has been crystallized and the X-ray structure should soon be published, providing additional information on the structure/function of the IL-5 receptor.

Expression of natural IL-5 antagonists has been detected in human eosinophils and in murine B cells. These proteins take the form of soluble IL-5Rα chains and can inhibit the proliferation of IL-5-dependent cell lines. Two soluble receptor transcripts are generated by differential splicing events that remove the soluble-specific exon from its position adjacent to the transmembrane region, producing a truncated protein consisting of the α chain extracellular domain. A soluble transcript accounts for around 90% of the transcripts in mature eosinophils from cord blood, and soluble IL-5Rα may act as a negative feedback mechanism during eosinophil activation. Recent work has suggested that IL-5 may regulate the production of its own receptor, determining the relative proportions of soluble and membrane-bound forms. Through the use of a minigene construct containing the human IL-5 gene it was shown that IL-5 could switch expression from soluble IL-5Rα to the transmembrane (TM)-bound TM-IL-5Rα form. In an eosinophil differentiation assay in the presence of IL-5, TM-IL-5Rα was up-regulated between days 5 and 7, returning to predominantly soluble IL-5Rα chain expression by day 14 of the assay, when mature eosinophils predominated. IL-5 may therefore positively regulate its receptor expression to promote IL-5 signaling during eosinophil differentiation. The mechanism by which expression changes between soluble and membrane-bound forms is not clear.

## V. IL-5 RECEPTOR SIGNALING

Although it is the IL-5Rβ chain that activates the majority of the intracellular signaling components, both the α and β chains of the IL-5 receptor are required to transmit the IL-5 signal. On binding of IL-5, the β chain becomes tyrosine phosphorylated, which provides binding sites for proteins that contain conserved Src homology 2 (SH2) domains such as the signal transduction and activator of transcription (Stat5) protein. Many signal transduction proteins utilize SH domains to mediate binding to receptor phosphotyrosine residues. Deletion studies of the β chain cytoplasmic domain have revealed two regions important for the mitogenic signal. The membrane region proximal to residue 517 is sufficient to induce expression of c-Myc and the pim-1 kinase. Residues 626 to 763 of the β chain mediate phosphorylation of the adapter protein Shc, activation of the Ras/Raf/-mitogen-activated protein kinase (MAPK) pathway, and induction of the transcription factors c-Fos and c-Jun. The adapter protein Shc mediates activation of Ras by recruiting the adapter protein Grb2, which binds the son-of-sevenless (Sos) guanine nucleotide exchange factor, which then activates Ras. The region from 626 to 763 also mediates activation of two kinases with key roles in cell division and survival. Deletion of this region abrogates the ability of the β chain to activate phosphatidylinositol 3-kinase (PI3K) and p70S6 kinase. PI3K activates a number of signaling pathways, including protein kinase B (PKB), and contributes to the activation of p70S6 kinase. Activation of PKB protects numerous cell types from apoptosis and p70S6 kinase is not only involved in regulating protein translation but also contributes to regulation of the cell cycle. Although some of these pathways have not yet been shown to be directly downstream of IL-5, evidence from other systems that utilize the common β chain suggests that they are likely to be. For example, PKB, a downstream effector of PI3K, mediates survival downstream of the common β chain containing IL-3R.

The α and β chains both contain conserved proline-rich sequences known as box1 sequences; these are found in a large number of cytokine receptors. In the case of the β chain, the proline-rich sequence lies in the membrane-proximal region, which is crucial for activation of the genes encoding c-Myc and pim-1. Mutation of proline 352 or 355 inhibits the IL-5 mitogenic signal, thus the proline-rich box1 sequence of the α chain is essential for mediating growth signals. Mutation of these proline residues has been shown to abrogate activation of the kinases JAK1 and JAK2 and their downstream target, the transcription factor Stat5. A dominant negative Stat5 protein can inhibit proliferation induced by IL-3, IL-5, and GM-CSF, all of which use the βc chain and activate JAK kinases as part of their signaling function. In addition to Stat5, IL-5 signals have been reported to mediate activation of the Stat family members Stat1α and Stat3. Co-immunoprecipitation experiments have provided significant information on the role of JAK kinases in IL-5 signaling. JAK2 associates with the hIL-5Rα chain with or without IL-5 stimulation, showing that JAK2 is bound constitutively to the α chain. JAK1, on the other hand, constitutively associates with the βc chain but also becomes associated with the α chain on IL-5 stimulation. In both cases, it is only after IL-5 stimuli that the JAKs become phosphorylated and activated. Although abundant data on JAK/Stat function have appeared recently, the number of defined target genes is still relatively small. Stat5 target genes include JAK2-binding SH2-containing protein (JAB) and the cytokine-inducible SH2 protein (CIS). CIS is a member of the suppressor of cytokine signaling (SOCS) family of proteins, which act as negative feedback controls by suppressing JAK signaling.

It has been known for some time that the cytoplasmic domain of the α chain is required for IL-5 signaling, but it is only in the past few years that specific signaling functions have been defined. In addition to a role in JAK/Stat activation, the α chain has also been shown to interact with IL-5-specific signaling molecules. Using a two-hybrid approach, the protein syntenin was shown to interact with the last 15 carboxy-terminal residues of the IL-5Rα chain but, significantly, not with the IL-3 or GM-CSF α chains, thereby generating an IL-5 receptor-specific signal. Further two-hybrid and co-immunoprecipitation approaches showed that syntenin bound to the Sox4 transcription factor. Sox4 is known to play a role in B-cell development and IL-5 has been implicated in the development of the B1 subset of B cells in the mouse system. Although only published recently, these data may provide a link between the IL-5 receptor and B-cell development. In addition to JAK/Stats, a number of other kinases have been implicated in IL-5 signaling in B cells. For example, IL-5 signals in an early B-cell line were shown not only to activate JAK2 but also to activate B-cell-specific Bruton's tyrosine kinase (Btk). Addition of a gain-of-function mutant of Btk was able to confer IL-5 independence on this cell line. Mice deficient in Btk are immunodeficient (xid) and have vastly reduced levels of circulating Ig due to a block in B-cell development. These mice also have a defect in B1 cell development similar to the IL-5-deficient mice. The B cells from Btk-deficient mice respond poorly to IL-5 in an IgM induction experiment, but eosinophils from these mice respond normally, suggesting that Btk is specifically involved in IL-5 signaling in B cells.

## VI. EOSINOPHIL DIFFERENTIATION/PROLIFERATION AND APOPTOSIS

IL-5 was first identified by a number of activities in cell culture, one of which was its ability to promote eosinophil differentiation in bone marrow proliferation assays. Transgenic mice constitutively expressing IL-5 within the T-cell compartment develop a profound eosinophilia that infiltrates the blood, spleen, and bone marrow. These mice retain eosinophilia throughout their lives yet remain apparently normal. This is presumably because the large numbers of eosinophils do not degranulate and cause tissue damage. In the presence of secretory Ig, IL-5 has been shown to enhance eosinophil degranulation and the release of eosinophil-derived neurotoxin (EDN). IL-5 can also increase production of the superoxide anion, a cytotoxic weapon commonly used by the immune system. The reason why degranulation and tissue damage do not occur in IL-5 transgenic mice *in vivo* is unclear. The ability of eosinophils to lyse antibody-coated tumor cells is increased in the presence of IL-5, as is the phagocytic uptake of opsonized yeast particles.

In addition to modulating eosinophil activation, IL-5 can also promote eosinophil chemotaxis and survival. IL-5 has a marked dose-dependent chemotactic effect on eosinophils *in vitro*. In addition, it has also been reported that IL-5 "primes" eosinophils, which then become more responsive to other chemotactic molecules. A possible mechanism for this effect has recently been identified. Comparison of the signal transduction events downstream of chemotactic molecules, such as IL-8 and receptor-activated neutrophil T-cell expression and secretion (RANTES), has shown that activation of the extracellular signal-related kinases ERK1 and ERK2 (MAP kinases) is significantly increased in IL-5-primed eosinophils. Therefore, in addition to promoting eosinophil differentiation and activation, IL-5 may also be responsible for their recruitment to sites of inflammation.

Purified human eosinophils die by apoptosis in cell culture systems. Although IL-3 and GM-CSF promote survival in these systems, IL-5 has a much more potent eosinophil survival activity. Eosinophils from patients with eosinophilic pleural effusions exhibit in culture enhanced survival that can be blocked by addition of anti-IL-5 antibody. IL-5 can inhibit apoptosis induced by toxic agents such as staurosporine and by physiological mediators such as Fas. IL-5 strongly inhibits Fas-mediated eosinophil apoptosis and this correlates with the prevention of caspase activation. A further study found that IL-5 blocked translocation of the pro-apoptotic protein Bax to the mitochondria and subsequent cytochrome *c* release. Increased expression of Bcl-2 and its family member Mcl-1 has also been proposed as to mediate the IL-5 survival signal. However, although the IL-5-mediated survival effect is clear in these studies, the changes in Bcl-2 and Mcl-1 protein levels are small and their significance remains to be proved. The molecular link between the IL-5R and the apoptotic machinery has not yet been elucidated, although it may well involve PI3K, which is activated downstream of IL-5 and has several known survival functions.

Infection with the parasitic hookworm (*Necator americanus*) in humans and the nematode worm *Nippostrongylus brasiliensis* in mice leads to the profound development of eosinophilia and increased levels of IgE. Using monoclonal antibodies to inhibit IL-4 and IL-5, it has been shown that even though IL-4 is responsible for IgE induction, IL-5 mediates eosinophilia in *N. brasiliensis*-infected mice. IL-5-deficient mice fail to develop eosinophilia in response to *Mesocestoides corti* helminth infection, although basal levels of eosinophils are unaffected. IL-5 deficiency and the resulting lack of eosinophilia do not appear to affect worm expulsion, but potential functions of IL-5 could be hidden by the redundancy within the $T_H2$ cytokines. Recent evidence suggests that the individual roles of $T_H2$ cytokines in worm expulsion are only visualized on a background lacking other $T_H2$ cytokines. For example, deletion of IL-5 alone does not alter *N. brasiliensis* expulsion, but deletion of IL-5 in conjunction with deletion of other $T_H2$ cytokines has cumulative effects, indicating that each cytokine contributes to the overall response.

## VII. BASOPHILS/MAST CELLS

In addition to eosinophils, the IL-5 receptor is also expressed on human basophils. Cultures of human cord blood in the presence of IL-5 stimulates the growth of eosinophils, as expected, but also results in an increase in the numbers of basophils, although to a much lesser extent. IL-5 enhances basophil activation in a manner similar to its role in priming eosinophil activation. Basophils produce histamine and leukotriene, which are released at sites of inflammation. Complement C5a by itself triggers histamine release, although basophils primed with IL-5 show enhanced histamine release and also produce large quantities of leukotriene C4 in response to C5a. IL-5 is also able to promote histamine release in response to the basophil agonists complement C3a, neutrophil-activating peptide 1, and IgE.

Mast cells have also been reported to secrete IL-5 in response to cross-linking of the FcεRI receptor by IgE. In addition, IL-5 may prime mast cells and alter their cytokine production. A study looking at the ability of IL-4 and IL-5 to modify mast cell cytokine production in response to FcεRI cross-linking found that the two cytokines primed mast cells to produce different cytokine profiles. For example, IL-5 priming increased production of cytokines such as tumor necrosis factor α (TNFα), IL-5, and IL-13, whereas IL-4 additionally enhances histamine release. The ability of IL-5 to mediate eosinophil and basophil activation and to modify mast cell secretion indicates that IL-5 is an important mediator of inflammatory responses and may provide a target for anti-inflammatory treatments.

## VIII. B LYMPHOCYTES

IL-5 was originally identified due to its ability to induce differentiation and antibody production in murine B cells. Despite this fact, to date, human B cells have not been shown either to express the IL-5 receptor or to respond to IL-5. This is a major species difference that needs to be borne in mind when considering anti-IL-5 therapies. In the mouse, although not active on naive B cells, IL-5 acts in concert with antigen to promote the growth and development of IgM- and IgG-secreting B cells, and in combination with IL-4, IL-5 can promote IgG1 secretion. IL-5 has also been reported to enhance IgA production preferentially in lipopolysaccharide (LPS)-stimulated splenic B-cell cultures. However, in B cells isolated from the gut-associated lymphoid tissue, IL-5 appears to act by increasing IgA expression in switched cells rather than by inducing switching to the IgA isotype. A recent study has provided evidence of a role for IL-5 in switching to the IgG1 isotype. CD38 is widely expressed on B cells, and stimulation of CD38 with the antibody CS/2 protects B cells from apoptosis and induces expression of the IL-5Rα chain. Cross-linking of sIgD + splenic B cells (conventional B cells) with the CS/2 antibody in the presence of IL-5 has been shown to strongly induce IgG1 expression. Significantly, IgM to IgG1 isotype switching events are detected only after culture and not in the starting population, although this effect has not been proved to be a direct effect of IL-5.

The development of transgenic and knockout mice has enabled the role of IL-5 in antibody responses to be investigated *in vivo*. Deletion of the IL-5 ligand results in mice with a defect in B1 cell development, an inability to produce eosinophilia in response to helminth worm infection, but with apparently normal antibody responses. IL-5Rα-deficient mice also exhibit a decrease in B1 cells (a subset known to express IL-5Rα), but, unlike the IL-5 − /− mice, show reduced levels of circulating IgM and IgG3. Reciprocally, IL-5 transgenic mice show strongly enhanced levels of serum IgM and increased levels of IgA. However, in another line of transgenic mice, no differences in immunoglobulin levels are observed. Analysis of IgA production in IL-5Rα − /− mice reveals reduced levels in mucosal secretions. Closer examination of mucosal effector tissues, such as the intestinal lamina propria, reveals a reduction in the number of sIgA B1 cells at these sites. The B1 cells are a B cell subset that expresses Mac1, in the case of B1b cells, and Mac1 with CD5, in the case of B1a cells. The B1 cells reside mainly in the peritoneal cavity but are also resident in gut-associated lymphoid tissues and are predominantly responsible for the production of natural antibodies that have been linked to the development of autoimmunity. Transgenic overexpression of IL-5 not only increases CD5 + B1 cells but also results in the production of IgM anti-DNA autoantibodies. We have recently produced transgenic mice expressing IL-5 under the control of a B-cell-specific enhancer. These mice produce autoreactive IgG antibodies against nuclear antigens. IL-5 may therefore play a role in the development of autoantibody-mediated autoimmunity.

IL-5-deficient mice produce normal serum antibody levels and comparable specific antibody responses to influenza virus infection. Although the presence of IL-5 may not be essential for antibody responses, a wealth of *in vitro* and *in vivo* data point to a role for IL-5 in stimulating B cells and modifying antibody responses in mice. In contrast, there is no compelling evidence for an effect on B cells or antibody responses in humans.

## IX. IL-5 IN DISEASE

The most well-known link between IL-5 and human disease is in the inflammatory disease asthma. IL-5 plays a role in the activation of eosinophils, basophils, and mast cells, all of which are important mediators of inflammation. IL-5 mRNA has been detected in mucosal bronchial biopsies from asthmatic patients. Ablation of IL-5 function by gene knockout or by administration of neutralizing anti-IL-5 antibody abolishes eosinophilia and airway hyperreactivity in some mouse and primate models of asthma, providing a link between IL-5 and the asthmatic phenotype. Preliminary reports from clinical trials of humanized anti-IL-5 antibodies have reported substantially decreased blood eosinophilia in asthmatic patients in response to a single intravenous injection. Although blood eosinophilia is reduced in these patients, there is no effect on airway hyperresponsiveness to histamine. Surprisingly, there is no reduction in lung eosinophils, suggesting that the antibody is not entering the lung tissue and neutralizing locally produced IL-5. Monoclonal antibodies against the common βc chain have been developed such that they simultaneously inhibit IL-3, IL-5, and GM-CSF, and these may be more potent eosinophil inhibitors. Peptide antagonists that inhibit IL-5 have also been reported and together with specific antibodies provide a range of potential anti-IL5 therapeutic agents.

Expression of IL-5 has also been associated with a number of other diseases, including Hodgkin lymphoma and idiopathic hypereosinophilic syndrome (HES). Many cases of HES are believed to be due to defects at a primitive stage of eosinophil differentiation, but a significant number of cases are associated with aberrant T-cell populations expressing large amounts of IL-5. Hodgkin's lymphomas are characterized by malignant Hodgkin/Reed–Sternberg (H/RS) cells. These cells constitute only a small percentage of the tumor mass, with the remainder made up of nonmalignant T cells, B cells, eosinophils, and neutrophils. The reason for the accumulation of these cells around the Reed–Sternberg cells is not known, but studies have implicated the expression of cytokines by the H/RS cells as an important factor. Hodgkin lymphomas are classified according to the associated cellular infiltrates, and certain cases are associated with eosinophilia. Examination of a panel of samples from Hodgkin tumors with associated eosinophilia reveals that the malignant H/RS cells in these cases express IL-5. Expression of IL-5 has also been detected in a number of H/RS cell lines and it may be that IL-5 is important in the development of Hodgkin disease, especially in cases associated with eosinophilia.

IL-5 is an important molecule in inflammatory responses, largely due to its control of eosinophil production. The development of specific inhibitors of IL-5 function may therefore prove to be useful therapeutic agents in the treatment of diseases such as asthma. The apparent low efficacy of anti-IL-5 monoclonal antibody to decrease airway eosinophils and hyperresponsiveness in asthma patients emphasizes the importance of developing alternatives such as small-molecule antagonists.

## Glossary

**apoptosis** Form of cell death controlled by an array of intracellular molecules, resulting in nuclear DNA degradation and phagocytosis of the cell residua. This process is used in a widespread fashion during development and in the control of lymphocyte differentiation and immune responses.

**asthma** Atopic disease characterized by the tendency to produce hypersensitive responses to normally innocuous antigens, resulting in lung inflammation due to the degranulation of mast cells and eosinophils and leading to impaired breathing ability.

**eosinophilia** The presence of highly elevated numbers of eosinophils, white blood cells important in the defense against parasitic infections.

**helminth worms** Parasites such as *Nippostrongylus brasiliensis*, which pass through the intestine and provoke a strong $\mathrm{T_H}2$-mediated immune response that normally results in clearance of the worms from the system.

**isotype switching** Process by which a B cell switches from production of an immunoglobulin isotype, such as IgM, to production of another isotype, such as IgA; accomplished by switching to the transcription of an alternative antibody heavy chain C region.

**T helper 2 response** Immune response mediated by CD4 + T helper 2 ($\mathrm{T_H}2$) lymphocytes that produces the characteristic $\mathrm{T_H}2$ cytokines (interleukins-4, -5, -9, and -13). The $\mathrm{T_H}2$ branch of the immune system is mainly involved in promoting antibody production by B lymphocytes.

## See Also the Following Articles

**Apoptosis • Glucocorticoids and Asthma • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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TABLE 1 Summary of Chromosomal Localization and Homology among Species

| Molecule | Alternative name[a] | Species[b] | Amino acid length | | Homology[c] | Chromosome | Database accession |
|---|---|---|---|---|---|---|---|
| | | | Precursor | Mature | | | |
| IL-6 | IFNβ2, BSF-2, TRF, BCDF, BCDFII, 26-kDa protein, HPGF, HSF, MGI-2, IL-HP1 | Human | 212 | 185 | – | 7p21 | X04602 |
| | | Mouse | 211 | 187 | 42% | 5 | J03783 |
| | | Rat | 211 | 187 | 41% | 4 | M26744 |
| | | Pig | 212 | 184 | 62% | 9 | AF309651 |
| | | Cow | 208 | ~182 | 53% | 4 | X57317 |
| | | Sheep | 208 | 180 | 53% | 4q1.3–q1.4 | X62501 |
| | | vIL-6 | 204 | 182 | 25% | — | U67774 |
| IL-6Rα | CD126, gp80 | Human | 468 | 449 | — | 1q21 | X12830 |
| | | Mouse | 460 | 441 | 54% | 3 | X51975 |
| | | Rat | 462 | 443 | 53% | 2 | M58587 |
| gp130 | CD130, IL-6ST, IL-6Rβ | Human | 918 | 896 | — | 5q11 | M57230 |
| | | Mouse | 917 | 895 | 77% | 13 | M83336 |
| | | Rat | 918 | 896 | 78% | 2q14–q16 | M92340 |

[a] IFNβ2, Interferon β2; BSF-2, B-cell stimulating factor-2; TRF, T-cell replacing factor; BCDF, B-cell differentiation factor; HPGF, hybridoma/plasmacytoma growth factor; HSF, hepatocyte-stimulating factor; MGI-2, monocyte/granulocyte inducer type 2; IL-HP1, interleukin hybridoma plasmacytoma 1; IL-6ST, IL-6 signal transducer.

[b] Viral IL-6 encoded by Kaposi's sarcoma-associated virus.

[c] Overall homology to the human amino acid.

AF012908), beluga whales (*Delphinapterus leucas*, AF076643), and red-crowned mangabeys (*Cercocebus torquatus*, L26032). In addition, Kaposi's sarcoma-associated herpes-like virus encodes a functional IL-6 homologue called vIL-6 (U67774). The amino acid sequence deduced from the human *IL-6* cDNA reveals that human IL-6 consists of 212 amino acids, with a hydrophobic signal peptide of 27 amino acids and two potential N-linked glycosylation sites (Fig. 1a). Four cysteine residues are found in the human IL-6 amino acid sequence; this motif is conserved among species, including viruses (vIL-6), and also exists in human and mouse G-CSF. In contrast to the human IL-6, the mouse orthologue contains several potential O-linked glycosylation sites instead of the N-linked glycosylation site. The overall homology at the amino acid level in human, animal, or viral IL-6 is summarized in Table 1.

## D. Protein

The human IL-6 protein has a molecular mass ranging from 21 to 28 kDa and an isoelectric point of 5.4. IL-6 protein is posttranscriptionally glycosylated and phosphorylated on serine residues. Glycosylation seems not to be necessary for the biological activity of IL-6 because the recombinant IL-6 protein produced by prokaryotes (*Escherichia coli*) appears functional. Human IL-6 is functionally active in mouse cells, but mouse IL-6 is not active in human cells. As shown in Fig. 1b, the X-ray crystal structure of human IL-6 is a four-helix bundle, similar to the crystal structure of many cytokines, with an additional small "helix E." The A and B helices run in the same direction, and the C and D helices run in the opposite direction. The crystal structure of human IL-6 is similar to that of human G-CSF, growth hormone, and other IL-6 family cytokines (see later), including ciliary neurotropic factor (CNTF), leukemia-inhibitory factor (LIF), and oncostatin-M (OSM).

## E. Polymorphisms

Many nucleotide variations are found in the human *IL-6* gene. These variations sometimes encode functional polymorphisms. In the 5′ flanking region (Fig. 2), the C/G variation at position −174 influences IL-6 expression. After coronary artery bypass surgery, people with the −174C/C allele show higher plasma IL-6 levels than do people with the −174G/G allele. In addition, a lower frequency of the −174C/C genotype is observed in juvenile chronic arthritis patients than in the normal population. The C/G variation at position −174 is also associated with susceptibility to type 1 diabetes mellitus, but no association with systemic lupus erythematosus in caucasian Germans is observed. However, another *in vitro* study has suggested that IL-6 transcription is cooperatively controlled by complex allelic interactions among polymorphic

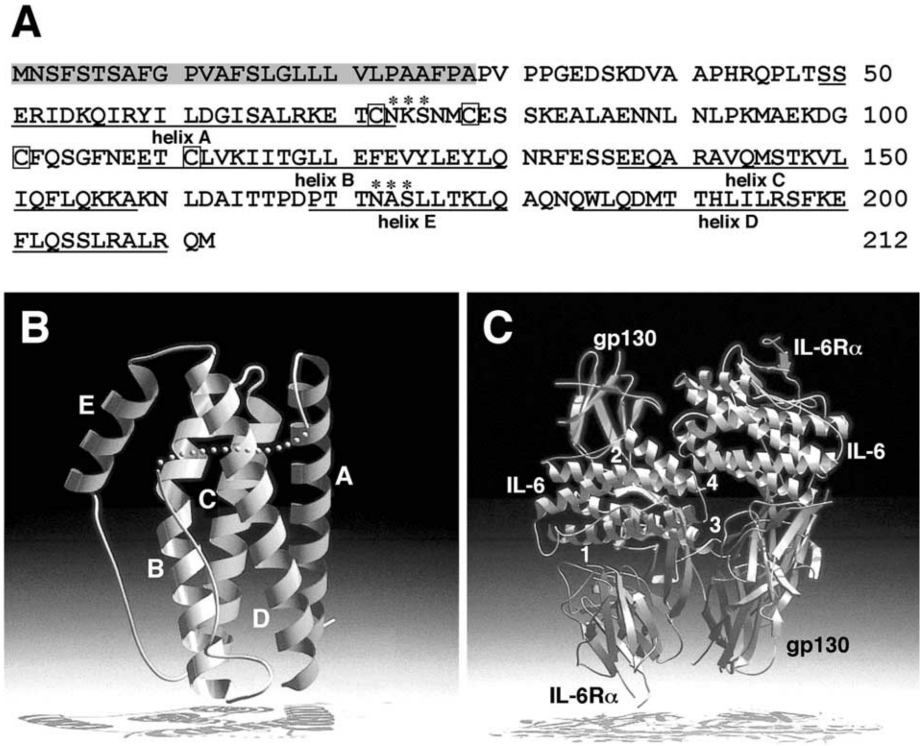
A
MNSFSTSAFG PVAFSLGLLL VLPAAFPAPV PPGEDSKDVA APHRQPLTSS 50
ERIDKQIRYI LDGISALRKE TCNKSNMCES SKEALAENNL NLPKMAEKDG 100
helix A
CFQSGFNEET CLVKIITGLL EFEVYLEYLQ NRFESSEEQA RAVQMSTKVL 150
helix B
helix C
IQFLQKKAKN LDAITTPDPT TNASLLTKLQ AQNQWLQDMT THLILRSFKE 200
helix E
helix D
FLQSSLRALR QM 212
B
E
A
C
B
D
C
gp130
IL-6Rα
IL-6
IL-6
2
4
3
1
gp130
IL-6Rα

**FIGURE 1** Amino acid sequence and X-ray crystallography of human IL-6. (A) The amino acid sequence of human IL-6. Amino acids are denoted using the single-letter code. The signal peptide is shaded, the four-cysteine motif is boxed, and the potential N-linked glycosylation sites are indicated with asterisks. Helices A to E are underlined. (B) The 1.9-Å X-ray crystallography structure of recombinant human IL-6. The N-terminal 18 amino acids of IL-6 that are not visible in the electron density map are indicated with a dotted line. (C) The hexamer model of the IL-6/IL-6Rα/gp130 complex. The proposed binding sites are labeled and correspond to sites 1 to 4 in IL-6. From Somers *et al.* 1.9 Å crystal structure of interleukin 6: Implications for a novel mode of receptor dimerization and signaling. *EMBO J.* **16**(5), (1997), 989–997, by permission of Oxford University Press.

regions in the IL-6 promoter (−596G to A, −572G to C, −392/373 AT runs, and −174G to C) (Fig. 2). Another polymorphism in the fourth intron (G to A at position 4470) appears to correlate with Crohn's disease and ulcerative colitis, although no significant alteration of IL-6 expression has been observed to result from the polymorphism.

## III. REGULATION OF IL-6 EXPRESSION

### A. Biological Events and Factors Regulating IL-6 Production

In young, healthy animals, serum IL-6 levels are normally low. IL-6 production is rapidly induced in the course of acute inflammatory responses that occur in association with injury, trauma, stress, and infection. Chronic inflammation, which is often observed in many autoimmune diseases, is also associated with increased IL-6 production. Aging also influences the production of IL-6. With advancing age, plasma IL-6 levels increase, a change that is explained at least in part by age-associated diseases. Because estrogens and androgens are known to inhibit IL-6 expression, however, a decrease in the sex hormones with aging may contribute to this increase. Physical exercise such as running and cycling is reported to induce many cytokines, including IL-6, which is the predominant cytokine released from skeletal muscle. Increased body weight (obesity) also correlates with the elevation of IL-6; this is partly explained by the increase in adipose tissue, which is also known to produce IL-6.

Many soluble factors promote IL-6 expression in various types of cells and cell lines. These include serum, cytokines (interferon β, IL-1, OSM, platelet-derived growth factor, TNFα and transforming growth factor-β), complement complex, bacterial components (e.g., lipopolysaccharide, lipoteichoic acid, and unmethylated CpG dinucleotide motif), viral components [e.g., double-stranded RNA

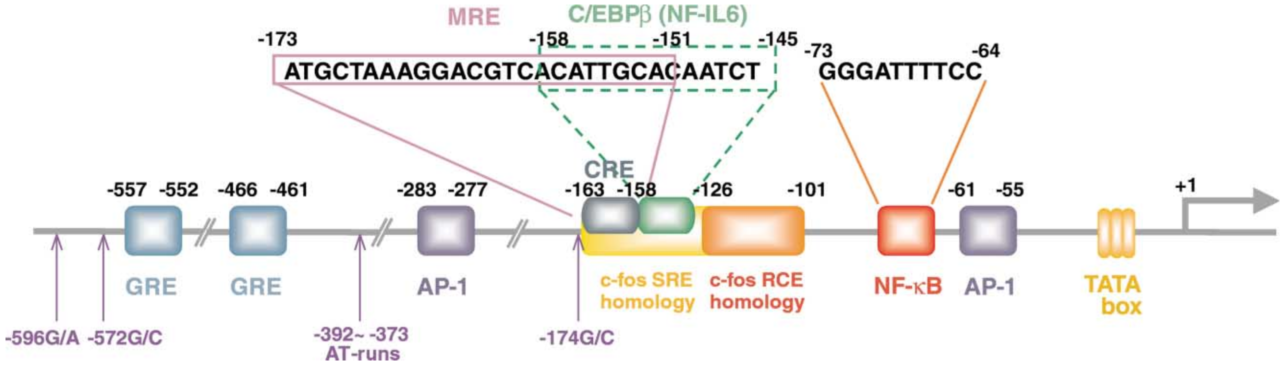

**FIGURE 2** A schematic illustration of the 5′ flanking promoter region of the human *IL-6* gene. The nucleotide position of the cis-regulatory elements and polymorphic regions is given relative to the major transcription start site (+1). Functional polymorphisms located in the 5′ flanking region are indicated with arrows. MRE, Multiple response element; see text for other abbreviations.

or poly(I):poly(C)], activators of the cyclic AMP/protein kinase A pathway (forskolin, cholera toxin, and dibutyryl cyclic AMP), phorbol 12-myristate 13-acetate, calcium ionophore A23187, eicosanoid lipid mediators (prostaglandins and leukotriene B4), neuropeptides/neurotransmitters (substance P, epinephrine, norepinephrine, vasoactive intestinal peptide, adenosine, calcitonin, serotonin, and histamine), thrombin, angiotensin II, and others. On the other hand, IL-10, somatostatin, 1,25-dihydroxyvitamin $D_3$, retinoic acid, glucocorticoids, estrogens, and androgens inhibit IL-6 production in certain cells. Interestingly, IL-4 and IL-13 inhibit IL-6 expression in monocytes, but enhance its expression in osteoblasts.

Cross-linking cell surface receptors, such as the Fcϵ and Fcγ receptors, T-cell receptor, CD40, CD95 (Fas), and certain members of the integrin superfamily, with an agonistic antibody or specific counter-receptors also stimulate IL-6 production.

### B. IL-6-Producing Cells

Many cell types produce IL-6. These include T cells, B cells, polymorphonuclear cells, eosinophils, monocyte/macrophages, mast cells, dendritic cells, chondrocytes, osteoblasts, endothelial cells, skeletal and smooth muscle cells, islet beta cells, thyroid cells, fibroblasts, mesangial cells, keratinocytes, and certain tumor cells. In addition, adipose tissue is a source of IL-6. Neuronal cells, including microglial cells and astrocytes, are also IL-6 producers.

### C. Promoter Region of the *IL-6* Gene

As shown in Fig. 2, several potential transcription control elements are found in the 5′ flanking region of the human *IL-6* gene. These include the glucocorticoid-responsive element (GRE), activator protein 1 binding site (AP-1), multiple response element (MRE), c-fos serum-responsive element (SRE) homology, c-fos retinoblastoma control element (RCE) homology, cyclic AMP response element (CRE), nuclear factor for IL-6 expression (NF-IL-6; also known as the CCAAT/enhancer binding protein β, C/EBPβ) binding sites, and the NF-κB binding site. The products of tumor-suppressor genes, p53, and retinoblastoma proteins repress the activity of an *IL-6* promoter fragment containing nucleotides −225 to +13. The human *IL-6* gene contains three copies of a TATA-box and transcription start sites. The preferential utilization of a specific transcription start site in a variety of cells suggests that distinct regulatory mechanisms may be responsible for the *IL-6* gene expression in different tissues.

## IV. RECEPTOR AND SIGNAL TRANSDUCTION

### A. Molecular Properties of the IL-6 Receptor Complex, IL-6Rα and gp130

IL-6 has high-affinity ($K_d = 40$ to 70 p$M$) and low-affinity ($K_d = 1$ to 6 n$M$) receptors. IL-6 binds IL-6Rα (CD126) alone with low affinity. In the presence of another receptor component, gp130 (CD130), which cannot itself bind IL-6, the ligand/receptor complex shows a 100-fold higher affinity and generates signal transduction. Both receptors possess an immunoglobulin-like domain, a four-cysteine motif, and a Trp-Ser-X-Trp-Ser (WSXWS, where X is any amino acid) motif in the extracellular region. These motifs are conserved among members of the type I cytokine receptor family, including the receptors for prolactin, growth hormone, many interleukins, leptin, erythropoietin, thrombopoietin, LIF, OSM, CNTF, G-CSF, and granulocyte/macrophage-CSF. The four-cysteine and WSXWS motifs are responsible for the ligand binding and are known as the cytokine-binding module (CBM) (Fig. 3A).

### 1. *IL-6Rα: Necessary for Ligand Binding*

IL-6Rα, also known as gp80 and CD126, is an 80-kDa single polypeptide chain. Molecular cloning of the human *IL-6R*α cDNA was achieved in 1988 (database accession, X12830). Mouse (X53802) and rat (M58587) *IL-6R*α cDNAs have also been identified. The human IL-6Rα consists of 468 amino acids with six potential N-linked glycosylation sites and 11 cysteine residues. The putative mature form of IL-6Rα contains 449 amino acids and consists of an extracellular region of 339 amino acids, a transmembrane domain of 28 amino acids, and a cytoplasmic tail of 82 amino acids. Although the short cytoplasmic domain of IL-6Rα is not important for IL-6 signal transduction in a myeloid leukemic cell line (M1), it functions in the expression of the receptors on the basolateral side of polarized Madin–Darby canine kidney cells. The human *IL-6R*α gene is located on chromosome 1q21. The mouse and rat *IL-6R*α genes are on chromosomes 3 and 2, respectively (Table 1). IL-6Rα is expressed on the cell surfaces of leukocytes, hepatocytes, intestinal epithelial cells, cells of the endocrine glands (such as the pituitary and adrenal cortex), and certain cell lines. In addition, dexamethasone treatment up-regulates IL-6Rα expression in

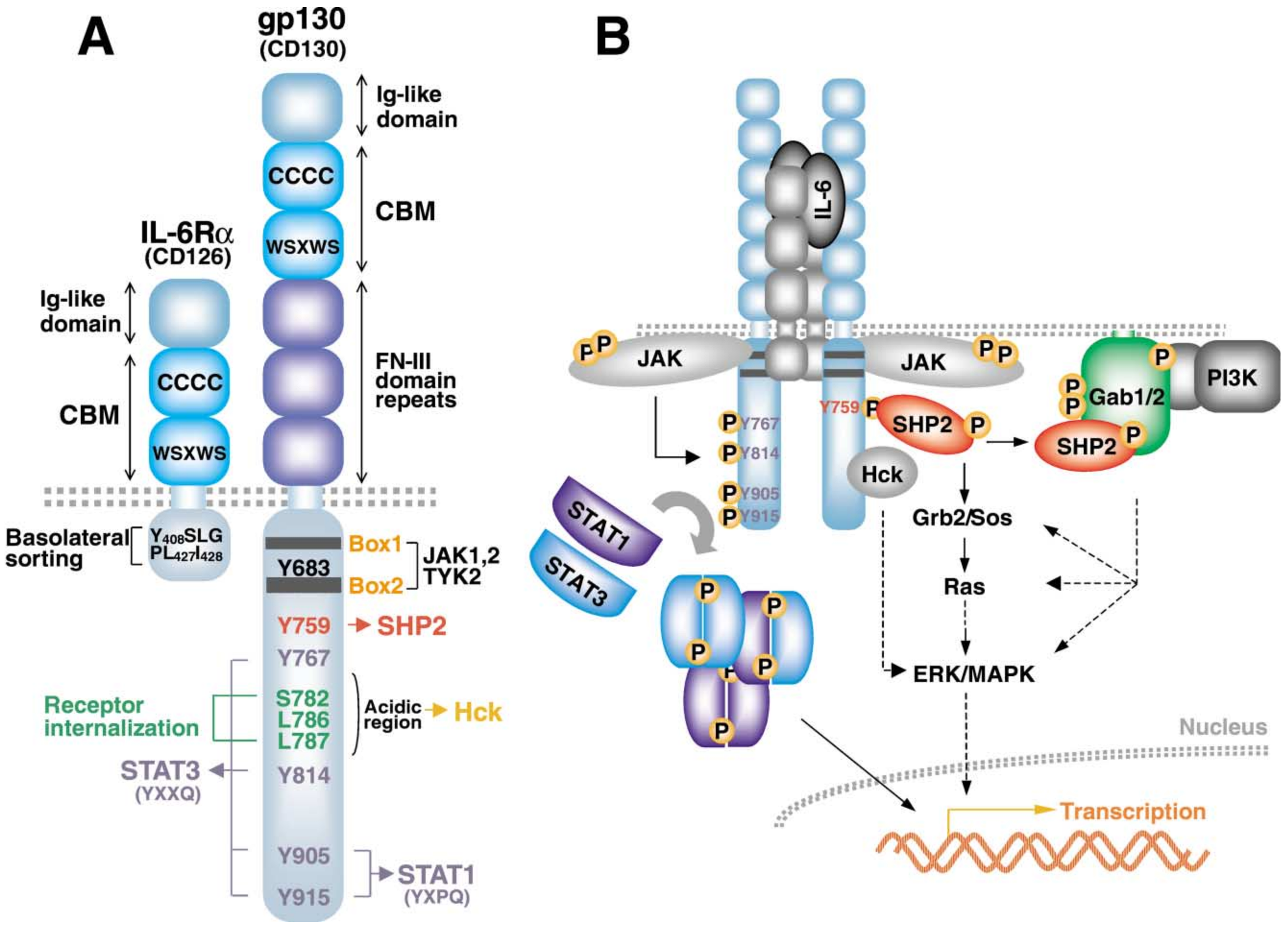
A
gp130
(CD130)
Ig-like domain
CCCC
CBM
WSXWS
IL-6Rα
(CD126)
Ig-like domain
FN-III domain repeats
CCCC
CBM
WSXWS
Basolateral sorting
Y408SLG
PL427I428
Box1
JAK1,2
Y683
TYK2
Box2
Y759
SHP2
Y767
Receptor internalization
S782
L786
L787
Acidic region
Hck
STAT3
(YXXQ)
Y814
Y905
Y915
STAT1
(YXPQ)
B
IL-6
P
P
JAK
JAK
P
P
P
Gab1/2
PI3K
P
Y767
Y759
P
SHP2
P
P
SHP2
P
P
Y814
Hck
P
Y905
P
Y915
Grb2/Sos
STAT1
STAT3
Ras
P
P
P
P
ERK/MAPK
P
P
Nucleus
Transcription

**FIGURE 3** Signal transduction. (A) Schematic illustrations of the structures of IL-6Rα and gp130. Amino acid residues are denoted using the single-letter code, followed by the positions of the residues in the human gp130 amino acid sequences. Box 1 and box 2 domains in human gp130 are $I^{651}$WPNVDP and $V^{691}$SVVEIEANDKKP, respectively. The acidic domain required for Hck association extends from amino acid 771 to amino acid 811; CCCC is the four-cysteine motif. FN-III, Type III fibronectin; Ig-like, immunoglobulin-like. (B) The intracellular signal transduction pathway generated by IL-6. P, Phosphotyrosine residue; see text for details and abbreviations.

osteoblasts. IL-6Rα expression in naive resting B cells is not detectable but is induced in activated B cells. IL-6Rα has soluble forms that are normally found in the sera and cerebrospinal fluid of healthy individuals, at ~0–50 ng/ml (in women) and ~0.8–1.6 ng/ml, respectively. The soluble forms of IL-6Rα are generated by proteolytic cleavage or alternative splicing of its mRNA, and act in an agonistic manner with IL-6 on cells expressing only gp130. To date, no report describes a phenotype of IL-6Rα knockout mice.

### 2. *gp130: Necessary for Signal Transduction*

Glycoprotein gp130 has a molecular mass of 130–150 kDa; gp130 has the alternative names IL-6Rβ, CD130, and IL-6 signal transducer. The molecular cloning of human *gp130* cDNA was reported in 1990 (database accession, M57230). The cDNA sequence for mouse (M83336), rat (M92340), rhesus monkey (AF227555, partial sequence), chicken (AJ011688), and African clawed frog (*Xenopus laevis*, AF041845) gp130 has also been reported. The human gp130 precursor contains 918 amino acids. The mature human gp130 contains 896 amino acids and consists of an extracellular domain of 597 amino acids, a membrane-spanning region of 22 amino acids, and a cytoplasmic domain of 277 amino acids. There are 14 potential N-linked glycosylation sites. gp130 has three fibronectin type III domains in the extracellular region. The cytoplasmic domain contains important elements for signal transduction: (1) box motifs, which are necessary for the association with non-receptor tyrosine kinases called Janus kinases (JAKs), and (2) six tyrosine residues (Fig. 3). In humans, there are two chromosomal loci for gp130; one is the genuine *gp130* gene, located on chromosome 5q11, and the other is a pseudogene localized to chromosome 17. The murine and rat *gp130* genes are located on chromosomes 13 and 2q14–q16, respectively (Table 1). The human *gp130* gene has 17 exons separated by 16 introns. The X-ray structure of the extracellular domain of the human gp130 has recently been reported. In contrast to IL-6Rα, gp130 is ubiquitously expressed on the cell surface. Alternative splicing of *gp130* mRNA gives rise to soluble forms; one of these is found in sera at ~210–290 ng/ml (in women) and acts in an antagonistic manner. Another splicing variant of gp130, termed gp130-RAPS (database accession, AB015706), is an autoantigen in rheumatoid arthritis patients. The variant gp130-RAPS also antagonizes IL-6 activities *in vitro*. The disruption of the *gp130* gene in mice leads to embryonic or perinatal lethality, depending on the genetic background of the mouse. Knock-in mutant mouse strains that are defective in gp130-mediated activation of the signal transducer and activator of transcription, Stat3, also die within 1 day after birth. On the other hand, another knock-in mouse that lacks the SH2 domain-containing protein tyrosine phosphatase (SHP2)-binding site of gp130 (Y759, where Y is the one-letter code for tyrosine; see Fig. 3) is born normally and apparently healthy, but displays splenomegaly, lymphadenopathy, and enhanced immunoglobulin production, indicating that Y759 of gp130 has a negative regulatory role(s) *in vivo*.

## B. IL-6 Family Cytokines

Glycoprotein gp130 is used as a signal-transducing receptor component by several other cytokines. These include IL-11, CNTF, OSM, cardiotoropin-1, and LIF. Thus, these cytokines are categorized as "IL-6 family cytokines." In addition, a recently identified molecule, novel neurotropin-1 (NNT-1)/BSF-3/cardiotropin-like cytokine (CLC), also uses gp130 as a component for its functional receptors. Because the gp130 signaling component is shared by the IL-6 family cytokines, their functions are largely overlapping.

## C. Binding of IL-6 to the Receptor

When IL-6 binds to IL-6Rα and gp130, the molecules form a hexameric complex with a stoichiometry of 2:2:2 (see Fig. 1c). Analysis by means of site-directed mutations of the IL-6 protein has identified the regions required for interaction with the receptor complex and for signal transduction. The region in IL-6 termed "site 1" is important for binding with IL-6Rα. IL-6 mutated at "site 2" or "site 3" can bind to the receptor but cannot generate intracellular signals. The X-ray crystallography of IL-6 has led to a predicted role for the region, designated as "site 4" in the interaction between two IL-6/IL-6Rα/gp130 complexes, in stabilizing the architecture of the signaling complex. A model of the ligand/receptor interaction cascade is as follows: (1) IL-6 binds to IL-6Rα via site 1 in IL-6, forming a heterodimer; (2) the binary IL-6/IL-6Rα complex contacts the gp130 CBM through site 2 and also forms contacts between the C-terminal cell surface domain of IL-6Rα and gp130, resulting in a trimolecular complex with a 1:1:1 stoichiometry that is not yet able to generate intracellular signals; (3) two trimolecular complexes are assembled together via site 3 and the immunoglobulin-like domain of gp130, as well as through site 4, completing the hexamer complex, which is competent for signaling.

### D. Signal Transduction Generated by IL-6

Engagement of IL-6 with the receptor induces activation of tyrosine kinases (JAK1, JAK2, and TYK2) that are constitutively associated with gp130 via the box domains. The activated JAKs phosphorylate tyrosine residues in the cytoplasmic domain of gp130 (Fig. 3b). The second phosphotyrosine residue from the plasma membrane (Y759 in humans) is necessary for the recruitment of SHP2. After being recruited, SHP2 is phosphorylated and can then interact with Grb2 (growth factor receptor-bound protein 2), which is constitutively associated with Sos (son of sevenless), a GDP/GTP exchanger for Ras. The activated Ras (i.e., the GTP-form) transmits signals, leading to the activation of an extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade. In fact, a point mutation in the SHP2-binding site (Y759) of gp130 leads to a defect in the ERK/MAPK activation. SHP2 also binds to another adapter protein, Gab1/2 (Grb2-associated binder 1/2), which associates with the p85 subunit of phosphatidylinositol 3′-kinase (PI3K) in a stimulation-dependent manner, resulting in the formation of a tertiary complex, SHP2/Gab/PI3K. The activity of ERK/MAPK is severely impaired in Gab1 knockout fibroblasts stimulated with IL-6/IL-6Rα, indicating a requirement for Gab1 in the gp130 Y759-mediated pathway(s). An Src family tyrosine kinase, Hck, is associated with an acidic region of the cytoplasmic domain of gp130 and modulates the ERK/MAPK activity.

Phosphorylation of any of the four tyrosine residues (Y767, Y814, Y905, and Y915 in humans), all of which lie within a YXXQ motif, is required for the activation (tyrosine phosphorylation) of a transcription factor, Stat3. In addition, Stat1 is activated through phosphotyrosines that lie within a YXPQ motif (Y905 and Y915 in humans). Following phosphorylation, Stat proteins are homodimerized (Stat1/Stat1 and Stat3/Stat3) or heterodimerized (Stat1/Stat3) and subsequently enter the nucleus and activate the transcription of many genes. Stat3 is also phosphorylated on serine residues by an H7-sensitive kinase pathway. This phosphorylation is necessary for the full transcriptional activity of Stat3. Glycoprotein gp130 also receives serine phosphorylation on its cytoplasmic domain (S782 in humans), and the surface expression of gp130 is increased when this serine residue is mutated. Other signaling cascades generated directly through JAKs, such as those involving Stat5 activation, have also been reported.

After binding IL-6, the receptor complex is internalized, independent of its signaling capability. A dileucine motif in the cytoplasmic domain of gp130 (L786 and L787 in humans; L is leucine in the one-letter code) is involved in the efficient internalization of the complex. Molecules that negatively regulate IL-6 signaling have been identified. A protein inhibitor of activated Stat3 (PIAS3) directly interacts with the activated (i.e., phosphorylated) Stat3 and down-regulates the transcriptional activity of Stat3. The suppressor of cytokine signaling (SOCS) family proteins are also negative regulators of IL-6 signaling.

## V. BIOLOGICAL ACTIVITIES OF IL-6

### A. Molecules Regulated by IL-6

IL-6 stimulates the transcription of a variety of genes through the signal transduction pathways depicted in Fig. 3b. Genes encoding acute-phase proteins are directly regulated by IL-6 via two types of IL-6-responsive elements (see later). The *jun B* gene promoter contains an IL-6-responsive element that consists of a putative Ets-binding site, which has been found to be a Stat3-binding site, and a cyclic AMP-responsive element, both of which are essential for IL-6-induced *jun B* expression. IL-6-responsive elements have also been identified in the promoter region of the *IRF1* and *stat3* genes. Many other genes, such as *C/EBPβ*, *C/EBPδ*, c-*myc*, c-*myb*, *bcl-2*, *p19IND4D*, *pim1/2*, *SOCS/JAB/SSI-1*, and *gp130*, are controlled by the activation of gp130. In addition, metallothionein I, an adapter molecule for the IL-1R/Toll-like-R family (MyD88), IL-4Rα, and tissue-inhibitor of metalloproteinase I are reported targets of IL-6 signaling.

### B. Effects on the Immune System

As suggested by its former name, BSF-2, IL-6 acts on activated B cells to promote cell growth and immunoglobulin production, but does not affect naive resting B cells due to a lack of IL-6Rα expression. Consistent with this, IL-6 transgenic mice show massive plasmacytosis and hypergammaglobulinemia. The effect of IL-6 on immunoglobulin production in B cells is dependent on Stat3 activation. In T cells, IL-6 is involved in cell activation, proliferation, and survival. Some of these effects are attributable to the up-regulation of IL-2Rα (CD25) and the induction of anti-apoptotic Bcl-2 family molecules. IL-6 is sometimes categorized as a T helper ($T_H$) type 2 ($T_H2$) cytokine, but its effect on the balance between $T_H1$ and $T_H2$ is still controversial.

IL-6 in synergy with IL-2 increases the expression of mediators for target cell killing, such as serine esterase and perforin, and thus plays an important role in the functions of CD8-positive cytotoxic T cells. In agreement with these observations, the disruption of the *IL-6* gene in mice results in a reduction in T-cell/thymocyte numbers, a decrease in immunoglobulin production, and a compromised cytotoxic activity of CD8-positive T cells.

IL-6 plays a critical role in protection against both bacterial (e.g., *Listeria monocytogenes*, *Escherichia coli*, and *Candida albicans*) and viral (vaccinia virus) infection, given that IL-6 knockout mice are highly sensitive to these pathogens, and the injection of recombinant IL-6 enhances their resistance. IL-6 is generally grouped as a member of the pro-inflammatory cytokines, but it also has anti-inflammatory characteristics: antigen-induced pulmonary inflammation is exaggerated in mice lacking IL-6 and a lung-specific overexpression of IL-6 protein ameliorates these symptoms. In addition, endotoxin-induced acute inflammatory responses are enhanced in IL-6 knockout mice compared to wild-type animals.

## C. Effects on Hematopoiesis

IL-6 induces proliferation of murine pluripotent stem cells in synergy with the action of IL-3. IL-6 triggers dormant progenitor cells to enter the cell cycle, whereas IL-3 supports the continuation of the cell cycle progression. IL-6 also stimulates megakaryopoiesis in concert with stem cell factor and thrombopoietin. IL-6 knockout mice have a reduced number of megakaryocyte progenitors. In addition, IL-6 is involved in granulopoiesis. Mice doubly deficient for IL-6 and G-CSFR show neutropenia that is more severe than that seen in G-CSFR knockout mice. In addition, injection of IL-6 into G-CSFR knockout mice improves granulopoiesis.

IL-6 can arrest the cell cycle and induce differentiation in human (U937) and mouse (M1) myeloid cell lines. The murine M1 cells differentiate into macrophages in response to IL-6. This signal is mediated by IL-6-induced Stat3 activation. Mice overexpressing both IL-6 and IL-6Rα show extramedullary hematopoiesis, which supports the preceding observations.

## D. Effects on the Acute-Phase Reaction and Liver Regeneration

The acute-phase reaction is elicited by inflammation associated with infection, injury, and other factors. This reaction aids the body in recovery and helps maintain homeostasis. The acute-phase reaction consists of fever, an increase in vascular permeability, and the production of acute-phase proteins by hepatocytes. The acute-phase proteins are divided into two groups based on their regulating cytokines. IL-6 directly up-regulates the mRNA expression of type II acute-phase proteins through Stat3 activation. IL-6 also contributes to the increase in type I protein levels, which are mainly regulated by IL-1. Intravenous injection of IL-6 rapidly induces the production of the acute-phase proteins in animals. In IL-6 knockout mice, the acute-phase response is lower than in wild-type mice.

IL-6 is known to play a fundamental role in liver regeneration. After hepatectomy in rats, a prompt increase in tumor necrosis factor a (TNFα), followed by an elevation of serum IL-6, is observed. Mice deficient for TNF receptor type I (TNF-RI) are unable to induce the IL-6 elevation after partial hepatectomy. IL-6 or NF-IL-6 (C/EBPβ) knockout mice show an impaired proliferative response during liver regeneration. Injection of IL-6 restores the capacity of the liver to regenerate in TNF-RI or IL-6 knockout mice. These observations indicate that TNFα-induced IL-6 production is essential for liver regeneration.

## E. Effects on Bone Metabolism

IL-6 is implicated in osteoclastogenesis. Osteoclast formation is enhanced by ovariectomy in mice, an effect that is negated when an anti-IL-6 antibody or 17β-estradiol is administered to the mice. Consistent with this finding, IL-6 knockout mice are resistant to the bone loss induced by ovariectomy. These findings suggest an important role for IL-6 in the osteoporosis found in postmenopausal women and patients with rheumatoid arthritis.

## F. Effects on the Nervous and Endocrine Systems

*In situ* mRNA hybridization reveals that IL-6 is transcribed at high levels in the hippocampus, hypothalamus, and subcortical structures of the rat brain. In addition, soluble IL-6Rα is detectable in the cerebrospinal fluid in humans. These findings suggest a functional importance of IL-6 in the central nervous system (CNS). In fact, an *in vitro* study showed that IL-6 can induce neurite outgrowth in a rat pheochromocytoma cell line (PC12) pretreated with nerve growth factor. This effect is mediated by IL-6-induced ERK/MAPK activation. The CNS-specific overexpression of IL-6 protein in mice results in the development of reactive gliosis, and these mice exhibit significant neurodegeneration, which leads to motor problems such as ataxia, seizure, and tremor. In addition, IL-6 knockout mice exhibit delayed recovery of the sensory functions after crush lesions of the sciatic nerve.

IL-6 is reported to modulate the endocrine system via the hypothalamic–pituitary–adrenal axis. Injection of recombinant IL-6 stimulates the release of adrenocorticotropic hormone in a manner independent of the action of corticotropin-releasing hormone. Expression of IL-6Rα on pituitary corticotrophs and in the adrenal cortex supports the direct action of IL-6 on hormone release.

## VI. IL-6 AND DISEASES

A number of diseases are reportedly associated with the aberrant expression of IL-6. These include Alzheimer's disease, bronchial asthma, cardiac myxoma, Castleman's disease, cystic fibrosis, inflammatory bowel disease, multiple sclerosis, Paget disease, psoriasis, rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus, and type 2 diabetes mellitus. IL-6 is also implicated in the pathogenesis of osteoporosis. In addition, some other diseases are associated with IL-6 gene polymorphisms. Many tumors, including Kaposi's sarcoma, melanoma, multiple myeloma, and prostate cancer, produce IL-6, which can act as an autocrine growth factor for the neoplasm.

Involvement of IL-6 in the pathogenesis of some diseases has been demonstrated using animal models of the diseases. IL-6 transgenic mice on a BALB/c background exhibit symptoms similar to multiple myeloma. The disease severity of murine models for multiple sclerosis (experimental autoimmune encephalomyelitis), rheumatoid arthritis (collagen-induced arthritis), osteoporosis (induced by ovariectomy), and inflammatory bowel disease (dextran sulfate sodium-induced colitis) is significantly improved in IL-6 knockout mice. Anti-IL-6 antibody treatment ameliorates autoantibody production and prolongs life in an autoimmune-prone (NZB × NZW)F1 mouse strain. By contrast, when twitcher mice, a model of globoid cell leukodystrophy (Krabbe's disease), are crossed with IL-6 knockout mice, the disease symptoms are enhanced. Similarly, antigen-induced pulmonary eosinophilia, an animal model of bronchial asthma, is exaggerated in IL-6 knockout mice.

Results from all of these animal studies indicate that IL-6 and its receptor are likely therapeutic targets for various diseases, and attempts to identify therapeutic approaches are underway. One potential therapeutic agent, an antagonistic, humanized anti-IL-6Rα antibody, is currently being used in a Phase II clinical trial for the treatment of rheumatoid arthritis, Crohn's disease, and Castleman's disease. In addition, adenovirus-mediated expression of IL-6/IL-6Rα fusion protein, which is 100- to 1000-fold more active and thus is called "hyper-IL-6," is being tested for use in clinical treatment of fulminant hepatic failure.

## VII. SUMMARY

IL-6 is a multifunctional cytokine that acts on and is produced by many cell types in the body. Although the name "interleukin" literally indicates a substance that is a mediator between leukocytes, IL-6 not only acts on the immune system but also functions in the endocrine and nervous systems. The collective effort of many investigators in different fields has been necessary to disclose the multifunctional aspects of IL-6. In particular, details of the intracellular signaling cascade have been rapidly clarified by many investigators. However, accumulating evidence sometimes reveals contradictory observations; for instance, IL-6 is found to act both as a pro-inflammatory and an anti-inflammatory mediator. The authors of a recent publication on gp130 knock-in mouse strains suggest that these discrepant observations can be explained by the idea that there are variations in the balance between positive and negative intracellular signals generated by a given ligand, leading to different, and sometimes opposite, outcomes *in vivo*.

IL-6 is implicated in the pathogenesis of various kinds of diseases. Elucidation of the detailed mechanism of action of IL-6 will continue to be beneficial for understanding these disorders and for the development of clinical treatments.

## Glossary

**adapter protein** Contains multiple docking sites for other proteins but lacks enzymatic activity; functions as an "adapter" by allowing the assembly of complexes of signaling proteins that generate signal transduction.

**5′,3′ flanking region** Noncoding region upstream (5′ flanking) or downstream (3′ flanking) of a gene. A cis-regulatory element for gene expression is often found in the 5′ flanking region.

**knockout/knock-in mice** Mutant mice in which certain genes are disrupted using gene-targeting technology (knockout), or mice in which native genes are changed by the introduction of artificially altered genes (e.g., point mutations) into the endogenous locus (knock-in).

**signal transduction** Series of intracellular events that link extracellular stimuli (e.g., ligand binding) to the activation of gene transcription.

**tyrosine kinase** Enzyme that phosphorylates protein tyrosine residues. There are two major types: (1) receptor tyrosine kinases, which penetrate the plasma membrane and possess a kinase domain in the cytoplasmic region, such as the receptors for many growth factors, and (2) nonreceptor tyrosine kinases, which reside in the cytoplasm, such as Src family tyrosine kinases and Janus kinases.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

## Further Reading

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# Interleukin-7

Hergen Spits
*Netherlands Cancer Institute, Amsterdam*

I. THE IL-7 GENE AND PROTEIN
II. THE IL-7Rα GENE AND THE IL-7 RECEPTOR COMPLEX
III. PRODUCTION OF IL-7
IV. EXPRESSION OF THE IL-7R COMPLEX
V. SIGNAL TRANSDUCTION THROUGH IL-7Rα
VI. THE FUNCTION OF IL-7 IN DEVELOPMENT AND ACTIVITIES OF T AND B CELLS
VII. EFFECTS OF *IN VIVO* ADMINISTRATION OF IL-7
VIII. IL-7 AS A POSSIBLE THERAPEUTIC AGENT

**Interleukin-7 (IL-7) is a single-chain protein of 25 kDa produced predominantly by epithelial cells, especially keratinocytes and thymic epithelial cells. The functional interleukin-7 (IL-7) receptor consists of two chains, an α chain (IL-7Rα) and a γ chain. The latter polypeptide is denoted γ common (γc) because it is also a component of the receptors for IL-2, IL-4, IL-9, and IL-15. IL-7 plays an essential, nonredundant role in the development of T and B cells in the mouse and of T cells in humans. IL-7 promotes the survival and proliferation of T-cell precursors in humans and mice and of B-cell precursors in mice. IL-7 affects the survival and proliferation of mature T cells but not of mature B cells. IL-7 is important for homeostasis of naive T cells and probably also for the function of mature T cells in the periphery. IL-7 is also essential for the formation of Peyer's patches in the mouse. Data in the mouse suggest that IL-7 may be of clinical benefit by stimulating T-cell development in recipients of hematopoietic stem cell transplantation, but thus far no clinical trials have been performed in humans.**

## I. THE IL-7 GENE AND PROTEIN

### A. IL-7 Gene

Accession numbers for the IL-7 gene are as follows: human gene, M29048; human cDNA, NM_000880; mouse gene, AH_001973; mouse cDNA, X07962; bovine gene, X64540; and swine gene, AB035380.

The human IL-7 gene is located on chromosome 8, bands q12–q13. It is approximately 33 kb long and contains six exons. Murine IL-7 is 56 kb long and maps to chromosome 3.

## B. IL-7 Protein

IL-7 is a single-chain glycoprotein of 25 kDa that contains six cysteine residues. The disulfide bonds are essential for the biological activity of the protein. Human IL-7 (152 amino acids and 17.4 kDa) and murine IL-7 (127 amino acids) show 60% sequence homology at the protein level. The human IL-7 gene contains an open reading frame of 534 nucleotides encoding a polypeptide of 177 amino acids with a predicted molecular weight of 17.4 kDa with three potential N-linked glycosylation sites and a signal peptide of 25 amino acids. The murine gene contains an open reading frame encoding a signal peptide of 25 amino acids and a functional protein of 127 amino acids with a predicted molecular weight of 14.9 kDa. The molecular weight of the expressed protein is 25 kDa, due to glycosylation of two potential N-linked glycosylation sites. Both human and mouse proteins contain three disulfide bonds that are indispensable for biological activity.

# II. THE IL-7Rα GENE AND THE IL-7 RECEPTOR COMPLEX

## A. The IL-7Rα Gene

Accession numbers for the IL-7Rα gene are as follows: human gene: AF043123-9, AF043124-9, AF043125-9, AF043126-9, AF043127-9, and AF043128-9 (these accession numbers denote the different exons); human cDNA, NPI_002185; and mouse cDNA, M29697.

Human IL-7Rα maps to chromosome 5p13. The chromosomal localization of murine IL-7Rα is unknown. Both human and mouse genes contain eight exons and seven introns with a total size of 19 kb in the human and 24 kb in the mouse. The first exon contains the 5′-untranslated region, the signal peptide, and the amino-terminus of the mature protein. The remainder of the extracellular region is encoded by exons 2 to 6. In addition, exon 6 encodes the transmembrane portion of the cytoplasmic region and exons 7 and 8 encode the rest of the cytoplasmic region. The entire 3′-untranslated region is encoded by exon 8. Differential splicing results in mRNA encoding a secreted form of the human IL-7Rα receptor. This form lacks the sequences in exon 6 that encode the transmembrane region.

## B. IL-7R Complex

The murine IL-7Rα gene encodes a protein of 439 amino acids with a calculated MW of 49.5 kDa. On T cells, the protein has a MW of 90 kDa. The associated γc chain has a MW of 74 kDa. IL-7Rα is a type I membrane protein with a single transmembrane domain. The extracellular domain contains features of the cytokine receptor superfamily. The cytoplasmic domain with 195 amino acids does not contain consensus sequences of protein kinases. The crystal structure of IL-7Rα has not been determined. Based on the model of the prolactin receptor, intrachain disulfide bridges are most probably formed between the first and second Cys residues and between the third and fourth Cys residues. The receptor contains a WS motif (WSXWS) at the COOH-terminus of the extracellular domain.

# III. PRODUCTION OF IL-7

The main cell types that express the gene are bone marrow stroma, thymic stroma, intestinal epithelial cells, and keratinocytes. IL-7 mRNA has furthermore been detected in mature but not immature dendritic cells and dendritic cells derived from CD34 cord blood cells cultured with granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α, but it is not clear whether these cells secrete IL-7. In addition, the gene was expressed in platelets and a megakaryocyte cell line. Various malignant T-cell types express the gene (Table 1).

# IV. EXPRESSION OF THE IL-7R COMPLEX

Since IL-7 is important for early T- and B-cell development (see below), it is not surprising that the receptor complex is expressed on T- and B-cell progenitors. In addition, this complex is expressed on mature T cells and furthermore on malignant cells originating from different cell types. They are summarized in Table 2.

# V. SIGNAL TRANSDUCTION THROUGH IL-7Rα

## A. Kinases Activated by IL-7

The IL-7R complex requires both the α chain and γc for relaying signals. These two chains associate with tyrosine phosphokinases that relay signals triggered by binding of IL-7 to the receptor complex. Tyrosine kinases belonging to the family of Janus kinases (JAKs) are essential for IL-7R signaling. Two Janus kinases, JAK1 and JAK3, associate with the complex. JAK1 binds to IL-7Rα and JAK3 to γc. Both kinases become phosphorylated when IL-7 binds to its receptor, but the extent of phosphorylation of JAK3 is much higher than that of JAK1. In contrast to

TABLE 1

| Tissue or cell type | mRNA (human, mouse) | Protein |
|---|---|---|
| Bone marrow stromal cells | + (hu, mo) | + (hu, mo) |
| Thymic stromal cells | + (hu, mo) | + (hu, mo) |
| Keratinocytes | + (hu, mo) | + (hu, mo) |
| Intestinal epithelial cells | + (hu) | + (hu) |
| Follicular dendritic cells | + (hu) | + (hu) |
| DCs derived from CD34$^+$ cells cultured with granulocyte macrophage CSF and TNFα | + (hu) | n.d. |
| CMRF-44$^+$/CD14$^-$/CD19$^-$ Peripheral blood low-density DCs, purified after overnight tissue culture | + (hu) | + (hu) |
| Adult liver | + (rat) | n.d. |
| Uterus | + (mo) | n.d. |
| Brain | + (hu) | n.d. |
| Vascular endothelial cells | + (hu) | + (hu) |
| Fibroblasts | + (hu) | n.d. |
| Oral mucosa | + (hu) | + (hu) |
| Psoriatic plaques | + (hu) | n.d. |
| Lesions from tuberculoid lepra | + (hu) | + (hu) |
| Colorectal cancer cells | + (hu) | + (hu) |
| Renal call cancer tissues and cells | + (hu) | + (hu) |
| Bladder cancer | + (hu) | n.d. |
| Burkitt's lymphoma (American) | + (hu) | + (hu) |
| Epstein–Barr virus B-cell lines | + (hu) | + (hu) |
| Chronic B-cell leukemia cells | + (hu) | + (hu) |
| Hepatocarcinoma | + (hu) | n.d. |

*Note.* CSF, colony-stimulating factor; DCs, dendritic cells; hu, human; mo, mouse; n.d., not determined; TNFα, tumor necrosis factor α.

TABLE 2

Fetal NK/DC precursors in fetal lymph nodes
Common T/NK/B lymphoid precursor cells
Cryptopatch-associated lymphoid precursors
Developing T cells
  All subsets of CD3$^-$CD4$^-$CD8$^-$ thymocytes
  A fraction of CD4$^+$CD8$^+$ DP cells
  SP CD4$^+$ or CD8$^+$ thymcytes
  TCRγδ cells
  Thymic NK$^-$1.1$^+$ T cells
Developing B cells
  All pre-pro-B, pro-B, and pre-B-cell stages
Mature T cells
Bone marrow-derived macrophages
Malignant cell types
  Colorectal cancer cells
  Renal cancer cells
  Cutaneous T-cell lymphomas
Human intestinal epithelial cells

*Note.* DC, dendritic cell; DP, double positive; NK, natural killer; SP, single positive; TCR, T-cell receptor.

JAK3, which is specifically expressed in lymphoid cells, JAK1 is ubiquitously expressed. JAK3 activity and most likely also JAK1 activity are required for IL-7R-mediated signaling during T-cell development. Another kinase that associates with the IL-7R complex is phosphatidylinositol 3-kinase (PI3K). JAK3 appears also to control PI3K activity. PI3K phosphorylates the D3 position of the inositol group of phosphoinositide lipids to generate phophatidylinositol 3-phopsphate [PtdIns(3)P], PtdIns(3,4)P2, and PtdIns(3,4)P3. The last two products are important for cellular proliferation. The enzyme consists of an 85 kDa adapter and a 110 kDa catalytic unit. Upon ligand binding, the p85 unit is tyrosine-phosphorylated and associates with many receptors including the IL-7Rα chain. Stimulation of human thymocytes with IL-7 activates both isoforms of p85, 1 and 2, in human thymocytes. Interestingly, phosphorylation of p85 required JAK3, which was shown to associate with p85. It is probable that two pools of PI3K are activated by IL-7; one is associated with the IL-7Rα chain and another with insulin receptor substrate-1 (IRS-1) and IRS-2. PI3K appears to be important for the survival and proliferation of both T- and B-cell precursors. PI3K associates with the IL-7α chain through an SH2 domain recognition motif (YXXM) spanning residue Y449 in its cytoplasmic domain. Interestingly, this residue was found to be critical for IL-7-mediated proliferation of murine pre-B cells. Induction of immunoglobulin H (IgH) VDJ gene recombination, however, did not require the Y449 residue. These findings indicate that IL-7Rα-transduced signals can control survival as well as proliferation and differentiation through distinct pathways. More recently it was demonstrated that PI3K also mediates the survival and proliferation of human T-cell precursors. The Y449 in the cytoplasmic domain of the IL-7R is important for the survival and expansion of human thymocytes. A dominant-negative mutant of p85 that binds to the receptor but fails to interact with p100 was introduced into human T-cell precursors by retrovirus-mediated gene transfer. This mutant was found to inhibit the survival and expansion of the T-cell precursors in a fetal thymic organ culture but did not inhibit differentiation of these cells.

Recently it was demonstrated that protein kinase B (PKB) can be activated by IL-7. PKB seems to play a central role in PI3K-mediated protection against apoptosis in a wide range of cell types. As activation of PKB as well of PI3K requires residue 449Y, it is very likely that in human T-cell precursors PKB is a downstream effector of PI3K as well. The substrates of IL-7-activated PKB are not yet known. Two sets of observations suggest a role for the p38 mitogen-activated protein kinase (MAPK) in IL-7-mediated proliferation of mature T cells. First, activation of a murine T-cell line by IL-7 resulted in phosphorylation of p38 MAPK, and second, the proliferative response of human T cells was inhibited by the highly selective p38 MAPK inhibitor SB203580. Which signaling pathway activates p38 MAPK is unknown. p21ras is not activated by IL-7 and therefore does not act in this pathway. The same report also documented activation of the stress-activated protein kinase/Jun-N-terminal kinase by IL-7, but there is no evidence that activation of this enzyme is important for the proliferative response induced by IL-7.

### B. Transcription Factors Activated by IL-7R Signaling

In general, JAKs phosphorylate the receptor chains, providing docking sites for the SH2 domains of STATs. STATs are recruited to the phosphorylated sites of the receptor and become phosphorylated. The phosphorylated STATs can dimerize, translocate to the nucleus, and stimulate the expression of cytokine-inducible genes. Two studies documented the activation of STAT1 by IL-7. The phenotype of STAT1-deficient mice, however, makes it unlikely that STAT1 plays an essential role in IL-7R signaling *in vivo* as the thymus of these mice is normal and T- and B-cell development proceeds undisturbed. STAT3 and STAT5 have been shown to be activated by IL-7 in several cell types. STAT3 is probably not involved in IL-7R signaling *in vivo*. STAT3 deficiency results in embryonic lethality, preventing analysis of the role of STAT3 in T-cell development in conventional STAT3 − /− mice. Recently, however, mice were generated with a STAT3 deficiency specifically in the T-cell lineage by conditional gene targeting using the Cre-loxP system. Floxed-STAT3 mice were mated with transgenic mice with Cre recombinase under control of the T-cell-specific Lck promoter. Although STAT3 was not expressed in the thymus of these mice, the cellular size of the thymus and distribution of thymic subsets were the same as in control mice. Thymocytes of these mice responded normally to IL-7 *in vitro*. Together these findings indicate that STAT3 is not involved in IL-7-mediated control of T-cell development. IL-7 activates STAT5 in human peripheral blood mononuclear cells and thymocytes. STAT5 was originally identified as a prolactin-induced mammary gland transcription factor. Two STAT5 genes, 5a and 5b, encode proteins that are approximately 95% identical in amino acid sequence. STAT5a and STAT5b differ in their COOH-terminal transactivation domains and display relatively specific actions. STAT5-deficient mice display immunological defects. STAT5a − /− mice have a reduced expansion of peripheral T cells, which is associated with a diminished IL-2-mediated induction of the IL-2Rα chain. STAT5b-deficient mice exhibit a thymus that is slightly reduced in size. In contrast, mice deficient for both STAT5a and STAT5b were reported to have no major decrease in size of the thymus. In addition, the distribution of CD4$^+$, CD8$^+$ single-positive (SP), and double-positive (DP) cells were normal. The reported findings on the phenotype of mice deficient for STAT5a, STAT5b, or both argues against a role for STAT5 isoforms in IL-7-mediated control of T-cell development.

## VI. THE FUNCTION OF IL-7 IN DEVELOPMENT AND ACTIVITIES OF T AND B CELLS

### A. The Role of IL-7 in Development of TCRαβ Cells

All lymphocytes are derived from a common lymphoid precursor. Progeny of these cells migrate to the thymus, where they develop into T cells. The earliest murine thymic precursors are characterized by expression of high levels of CD44, but lack the differentiation antigens CD3/TCR, CD4, and CD8. The CD4$^-$CD8$^-$ double-negative (DN) compartment contains at least four cell populations that can be discriminated on the basis of expression of CD25 and CD44. Development proceeds as follows: CD25 CD44 → CD25CD44 → CD25CD44 → CD25CD44. The latter cells acquire first CD8, followed by CD4 to attain the CD4$^+$CD8$^+$ DP phenotype.

The thymus of IL-7 mice is reduced in size, but the percentages of CD4CD8 cells and mature CD4 or CD8 single-positive T cells are the same as in the thymus of wild-type mice. The reduced size of the thymus is caused by an inhibition of the transition of CD44CD25 to CD44$^-$CD25 cells. IL-7 is not required at earlier, prethymic stages in the development of lymphoid cells. There is a consensus that IL-7 regulates the size of the early thymic compartment by promoting the survival and probably also the proliferation of early thymocyte precursors. Earlier *in vitro* studies have shown that IL-7 acts as a survival factor for CD4$^-$CD8$^-$, in particular, of the CD44CD25 thymocytes. Consistent with these results, IL-7 mice have an increased proportion of DN thymocytes binding annexin-V, a marker for cells undergoing apoptosis. DN thymocytes of IL-7 mice are compromised in expression of the anti-apoptotic protein Bcl-2. In a normal thymus, Bcl-2 shows a biphasic expression pattern: expression is high in DN thymocytes and is down-regulated in DP cells. Bcl-2 is reexpressed in mature SP cells. Bcl-2 expression in the earliest CD44CD25 thymocytes in IL-7 and wild-type mice is the same but IL-7 mice lose Bcl-2 upon progression of differentiation through the CD44CD25 and subsequent stages. The increase in apoptotic cells in IL-7 mice coincides with the decrease in expression of Bcl-2. Moreover, culturing thymocytes of IL-7 mice with IL-7 leads to restoration of Bcl-2 levels. To provide direct evidence for a role for Bcl-2 in IL-7-mediated T-cell development, Bcl-2 has been expressed as a transgene in mice deficient for the IL-7 receptor components IL-7Rα and γc. Although it was found in some studies that transgenic expression of Bcl-2 increased the size of the thymus marginally (two- to fourfold) or even completely compared to the IL-7Rα- or γc-deficient mice without Bcl-2, a recent analysis of a large number of γc − /−, ckit − /− or ckit − /− γc − /− mice failed to observe a rescue of thymic cells by a Bcl-2 transgene. Thus, it is unclear whether Bcl-2 is involved in the IL-7-mediated survival of T-cell precursors.

IL-7 is probably not important for rearrangement at the TCRα or TCRβ loci. Although in two studies it was found that the presence of IL-7 in cultures of T-cell precursors was required for TCRβ rearrangements, these studies could not exclude the possibility that the absence of TCR gene rearrangements was due to a failure of the pre-T cells to survive in the absence of IL-7. Moreover, although there is a delay in the onset of TCRβ rearrangement during embryonic life of IL-7 mice, TCRβ gene rearrangements are normal at birth. In addition, IL-7 mice have TCRαβ cells, albeit in reduced numbers in the spleen. Together these findings argue against an essential role for IL-7 in TCRβ (and α) gene rearrangements.

DN cells in the thymus acquire CD4 and CD8 following stimulation via the pre-TCR. DP cells undergo rearrangements at the TCRα locus and start to express low levels of TCRαβ. These cells are subjected to positive and negative selection through interactions of the TCRαβ and major histocompatibility complex/peptide complexes. This results in the expression of the activation antigen CD69 but also of IL-7Rα and the receptor for stem cell factor. IL-7, probably in cooperation with stem cell factor, maintains survival and induces proliferation of TCRαβ cells that have been subjected to positive selection.

IL-7 is also important for development of human T cells. IL-7 acts as a growth/maturation factor for human thymocyte precursors. Moreover, addition of anti-IL-7 and anti-IL-7R antibodies strongly inhibited human T-cell development in a fetal thymic organ culture system. Development was inhibited at an early stage of development. Genetic defects in the IL-7 system in humans cause severe combined immunodeficiency (SCID) syndromes. SCID patients with defects in γc, IL-7Rα, and JAK3 have been described. A complete γc deficiency in these patients is characterized by an absence of T and natural killer (NK) cells. However, normal numbers of B cells are present in γc-deficient patients. The same characteristics are also observed in JAK3-deficient patients. Two IL-7Rα-deficient patients were reported with normal numbers of NK and B cells but an absence of T cells in their peripheral blood. These data indicate that IL-7 is required for T-cell development, but is dispensable for NK- and B-cell development in humans.

### B. The Role of IL-7 in Development of TCRγδ Cells

Although the TCRαβ cells are reduced in number, there are virtually no TCRγδ cells present in IL-7- and IL-7Rα-deficient mice. Both intraepithelial lymphocyte and thymic TCRγδ cells are affected. Defects in rearrangements at the TCRγ locus, in transcription of germ-line and rearranged TCRγ genes and survival of TCRγδ precursors, contribute to the lack of TCRγδ cells in IL-7 and IL-7R − /− mice. TCRγ rearrangements are strongly reduced in two independently generated IL-7Rα mice due to the fact that IL-7 controls TCRγ locus accessibility. It was also reported that transcription of the germ-line and rearranged TCRγ was impeded in IL-7Rα mice. Thus, not only TCRγ locus accessibility but also inhibition of transcription of those TCRγ genes that are rearranged contribute to the lack of TCRγδ cells in IL-7 − /− mice. In addition, IL-7 is important for survival of TCRγδ precursors.

## C. The Role of IL-7 in the Function of Mature TCRαβ Cells

It has been reported that mature TCRαβ T cells are compromised in their functional capacities in the absence of the IL-7Rα. The majority of IL-7Rα mice failed to reject an allogeneic tumor, due to a strongly diminished frequency of cytotoxic T lymphocytes (CTL). IL-7Rα T cells were hyporesponsive to TCR-independent stimuli, such as the combination of the phorbol ester phorbol myristate acetate (PMA) and ionomycin. In IL-7 mice, however, T cells responded normally to polyclonal stimuli such as concanavalin A and PMA plus ionomycin on a per cell basis. The T cells of IL-7 mice have not been extensively analyzed for their responses to more physiological stimuli.

IL-7 has strong effects on mature human T cells *in vitro*. It acts as a survival and growth factor for human T cells. Interestingly, IL-7 is directly mitogenic for CD45RA naive human T cells, and culture of these cells results in maintenance of the CD45RA phenotype. IL-7 is involved in the induction of the survival and expansion of T cells after their export from the thymus. IL-7 has the capacity to counteract apoptotic stimuli. For example, it rescues human activated T cells from apoptosis induced by glucocorticosteroids and regulates Bcl-2 and CD25 expression. IL-7 was furthermore shown to up-regulate the expression of CD80 (B7.1) on human T cells, resulting in enhancement of T-cell stimulation by these cells. IL-7 can co-stimulate TCR-mediated activation of T cells, resulting in enhanced IL-2 mRNA accumulation and IL-2 secretion. The effect of IL-7 is in part mediated at the transcriptional level and involves the up-regulation of the DNA-binding activities of nuclear factor of activated T cells and activator protein-1.

It was recently documented that IL-7 mediates homeostasis of naive T cells *in vivo* since IL-7 was required for homeostatic expansion of naive $CD4^+$ and $CD8^+$ T cells in lymphopenic hosts. IL-7 is also required for survival of $CD8^+$ T cells in normal hosts. In addition, IL-7 seems to be involved in homeostasis of memory $CD8^+$ T cells but not of memory $CD4^+$ T cells. Also, in humans there is evidence for a role of IL-7 in T-cell homeostasis. The homeostatic effect of IL-7 may be mediated by the transcription factor lung Krüppel-like factor, since this factor, which is essential for the survival of naive T cells, is induced by IL-7.

## D. The Role of IL-7 in Development of B Cells

IL-7 and IL-7Rα − /− mice show severe defects in the development of B cells. A phenotypic analysis of different stages in B-cell development revealed that development of B cells in the bone marrow is arrested in the transition of pro-B cells (B220 CD24 CD43) to pre-B cells (B220 CD24 CD43). Mature B cells that express both B220 and surface IgM were present in these mice but at strongly reduced numbers. IL-&Rα mice have a block at an earlier stage of B-cell development, namely, the transition of pre-pro-B to pro-B cells. Transgenic expression of Bcl-2 does not reconstitute B-cell development in IL-7Rα and γc mice, suggesting that IL-7 induces the differentiation of B cells. Indeed, IL-7 appears to be required for IgH gene rearrangements. It is likely that IL-7 is also necessary for protection against apoptosis and for expansion of early B-cell precursors. Surprisingly, IL-7 is not essential for development of human B cells. SCID patients deficient for either γc or IL-7Rα lack T cells but have near normal numbers of B cells. The reason for this species difference is unknown as a cytokine that controls human B-cell differentiation has yet to be found.

## VII. EFFECTS OF *IN VIVO* ADMINISTRATION OF IL-7

The effects of administration of exogenous IL-7 have been studied in various animal models. A general observation in these experiments is a marked stimulation of B-lymphocyte production but increases in T cells, NK cells, and macrophages were also observed. Exogenous IL-7 at an optimal dose can markedly stimulate *in vivo* B lymphopoiesis, eventually resulting in elevated levels of pre-B and B cells. Changes in myelopoiesis were observed after *in vivo* administration of recombinant human (rh)IL-7 to mice. These resulted mainly from the emigration of myeloid progenitors from the bone marrow through the blood to the spleen, liver, and possibly other peripheral organs. This observation suggests that IL-7 modifies the expression of chemokine receptors on myeloid progenitors. It is, in this respect, noteworthy that IL-7 increases the expression of CXCR4, the receptor for stromal-derived factor-1, on thymocytes. In another study, rhIL-7 administration to normal mice caused a pronounced leukocytosis in the spleen and lymph nodes, with increases in B cells, T cells, NK cells, and macrophages. The increased numbers of T cells after IL-7 treatment were primarily the result of an expansion of the peripheral T-cell population. Interestingly, the T cells from rhIL-7-treated mice have enhanced proliferative responses to various stimuli *in vitro* and were able to enhance an allogeneic CTL response *in vivo*. It was also documented that administration of rhIL-7 to tumor-bearing mice profoundly increased the number of B and T cells and reduced the number of early renal adenocarcinoma pulmonary metastases.

An adverse effect of IL-7 administration in female mice is increased bone resorption. The effect of IL-7 was similar to that observed with estrogen deficiency. Bone resorption is caused through activation of osteoclasts. A recent report documented that IL-7 induced the expression of the osteogenic cytokine receptor activator of nuclear factor κB ligand and possibly other osteogenic factors, leading to the stimulation of osteoclast formation.

IL-7 has also been administered by gene transfer. Murine bone marrow infected with a helper-free recombinant retrovirus expressing the murine IL-7 (mIL-7) gene was used to reconstitute lethally irradiated hosts. Twenty-three percent of mIL-7 retrovirus-infected recipients became moribund within 4–16 weeks posttransplant, with splenomegaly and enlarged lymph nodes. Marked changes in T-cell subsets of spleen and lymph nodes were observed. The size of the thymus was not altered and the proportion of CD4CD8 cells was generally decreased, with corresponding increases in CD4, CD8, or CD4CD8 cells.

## VIII. IL-7 AS A POSSIBLE THERAPEUTIC AGENT

The fact that IL-7 stimulates T-cell development and can augment T-cell reactivities *in vitro* has led to the idea that this cytokine may be used to boost development and function of T cells in various clinical settings. IL-7 has been tested in mouse models of infectious diseases and bone marrow transplantation.

### A. Infectious Diseases

IL-7 has been tested in various models for its efficacy in treating infectious diseases. IL-7 augments protective immunity against *Toxoplasma gondii* in A/J mice and *Mycobacterium tuberculosis*in Balb-c mice. Moreover, a combination of IL-1 and IL-7 augments immunity against *Listeria monocytogenes*, due to enhanced responsiveness of TCRγδ cells to IL-7. These observations indicate that exogenous administration of human rIL-7 is able to protect mice against acute parasite challenge by stimulating interferon-γ production and augmenting the response mediated by CD8 CTL. However, IL-7 does not always confer a beneficial effect. Treatment of genetically susceptible Balb/c mice with IL-7 at the onset of the infection with *Leishmania major* led to enhanced lesion development and a significantly accelerated death of the animals. This was correlated with a 40-fold increased parasite burden in spleens and lymph nodes. A strong increase in the number of B cells was noted. It was suggested that an enhanced $T_H2$ response was responsible for the aggravating effect of IL-7 in this model. Administration of IL-7 also aggravated the course of infection with *Schistosoma mansoni*. Consistent with this finding, the growth of the parasite was blocked in IL-7 − /− mice.

### B. Bone Marrow Transplantation

Bone marrow transplantation (BMT) is increasingly used in the clinic to promote hematopoietic recovery. The activities of IL-7 on T cells have led to the proposal that IL-7 can be used to overcome immunodeficiencies in certain situations by accelerating T-cell development, by expanding the pool of newly developed T cells, and by promoting the functional maturation of T cells. Administration of IL-7 in this setting would also overcome the blocking effect of irradiation on IL-7 production, presumably by thymic stromal cells. Indeed, several preclinical studies have found that IL-7 could accelerate murine lymphocyte regeneration and acquisition of immune competence following chemotherapy and bone marrow transplantation. IL-7 boosted survival after challenge with influenza virus following syngeneic BMT. Both B- and T-cell responses were stimulated by IL-7. It was also reported that the BMT recipients had thymic hypoplasia and strongly reduced numbers of mature T cells 28 days after syngeneic BMT. When these mice were treated with IL-7, thymic cell numbers were normalized. Moreover, the function of antigen-specific T and B cells was improved. Recombinant human IL-7 was found to accelerate bone marrow engraftment affecting both myeloid and lymphoid compartments. These studies indicate a beneficial effect of IL-7 in autologous BMT. One study, however, suggested that in an allogeneic H2-matched BMT, IL-7 enhanced the responses of the allogeneic T cells posttransplant. This may result in graft-versus-host disease.

### C. Anti-tumor Immunity

Most IL-7Rα failed to reject an allogeneic tumor, due to a diminished frequency of CTL, indicating that IL-7 is needed to enable the mature T cells to reject tumor cells. In addition, IL-7 induces *in vitro* growth of murine anti-tumor CTL. A glioma cell line transfected with IL-7 reduced tumorigenicity *in vivo* depending on the amount of IL-7 produced. This reduction in tumorigenicity could be reversed in a dose-dependent fashion by injection of anti-IL-7-neutralizing monoclonal antibody at the tumor site. The response was specific, as other tumors were not rejected and rejection was mediated by CD8 T cells. Co-expression of IL-7 and the co-stimulatory molecule CD80 (B7.1) in mammary adenocarcinoma and a plasmacytoma resulted in a stronger protective response than with either IL-7 or CD80 alone. A protective effect of immunization with IL-7-transfected tumor cells was observed with the plasmacytoma cell line J558L, but in that model CD4 cells rather than CD8 cells were responsible for the protection. Injection of dendritic cells infected with an adenoviral vector harboring IL-7 resulted in a potent anti-tumor response in two murine lung cancer models.

## Glossary

**interleukin-7** A polypeptide responsible for the differentiation and growth of lymphocytes, in particular of T and B lymphocytes.

**interleukin-7 receptor** A cell surface-expressed complex consisting of two polypeptide chains that are designated IL-7Rα and γ comma (γc). γc is also part of the receptors for other cytokines.

**phosphatidylinositol 3 kinase** Kinase that phosphorylates the D3 position of the inositol group of phosphoinositide lipids to generate phophatidylinositol-3 phosphate [PtdIns(3)P], PtdIns(3,4)P2, and PtdIns(3,4)P3. The enzyme associates with the interleukin-7 (IL-7) receptor upon interaction with IL-7. It is important for survival and growth.

**signal transducer of activation and transcription (STAT)** Following interaction of interleukin-7 with interleukin-7 receptor, STAT5 is activated. Activated STAT5 forms a dimer and is translocated to the nucleus, where it activates several genes important for differentiation.

## See Also the Following Articles

**Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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# Interleukin-9

JEAN-CHRISTOPHE RENAULD
*Ludwig Institute for Cancer Research, Belgium*

I. INTRODUCTION
II. BIOLOGICAL SOURCES OF IL-9
III. IL-9-RESPONSIVE CELLS
IV. IL-9 AND DISEASES
V. THE IL-9 RECEPTOR STRUCTURE AND SIGNALING
VI. SUMMARY

**Interleukin-9, a cytokine produced mainly by T lymphocytes, was originally identified as a T-cell/mast cell growth factor with a narrow specificity. Interleukin-9 has since been found to be a broadly active cytokine with various targets, including B cells, macrophages, eosinophils, hematopoietic precursors, and epithelial cells. Its main physiological activity seems to be part of antiparasite immune responses.**

## I. INTRODUCTION

Interleukin-9 (IL-9) was originally purified from helper T-cell supernatants based on its ability to support the growth of a murine T-cell line. Human IL-9 was cloned both by expression cloning of a factor stimulating the growth of a human megakaryoblastic leukemia and by cross-hybridization with the mouse gene. A comparison of the mouse and human IL-9 protein sequences, which are 50% identical, is shown in Fig. 1. Both deduced protein sequences contain 144 residues having a typical signal peptide of 18 amino acids and four potential N-linked glycosylation sites. This glycosylation is responsible for the discrepancy observed between the predicted relative molecular mass (14,150 Da) and that measured for native IL-9 (30,000–40,000 Da). Although recombinant IL-9 produced in *Escherichia coli* recapitulates all *in vitro* IL-9 activities, glycosylation plays an important role in the *in vivo* activity of the protein. Indeed, the half-life of natural IL-9 is reduced more than threefold on desialidation. The sequence is also characterized by the presence of 10 cysteines that are perfectly matched in both mature proteins and a strong predominance of cationic residues, which explains the elevated p*I* measured with purified IL-9.

The human IL-9 gene is a single-copy gene that maps to chromosome 5, in the 5q31–q35 region, in a region of the genome that contains other cytokine genes, including those for other interleukins (IL-3, IL-4, IL-5, and IL-13) and granulocyte/macrophage colony-stimulating factor (GM-CSF). However, in the mouse, the IL-9 gene is not linked to the same gene cluster; it has been localized on mouse chromosome 13, and the IL-3, IL-4, IL-5, and GM-CSF genes are located on chromosome 11. The human and murine IL-9 genes share a similar structure, with five exons and four introns stretching over about 4 kb. The five exons are identical in size in both mice and humans and show homology levels ranging from 56 to 74%. In contrast, no significant sequence homology is found in the introns (except for intron 2, which is also the smallest one). However, 3′ and 5′ untranslated regions show a high level of identity, supporting a possible involvement of these sequences in the transcriptional or posttranscriptional regulation of IL-9 expression. In particular, numerous ATTTA motifs were found in the 3′ untranslated region of both genes. These sequences, frequently noted in cytokine mRNAs, may modulate the stability of the mRNA and thus are thought to be related to the short half-life of these messengers.

The promoter of the IL-9 gene contains potential recognition sites for several transcription factors,

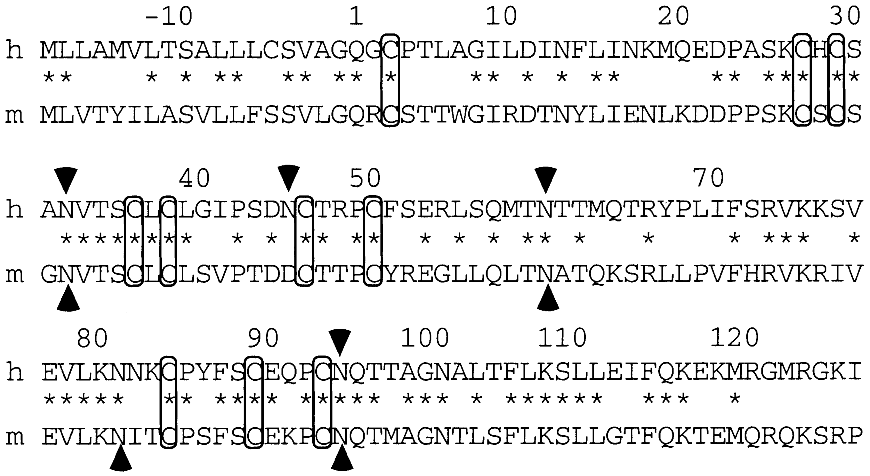
-10
1
10
20
30
h MLLAMVLTSALLLCSVAGQGCPTLAGILDINFLINKMQEDPASKCHCS
** * * ** ** ** * ** * * ** ** *** **
m MLVTYILASVLLFSSVLGQRCSTTWGIRDTNYLIENLKDDPPSKCSCS
40
50
70
h ANVTSCLCLGIPSDNCTRPCFSERLSQMTNTTMQTRYPLIFSRVKKSV
******** * * ** ** * * * ** * * * *** *
m GNVTSCLCLSVPTDDCTTPCYREGLLQLTNATQKSRLLPVFHRVKRIV
80
90
100
110
120
h EVLKNNKCPYFSCEQPCNQTTAGNALTFLKSLLEIFQKEKMRGMRGKI
***** ** **** ****** *** * ****** *** *
m EVLKNITCPSFSCEKPCNQTMAGNTLSFLKSLLGTFQKTEMQRQKSRP

**FIGURE 1** Alignment of human and mouse IL-9 protein sequences. Amino acids are indicated in the one-letter code. The 10 cysteine residues of the mature protein are boxed and arrows indicate the potential N-linked glycosylation sites. Amino acids number 1 refers to the N-terminus of the mature mouse protein.

such as activator proteins (AP-1 and AP-2), which could provide a structural basis for the induction of IL-9 expression by phorbol esters. A nuclear factor (NF-κB)-binding site has been also reported to actively regulate IL-9 expression.

## II. BIOLOGICAL SOURCES OF IL-9

Activated T helper ($T_H2$) lymphocytes are the main source of IL-9. Its *in vitro* production by activated spleen cells is inhibited, like that of other $T_H2$ cytokines, by IL-12, interferon γ (IFNγ), or CpG oligonucleotides. *In vivo*, potent $T_H2$ stimuli such as anti-immunoglobulin D (IgD)-triggered polyclonal activation and helminth infections also induce IL-9 expression. Conversely, $T_H1$-promoting factors such as IL-12, type I and type II interferons, and viral infection suppress IL-9 expression in various *in vivo* models. Finally, IL-9 expression nicely reflects $T_H2$ responses in *Leishmania major* infection, in which susceptible $T_H2$ responder mice produce IL-9 but resistant $T_H1$ strains do not.

The mechanisms involved in the regulation of IL-9 production by human T cells have been studied quite extensively *in vitro*. After stimulation of peripheral T cells, IL-9 mRNA expression peaks at 28 h and is completely abrogated by cycloheximide, an inhibitor of protein synthesis, pointing to the involvement of secondary signals in this process. A complex cascade of factors acting in synergy seems to be involved, with IL-2 being required for IL-4 production, both IL-2 and IL-4 being needed for IL-10 production, and eventually IL-4 and IL-10 being required for IL-9 biosynthesis. Other regulatory mechanisms, including IL-1 and transforming growth factor-β (TGF-β) could be involved in IL-9 expression by some T cell lines and tumors.

Another characteristic of IL-9 expression is its association with human T-cell lymphotropic virus type I (HTLV-I), a retrovirus involved in adult T-cell leukemias, which often produces IL-9 constitutively. The tax transactivator of HTLV-I might be implicated in this process through the NF-κB consensus site in the IL-9 promoter. Interestingly, in another system of T-cell transformation by murine polytropic retroviruses, viral infection also resulted in IL-9 expression.

Besides T cells, mast cells represent another source of IL-9. These cells produce IL-9 in response to ionomycin or IgE-antigen complexes. The levels of IL-9 produced by these cells are strongly enhanced in the presence of added IL-10, IL-1, or lipopolysaccharide.

## III. IL-9-RESPONSIVE CELLS

### A. Mast Cells

Mast cells were among the first identified targets for IL-9 in the mouse system. When primary mast cell lines are derived from hematopoietic progenitors, IL-9 is not sufficient to sustain mast cell growth, but synergistically enhances the proliferation induced by IL-3 or the combination of IL-3 and IL-4. Similar results are obtained by combining IL-9 with stem cell factor (SCF).

In addition to its activity on growth and survival, IL-9 is a potent regulator of mast cell function and differentiation. IL-9 up-regulates expression of the high-affinity IgE receptor in murine mast cell lines. IL-9 is also a major inducer of proteases, including granzyme B and mast cell-specific proteases of the mouse mast cell protease (MMCP) family, particularly MMCP-1, MMCP-2, and MMCP-4. IL-9-induced expression of MMCP-1 may be due to endogenously expressed TGF-β1, because anti-TGF-β antibodies block the effect of IL-9 on the percentage of MMCP-$1^+$ cells in primary bone marrow-derived mast cell (BMMC) cultures. Other cytokines induced by IL-9 in murine mast cell lines include IL-6, IL-13, and IL-22.

*In vivo*, IL-9 overexpression induces a massive mast cell infiltration of the gastric and intestinal epithelium, as well as in the upper airways and in kidneys. Injections of antibodies directed against c-kit, the SCF receptor, block mastocytosis in IL-9 transgenic mice. Because a constitutive SCF expression was observed in both IL-9 transgenic and control mice, these observations indicate that neither SCF nor IL-9 is sufficient to induce mastocytosis but that the synergistic activity of these cytokines is responsible for the *in vivo* amplification of this cell population in IL-9 transgenic mice. In a model of *Schistosoma* infection, a decrease in mast cells present in granulomas was reported in IL-9-deficient mice, suggesting that, at least under certain circumstances, IL-9 is required for optimal mast cell responses.

### B. T Lymphocytes

Although T lymphocytes were the first identified targets for IL-9, the physiological role of IL-9 for T cells remains puzzling. Human T lymphocytes need preactivation to respond to IL-9, and a significant degree of proliferation can be induced by IL-9 when human peripheral blood mononuclear cells (PBMCs) are preincubated for 10 days with phytohemagglutinin (PHA). These results suggest that responses to IL-9 require previous T-cell activation.

Several observations point to IL-9 as a potential T-cell oncogene. For instance, 5–10% of FVB mice expressing an IL-9 transgene spontaneously develop lymphoblastic lymphomas. Transfection of mouse IL-9-dependent T-cell lines with an IL-9 cDNA expression vector and injection of these cells into syngeneic animals result in widespread lymphoma development. Similar observations have been reported in a rat model, using an IL-2-dependent T-cell lymphoma that, after infection with murine polytropic retroviruses, became IL-2 independent by induction of an autocrine loop involving IL-9 and its receptor.

In humans, a link between dysregulated IL-9 production and lymphoid malignancies has been initially suggested by the observation that lymph nodes from patients with Hodgkin disease or large-cell anaplastic lymphomas constitutively produce IL-9. Constitutive IL-9 expression is also detected in HTLV-I-transformed T cells and in Hodgkin cell lines.

The role of IL-9 in thymic maturation was studied using human thymic precursor organ cultures, taking advantage of the fact that human progenitor cells from fetal liver or from cord blood can be cultured *in vitro* in thymic lobes from nude mice. Under these conditions, anti-IL-9 receptor antibodies inhibit the growth and differentiation of immature thymocytes, suggesting that IL-9 is involved in normal T-cell development. This observation contrasts with the fact that IL-9 -deficient mice are reported to have a normal thymus. Several possibilities could explain this discrepancy. IL-9 might be critical for human but not for mouse thymus development. The effect of cytokines may also be less redundant in an *in vitro* experimental setting using human precursors and the mouse thymus environment, compared to in a pure murine model. Alternatively, the role of IL-9 in thymic development might also vary between stages of fetal and adult life. In line with this hypothesis, it was shown that fetal murine thymocytes, but not adult thymocytes, proliferate in the presence of IL-9 *in vitro*.

Although IL-9 is specifically produced by $T_H2$ but not $T_H1$ cells, its role in the tuning of the $T_H1/T_H2$ balance *in vivo* remains more elusive. Stimulation of lymph node cells *in vitro* with plate-bound anti-CD3 antibodies induced similar IL-4, IL-5, and IFNγ production in control and IL-9-deficient mice. Also, *in vivo* T-cell-dependent anti-ovalbumin antibody and cytokine production was not altered by IL-9 gene disruption. In IL-9 transgenic mice, IgG1 and IgE concentrations are preferentially increased, as expected from increased $T_H2$ responses, but IgG2a concentrations are also significantly increased, as in $T_H1$-like responses. By contrast, in broncho-alveolar lavage from silicotic mice, IL-9 overexpression surprisingly leads to a decreased IgG1/IgG2a ratio, suggesting that $T_H1$ responses are favored. Finally, analysis of *in vivo* cytokine expression in transgenic mice shows that IL-13 expression is strongly up-regulated in lungs and gut. Altogether, these observation indicate that, if IL-9 actually modulates cytokine production by T cells, this modulation does not fit with a simple inhibition or promotion of a particular T helper subset.

## C. Hematopoietic Progenitors

Although human IL-9 was identified as a growth factor for megakaryoblastic leukemia Mo7E, its activities as a hematopoietic growth factor have remained fairly modest. In fact, IL-9 does not seem to be active on normal megakaryoblastic precursors but supports the clonogenic maturation of erythroid progenitors in the presence of erythropoietin. This activity was confirmed by several groups and is reproducibly observed with highly purified progenitors, particularly in synergy with SCF.

## D. Monocytes and Macrophages

An anti-inflammatory activity of IL-9 has been demonstrated in experimental septic shock models. Mice infected with *Pseudomonas aeruginosa* are protected against a lethal dose of these bacteria by administration of IL-9. This protection is accompanied by dramatic reductions in serum concentrations of tumor necrosis factor α (TNFα), IL-12, and IFNγ and by a strong induction of IL-10. *In vitro*, IL-9 inhibits the oxidative burst induced by lipopolysaccharide (LPS) in freshly isolated human monocytes and bronchial alveolar macrophages. This activity results from an induction of TGF-β, which in turn inhibits activation of extracellular signal-related kinase (ERK) by LPS.

## E. B Lymphocytes

*In vitro*, IL-9 has only a modest effect on immunoglobulin production by activated B lymphocytes. *In vivo*, although IL-9-deficient mice do not show obvious defects in antibody responses, observations made in IL-9 transgenic mice illustrate the potential activity of this cytokine in humoral responses. Basal titers of all Ig classes and antigen-specific antibody responses are indeed increased in the serum of transgenic animals. In addition, these mice are characterized by a dramatic increase in the number of peritoneal B1 cells, but not in conventional B cells, suggesting that IL-9 may act preferentially on this particular B-cell population, which is predominantly involved in autoimmunity. However, IL-9 transgenic mice do not have increased levels of autoantibodies, and the expansion of peritoneal B cells in these mice is mainly due to B1b cells, a functionally ill-defined B cell subset that differs from B1a cells by the absence of the CD5 surface marker.

## F. Eosinophils

IL-9 overexpression in transgenic mice leads to modest but significant eosinophilia in the blood, peritoneal cavity, and lungs. The mechanisms underlying the effect of IL-9 on eosinophils remain unclear. IL-9 does not induce any eosinophilia in IL-5-deficient mice. This raises the hypothesis that IL-9 induces eosinophilia by up-regulating the expression of IL-5 or its receptor. However, this observation might simply reflect the fact that IL-5 plays a nonredundant role in eosinophil development, independently from any effect of IL-9.

## G. Epithelial Cells

Data pointing to lung epithelial cells as important IL-9 targets have been obtained both from IL-9 transgenic mice and from primary lung cultures stimulated *in vitro* with IL-9. Such stimulation induced chemokine secretion and triggered enhanced eosinophil chemotaxis. Moreover, increased levels of eotaxin and MCP-1 were found in lungs from IL-9 transgenic mice by Western blot.

IL-9 action on lung epithelial cells also results in mucin secretion. Lung cells from IL-9 transgenic mice show enhanced expression of the mucin genes *MUC2* and *MUC5AC*, a finding confirmed with human primary lung cultures. The mechanisms underlying up-regulation of mucus production by IL-9 are not clear. The regulation of a murine calcium-activated chloride channel (mCLCA3) gene, *gob-5,* might play a role in this process. Subtraction cloning showed that the *gob-5* gene is up-regulated in the lungs of IL-9 transgenic mice. This gene is also induced in the lungs of allergen-exposed mice, and its human homologue, *Clca1*, is up-regulated by IL-9 *in vitro* in primary lung cultures. Interestingly, transfection of the *gob-5* cDNA in a human mucoepidermoid cell line induced mucus production and *MUC5AC* expression.

# IV. IL-9 AND DISEASES

## A. IL-9 and Parasite Infections

Infections by helminths induce an immune response characterized by strong IgE production and mucosal mastocytosis resulting from $T_H2$ cytokine production. During infection with intestinal nematodes, IL-9 production is induced in mesenteric lymph nodes, as are other $T_H2$ cytokines such as IL-3, IL-4, and IL-5. Interestingly, IL-9 transgenic mice were found to be particularly resistant to these intestinal worms. Conversely, inhibition of IL-9 activity affects the capability of infected mice to expel the nematode *Trichuris muris*.

*Schistosoma mansoni* infection is another parasite infection model in which IL-9 might play a role. In infected mice, tissue damage is primarily caused by granulomatous inflammation surrounding parasite eggs, whereas the intestine is subject to inflammation elicited by translocation of parasite eggs through the intestinal wall. In this model, IL-9 expression correlates with increased mast cell progenitors during the chronic phase of injection. Analysis of IL-9-deficient mice showed that IL-9 plays an essential role in goblet cell hyperplasia and mastocytosis in pulmonary granulomas induced by *S. mansoni* eggs. However, IL-9 overexpression results in a dramatic increase in mortality of infected mice. Dying animals showed ileum enlargement with muscular hypertrophy, mastocytosis, eosinophilia, goblet cell hyperplasia, and increased mucin expression, pointing to an overwhelming immune response.

## B. IL-9 and Asthma

Studies on the inheritance of susceptibility to asthma have pointed to a cluster of candidate genes, including IL-9, on human chromosome 5q31–q33. When DBA/2 and C57BL/6 mice, respectively, susceptible and resistant to experimental asthma, are crossed, susceptibility to the disease correlates with inheritance of the IL-9 gene from the asthma-prone strain. Moreover, IL-9 expression is higher in bronchial hyperresponsive animals. Further support for the role of IL-9 in asthmatic reactions is provided by studies of IL-9 transgenic mice, which show massive airway inflammation with infiltration by mast cells, eosinophils, and lymphocytes. Moreover, a striking epithelial cell hyperplasia is noted with mucus accumulation and subepithelial deposition of collagen. As a result, these mice show strongly increased airway response to methacoline or 5-hydroxytrytamine, a typical feature of asthma called hyperresponsiveness. Direct evidence for selective increased production of IL-9 in asthmatic airways has been reported using *in situ* hybridization and immunocytochemistry. *In vitro*, allergic children show increased IL-9 expression in response to allergens, as compared to nonallergic controls.

Contrasting reports have been published concerning the possibility of interfering with asthma by blocking IL-9 activity. On the one hand, administration of anti-IL-9 antibodies in a mouse model of allergic asthma significantly inhibited IgE production, pulmonary eosinophilia, goblet cell hyperplasia, and airway hyperreactivity. On the other hand, IL-9 deficient mice showed normal antigen-induced bronchial responsiveness in a similar experimental model.

## V. THE IL-9 RECEPTOR STRUCTURE AND SIGNALING

A single class of high-affinity binding sites for IL-9 ($K_d \backsim 100$ p$M$) has been detected on IL-9-responsive murine cells. IL-9 binds to a heterodimeric complex composed of the IL-9 receptor (IL-9R) and the γ-chain of the IL-2 receptor (also called γc, for γ common). The human IL-9R consists of a 522-amino-acid protein with an extracellular domain composed of 233 amino acids, including a WSEWS (W, tryptophan; S, serine; and E, glutamate) motif and typical conserved residues from the hematopoietin receptor superfamily. Antibodies directed against γc completely inhibit the activity of IL-9 without affecting the $K_d$ of IL-9 binding, indicating that γc is required for signal transduction but not for IL-9 binding.

So far, the main function of γc seems to consist of recruiting the tyrosine kinase Jak3, whereas the IL-9R is associated with Jak1. This association of Jak1 with the IL-9R was ascribed to a 98-residue juxtamembrane region of the receptor. On IL-9 binding, both Jak1 and Jak3 become phosphorylated and catalytically active and induce IL-9R phosphorylation on one out of its five tyrosine residues. This single phosphorylated residue acts as a docking site for Stat1, Stat3, and Stat5, three signal transduction and transcription activation factors that, after phosphorylation by the Jak kinases associated with the receptor, form hetero- or homodimers and migrate to the nucleus. Interestingly, mutation of this single tyrosine of the IL-9R abolished both Stat activation and cell growth control by IL-9, including protection against apoptosis and positive as well as negative effects on proliferation.

The role of other signal transduction pathways for IL-9 activities remains more elusive. Opposite observations have been reported concerning the involvement of the ras/mitogen-activated protein kinase (MAPK) pathway. It seems that IL-9 can indeed induce ERK phosphorylation, but that this effect of IL-9 is highly dependent on the cell lines. In Baf3 cells transfected with the human IL-9R, IL-9 induces ERK phosphorylation, and inhibition of this pathway by MAPK kinase (MEK) inhibitors or by dominant-negative Ras isoforms partially inhibits IL-9-induced proliferation.

IL-9 stimulation also involves the phosphorylation of an adapter protein called IRS-2, a feature shared with IL-4 signal transduction, and this pathway has been shown to be critical for growth regulation. Phosphorylation of IRS-2 is not dependent on the phosphorylation of the IL-9 receptor, contrasting with the IL-4 system in which IRS-2 associates with the IL-4 receptor through a phosphotyrosine residue. On IL-9 activation, IRS-2 might become phosphorylated by interacting directly with the Jak1 tyrosine kinase. After phosphorylation, IRS-2 binds the SH2 domain of various signaling proteins, including the p85 subunit of the phosphatidylinositol 3-kinase. The IRS-2 pathway is required for optimal IL-9-dependent proliferative responses, although activation of this pathway is not sufficient to induce proliferation.

IL-9 can also modulate other signaling cascades by regulating the expression of proteins involved in these pathways. This is the case for the NF-κB pathway, because IL-9 induces expression of Bcl-3, a protein that associates with p50/p50 NF-κB dimers. Bcl-3 expression has been shown to inhibit apoptosis in IL-9-dependent cell lines, in line with well-described anti-apoptotic activities of NF-κB. Another example of indirect modulation of signaling cascades by IL-9 is the induction of M-Ras/R-Ras3 in murine T cell lines. This protein shows an amino acid identity of 49 and 46% with p21 H-Ras and p23 K-Ras. Like other members of the Ras family, activated M-Ras is able to regulate cell proliferation and MAP kinase activation.

Negative feedback for signal transduction is a critical process in the regulation of cytokine activities. Cytokine-induced activation of phosphatases is a classical negative feedback mechanism. IL-9 induces the phosphorylation of the SHP2 phosphatase in a hematopoietic immature cell line. However, IL-9 signaling seems less sensitive to phosphatase-mediated down-regulation than does erythropoietin (EPO) signaling, a characteristic that can lead to more sustained STAT-5 activation and induction of distinct sets of genes.

Another mechanism responsible for negative feedback regulation consists of the induction of genes that code for inhibitors of Jak kinases. A family of such inhibitors has been described, including cytokine-inducible SH2 (CIS)-containing protein, and suppressors of cytokine signaling (SOCS-1, SOCS-2, and SOCS-3). Other SOCS-related proteins have been described but their function remains to be established. In T-cell lymphomas, IL-9 induces the rapid induction of CIS, SOCS-2, and SOCS-3, with a peak after 2 h of stimulation. However, only SOCS-3 is able to inhibit IL-9 signaling, and this effect is seen only when SOCS-3 is overexpressed to IL-9-responsive cells, suggesting that this process is not very efficient in down-regulating IL-9 activities. However, induction of these genes by IL-9 might lead to inhibition of signaling by more sensitive cytokine receptors, suggesting a role in cytokine cross talk rather than in feedback inhibition. A schematic representation of the currently described signal transduction pathways triggered by IL-9 is shown in Fig. 2.

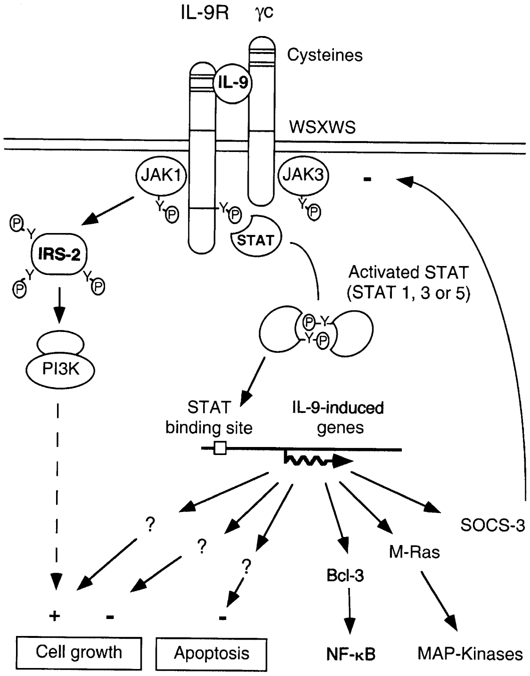

FIGURE 2 Schematic representation of the signal transduction pathways triggered by IL-9.

## VI. SUMMARY

IL-9 is a broadly active cytokine with various targets, including B cells, macrophages, eosinophils, hematopoietic precursors, and epithelial cells. Its aggravating role in asthma is probably due to its broad action on the different cells involved in this disease. A potential use of IL-9 *in vivo* stems from its ability to dampen excessive production of inflammatory cytokines by monocytes and macrophages, as has been shown in septic shock following infection with *Pseudomonas aeruginosa*. In view of the induction of IL-10 and TGF-β by IL-9, the ability of this cytokine to modulate autoimmune reactions opens further interesting perspectives for the use and study of this molecule. At the molecular level, IL-9 binds to a complex composed of IL-9R and γc, and most of its activities are mediated by the Jak–Stat pathway.

## Glossary

**eosinophils** White blood cells thought to be important in asthma and in defense against parasitic infections.

**helminths** Parasitic worms (nematodes) that infect the intestinal tract.

**Janus kinases** Enzymes that are activated by cytokine receptors; phosphorylate proteins known as signal transducers and activators of transcription, which are normally found in the cytosol but move to the nucleus on phosphorylation and activate a variety of genes.

**lymphocytes** White blood cells that bear variable cell surface antigen receptors. The two main classes of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells), mediate humoral and cell-mediated immunity, respectively.

**mast cells** Large cells containing granules that store a variety of mediator molecules, including histamine. They have high-affinity IgE receptors, which can trigger degranulation and play a crucial role in allergies.

**T ($T_H$) helper cells** Subsets of T cells that are characterized by the cytokines they produce. $T_H1$ cells are mainly involved in activating macrophages, whereas $T_H2$ cells are mainly involved in activating B lymphocytes.

## See Also the Following Articles

**Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

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# Interleukin-10

YaoZhong Ding, Shuang Fu, Dmitriy Zamarin, and Jonathan Bromberg
*Mount Sinai School of Medicine, New York*

I. INTRODUCTION
II. GENE AND PROTEIN STRUCTURE
III. VIRAL IL-10
IV. IL-10 EXPRESSION
V. IL-10 IN DISEASE STATES
VI. IL-10 SIGNALING

**Interleukin-10 is a soluble protein factor secreted by T cells, B cells, monocytes/macrophages, mast cells, and keratinocytes. It is involved in regulation of immunoreactivity. The general mode of action is in immune suppression, but interleukin-10 may also function in immune stimulation.**

## I. INTRODUCTION

Interleukin-10 (IL-10) is a key regulator of immune responses. Because of its ability to turn off cytokine production by T cells, IL-10 was originally described as cytokine synthesis inhibitory factor (CSIF). T-Cell stimulation *in vitro* in the presence of IL-10 was also known to lead to long-term anergy and the production of a negative regulatory T-cell subset. Further investigations have demonstrated that the immunosuppressive effects of IL-10 are often at the level of the antigen-presenting cell (APC) and not directly at the level of the T cell. It has also been demonstrated that IL-10 inhibits the immune function of other cell types IL-10 inhibits natural killer (NK) cell production of interferon γ (IFNγ), intercellular adhesion molecule-1 (ICAM-1) expression on activated vascular endothelial cells, and T-dependent responses of B cells. In sum, it has been shown that the predominant effect of IL-10 is to suppress multiple immune responses through individual actions on T cells, B cells, APCs, and other cell types, and to skew the immune response from one T helper ($T_H$) subset to another ($T_H1$ to $T_H2$).

A number of unexpected findings suggest that IL-10 has actions that are more complex than originally proposed. IL-10 can act as a proliferative cofactor for immature and mature thymocytes stimulated by IL-2 plus IL-4. IL-10 can inhibit T-dependent B-cell responses, but not T-cell-independent responses. In fact, IL-10 can act as a B-cell growth factor and support the autocrine growth of B-cell lymphomas. In NZB/W F1 mice, IL-10 stimulates the development of systemic autoimmune disease, which is mediated primarily by B cells, whereas anti-IL-10 monoclonal antibody (mAb) delays the onset of autoimmunity. Likewise, the $T_H2$ polarization induced by IL-10 enhances the development of granulomata and chronic inflammation. Mice with an IL-10 transgene regulated by an insulin promoter have a pronounced leukocytic infiltrate of CD4 + and CD8 + T cells, B cells, and macrophages, along with activation of the vascular endothelium. Transgenic IL-10 expression in these mice does not prevent or delay autoimmune or alloimmune disease. All of these data suggest proinflammatory functions for IL-10 under some circumstances. In sum, IL-10 is generally considered an immunosuppressive cytokine, but IL-10 may have either immunostimulatory or immunosuppressive effects, depending on the assay, the cell types involved, or other concomitant immune events (see Tables 1–3).

of IL-23 as a p19/p40 heterodimer with potent IL-12-like functions explained why the deficit of $T_H1$-type responses and cell-mediated immunity is strongly pronounced in p40 knockout mice but is less evident in p35 knockout animals. The IL-12 receptor antagonist (IL-12RA) p40/p40 homodimer plays a $T_H2$-driving role. Most recently, IL-27 was identified as a member of IL-12 family active at the earliest stages of immunity (Table 1).

## II. DENDRIKINES REGULATE IMMUNE REACTIVITY

Studies of the development and regulation of immunity have focused on the critical role of dendritic cells (DCs) in antigen capture and presentation. A second critical aspect of DC function is the induction via cytokine messages of the appropriate immune reactivity, depending on the nature of the pathogen or injury. DCs recruit the appropriate T and B cells, either within secondary lymphoid tissue or at chronic inflammatory sites, based on these additional messages. The array of cytokines and chemokines that are secreted are collectively known as “dendrikines”. Many of the approximately 800 cytokines identified to date can directly affect or are made by DCs. Several novel factors including the recently identified IL-12 family members IL-23 and IL-27 have been identified in the past few years. Integrating insights concerning these cytokines into a critical interpretation of the protean biology mediated by these important cells will be necessary to fully limn the context and meaning of the messages delivered by DCs. The cytokines or dendrikines secreted by DCs during the initiation or effector phase of the immune response promote the adaptive immune response. DCs can signal T cells in the absence of exogenous antigen, perhaps using the high surface expression of major histocompatibility complex (MHC) class II molecules to promote interleukin-12 receptor expression on $CD4^+$ T cells and enhance their survival as well as by prompting antigen-independent cytokine gene expression.

Dendrikines and antigens are not unlinked. For example, the development of inducible immunoproteasomes is enhanced in DCs derived from human peripheral blood monocytes after stimulation by maturation signals. These include T-cell mimics such as CD40L (CD154), bacterial mimics such as lipopolysaccharide (LPS) or IL-18, viral mimics such as interferon-α (IFN-α), or pro-inflammatory cytokines [tumor necrosis factor α (TNFα), IL-6, and IL-1β], reflecting presumably those factors that are predominant at the site of danger, damage, or injury. DCs and dendrikines drive naive T-cell expression of the IL-2/IL-15β chain and the common γ chain ($\gamma_C$), allowing them to proliferate in response to IL-2 and enhance the response of so-called central memory T cells to IL-7 and IL-15, thus permitting subsequent response to other T-cell growth factors. The distributed network of cells constituting the immune system supports a complex set of interactions dictated in large part by DC production of, and response to, cytokines, regulating the flow of information transfer and recruitment of cells to inflammatory sites.

## III. INTERLEUKIN-12p40/p35 HETERODIMER

The IL-12 genes are located on chromosome 5 (5q31–q33) (p35) and on chromosome 3 (3p12–p13.2) (p40). Expression of each subunit is independently regulated. Most cells express p35, either constitutively or after stimulation. In contrast, the IL-12 p40

TABLE 1 IL-12 Family Members

| Subunit composition | Name(s) | Receptor interactions | Predominant activity |
|---|---|---|---|
| p35/p40 | IL-12; IL-12p70; TSF; CLMF; NKSF | IL-12 β1/β2 | Pro-inflammatory NK, CTL, $T_H1$; enhances IFN-γ production; enhances cytotoxicity; enhances hematopoiesis directly, inhibits indirectly through enhanced IFN-γ production |
| p19/p40 | IL-23 | IL-12β1; additional receptor chain | Pro-inflammatory; IL-12-like |
| p40/p40 | p40 homodimer, IL-12 receptor antagonist | IL-12β1/β2, competitive inhibitor of IL-12R | Antagonizes IL-12 receptor interactions |
| p28/EBI-3 | IL-27 | WSX-1/TCCR; additional receptor chain | Mediates $T_H2$-type inflammation (?); promotes clonal expansion of naïve $CD4^+$ T cells |

gene is expressed in a far more restricted manner, primarily by antigen-presenting cells (APCs). Biologically active IL-12 can be produced only in cells expressing both the p35 and the p40 chains. APCs, including monocytes, macrophages, and DCs, require additional stimuli to produce the p35 chain, with the presence of the p40 subunit determining its ability to produce IL-12. The amount of p35 produced, however, dictates the amount of IL-12 produced. This complex regulatory pattern is consistent with the crucial role of IL-12 in regulating APC function as well as the subsequent activation, expansion, differentiation, and effector and regulatory functions of T and B cells.

IL-12 production is induced following: (1) direct induction by pathogens. Targeting molecules include CD14, Toll-like receptor-2 (TLR-2), TLR-9, and CD11b/CD18. IL-12 is also produced following phagocytosis of bacteria. Bacterial LPS, whole bacteria (SAC, BCG), nucleic acids including CpG motifs, double-stranded RNA, or poly(IC), particle ingestion, and products from several organisms including *Staphylococcus*, *Mycobacterium tuberculosis*, *Neisseria*, *Yersinia*, *Candida*, *Toxoplasma gondii*, *Listeria monocytogenes*, or *Plasmodium chabaudi* induce IL-12 production. (2) In addition, IL-12 is induced during the interaction of APCs with $T_H$ cells. CD40–CD40L (CD154) interaction induces IL-12 following interaction of CD40L-expressing $T_H$ cells with monocytes, macrophages, or DCs. $CD40^+$ human and murine macrophages interacting with activated $CD40L^+$ T cells or clones are induced to produce and secrete IL-12. Induction of IL-12 production also requires additional surface-bound and soluble factors. TRANCE/TRANCER, RANK/RANKL interactions, and cross-linking of MHC class II by the T-cell receptor or CD4 will induce IL-12 production. IFN-γ and IL-4 do not induce IL-12 by themselves but provide powerful co-stimulating signals, allowing efficient production of this factor. IFN-γ enhances IL-12 production following CD40 engagement or interaction with bacterial products. It has been shown that the presence of IFN-γ during DC maturation results in type 1 polarized DCs with a strongly enhanced capacity to produce IL-12 following subsequent CD40L stimulation. Both IL-1 and IL-4 also enhance CD40L-induced IL-12 production. Paradoxically, however, IL-4 can suppress production of IL-12 and other cytokines following LPS stimulation (Table 2).

The effective induction of high levels of bioactive IL-12 by DCs requires two signals: (1) CD40 ligation and a co-stimulatory cytokine, (2) a bacterial product and IFN-γ, or (3) CD40 ligation and a bacterial product. Such regulation of IL-12 production limits the adventitial induction of autoimmunity. Inhibitors of IL-12 production include cyclic AMP-elevating agents, glucocorticoids, histamine, IFN-α, IL-10, prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β), and several neuropeptides. These peptides all directly suppress IL-12 production in a stable fashion and include calcitonin gene-related peptide, vasoactive intestinal peptide, and pituitary adenylate cyclase-activating polypeptide. IL-12 is enhanced in IL-10-deficient animals and diminished in IFN-γ knockouts.

**TABLE 2 Production of IL-12 Family Members in Different Cell Types**

| Cell type | Production of IL-12 family members |
|---|---|
| Blood monocytes | Inducible production of small amounts of p35 and p40; secretion of small amounts of p70; inducible expression of EBI-3 and p28 |
| Blood-isolated DCs | Expression of p40 and p35; inducible secretion of p70 enhanced following culture with IFN-γ |
| Macrophages | Inducible expression of p19, p28, p35, p40, and p70 secretion |
| Langerhans cells | Secretion of p40 only |
| T cells | Both p19 and p35; no p40 or p70 |
| Splenic DCs | p40, p35, and p70; low in freshly isolated DCs; high in cultured DCs |
| Monocyte-derived DCs and bone marrow derived DCs | Inducible p19, p35, p40, p70, EBI-3 and p28; human and mouse secrete IL-23 (p19/p40) or IL-12 (p34/p40) after activation with TNFα, LPS, and anti-CD40 antibody |
| Neutrophils | Mostly IL-12p40, small amounts of p70 |
| Mesoglia | Low-level production of p40, p35, and p70 |
| Keratinocytes | p40 and p35 expression, p40 secretion, lack of p70 expression |
| Endothelial cells | Inducible p40, p35, and p70 |
| Trophoblasts | EBI-3 and p28/IL-27 (spontaneous) |

## A. IL-12 Promotes $T_H1$ Immunity

IL-12 promotes and sustains $T_H1$ cell development in murine models where it can be more readily tested. IL-12 deficiency limits the development of protective $T_H1$ responses to antigens including several pathogens (*Leishmania major*, *Listeria*, and *Mycobacterium*), resulting in progressive uncontrolled infection. In the absence of signal transducer and activator of transcription 4 (STAT4), the distal signaling molecule that is important in IL-12 biology, similar susceptibility to these organisms is noted. Humans lacking the functional IL-12 receptor (IL-12R) demonstrate a similar deficit in $T_H1$ responses to intracellular pathogens. IL-12 is important for eradicating virus in several murine models, and several viruses including measles virus have developed ways to limit IL-12 production. Animals deficient in p40 or the IL-12Rβ2 demonstrate very limited $T_H1$ responses. Some elements of antiviral immunity are still intact in this setting, suggesting a number of compensatory mechanisms. Repeated administration of IL-12R attenuates the IFN-γ response, enhances IL-10 production, and reduces the acute toxicity of this agent both in mice and in cancer patients receiving experimental therapy with IL-12.

## B. IL-12 Promotes Generation of Cytolytic Activity

IL-12 enhances IL-2-driven lymphokine-activated killer (LAK) activity from human peripheral blood mononuclear cells (PBMCs) and is inhibited by antibodies to TNFα and IFN-γ. IL-12 together with CD80 and IL-6 co-stimulation induces antigen-specific murine cytolytic T cells and, like IL-2, promotes enhanced LAK activity in the PBMCs of human immunodeficiency virus (HIV)-infected individuals. IL-12 is more effective as an enhancer of antigen-specific cytolytic activity and survival (in part through enhanced bcl-2 expression) of cytolytic effectors than it is as a T-cell growth factor. IL-12 up-regulates the expression of perforin, serine proteases, and cytolytic granules.

## C. IL-12 Promotes B-Cell and DC Activation and Survival

DCs promote the differentiation of naive (but not memory) B cells in part through IL-12 production. IL-12 also enhances B-cell production of IL-10. IL-12 also enhances the production of IgE. It promotes the nuclear localization of nuclear factor κB (NF-κB), priming murine DCs to produce additional IL-12 and enhance survival. With Flt-3 ligand, it enhances the maturation of Langerhans cells and promotes macrophage recruitment as a chemoattractant.

## D. IL-12 Enhances Hematopoiesis and Inhibits Angiogenesis

IL-12 acts directly on early hematopoietic progenitor cells, promoting hematopoiesis. It indirectly suppresses hematopoiesis with associated anemia and neutropenia by inducing IFN-γ and TNFα production *in vivo*, inducing an extramedullary hematopoietic response. Other hematopoietic growth factors [stem cell factor, Flt-3 ligand, IL-3, IL-4, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor, and erythropoietin] synergize with IL-12 to promote the proliferation and differentiation of bone marrow-derived cells in both mouse and human. It has been shown that IL-12 promotes dendropoiesis within lymphoid and nonlymphoid organs. IL-12 also inhibits angiogenesis in mice, mediated by IFN-γ induction of MIG and IP-10. IL-18 synergizes with IL-12 to limit angiogenesis.

## E. Potent Anti-tumor Activity of IL-12

IL-12 is an important mediator of effective anti-tumor responses in virtually every murine model in which it has been tested. Systemic administration prolongs survival and inhibits tumor growth and metastasis formation even if treatment is delayed for up to 3 weeks after tumor injection. Surprisingly, early administration of IL-12 is usually less effective or ineffective. This suggests that T cells need to be selected before IL-12 can mediate its biologic effects. The anti-tumor effect mediated by IL-12 administration depends on local production of IFN-γ and TNFα, and requires both $CD4^+$ and $CD8^+$ T cells. Targeted delivery of IL-12 to the tumor microenvironment by gene therapy reduces the tumor and synergizes with the systemic delivery of IL-2. Using a polycistronic retroviral vector encoding the IL-12 p35 and IL-12 p40 subunits, as well as the neomycin phosphotransferase gene ($neo^r$), Hideaki Tahara engineered tumor cells to stably secrete nanogram quantities of IL-12; the tumor cells failed to grow when injected into mice. It has also been shown that IL-12 synergized with B7.1 gene delivery.

## F. IL-12 in Therapy of Infectious Diseases

IL-12 is effective in a variety of murine infectious diseases involving bacteria, protozoans, mycobacteria, and fungi. IL-12 also mediates resistance to viral infections including lymphocytic choriomeningitis, HIV, cytomegalovirus, vesicular stomatitis virus, encephalomyocarditis virus, and influenza. IL-12 promotes a protective $T_H1$ response as well as antibody-mediated protection of *Le. major* in mice, reducing the parasite burden and IL-4 production in the regional lymph nodes. IL-12 treatment of susceptible A/J mice infected with blood-stage *Plasmodium chabaudi* AS decreases parasitemia and enhances survival. IL-12 treatment enhances the resistance of mice to *M. tuberculosis* infection and promotes protective $CD4^+$ T-cell responses.

### G. IL-12 in Transplantation

In general, IL-12 treatment of wild-type murine cardiac allograft recipients fails to accelerate and, in some instances, significantly delays graft rejection. Blocking IL-12 promotes murine cardiac allograft rejection but promotes liver allograft acceptance and that of allogeneic myoblasts. IL-12 p40 mRNA expression is increased in heart allografts of tolerant rats conditioned by donor-specific blood transfusion. Tacrolimus and intraportal donor-specific bone marrow infusion down-regulate the expression of IL-12 within transplanted organs. APCs isolated from cervical lymph nodes of mice that accepted corneal allografts produce less IL-12. Acute graft-versus-host disease (GVHD) is a major complication of bone marrow transplantation (BMT) and is characterized by hematopoietic dysfunction, immunosuppression, and histopathological changes in the skin, intestinal mucosa, and liver. IL-12 delivery on the day of BMT inhibits GVHD while preserving graft-versus-leukemia effects and is dependent on expression of Fas by donor T cells. Macrophage IL-12 production after LPS stimulation increased in patients who develop acute GVHD. IL-12 p40 mRNA is expressed only in human kidney allograft biopsies showing acute cellular rejection and in all biopsies with evidence of focal interstitial necrosis. IL-12 levels are elevated in human ischemic lung and IL-12 is not found in stable human lung transplant recipients.

### H. Therapeutic Efficacy of Human IL-12 in Clinical Trials

The therapeutic efficacy of IL-12 has been evaluated in multiple clinical trials in patients with cancer (melanoma, renal cell carcinoma, cutaneous T-cell lymphoma, Kaposi's sarcoma) as well as in patients with acquired immune deficiency syndrome, viral hepatitis, and asthma. Its evaluation in patients by the two primary companies developing it, Wyeth and Hoffman LaRoche, has been discontinued because of toxicity and limited effectiveness observed using the dose, route, and schedule that are necessary when it is administered as an outpatient regimen. Objective responses in patients with renal cell cancer, melanoma, and cutaneous T-cell lymphoma were observed. Treatment in an early study, using IL-12 administered at 500 ng/kg daily for 5 consecutive days, resulted in severe neurotoxicity with somnolence and confusion and death in two patients. IL-12 tachyphylaxis is noted as late as 2 weeks after IL-12 administration to patients; similar experiments in mice have revealed apparent protection from acute *rm*IL-12 toxicity by pretreatment with *rm*IL-12. Vaccination of metastatic melanoma patients using *rh*IL-12 and peptide induces only modest enhancement of immunologic responses. Peritumoral injection of IL-12-producing autologous cells was tested with 13/29 patients experiencing a clinical response and with 2/29 having an objective clinical response in noninjected lesions. At doses ≥7000 ng/day, a Schwartzman reaction was noted in several patients associated with severe local pain, swelling, and tumor necrosis. This was defined as dose-limiting toxicity. In patients with chronic hepatitis C, antiviral activity of IL-12 comparable to that of other current treatments is noted. Low-dose treatment in HIV patients was not effective and administration of daily doses of 1000 ng/kg *rh*IL-12 resulted in severe adverse events. Trials in asthmatic patients demonstrated a reduction of blood and sputum eosinophil counts with no significant effects on airway hyperresponsiveness or the intensity of late asthmatic reactions.

## IV. INTERLEUKIN-12 RECEPTOR

The IL-12 receptor is a dimeric complex with an approximate $M_r$ of 135–210 kDa. Expression cloning studies reveal a β1 chain and a β2 chain, each conferring low/intermediate binding affinity of the complex for IL-12; their combination promotes high-affinity binding. The IL-12Rβ2 chain but not the IL-12Rβ1 chain contains tyrosine residues in its cytoplasmic domain and is required for IL-12 signaling. Co-transfection of the IL-12Rβ1 and IL-12Rβ2 chains into cells yields high-affinity IL-12 receptor sites, allowing IL-12 to promote cell proliferation with an $ED_{50} = 1$ p*M* IL-12 p70. PHA-stimulated T-cell blasts express 1000–10,000 IL-12-binding sites per cell with three different affinities consistent with low-, intermediate-, and high-affinity IL-12 receptors displaying $K_d$s in the range of 2–6 n$M$, 50–200 p$M$, and 5–20 p$M$, respectively. In mice, the IL-12Rβ1 chain is responsible for binding IL-12. In human cells, both chains participate in IL-12 binding. The β1 chain interacts with the IL-12 p40 chain and the IL-12Rβ2 chain associates with the IL-12 p35 chain. Antibodies to the receptor inhibit IL-12-induced IFN-γ production and activated T-cell proliferation. IL-23 binds IL-12R, but also acts via additional receptors. IL-27 (EBI-3-p28) binds the WSX-1/TCCR receptor and likely one other, as yet unidentified chain.

### A. Cell Types and Tissues Expressing IL-12 Receptor

High-affinity IL-12R is present on activated T and NK cells, on DCs, on human B-cell lines, and on activated human PBMCs or tonsillar B lymphoblasts. Both chains are induced on naive ($CD45RA^+$) and memory $CD4^+$ T-cell clones within 48 h of activation with antigen or anti-CD3 treatment. IL-7 treatment augments expression of the IL-12Rβ1 chain, whereas $T_H2$ cells express only low levels of the IL-12Rβ2 chain. Expression of the high-affinity IL-12R by activated T cells is reciprocally regulated by IL-4 and IFN-γ. IL-4 inhibits IL-12Rβ2 expression and signaling. IFN-γ promotes expression of IL-12Rβ2 on developing $T_H2$ cells, but does not elicit IFN-γ production following IL-12 stimulation. Individuals with a mutant IL-12Rβ1 gene display diminished IFN-γ production, and recurrent mycobacterial and *Salmonella* infections. Similarly, IL-12R-deficient mice show a pronounced deficit in the ability to control intracellular infections.

### B. Regulation of IL-12 Receptor Expression

IL-4, TGF-β, and IL-10 down-regulate IL-12Rβ2 chain expression, signaling, and biologic effects. PGE2 and dexamethasone inhibit IL-12Rβ1 and IL-12Rβ2 expression. Cholera toxin inhibits the expression of both chains. IFN-γ maintains the ability of cells to signal through the IL-12R *in vitro* and *in vivo*. Differential regulation of IL-12 receptor expression by these factors is involved in the development of $T_H1$ and $T_H2$ cells and their stability. The cytokines IL-2, IL-7, and IL-15 induce IL-12Rβ1 expression, whereas the natural killer T-cell ligand α-galactosylceramide induces mRNA for both IL-12Rβ1 and IL-12Rβ2.

### C. Signaling Pathways

Distal molecules in IL-12 signaling include Janus kinase 2 (JAK2) and tyrosine kinase 2 (Tyk2), STAT1, STAT3, STAT4, NF-κB, and c-Jun. Cross-linking of the IL-12 receptor results in the activation of the receptor-associated tyrosine JAK2 and Tyk2 kinases, mitogen-activated protein kinase, and the src family lck tyrosine kinase. Tyk2 interacts with the β1 chain of the IL-12 receptor and JAK2 interacts with the β2 subunit. STAT1 can dimerize with either STAT3 or STAT4, and STAT4 can dimerize with STAT3. Only STAT4, but neither STAT1 nor STAT3, is activated directly through the IL-12 receptor. Both IL-12 and IFN-α induce tyrosine phosphorylation and DNA binding of STAT4. IL-12R activates STAT4 in a direct fashion, whereas it binds indirectly via STAT2. STAT4 knockout mice are defective in IL-12 responses with profound defects in $T_H1$ immunity. $T_H2$ immune responses predominate. Although $T_H1$-associated immune responses do occur, they are weaker than those observed in control animals in response to IL-12 or to pathogenic organisms including *Li. monocytogenes*. IL-12 activation of NK cells does not occur in STAT4 knockout mice. IL-12-induced IFN-γ production is diminished and long-term antigen-specific immunity is impaired.

## V. INTERLEUKIN-12 RECEPTOR ANTAGONIST—p40/p40 HOMODIMER

The IL-12 p40 chain is expressed by APCs at 10- to 100-fold greater levels than the IL-12 p35 chain, being induced by stimuli similar to those inducing IL-12 p70 expression. Secreted p40/p40 homodimers (IL-12RA) bind to the IL-12R but do not elicit a signal, serving as functional antagonists of the IL-12 receptor. IL-12RA inhibits IL-12-induced murine concanavalin A-blast proliferation, splenocyte secretion of IFN-γ, and NK activation in the mouse *in vitro* and following *in vivo* administration, promoting a $T_H2$-type response and correlating with IL-10 and PGE2 production. In the mouse, IL-12RA inhibits with an $IC_{50}$ of 1–10 ng/ml. In humans, at least 10-fold higher concentrations are required. IL-12RA is less stable, dissociating in aqueous environments to inactive p40 monomers. Most IL-12-inducing stimuli also induce IL-12RA, sometimes by itself. Production of IL-12RA is noted in several murine tumors, at chronic inflammatory sites, and following ultraviolet irradiation. High levels of IL-12RA in peritoneal fluid from patients with endometriosis may play an IL-12-antagonistic role, locally inhibiting NK function. Bronchial epithelia, in the setting of asthma, produce IL-12RA, and serum levels increase with age. Production of IL-12RA is induced by PGE2, thereby enhancing the $T_H2$ response.

## VI. INTERLEUKIN-23—p19/p40 HETERODIMER AND ITS RECEPTOR, IL-12Rβ1/IL-23R

The existence of an alternative p40-binding partner interacting with the IL-12 receptor was suggested by the greater defect of p40 null mice than of p35 null mice. The gene encoding this novel partner, p19, is located on human chromosome 12q13 and encodes an 18.7 kDa, 189-amino-acid, four-helical cytokine with five cysteine residues and no N-glycosylation sites. Substantial (70%) homology of the mouse 197-amino-acid p19 protein with a MW of 19.8 kDa was subsequently determined. p19 is most closely related to other members of the extended IL-6 family including IL-6 itself and IL-12 p35 and G-CSF. Like IL-12 p35, p19 must be co-expressed with IL-12 p40; its message is expressed by polarized $T_H1$ cells and activated macrophages. Both human and mouse monocyte-derived DCs generated in the presence of granulocyte/macrophage colony-stimulating factor and IL-4 secrete IL-23 (p40/p19 complex) after activation (TNFα, LPS, and CD40 ligation).

IL-23 promotes IFN-γ production and strongly enhances the proliferation of activated mouse memory T cells ($CD4^+CD45RB^{low}$) but not naive T cells ($CD4^+CD45RB^{high}$). In humans, IL-23 similarly has a greater effect on activated CD45RO memory than on activated CD45RA naive T cells. Ubiquitous expression of the IL-23 (p19) transgene results in widespread inflammation in multiple organs, runting, infertility, and premature death at 3 months. Lymphocytes, macrophages, and neutrophils are found at inflammatory sites and in the peripheral blood. IL-23 transgenic animals had elevated serum cytokine levels (IL-1, TNFα, and IFN-γ) and increased acute-phase proteins. Limited hepatic expression of IL-23 had no apparent phenotype, whereas transfer of IL-23 transgenic bone marrow into lethally irradiated animals induced the same widespread inflammation.

The p19/p40 heterodimer IL-23 binds to IL-12Rβ1 along with another IL2Rβ homologue, the IL-23R, recently identified by Robert Kastelein and colleagues. IL-23 induces STAT4 signaling in phytohemagglutinin (PHA)-activated T cells.

## VII. INTERLEUKIN-27—p28/EBI-3 HETERODIMER AND ITS RECEPTOR, WSX-1/TCCR

The Epstein-Barr virus-induced gene 3 (EBI-3), identified by Elliott Kieff, Mark Birkenbach, and colleagues, is a hematopoietin receptor family member with homology to the p40 subunit of interleukin-12 and to the ciliary neurotropic factor. EBI-3 maps to chromosome 19 (19p13.2–p13.3), near genes encoding the erythropoietin receptor and Tyk2. This gene encodes a 34 kDa glycoprotein lacking a membrane-anchoring motif and is not secreted as a monomer but can be secreted to a very limited extent as a p35-linked heterodimer. The authentic binding partner is p28, a member of the long-chain four-helix cytokines, homologous to p38. The gene encoding human p28 is located on chromosome 16p11 and encodes a 243- or 234-amino-acid cytokine (human and mouse, respectively) with 13 negatively charged glutamic acid residues and no N-glycosylation sites. Several O-glycosylation sites are predicted. p28 is most closely related to IL-11 and CNTF, mimicking NNT-1/CLC binding to the CNTF receptor.

EBI-3 is detected in the spleen and tonsils and at high levels within syncitiotrophoblasts in the human placenta. It is found within the interfollicular zones of tonsil tissue and sinusoidal cells in the perifollicular areas of the spleen. PBMCs express EBI-3 following pokeweed mitogen stimulation. EBI-3 is one of the most prominent genes expressed in activated DCs. Interestingly, expression of the p28 chain peaks at 3–6 h following PBMC activation and then rapidly diminishes, whereas EBI-3 mRNA levels can be identified for up to 72 h.

High levels of expression of EBI-3 are observed in virtually all patients with active ulcerative colitis but only rarely in patients with Crohn's disease. An oxazolone-induced $T_H2$-mediated inflammatory bowel disease model in the mouse has been noted by Richard Blumberg and Mark Birkenbach to be associated with high levels of EBI-3. EBI-3 knockout mice are protected from $T_H2$-associated colitis but not $T_H1$ disease. This suggests that p27 has an active role in sustaining or inducing $T_H2$-type autoimmunity. Robert Kastelein suggests that IL-27 plays a major role inducing $T_H1$ immunity by enhancing the proliferation and survival of activated naive $CD4^+$ T cells. He believes its most critical role may be early in T-cell biology during the afferent arm of the immune response and proximal to that of IL-12 and IL-23, which may be sequentially important in $T_H1$ immunity.

WSX-1/TCCR, expressed on the same chromosome as the IL12R$\beta$1 chain (chromosome 19), is one of the chains of the functional IL-27 receptor. Initiation of $T_H1$ responses but not their continuation requires expression of this receptor. Another chain is likely required for signaling but has not been identified.

## Glossary

**cytotoxic lymphocyte maturation factor** This term was initially used to define the ability of interleukin-12 to synergize with interleukin-2, particularly in the presence of corticosteroids, to stimulate cytolytic activity from natural killer (lymphokine-activated killer activity) and T cells.

**dendrikines** Factors made by dendritic cells and acting on themselves (autocrine activity), on other cells locally in the lymph node or tissues where they reside (paracrine activity), or on immune cells at other distant sites or tissues (endocrine activity). Dendrikines are like lymphokines, soluble factors elaborated by lymphocytes, and like monokines, soluble factors elaborated by monocytes; all of these would be considered cytokines, soluble factors made by one cell and acting on other cells and occasionally acting on themselves.

**directed exocytosis** The mechanism by which cells can communicate via soluble mediators across an immunologic synapse when they are in close proximity to other cells; cytokines are delivered from a region of one cell adjacent to another, usually after a series of triggering events between the two cells.

**Epstein–Barr virus (EBV)-induced gene 3** Dendrikine whose gene product has homology to the p40 chain of interleukin-12, pairing with p19 to create interleukin-27. Induced in B cells following EBV infection and expressed in macrophages and dendritic cells; appears to be important in some chronic inflammatory bowel diseases including Crohn's colitis and possibly associated with pregnancy since it is found in large amounts in human placenta.

**interleukin-12** Heterodimeric cytokine of the extended interleukin-6 (IL-6) family of hematopoietins; consists of p35 and p40 noncovalently linked chains with homology to the IL-6 molecule interacting with an extracellular domain of the IL-6 receptor; initially identified in Epstein–Barr virus-transformed B cells and subsequently in macrophages, dendritic cells, and keratinocytes.

**interleukin-12 receptor antagonist** Homodimeric p40 molecule serving to bind to the interleukin-12 receptor but without agonist activity and blocking the active heterodimer.

**interleukin-23** Novel interleukin-12 family member consisting of a common p40 chain as well as a novel p35 homologue, p19; appears to be important in the late stages of inflammation and autoimmunity as a monokine/dendrikine promoting chronic inflammation.

**interleukin-27** Novel interleukin-12 family member consisting of p28 and EBI-3 as a heterodimer; appears to be important in the earliest stages of inflammation, particularly in ulcerative colitis, as a monokine/dendrikine promoting activation of naive CD4$^+$ T cells and promoting a $T_H1$ response.

**natural killer stimulatory factor** Original functional definition of the biologic activity identified in the laboratory of Dr. Giorgio Trinchieri at the Wistar Institute, capable of enhancing natural killer (NK) cytolytic activity and interferon-$\gamma$ production from NK cells; subsequently termed interleukin-12.

**T-cell-stimulating factor** Originally identified by Hermann and Rude as a factor promoting T-cell function, survival, and cluster formation; subsequently identified as being identical to interleukin-12.

## See Also the Following Articles

**Interleukin-1, Interleukin-2, etc. (multiple Interleukin entries)**

## Further Reading

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# Interleukin-13

Jan E. de Vries and José M. Carballido
*Novartis Research Institute, Vienna, Austria*

I. INTRODUCTION
II. IL-13 PROTEIN AND IL-13 GENE
III. IL-13 RECEPTOR COMPLEXES
IV. IL-13 SIGNAL TRANSDUCTION
V. IL-13 PRODUCTION
VI. IMMUNOSTIMULATORY AND PRO-INFLAMMATORY EFFECTS OF IL-13 *IN VITRO*
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IX. EFFECTS OF IL-13 ON TUMOR CELLS
X. SUMMARY

**Interleukin-13 (IL-13) was first described in 1989 as P600, an mRNA selectively expressed by mouse T helper 2 (T$_H$2) cells. Its human homologue was cloned 3 years later. Expression of the human cDNA led to the discovery that P600 encoded a cytokine that was designated interleukin-13. IL-13 is produced by various cells of the immune system following activation but is generally considered a T$_H$2 cytokine. In addition, basophils and mast cells are major producers of IL-13.**

## I. INTRODUCTION

Interleukin-13 (IL-13) shares many functional characteristics with IL-4, which is related to the fact that the IL-13 receptor (IL-13R) is a heterodimeric receptor consisting of an IL-13-binding chain (IL-13R$\alpha$1) and the IL-4R$\alpha$ chain that binds IL-4. IL-4 utilizes this IL-4R$\alpha$/IL-13R$\alpha$1 complex (called type II IL-4R) as an alternative IL-4R (Fig. 1). These shared receptor components account for the many overlapping activities of IL-13 and IL-4. However, IL-13 and IL-4 also have clearly distinct activities that are determined by the differential distribution of IL-13R and IL-4R on various cell types. IL-13 plays a critical role in the pathogenesis of asthma, particularly in the induction of airway hyperresponsiveness and overproduction of mucus. IL-13 is also the dominant cytokine in the induction of granuloma formation and tissue fibrosis associated with chronic inflammation. On the other hand, IL-13 has protective effects against gastrointestinal nematode infections

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**compromise thyroid hormone production, resulting in pathological states of hypothyroidism such as cretinism and goiter.**

## I. INTRODUCTION

Thyroid hormones, triiodothyronine (T3) and tetraiodothyronine or thyroxine (T4), are essential for normal growth, development, and metabolism, and the effects of deficient or excessive thyroid hormone concentrations are manifested throughout the body. The fundamental components of T3 and T4 are iodine and specific tyrosine residues within thyroglobulin (TG) that provide substrates for iodine attachment. To synthesize thyroid hormones, the thyroid concentrates iodide ($I^-$) from the circulation and organifies it at the apical membrane to generate iodotyrosines, which are then coupled to form iodothyronines (T3 and T4). The iodinated TG subsequently undergoes endocytosis and proteolysis for release of T3 and T4 into the bloodstream.

The structural organization of the thyroid gland and the distribution of key transporter proteins and enzymes within it ensure that iodine and TG will yield T3 and T4 upon the execution of numerous steps (Fig. 1). The thyroid gland is organized into follicles composed of polarized thyrocytes lining a lumen filled with colloid. The basal aspect of the thyrocyte is in contact with intervening supporting tissue that is rich in blood and lymphatic capillaries, facilitating the uptake of iodide from the blood by the sodium/iodide symporter (NIS) and the release of T3 and T4 into the circulatory system. Pendrin and thyroid peroxidase (TPO) are strategically located within the apical membrane of the thyrocyte, which borders the colloid-filled lumen. Pendrin transports iodide into the follicular lumen positioning it near TG and TPO. In conjunction with hydrogen peroxide, TPO catalyzes the iodination of tyrosine residues on TG to make 3-monoiodotyrosine (MIT) and 3,5-diiodotyrosine (DIT) and then couples the iodotyrosines to form T3 and T4. Hormone-containing TG in the follicular lumen becomes the macromolecular storage site for iodine as well as for thyroid hormone. The thyroid gland is unique among endocrine organs in that the final assembly and storage of hormone occur extracellularly within the colloid of the follicular lumen.

The biosynthesis and secretion of T3 and T4 are stimulated mainly by thyroid-stimulating hormone (TSH) through cyclic adenosine monophosphate (cAMP), in conjunction with insulin and/or insulin-like growth factor 1 (IGF-1). TSH increases the expression of NIS, TG, and TPO and, therefore, T3 and T4 production. Recent studies demonstrate that TG inhibits NIS, TG, and TPO expression, which consequently counteracts the stimulatory effects of TSH to induce T3 and T4 formation.

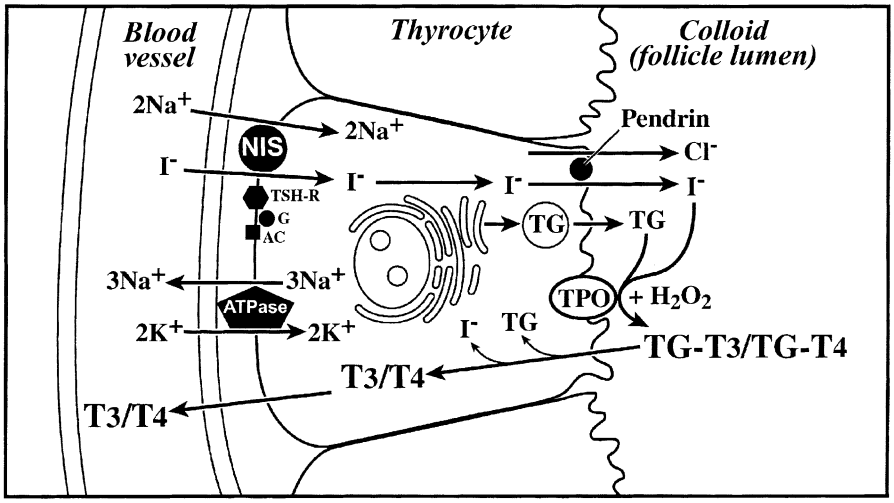

FIGURE 1 Key steps in thyroid hormone biosynthesis including basolateral iodide uptake (NIS, sodium/iodide symporter), apical iodide transport (pendrin), iodination and coupling (TPO, thyroid peroxidase) within thyroglobulin (TG), endocytosis and proteolysis, and hormone release. AC, adenylate cyclase; ATPase, sodium/potassium ATPase; G, G-protein; TSH-R, thyroid-stimulating hormone receptor.

## II. SODIUM/IODIDE SYMPORTER: BASOLATERAL IODIDE UPTAKE

The sodium/iodide symporter is a membrane glycoprotein that mediates the uptake of $I^-$ along the basolateral membrane of thyrocytes. NIS couples the inward translocation of two sodium ions down their electrochemical gradient with the inward translocation of one iodide ion against its electrochemical gradient. The energy required to drive this process is provided by the sodium gradient generated by sodium ($Na^+$)/potassium ($K^+$) ATPase. NIS is believed to span the plasma membrane 13 times, exhibiting an extracellular amino-terminus and a cytoplasmic carboxy-terminus. The protein exists primarily as a mature ~87 kDa glycosylated peptide. However, the function, membrane targeting, and stability of NIS do not appear to be affected in the absence of glycosylation. Although the exact mechanisms remain to be elucidated, several hydroxyl-containing amino acid residues located in transmembrane segment IX (Ser-353, Thr-354, Ser-356, and Thr-357) appear to play an important role in NIS activity.

In addition to its physiological importance, the ability of NIS to concentrate iodide holds clinical relevance by providing the basis for using radioactive iodine in the treatment and management of various thyroid diseases. For example, radioactive iodine is commonly used to destroy overactive thyrocytes in patients with thyrotoxicosis, to ablate normal and malignant thyroid tissues in patients who have undergone total thyroidectomy for thyroid carcinoma, and to perform whole-body scans for the detection of recurrent and metastatic thyroid cancer. In addition to the thyroid gland, there are several extrathyroidal tissues, including lacrimal and salivary glands, gastric mucosa, and lactating mammary glands, with functional NIS expression. Although the exact role that iodide plays and the factors that modulate NIS expression in these tissues have not been established, the application of radioactive iodine for the treatment of cancers occurring in these extrathyroidal sites is promising.

## III. PENDRIN: APICAL IODIDE TRANSPORT

Once within the thyrocyte, $I^-$ is passively transported into the follicular lumen via pendrin. Pendrin is an ~86 kDa chloride/iodide transport protein and is located exclusively at the apical membrane of thyrocytes. Pendrin is the product of the gene responsible for Pendred syndrome, a genetic disorder characterized by sensorineural hearing loss and goiter. Pendrin is characterized by 11 or 12 putative transmembrane domains and is closely related to a family of sulfate transport proteins. However, pendrin does not transport sulfate and, unlike NIS, does not require sodium for its transport of iodide.

## IV. THYROGLOBULIN: PROHORMONE

Mature thyroglobulin existing as a dimer is a 660 kDa glycoprotein containing 132 tyrosine residues, some of which are the substrates for iodine attachment to generate T3 and T4. Carbohydrate chain synthesis is initiated once the polypeptide chain of thyroglobulin is produced on polyribosomes of rough endoplasmic reticulum (RER) within the thyrocyte. Enzymes such as protein disulfide isomerase and peptydylprolyl isomerase, as well as several molecular chaperones, including calnexin, BiP, GRP94, and Erp72, ensure that newly synthesized TG leaves the ER properly folded and migrates to the Golgi complex where it undergoes glycosylation and sulfation. Only properly folded and glycosylated thyroglobulin will be allowed to reach the apical membrane of the thyrocyte.

There are various forms of thyroglobulin found within the thyroid gland, including 12S monomers, 17S dimers, and mature iodinated 19S dimers and 27S tetramers. The two monomeric polypeptide chains comprising a 17S dimer are believed to be identical but become heterogeneous following glycosylation and iodination as a 19S dimer. The tetramer is similar in amino acid content to the 19S dimer but contains more iodine and accounts for only 10% of all mature TG. Within the colloid, TG primarily exists as soluble 19S dimers or 27S tetramers. However, 30% of the thyroglobulin contains 40% more iodine and is compacted for storage, rendering it insoluble. This demonstrates that colloid is the extracellular storage site for iodine as well as thyroid hormone.

Tyrosine residues within three consensus sequences (Asp/Glu-Tyr, Ser/Thr-Tyr-Ser, and Glu-X-Tyr) are favored for iodination. There are four major (A through D) and three minor (G, N, and R) hormonogenic sites within TG. Utilization of the different tyrosine residues within the known hormonogenic sites varies with iodine availability, TSH stimulation, and species. Subsequent iodination by thyroid peroxidase at these favored tyrosine residues stabilizes thyroglobulin by oxidation of free sulfhydryl groups and formation of interchain disulfide bonds. This is evidenced by the fact that iodine-poor thyroglobulin dimers readily dissociate.

## V. THYROID PEROXIDASE: IODINATION AND COUPLING

Thyroid peroxidase is an ~105 kDa heme-containing glycoprotein that belongs to a family of mammalian peroxidases that includes myeloperoxidase, lactoperoxidase, eosinophil peroxidase, and salivary peroxidase. TPO is located on the apical membrane of thyrocytes where it catalyzes iodination of TG and coupling of iodotyrosines to form iodothyronines attached to TG. However, *in vitro* studies have shown that such functions are not unique to TPO in that lactoperoxidase and myeloperoxidase can also serve as catalysts for both iodination and coupling.

There are currently three forms of TPO that are generated through alternative splicing of the same gene. TPO-1 is the full-length enzymatically active glycoprotein characterized by a catalytic arginine, proximal and distal histidines, and asparagine-linked glycosylation sites. TPO-2, which lacks 57 amino acids from the middle of the sequence, is enzymatically inactive due to the loss of essential histidine and asparagine residues. The third alternatively spliced form is TPOzanelli, which, like TPO-1, is enzymatically active. However, TPOzanelli has a shorter half-life and is less stable than TPO-1 due to a modified carboxy-terminus.

Iodination of tyrosine residues and coupling of iodotyrosines on TG by TPO occur near the apical surface of the thyrocyte at the cell–colloid interface within the follicular lumen and require close proximity of TPO, hydrogen peroxide, TG, and $I^-$. The oxidation of iodide is coupled to the reduction of hydrogen peroxide because TPO is catalytically inactive in the absence of hydrogen peroxide. Three primary mechanisms of TPO-catalyzed iodide oxidation have been proposed but none has been established. The first mechanism suggests that TPO-bound free radicals of iodide and tyrosine are produced by oxidation to form iodotyrosines. Alternatively, the initial oxidation of $I^-$ to $I^+$ is followed by iodination of tyrosine. In the third proposal, tyrosine iodination follows oxidation of iodide to hypoiodite. Regardless of the mechanism employed, TPO attaches oxidized iodide to tyrosine residues within thyroglobulin producing MIT and DIT (Fig. 2). Subsequently, TPO couples one MIT residue and one DIT residue to form T3 or two DIT residues to form T4 (Fig. 2). Under conditions of normal thyroid activity and normal iodine supply, a typical molecule of mature 19S TG contains 2.5 T4 residues, 0.7 T3 residues, 4.5 DIT residues, and 5.0 MIT residues. However, the ratio of MIT to DIT is greatly increased during iodine deficiency, favoring T3 formation.

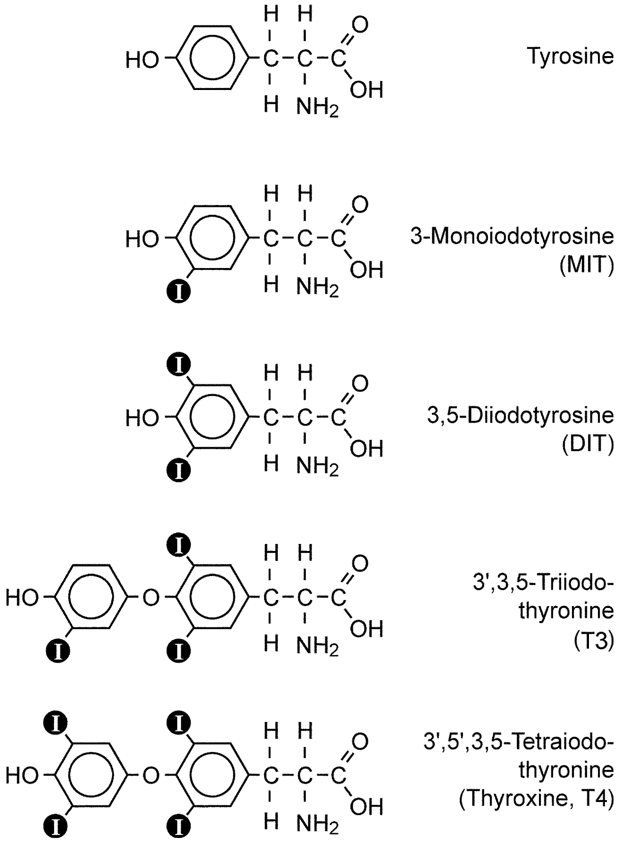
Tyrosine
3-Monoiodotyrosine (MIT)
3,5-Diiodotyrosine (DIT)
3',3,5-Triiodo-thyronine (T3)
3',5',3,5-Tetraiodo-thyronine (Thyroxine, T4)

**FIGURE 2** Chemical structures of the iodinated tyrosine residues, MIT and DIT, within thyroglobulin, which are then coupled to form T3 and T4.

## VI. ENDOCYTOSIS, PROTEOLYSIS, AND HORMONE RELEASE

To facilitate the secretion of T3 and T4 into the bloodstream, thyroglobulin must be processed further within the thyrocyte. Thyroglobulin gains reentry into the thyrocyte primarily by receptor-mediated or nonselective pinocytosis. Coated pits containing thyroglobulin are invaginated to form internalized, coated vesicles. Some species, particularly rats, develop elongated microvilli and pseudopodia, which extend into the follicular lumen to indiscriminately phagocytize and internalize a portion of the adjacent colloid, resulting in the formation of colloid droplets. Once inside the thyrocyte, thyroglobulin takes one of three pathways: proteolysis to release T3/T4, recycling back to the follicular lumen, or transcytosis to the bloodstream. To break down TG and release T3/T4, TG migrates within the cell to fuse with lysosomes. Lysosomal enzymes such as cathepsin D (aspartic endopeptidase), cathepsins B, H, L, and S (cysteine endopeptidases), and lysosomal dipeptidase I and dipeptidyl peptidase II (exopeptidases) proteolytically cleave iodoamino acids from thyroglobulin. Iodine is then removed from MIT and DIT by an iodotyrosine-specific deiodinase in the thyroid, and the iodide and tyrosine are recycled. A small proportion of T4 is also converted to T3 within the thyrocyte by a thyroidal 5′-deiodinase similar to that found in peripheral tissues. T3 and T4 are then released into the bloodstream where they are transported in association with specific plasma-binding proteins. During pinocytosis, immature thyroglobulin is also internalized. This immature thyroglobulin is recognized by *N*-acetylglucosamine and/or asialoprotein receptors and is targeted for recycling. Finally, a small percentage of thyroglobulin is diverted directly to the bloodstream by a process known as transcytosis.

## VII. THYROID-STIMULATING HORMONE AND OTHER REGULATORY FACTORS

Virtually all aspects of thyroid hormone biosynthesis are directly regulated by thyroid-stimulating hormone. TSH is produced by thyrotrophs within the pars distalis of the pituitary gland in response to thyrotropin-releasing hormone (TRH) produced in the hypothalamus. Glucocorticoids, somatostatin, and dopamine inhibit TSH whereas α-adrenergic agonists increase TSH; however, these effects are of importance primarily during pathologic states. TSH increases the synthesis of key proteins expressed in the thyroid gland, including the sodium/iodide symporter, thyroglobulin, and thyroid peroxidase. TSH-mediated regulation occurs mainly by increased expression and activation of the thyroid-restricted transcription factors, thyroid transcription factor 1 (TTF-1), TTF-2, and Pax-8, which are responsible for expression of thyroid-restricted genes. The ultimate result is increased production and secretion of T3 and T4, which themselves inhibit the secretion of TSH and TRH. Most of the TSH effects within the thyroid are mediated by the cAMP–protein kinase A (PKA) cascade, which is initiated following binding of TSH to its receptor on the basal membrane of the thyrocyte. TSH–cAMP–PKA activity also requires the presence of insulin and/or IGF-1. In addition to the increased synthesis of NIS, TG, and TPO, specific consequences resulting from TSH stimulation include phosphorylation and trafficking of NIS to the plasma membrane, increased glycosylation of TG, increased production of hydrogen peroxide from increased nicotinamide adenine dinucleotide phosphate oxidase, increased pseudopod and colloid droplet formation, increased production of cathepsins B and L, and suppression of TSHR gene expression. In the thyroid gland, the effects of lithium, administered for the treatment of thyrotoxicosis, are believed to be due to the suppression of TSH-stimulated cAMP production.

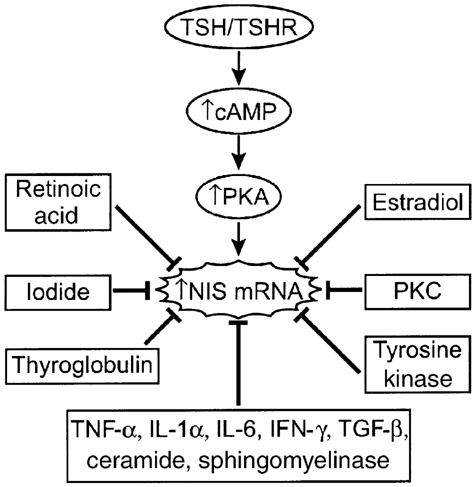

**FIGURE 3** Factors affecting sodium/iodide symporter (NIS) mRNA levels in normal thyroid cells. Factors within circles stimulate NIS expression and factors within boxes inhibit NIS expression. cAMP, cyclic adenosine monophosphate; IFN-γ, interferon-γ; IL-1α, interleukin-1α; IL-6, interleukin-6; mRNA, messenger ribonucleic acid; PKA, protein kinase A; PKC, protein kinase C; TGF-β, transforming growth factor-β; TNFα, tumor necrosis factor α; TSH/TSHR, thyroid-stimulating hormone/thyroid-stimulating hormone receptor.

Follicular thyroglobulin has been shown to counteract the stimulatory effects on the thyroid by TSH through suppressing the expression of TG, TPO, NIS, and TSHR. Follicular TG exerts its actions through suppression of TTF-1, TTF-2, and Pax-8. Follicular TG also inhibits the expression of vascular endothelial growth factor and vascular permeability factor. This results in decreased vascular flow through the thyroidal capillary bed, reducing the availability of iodide for subsequent concentration by NIS. It is reported that the 27S tetramer and poorly iodinated TG have greater suppressive effects than the 19S dimer and highly iodinated TG, respectively. In contrast, the expression of pendrin is up-regulated by follicular TG.

Factors that specifically influence the expression and function of NIS, pendrin, and TPO are important negative regulators in the production of thyroid hormones. For example, perchlorate and thiocyanate are specific inhibitors of NIS. Ouabain inhibits $Na^+/K^+$ ATPase, effectively removing the energy source necessary for NIS to transport iodide against its gradient. Other factors that affect NIS expression are depicted in Fig. 3. In addition to inhibiting NIS expression, excess iodide inhibits hormonogenesis by preventing TPO from iodinating further tyrosines. TPO is also specifically inhibited by thioureylene compounds such as propylthiouracil, methylmercaptoimidazole, and carbimazole, as well as sulfonamides. Pendrin-mediated chloride transport has been demonstrated to be inhibited by DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), furosemide, and probenecid.

## Glossary

**colloid** Gelatinous substance within the lumen of thyroid follicles that contains thyroglobulin with attached iodine and thyroid hormone.

**cretinism** Developmental condition due to congenital or early-onset hypothyroidism characterized by arrested physical and mental development, dystrophy of bones and soft tissues, and depressed basal metabolism.

**goiter** Enlargement of the thyroid gland causing a swelling in the front part of the neck that is due to hyperfunction or hypofunction of the thyroid.

**molecular chaperones** Molecules that are present in virtually all cell types and cellular compartments, and that aid in the proper folding and transport of proteins.

**organification** Oxidation of iodide and attachment to organic molecules such as thyroglobulin in the thyroid gland by thyroid peroxidase for iodine retention, storage, and hormone production.

**sodium/potassium ATPase** Physiological membrane-associated protein that pumps two potassium ions into the cell and three sodium ions out of the cell for every adenosine triphosphate that is split to form adenosine diphosphate and inorganic phosphate.

**thyrotoxicosis** Clinical condition due to overactivity of the thyroid gland and excess thyroid hormone production characterized by rapid heart rate, elevated basal metabolism, goiter, exophthalmia, nervous symptoms, and weight loss.

**thyrotropin** Hormone secreted by the anterior pituitary that is also known as thyroid-stimulating hormone.

**transcription factors** Proteins that control gene expression by binding to specific sequences on deoxynucleic acid (DNA) as DNA is transcribed into ribonucleic acid.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Thyroglobulin • Thyroid Hormone Receptor • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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acid (OPDA) reductase (see Fig. 1) normally expressed in the pistil, petals, and stamen filaments. Mutant plants dehisce only as the flowers are senescing, a phenotype that can be rescued with exogenous JA. Jasmonates also promote fruit ripening, a phenomenon that is sometimes exploited in the produce industry. Because fruit ripening is commonly attributed to other plant hormones, especially ethylene, plant physiologists were first puzzled by this revelation until it became apparent that jasmonates induce the ethylene-forming enzyme (1-aminocyclopropane-1-carboxylic acid oxidase). In this way, jasmonates could facilitate the coordination of flowering and fruit ripening in response to appropriate developmental and environmental conditions.

Since endogenous jasmonate levels are relatively high in vegetative sink tissues and in developing reproductive tissues, researchers surmised that jasmonates have important roles in regulating source–sink relationships. This role is supported by evidence that jasmonates induce tuberization and deposition of vegetative storage proteins. Jasmonates also effect a trade-off between primary and secondary metabolism, degrading photosynthetic enzymes and inactivating ribosomes while stimulating terpenoid, phenylpropanoid, and alkaloid biosynthesis. This trade-off has important implications for plant productivity and self-defense against herbivores and pathogens.

Many plants require jasmonates for induced systemic resistance to pathogens. Jasmonate-insensitive *coi1* mutants of *A. thaliana* display increased susceptibility to necrotrophic fungi. Spraying wild-type plants with exogenous MeJA increases their resistance to many of these pathogens. Whereas salicylates are primarily responsible for pathogen-induced systemic acquired resistance (SAR), jasmonates have a major role in non-pathogen-induced systemic resistance (ISR). Some studies suggest that SAR, ISR, and wound-induced responses are antagonistic, namely, that the last two compromise SAR against pathogens and vice versa. Such fitness trade-offs have been documented in field and transgenic investigations and are likely related to the fact that salicylates can inhibit jasmonate biosynthesis and vice versa. However, in 2000, van Wees *et al.* showed that simultaneous induction of SAR and ISR in *A. thaliana* results in an additive effect on the level of induced

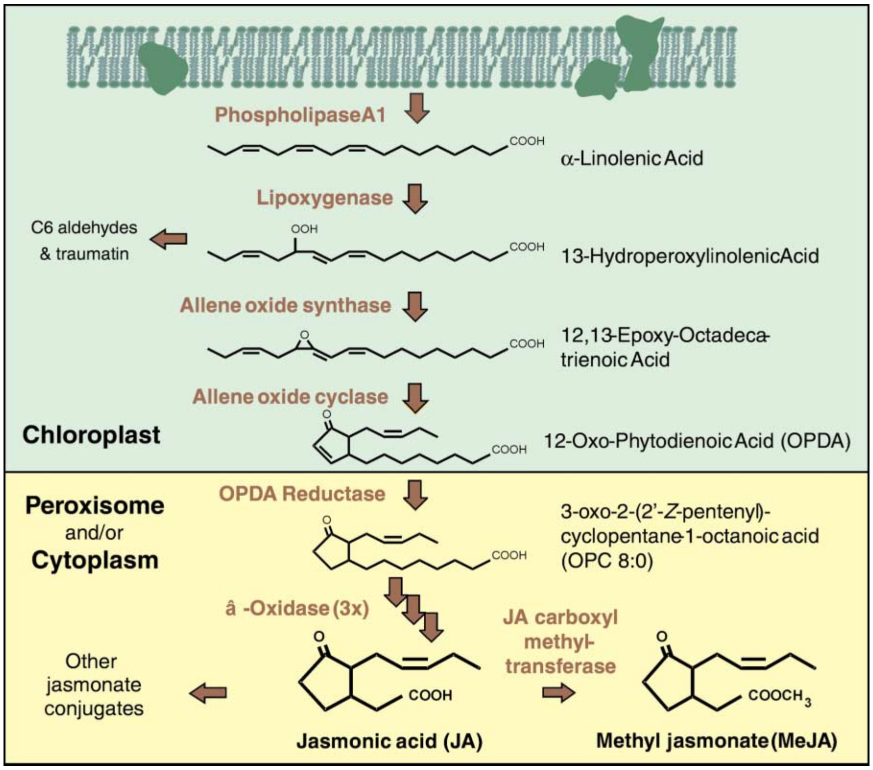
PhospholipaseA1
COOH
α-Linolenic Acid
Lipoxygenase
C6 aldehydes
& traumatin
OOH
COOH
13-HydroperoxylinolenicAcid
Allene oxide synthase
O
COOH
12,13-Epoxy-Octadeca-
trienoic Acid
Allene oxide cyclase
Chloroplast
O
COOH
12-Oxo-PhytodienoicAcid (OPDA)
Peroxisome
and/or
Cytoplasm
OPDA Reductase
O
COOH
3-oxo-2-(2'-Z-pentenyl)-
cyclopentane-1-octanoic acid
(OPC 8:0)
â -Oxidase (3x)
JA carboxyl
methyl-
transferase
Other
jasmonate
conjugates
O
COOH
Jasmonic acid (JA)
O
COOCH3
Methyl jasmonate (MeJA)

**FIGURE 1** Jasmonate biosynthesis via the octadecanoid (or oxylipin) pathway. This pathway involves two or three cellular compartments: chloroplasts, peroxisomes, and/or cytoplasm.

protection against *Pseudomonas syringae* pv. *tomato*. DNA microarray analyses have also revealed a set of 55 genes that are co-induced and another set of 28 that are co-repressed by SA and JA in *Arabidopsis*. Obviously, there is much to learn about the complex cross-talk between these two pathways in mediating plant self-defense.

Jasmonates also affect tritrophic interactions. MeJA and other volatile oxylipins can attract parasitic wasps to herbivorous insect larvae, upon which they lay their eggs. Once these hatch, the wasp larvae parasitize the host herbivore. Clearly, jasmonates have multiple roles in plant defense against multiple enemies.

## II. BIOSYNTHESIS

Jasmonates are synthesized from α-linolenic acid (18:3), which is derived from membrane phospholipids, via the octadecanoid pathway (Fig. 1). Enzymes of this pathway have recently been cloned and characterized. Several (phosopholipase A1, lipoxygenase, allene oxide synthase, and allene oxide cyclase) contain chloroplast transit peptides, providing strong evidence for a chloroplast location for the first part of this pathway. Protein localization experiments have confirmed this finding (see below). Evidence for a peroxisomal location of the final steps of this pathway comes mainly from the fact that this organelle is the site of beta-oxidation in plant cells. However, since there are cytoplasmic isoforms of many octadecanoid enzymes, in 1999, Wang *et al.* also proposed a cytoplasmic location of the pathway.

Because these enzymes degrade membrane phospholipids at defined cleavage sites, phospholipase activity is essential for initiating jasmonate biosynthesis. Various candidate phospholipases have been cloned and it has been proposed that they play a role in jasmonate biosynthesis. In 2000, Ishiguro *et al.* identified an *A. thaliana* mutant that was defective in anther dehiscence from which they cloned a phospholipase A1. That this phospholipase is primarily responsible for jasmonate biosynthesis is strongly supported by evidence that it is localized in chloroplasts and that the mutant phenotype can be rescued by exogenous application of linolenic acid or JA.

The release of free α-linolenic acid and addition of molecular oxygen by lipoxygenase results in the formation of 13-hydroperoxylinolenic acid, a precursor for the biosynthesis of jasmonates and other oxylipins. In fact, jasmonates can stimulate oxylipin biosynthesis by inducing the expression of certain isoforms of lipoxygenase and other enzymes of this pathway. The biosynthesis of characteristic combinations of oxylipins may induce specific plant responses to wounding or pathogenesis.

Allene oxide synthase (AOS), an atypical cytochrome P450 with a high turnover rate (1000 $\text{min}^{-1}$), catalyzes the putative committed step in jasmonate biosynthesis. Overexpression of chloroplastic AOS in transgenic flax leads to the accumulation of JA, but does not activate jasmonate-responsive genes. In contrast, overexpression of cytoplasmic AOS does not affect jasmonate levels in nonstressed tobacco plants, lending further support to a plastidic location of the octadecanoid pathway. In tomato, an AOS is targeted to the stromal side of the chloroplast inner envelope. Because 12,13-epoxy-octadecatrienoic acid, the product of the AOS-catalyzed reaction, is unstable, it is likely that AOS is in close proximity to allene oxide cyclase (AOC), the next enzyme in the pathway.

AOC catalyzes the stereo-specific cyclization of 12,13-epoxy-octadecatrienoic acid to specifically form 9*S*,13*S*-12-oxo-phytodienoic acid (OPDA), which has both side chains in the *cis* configuration. Immunohistochemical data suggest that tomato AOC is a soluble protein that resides in the chloroplast stroma. However, localization in the intermembrane space is also a possibility.

OPDA reductase catalyzes the reduction of the double bond in the pentacyclic ring of OPDA, forming 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC 8:0). Of the three isoforms identified in *A. thaliana*, only OPR3 catalyzes this reaction. Interestingly, *opr3* mutant plants are still capable of responding to wounding, demonstrating that OPDA and/or other oxylipins have important biological activities commonly attributed to jasmonates.

Three rounds of beta-oxidation culminate in the stereospecific formation of 3*R*,7*S*-jasmonic acid, the *cis* enantiomer. However, 3*R*,7*R*-jasmonic acid (a more stable *trans* configuration) is the biologically active form. Whether this epimerization occurs enzymatically or mainly nonenzymatically remains unknown. Volatile MeJA is produced via JA carboxyl methyltransferase, presumably in the cytoplasm since this *Arabidopsis* enzyme lacks a clear signal peptide. Wounding and exogenous MeJA systemically induce the expression of this gene in leaves and flowers.

Other hormones influence jasmonate biosynthesis by exerting their effects on the expression and activity of octadecanoid pathway enzymes. SA inhibits AOS gene expression, but reports of its effects on AOS enzyme activity are variable. In contrast, ethylene induces AOS expression and activity. Both of these effects are consistent with observed physiological responses: Salicyclic acid and jasmonates tend to have antagonistic roles, whereas ethylene and jasmonates tend to act synergistically.

## III. SIGNAL TRANSDUCTION

The complexity of plant defensive responses, involving extensive cross-talk among jasmonates, SA, and ethylene, has hampered efforts to elucidate jasmonate signaling pathways (see Fig. 2). Receptor and effector molecules remain elusive. It is known that several members of the mitogen-activated protein kinase (MAPK) family are up-regulated by biotic and abiotic stress. One of these is WIPK, a systemic wound-induced protein kinase in tobacco. Overexpression of WIPK in transgenic plants caused an increase in jasmonate levels and activation of proteinase inhibitor expression. However, this result could not be reproduced in another laboratory, perhaps due to the lack of activation by an upstream MAPK kinase. SIPK, a SA-induced protein kinase in tobacco, is induced at even higher levels in wounded plants, but its role(s) in the wound response remains unclear. Constitutive activation of NtMEK2, the upstream MAPK kinase activator of SIPK and WIPK, induces programmed cell death, indicating a role in plant responses to pathogens. Each of these MAPKs is thought to act upstream of jasmonate, inducing the octadecanoid pathway through a process that may also require transient $Ca^{2+}$ influxes.

MAPK cascades may also act downstream of external or internal jasmonates. An *Arabidopsis* MAPK mutant *mpk4* exhibits high SA levels and low-level expression of jasmonate-induced genes. Exogenous JA was unable to induce expression of these genes, suggesting that MPK4 acts downstream of jasmonate. However, in another study, MPK4 activity was up-regulated within 5 min of touching or

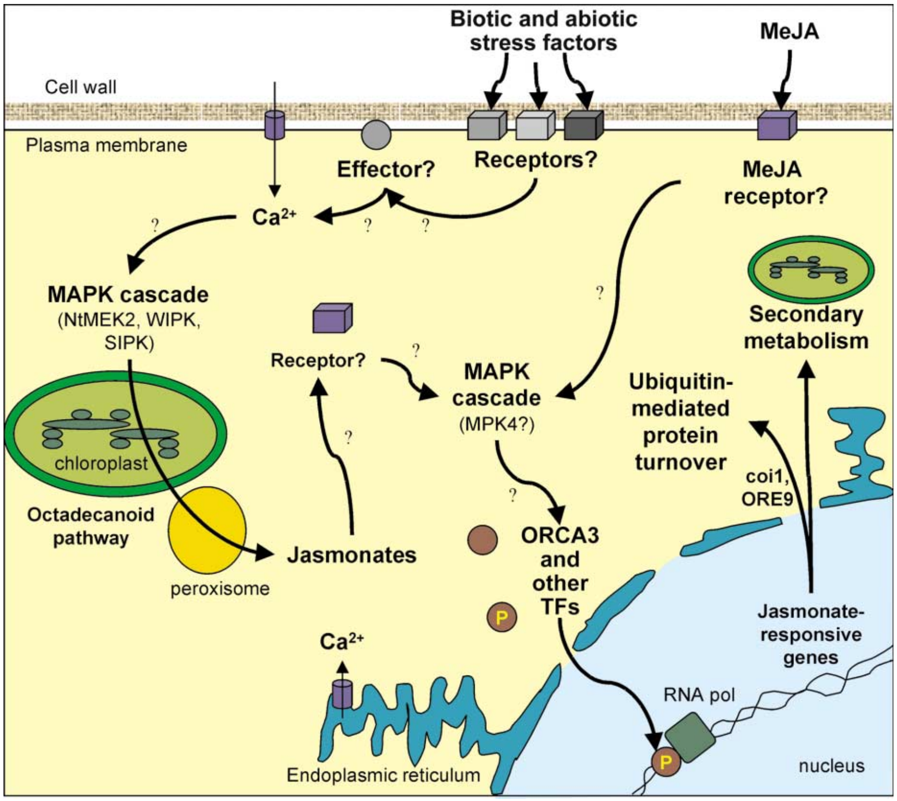
Biotic and abiotic stress factors
MeJA
Cell wall
Plasma membrane
Effector?
Receptors?
MeJA receptor?
Ca2+
?
?
?
MAPK cascade
(NtMEK2, WIPK, SIPK)
?
Secondary metabolism
Receptor?
?
MAPK cascade
(MPK4?)
Ubiquitin-mediated protein turnover
chloroplast
?
coi1, ORE9
Octadecanoid pathway
?
ORCA3 and other TFs
Jasmonates
peroxisome
P
Jasmonate-responsive genes
Ca2+
RNA pol
P
Endoplasmic reticulum
nucleus

**FIGURE 2** A current working model of jasmonate signal transduction indicates the involvement of MAPK cascades in activating jasmonate biosynthesis and action. Many upstream elements of this pathway are still unknown. Also unclear are the complex interactions of various MAPK cascades that are coordinately regulated by environmental stress, jasmonates, salicylates, ethylene, and possibly other hormones.

wounding (much faster than the kinetics of JA accumulation), suggesting that it functions upstream of jasmonate or in a jasmonate-independent pathway. These MAPK cascades may facilitate another level of cross-talk with signaling pathways involving similar cascades, including ethylene, SA, and possibly ABA.

Much of current jasmonate signal transduction research is focused on genomic analyses and characterization of *Arabidopsis* mutants. JA-insensitive mutants include *jar1*, *jin1*, *jin4*, and *coi1*. The affected *coi1* gene encodes an F-box receptor protein that contains a leucine-rich repeat. This family of receptors facilitates transfer of ubiquitin to targeted proteins for proteolysis in proteasomes. Targets of these F-box proteins putatively include proteins that normally repress jasmonate responses. Mutants with constitutively activated jasmonate responses also exist. These include *cev1* and *cex1*. Since the *cev1* phenotype requires *coi1* and *etr1* (the ethylene receptor), it likely encodes a protein that regulates an early step in plant defense involving jasmonate and ethylene signaling. Whether these mutations affect endogenous JA levels is still unknown. Complete elucidation of the pathway awaits characterization of these and other mutants.

Jasmonate signaling pathways converge on a set of transcription factors that regulate gene expression. Terpenoid indole alkaloid (TIA) biosynthesis in *Catharanthus roseus* (Madagascar periwinkle) is regulated by ORCA3, a jasmonate-responsive transcription factor that orchestrates primary and secondary metabolism, and other ORCA proteins. Gel mobility shift assays show that ORCAs interact with upstream elements at the promoters of jasmonate-responsive genes. Overexpression of ORCA3 results in increased expression of key enzymes in the biosynthesis of TIAs and TIA precursors. Plants likely make use of TIAs—including serpentine (a tranquilizer), vinblastine (an anti-tumor drug), strychnine (poison), and campothecin (an anti-neoplastic agent)—and other secondary metabolites in wound healing and self-defense.

## IV. SUMMARY

Jasmonates are phospholipid-derived hormones that regulate plant development and responses to environmental stress. They slow normal growth and developmental processes that are sensitive to environmental stress and promote multiple responses to stressful conditions. They also promote flower and fruit development. MeJA, a volatile signal, can alert neighboring plants to biotic and abiotic stresses or even summon parasitic wasps to afflict insect herbivores. Thus, it has interesting ecological and physiological implications. The enzymes that catalyze jasmonate biosynthesis have recently been cloned and characterized. Much of current jasmonate research is focused on elucidating the mechanisms of jasmonate signal transduction, clarifying the mechanisms of cross-talk with other hormones, and identifying the molecular mechanisms of plant self-defense.

## Glossary

**dehiscence** Release of mature pollen grains via degradation of the callose cell wall that surrounds a tetrad of microspores.

**elicitors** Compounds present in minute quantities in the saliva of herbivores, in the exudates of pathogens, or as degradation products of cell walls that are recognized by receptor proteins. Elicitors induce defensive responses in the affected plant.

**environmental stress** Biotic (insects, pathogens, etc.) and abiotic (drought, hail, temperature extremes, etc.) factors in the environment that can damage plants due to herbivory, disease, wounding, and physiological maladies (such as water stress).

**induced systemic resistance** Self-defense mechanism induced by nonpathogenic rhizobacteria that initiates the systemic production of pathogenesis-related proteins, leading to broad resistance against pathogens.

**oxylipins** Octadecanoid pathway products, including volatile six-carbon aldehydes (hexenal and hexenol), traumatin, cutin monomers, 12-oxo-dodecenoic acid, 9*S*,13*S*-12-oxo-phytodienoic acid, jasmonates, and methyl ester or amino acid conjugates of many of these. Oxylipins can function as signal molecules in plants and/or in attracting parasitic wasps.

**systemic acquired resistance** Self-defense mechanism induced by pathogen infection that initiates the systemic production of pathogenesis-related proteins, leading to broad resistance against pathogens.

**tritrophic interactions** Relationships involving species at three different trophic levels within an ecosystem. In the case of jasmonates, these interactions involve wounded plants, herbivorous insect larvae, and parasitic wasps.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Systemins • Salicylic Acid**

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# Juvenile Hormone Action in Insect Development

Kiyoshi Hiruma

*University of Washington*

I. INTRODUCTION
II. JUVENILE HORMONE TITERS AND POSTEMBRYONIC DEVELOPMENT
III. LARVAL–PUPAL COMMITMENT OF VARIOUS TISSUES
IV. ACTION OF JUVENILE HORMONE ON ENDOCRINE SYSTEMS
V. ROLES OF JUVENILE HORMONE IN EMBRYONIC DEVELOPMENT
VI. DIAPAUSE
VII. MOLECULAR ACTIONS OF JUVENILE HORMONE
VIII. SUMMARY

**Insect development is under the control of two hormones, ecdysone and juvenile hormone. Juvenile hormone is a sesquiterpenoid molecule that modulates ecdysone action and functions in prevention of adult differentiation, retention of larval structures, and regulation of ovarian maturation.**

## I. INTRODUCTION

Juvenile hormone (JH), a regulator of insect development, is synthesized and released by the glandular corpora allata. $\alpha$-Ecdysone (E) and its precursor are synthesized by the prothoracic glands following stimulation by prothoracicotropic hormone (PTTH), which is produced by neurosecretory cells in the brain and is released from their terminals either in the corpora cardiaca or in the corpora allata in the Lepidoptera. The discussion here focuses on the role of JH in insect development, with particular emphasis on the Lepidoptera, in which this role has been well studied.

The activity of the corpora allata is regulated by humoral factors such as allatotropin and allatostatin as well as by nervous connections. In addition to having multiple functions in blocking adult differentiation, retaining larval structures, and regulating ovarian maturation, JH is a key player for phase polymorphism in armyworms, aphids, and locusts, and for caste differentiation in termites and ants. Molting is caused by 20-hydroxyecdysone (20E). The action of JH on development is always associated with ecdysone action. JH does not prevent ecdysone action in inducing the molt, but modulates its action. JH in the hemolymph usually binds to the hemolymph JH-binding protein, so that it is protected from metabolism by the general esterases.

## II. JUVENILE HORMONE TITERS AND POSTEMBRYONIC DEVELOPMENT

JH is present in the hemolymph throughout the larval (nymphal) life, through the penultimate instar, and its presence causes a larval (nymphal) molt when the 20E titer increases. In the insect subclass Hemimetabola, JH is absent during adult development in the last instar nymph. Its role in the regulation of pupation in the Holometabola is much more complicated (Fig. 1).

### A. Hemimetabola

In the cockroach, *Nauphoeta cinerea*, nymphal molts occur when the ecdysteroid titer rises in the presence of JH (Fig. 1). JH application between 5 and 10 days after this ecdysis causes a supernumerary nymphal molt instead of the nymphal–adult molt. Therefore, JH must be absent during the critical period if there is to be a normal transformation to the adult stage. Subsequent exposure to the high level of 20E seen before the adult ecdysis is required for the actual adult development.

### B. Holometabola

#### 1. *Larval Molt*

In the lepidopteran the tobacco hornworm, *Manduca sexta*, rising ecdysteroid titers in the presence of JH cause a larval molt, and the critical period for the presence of JH is at the initiation of the ecdysteroid rise. When the corpora allata are removed from the larva before the critical period of JH for molting (Fig. 1), the larva skips the fifth instar and pupates precociously. The application of JH to the allatectomized larva restores the larval molt, indicating that JH is necessary for this molt.

#### 2. *Larval–Pupal Commitment*

In the last instar *Manduca* larva, the JH titer declines precipitously because of the cessation of secretion of JH by the corpora allata and the increase of JH esterase activity (Fig. 1), which metabolizes JH to JH acid. JH becomes undetectable by 2 days after the last larval ecdysis. The high JH esterase activity on day 3 is important to remove the trace amount of JH from the hemolymph, allowing the brain to release PTTH. This PTTH is responsible for activation of the prothoracic gland so that it releases a small pulse of ecdysteroid on day 3 to day 4 (consisting of a mixture of α-ecdysone:20E in a 1:1 ratio). The 20E, acting in the absence of JH, causes larval–pupal commitment of the abdominal epidermis. Therefore, this small ecdysteroid peak is also called the commitment peak. In the epidermis, this is accompanied by loss of mRNAs for larval cuticle proteins and synthesis of new mRNAs. In addition, this small ecdysteroid pulse in the absence of JH causes the cessation of the feeding, followed by emptying of the gut contents, which causes the onset of wandering behavior. When a small amount of JH is applied before the peak occurs, the small peak does not appear until the applied JH degrades, and all the events mentioned above are delayed. When a large amount of JH is applied, the larvae molt to gigantic sixth instar larvae (supernumerary larvae). The application of JH during the peak gives rise to various larval–pupal intermediates, but JH has no effect on pupation when applied after the peak. Thus, the switching of the program of larval to pupal occurs during this small ecdysteroid peak in the absence of JH.

#### 3. *Pupal Molt*

Pupally committed abdominal epidermis can no longer make larval cuticle even if exposed to JH, nor can it make pupal cuticle without exposure to high levels of ecdysteroid. Two days after the larval–pupal commitment of the epidermis and entry into the wandering stage in *Manduca*, there is an ecdysteroid surge along with that of JH (Fig. 1). This molting surge of ecdysteroid causes actual pupal differentiation. The corpora allata at this stage in *Manduca* synthesize and release JH acid, but peripheral tissues such as wing discs produce O-methyl transferase, which converts JH acid to JH. This JH is important in coordinating the development of various tissues for normal pupation; some of the tissues, such as wing discs and eyes, develop into adult structures if the JH is removed by allatectomy.

#### 4. *Adult Molt*

Shortly after pupation in *Manduca*, there is a JH critical period that prevents adult development (Fig. 1). During this period, pupal–adult commitment is completed, probably by the small amount of

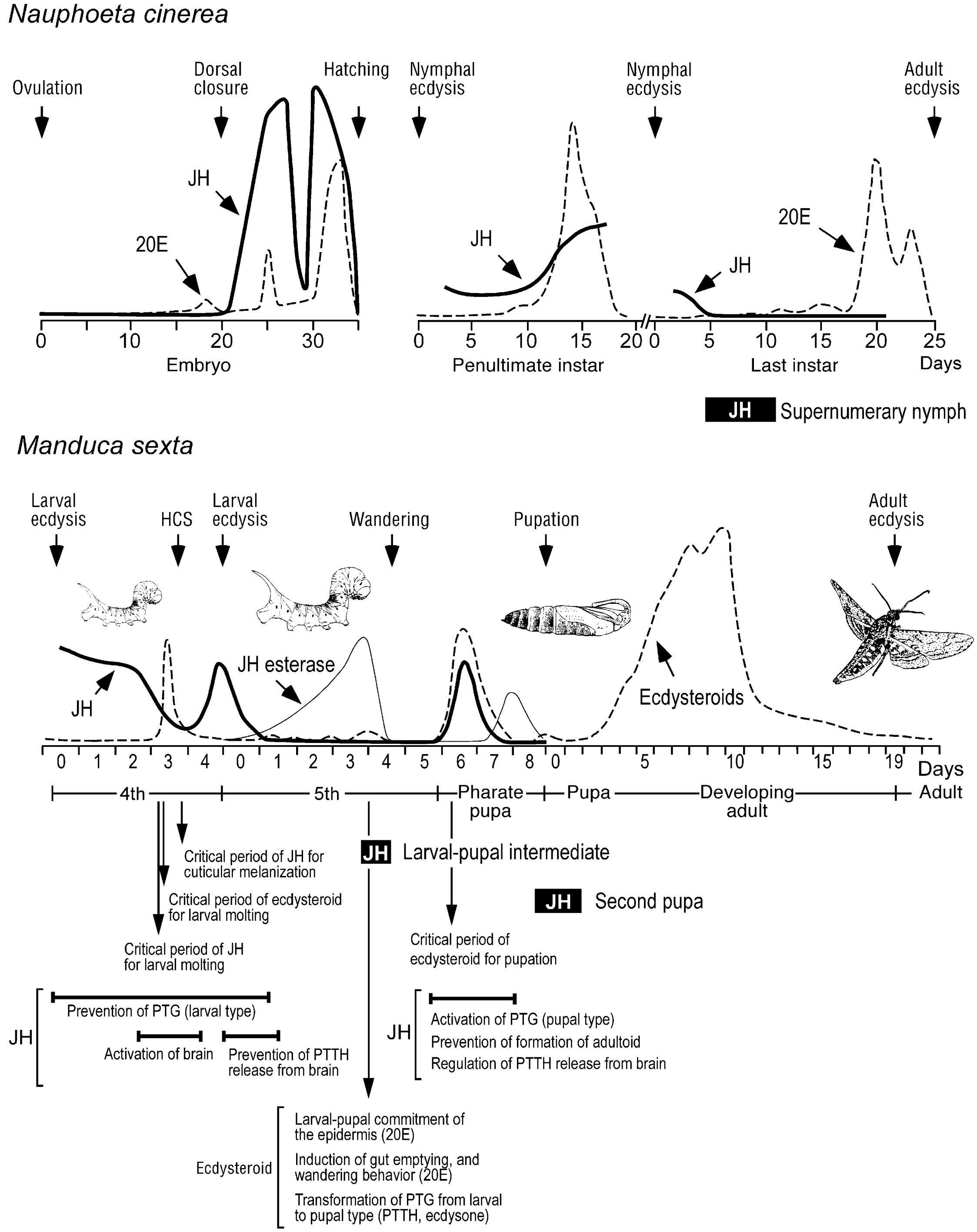

FIGURE 1 Schematic representation of juvenile hormone (JH) and ecdysteroid titers during development in Hemimetabola (*Nauphoeta cinerea*) and Holometabola (*Manduca sexta*), and various events that are caused by each hormone. All of the events in Holometabola are results from studies of both *Mamestra brassicae* and *M. sexta*. The horizontal black bars indicate the critical periods of JH for commitments. 20E, 20-Hydroxyecdysone; PTG, prothoracic gland; PTTH, prothoracicotropic hormone; HCS, head capsule slippage. Redrawn from Lanzrein *et al.* (1985), with permission.

ecdysteroid found at this stage in the absence of JH, and JH prevents this commitment process. After the commitment, the actual adult structures are made by the action of the subsequent ecdysteroid surge, 5 to 10 days after pupation.

## III. LARVAL–PUPAL COMMITMENT OF VARIOUS TISSUES

In addition to abdominal epidermis, many other tissues commit to pupal development during the final instar stage. The timing of the pupal commitment and their mechanisms are different from tissue to tissues. In this section, some of the most well-studied representative tissues are discussed.

### A. Wing Discs

The cells of imaginal discs, such as wing discs, proliferate throughout larval life; in the final instar they are committed to pupal development. The wing discs are usually pupally committed much earlier than the abdominal epidermis. Thus, when the final larvae are given JH to cause supernumerary larval molt, the resultant larvae very often contain wing discs, which have deposited pupal cuticle. This is because they have already become pupally committed by the time of the application of JH. The pupal commitment of some cells in wing discs of *Manduca* begins 12 to 18 h after the final ecdysis, and all the cells are pupally committed by day 3. The wing discs of the greater wax moth, *Galleria mellonella*, contain high levels of JH esterase activity early in the feeding stage of the last instar larvae, and this esterase breaks down the JH in the discs so that the pupal commitment of the wing discs occurs when the JH titer is declining. The detailed mechanisms of pupal commitment of the wing disc cells are still unknown.

### B. Verson's Gland (Dermal Gland)

Dermal glands were first described in the silkworm, *Bombyx mori*, by Verson. Therefore, they are also called Verson's glands in Lepidoptera. They are epidermal derivatives. Verson's glands are composed of three cells (a large secretory cell, a saccule cell, and a duct cell),which are found in each segment and which secrete a cement layer over the epicuticle just at or after ecdysis. The secretory cell produces stage-specific proteins during larval and pupal molts. In *Manduca*, before pupal commitment (ability to make pupal proteins) of the secretory cell, exposure to JH acid in the presence of very small amount of ecdysteroid is required to cause the cells to become competent to commit to make pupal-specific proteins. The actual pupal commitment then occurs between 12 and 36 h after the final larval ecdysis during the decline of JH, possibly in response to a small amount of ecdysteroid. Thus, the cells become committed to pupal development earlier than the abdominal epidermis.

### C. Crochet and Proleg

Crochets are tiny hooks on the tips of the prolegs of lepidopteran larvae; they are used to grasp the food plant and to prevent falling away from the stalk, leaf, flower, or fruit. After a larva finds its pupation site during the wandering stage, the prolegs and crochets are no longer necessary. They are larval-specific tissues composed of cells that are destined to die before pupation occurs. In *Manduca*, the crochet-forming cells die before the pupal commitment of the abdominal epidermis, coincident with the decline of JH titer. Because of this earlier commitment, some supernumerary larvae do not have their crochets.

The prolegs are controlled by a set of retractor muscles, the largest of which is the principal planta retractor muscle (PPRM). The PPRM is driven by the PPR motoneuron, but the fate of the PPRM is not influenced by interactions with the PPR, as evidenced by the fact that denervation does not cause its degeneration. Both PPR and PPRM die in response to the molting surge of ecdysteroid, but they are committed to die, at the same time as the larval–pupal commitment of the abdominal epidermis, by ecdysteroid acting in the absence of JH.

## IV. ACTION OF JUVENILE HORMONE ON ENDOCRINE SYSTEMS

JH not only modulates ecdysone action on various tissues, but also affects the activities of other endocrine organs that are responsible for molting and metamorphosis—in particular, the brain and the prothoracic gland.

### A. Larval Molt

The prolonged high JH titer seen during the penultimate larval instar (Fig. 1) is necessary to cause an increase in ecdysteroid titer at a proper time in both hemimetabolous (*Rhodnius prolixus*) and holometabolous (*Mamestra* and *Manduca*) insects. When JH is removed by allatectomy, the large molting surges of ecdysteroid are drastically depressed, but the application of JH restores these surges. In *Mamestra*, JH apparently activates the brain to synthesize and/

or release PTTH, which then stimulates ecdysteroid synthesis and release by the prothoracic glands.

### B. Larval–Pupal Commitment

After the last larval ecdysis, the nature of the brain and prothoracic gland changes dramatically. During the feeding stage in *Manduca*, the secretion of PTTH is strongly inhibited by JH and growth continues if JH is present. Therefore, the decline of JH is important for the brain to release PTTH, which is responsible for the commitment peak of ecdysteroid. This commitment peak does not appear until JH disappears completely. The signal for the decline of JH depends on the weight of the larvae, but the means of recognition of the critical weight is unknown.

After exposure of the commitment peak of ecdysteroid, the responsiveness of prothoracic glands to JH dramatically changes in *Mamestra*, *Manduca*, and *Spodoptera littoralis*. The prothoracic glands before this exposure are inhibited by JH (larval type), but afterward they are activated by JH (pupal type). In *Mamestra*, this transformation is caused by PTTH, which is responsible for the commitment peak and/or α-ecdysone from the prothoracic glands in the absence of JH. Unlike PTTH, the stimulatory action of JH on prothoracic glands appears to be indirect. In *Manduca*, JH causes the fat body to produce a hemolymph protein that enhances the production of ecdysteroids by the prothoracic glands.

### C. Pupal Molt

During the pupal molt, JH plays an important role not only for normal pupation but also for the timing of pupation. The removal of this JH by allatectomy delays pupal ecdysis by a day or so in both *Mamestra* and *Manduca*. Because the molting-peak ecdysteroid is also delayed under these conditions, the reappearance of JH at this time is likely important in conjunction with environmental signals that stimulate PTTH release.

## V. ROLES OF JUVENILE HORMONE IN EMBRYONIC DEVELOPMENT

Both ecdysteroids and JH are found in freshly laid eggs in the locust, *Locusta migratoria*. The ecdysteroids are inactive conjugates; at times corresponding to the early embryonic molts, these inactive conjugates are converted to 20E and other metabolites. The later ecdysteroid surges are likely a result of the prothoracic glands of the embryo. The JH is wiped out by the JH esterases that appear with the onset of embryonic development, then JH reappears late in embryogenesis, when it is secreted by the embryonic corpora allata. In *Nauphoeta*, two surges of JH appear shortly after dorsal closure (Fig. 1). Application of precocene to destroy the corpora allata of *Nauphoeta* embryos leads to a delay in development, reduced pigmentation of the mandibles and leg bristles, and defects in gut formation, thus the JH in late embryogenesis is necessary for normal development. JH application restores normal development.

JH mimetics applied to insect eggs during early embryogenesis in most insects cause disruption of blastokinesis (the movement of the embryo within the egg so that its dorsal surface is toward the egg shell; more properly known as katatrepsis) and often cause defects in dorsal closure. In the Hemimetabola, such as the locust, the presence of JH at the time of katatrepsis, when JH is not normally present, also causes premature termination of patterning, suppression of growth, and precocious differentiation of the nymphal stage. In the Holometabola, as exemplified by lepidopterans, despite the effect of JH on blastokinesis, there is little effect on growth and differentiation. This lack of effect of applied JH is thought to be due to the earlier appearance of JH during katatrepsis in these embryos. These effects of JH and other considerations have led Truman and Riddiford to hypothesize that during the evolution of complete metamorphosis, the embryos of holometabolous insects showed an advancement in the timing of JH secretion by the embryonic corpora allata. The resultant alteration in tissue patterning and precocious differentiation was then important for the evolution of the novel form of the larva.

## VI. DIAPAUSE

Many insects enter diapause at different stages; entry is triggered by environmental cues such as temperature, day length, and humidity. During diapause, the normal progression of growth ceases, yet diapause is not a simple arrest of development, but is an alternative developmental pathway. Usually, diapausing insects are physiologically and biochemically different from the nondiapausing individuals. The programming of diapause is also under the control of hormones. The embryonic diapause in *Bombyx* is induced by a subesophageal ganglion diapause hormone that acts on the ovarioles of females during egg maturation, and the females lay diapausing eggs.

Pupal diapause is caused by arrest of PTTH release, so that the prothoracic glands are not stimulated. JH is a key player in larval and adult diapause. Adult diapause is characterized by the halt of reproduction and is basically due to the cessation of secretion of JH by the corpora allata.

Diapause in last-instar lepidopteran larvae has been well studied. In both the rice stem borer, *Chilo suppressalis*, and the southwestern corn borer, *Diatraea grandiosella*, the JH titer in the hemolymph is high during the diapause, which not only induces diapause but also maintains its status. In addition, the prothoracic glands seem to be inactive. Allatectomy breaks larval diapause and the larvae pupate within 30 days. Larval diapause is artificially induced by the application of JH in these species. In *Diatraea*, the fat body synthesizes a diapause-associated protein in prediapausing and diapausing larvae with high JH titers. This protein has a high affinity for JH, so it may function as a JH carrier protein; furthermore, it is induced by JH application in nondiapausing larvae. A high JH titer also induces the prepupal diapause in the slug moth, *Monema flavescens*.

## VII. MOLECULAR ACTIONS OF JUVENILE HORMONE

JH enters the larval epidermal cell in *Manduca* and one-third goes to the nucleus. Photoaffinity-labeled JH I, JH II, and methoprene (a JH mimic) bind to a 29 kDa protein that is found in the larval epidermis. This protein was once considered to be a JH receptor, but further studies have shown only a low-affinity binding protein. It has been reported recently that *Drosophila* ultraspiracle protein (USP) binds JH III and JH III bisepoxide with low affinity (about $4 \times 10^{-7}$ *M*). JH III and methoprene cannot replace the bacterial phospholipid found in the ligand-binding pocket of recombinant USP, but JH I acid is predicted to fit into this pocket. However, no functional studies of the role of USP as a JH receptor have been reported. There are a number of reports regarding JH-binding proteins. Particularly interesting are the *methoprene-tolerant* (*Met*) gene in *Drosophila* mutants, which have an intracellular JH-binding protein with reduced JH-binding activity. The Met protein is found in the nucleus and has a high degree of similarity to the basic helix–loop–helix Per, Arnt, and Sim (PAS) domain family members. How this is related to JH action is not known. Null mutants show no defect in development, but have delayed and reduced vitellogenesis. Therefore, a JH receptor for insect development is still elusive.

### A. Regulation of Cuticular Melanization

Larval pigmentation is under the control of JH in many insects, but there are not many studies at the molecular level. Normally, *Manduca* larvae have a transparent cuticle with black markings. Yet when JH is removed by allatectomy about 30 h before the last larval ecdysis, cuticular melanization occurs in the newly synthesized fifth instar larval cuticle and can be prevented by application of JH (Fig. 1). The melanin is dopamine melanin and is found in granules deposited to the cuticle. Accordingly, dopa decarboxylase (DDC) (converts dopa to dopamine) and granular phenoloxidase (PO) (oxidizes dopamine in the first step of the oxidative path to melanin) are crucial enzymes for this process.

In allatectomized melanizing larvae, the abdominal epidermis produces a granular pro-PO that is incorporated to the new cuticle in the premelanin granules. The pro-PO is later activated by the decline of ecdysteroid titer. The application of JH 25 to 30 h before ecdysis completely prevents the production of this enzyme (Fig. 2). DDC is essential for not only melanization but also for cuticular sclerotization, and its activity and its mRNA increase in response to the decline in the ecdysteroid titer. In allatectomized larvae, the levels are twofold higher than those in nonmelanizing normal larvae (Fig. 2). The excess dopamine is incorporated to the granules deposited in

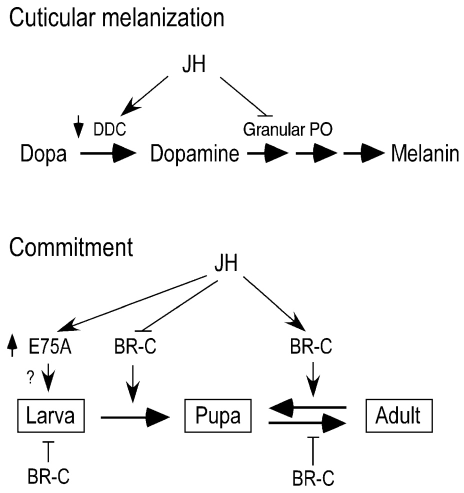

FIGURE 2 Models for molecular actions of juvenile hormone (JH). DDC, Dopa decarboxylase; PO, phenoloxidase; BR-C, broad complex; ↓, induction; ⊥, inhibition.

the cuticle, where activated granular PO is present, so that melanization occurs within the granules. The action of JH on DDC gene expression is thus to modify the amount of gene expression, as is also seen in E75A (see next section), rather than the suppression of new gene expression, as seen in granular PO. The mode of action of JH in both cases is unknown.

## B. Regulation of the Ecdysone-Induced Transcription Factor Cascade

Most of the events caused by 20E are through the control of the ecdysone receptor (EcR), but EcR is an inactive receptor and it needs to partner with ultraspiracle protein to be functional. 20-Hydroxyecdysone enters the cells and then the nucleus, where EcR and USP are bound to the DNA. Once 20E binds to EcR, the 20E/EcR/USP complex directly activates the early genes [e.g., E75 and broad complex (BR-C)], the protein products of which in turn activate the late tissue-specific genes (e.g., L71 in the salivary gland) and inhibit the early genes.

Two to three isoforms of EcR and USP are known from various insects, and the receptor levels fluctuate with development. The primary role of JH in morphogenesis is to modulate the ecdysone action, and JH also modulates the ecdysone-inducible EcR and USP expression. Expression of both EcR and USP is up-regulated by 20E in a complex fashion. In the *Manduca* epidermis, the expression of all of the EcR (EcR-A and EcR-B1) and USP (USP-1 and USP-2) isoforms is induced by 20E, but JH prevents this action. The one exception is USP-2, which is unaffected by JH. JH is also known to prolong the half-life of both EcR-A and EcR-B1 proteins.

## C. Larval–Pupal Commitment

In the ecdysone cascade, in addition to EcR and USP, JH affects the expression of a few other transcription factors. In *Manduca*, studies of the metamorphic role of these factors show that JH enhances the expression of one of the 20E-induced transcription factors, E75A, in larval epidermis, where it prevents pupal commitment of the epidermis, suggesting that a high titer of E75A is important for maintenance of the larval state (Fig. 2). Another 20E-induced transcription factor, BR-C, is expressed only from the time of pupal commitment through the time of pupation in both *Manduca* and *Drosophila*. In *Manduca*, the appearance of BR-C protein correlates with pupal commitment of the abdominal epidermis on day 3 of the final larval instar, and the prevention of pupal commitment by JH prevents BR-C expression both *in vivo* and *in vitro* (Fig. 2). Expression of BR-C in the abdominal epidermis occurs in a strict spatial pattern that coincides with the loss of the sensitivity to JH. Furthermore, once epidermis is exposed to 20E for 6 h, at which time BR-C mRNA is first detectable, JH can no longer prevent BR-C expression. Similarly, JH cannot prevent the pupal commitment once the cells are exposed to 20E, in the absence of JH, for more than 6 h. The appearance of BR-C mRNA in wing discs also correlates with their pupal commitment. Therefore, BR-C expression is one of the first molecular events underlying pupal commitment of both abdominal epidermis and wing discs. In addition, *Drosophila* mutants that lack BR-C entirely develop normally until metamorphosis, but die before pupation. These observations suggest that BR-C is a key factor for metamorphosis.

A recent breakthrough on the molecular mechanism of pupal commitment has been reported by Zhou and Riddiford, who clearly show in *Manduca* and *Drosophila* that BR-C is a pupal-specifying transcription factor (Fig. 2). Normally, BR-C is present during pupal cuticle formation but is not present during the adult molt. When JH is given just before or after pupal ecdysis in *Manduca*, BR-C mRNA is expressed during the production of a second pupal cuticle. This re-expression of BR-C mRNA can be also induced *in vitro* by exposing the pupal wing to 20E in the presence of JH. In *Drosophila*, the application of JH at pupariation causes the formation of a “pharate adult” with a normal adult head and thorax but a pupal-like abdomen. In these JH-treated insects, BR-C mRNA is re-expressed during adult development in the abdomen but not in the head and thorax. Moreover, pupal cuticle genes are re-expressed and an adult cuticle gene is suppressed in the JH-treated abdomen. These findings show that the abdominal cells are making a second pupal cuticle. The use of transgenic *Drosophila* carrying the various BR-C isoforms under a heat-shock promoter allows determination of the effects of misexpression of each of the four isoforms (Z1–Z4) on pupal cuticle production. Expression of the Z1 isoform at the onset of cuticle production induces re-expression of two pupal cuticle genes and reduces expression of an adult cuticle gene, causing the deposition of a second pupal cuticle. Unlike the JH-treated flies, this induction of a new pupal cuticle occurs in the head and thorax as well as in the abdomen. Interestingly, misexpression of the Z1 isoform during the second larval molt causes premature expression of a pupal cuticle gene and suppresses expression of a larval cuticle gene. The other isoforms have similar effects on some, but not all, cuticle genes. These findings clearly show that BR-C is indeed a pupal-specifying transcription factor.

## VIII. SUMMARY

JH has multiple functions, and a primary role of JH in insect development is to modulate ecdysone action. JH maintains the current commitment of the tissues and cells, whereas ecdysone causes both predifferentiative and differentiative cellular events that are necessary for the molt. Thus, when JH is present, a molt to a larval stage ensues. If JH is absent at the onset of the molt, metamorphosis occurs. Studies of the molecular mechanisms of JH action have been hampered by the failure to isolate its receptor(s). Recently, detailed studies of JH action have begun, and further studies should elucidate new aspects of the action of this unique molecule.

## Glossary

| | |
|---|---|
| **allatectomy** | Removal of the corpora allata, the source of insect juvenile hormone. |
| **allatostatin** | Neuropeptide that inhibits the biosynthesis of juvenile hormone by the corpora allata. |
| **allatotropin** | Neuropeptide that activates the biosynthesis of juvenile hormone by the corpora allata. |
| **commitment** | Capability of the cell to enter a particular stage-specific differentiative program that is elicited during an insect molt. For example, if the epidermis is pupally committed, the epidermis can no longer make larval cuticle, but requires 20E to make actual pupal cuticle. |
| **corpus allatum** | Gland that synthesizes juvenile hormone. |
| **ecdysis** | Process of shedding the old cuticle. |
| **ecdysone** | Generic term denoting the biologically active ecdysteroids that initiate and coordinate an insect molt. |
| **ecdysteroids** | Compounds in which the steroid nucleus bears a cis-fused A/B ring junction, a 7-ene-6-one chromophore, and a 14$\alpha$-OH. |
| **Hemimetabola** | Insect subclass in which insects go through several nymphal stages, then molt directly to the adult stage. |
| **Holometabola** | Insect subclass in which insects have a pupal stage in their life cycle. There usually are several larval stages followed by a pupal stage, then the adult stage. |
| **20-hydroxyecdysone** | Biologically active ecdysteroid that induces molting and metamorphosis. |
| **juvenile hormone** | Sesquiterpenoid compound in insects that has multiple functions, such as the prevention of adult differentiation, the retention of larval structures, and the regulation of ovarian maturation. |
| **molting** | Process of producing a new cuticle and culminating in ecdysis. |
| **prothoracic gland** | Tissue that synthesizes ecdysteroids; located mainly in the prothoracic segment in Lepidoptera. |
| **prothoracicotropic hormone** | Neuropeptide that stimulates prothoracic glands to synthesize ecdysteroids. |
| **transcription factor** | Protein that regulates the transcription of a gene. |

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysone Action in Insect Reproduction • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormone Biosynthesis • Juvenile Hormones, Chemistry of • Neuropeptides: Roles in Regulation of Juvenile Hormone Production • Pheromone Production in Insects**

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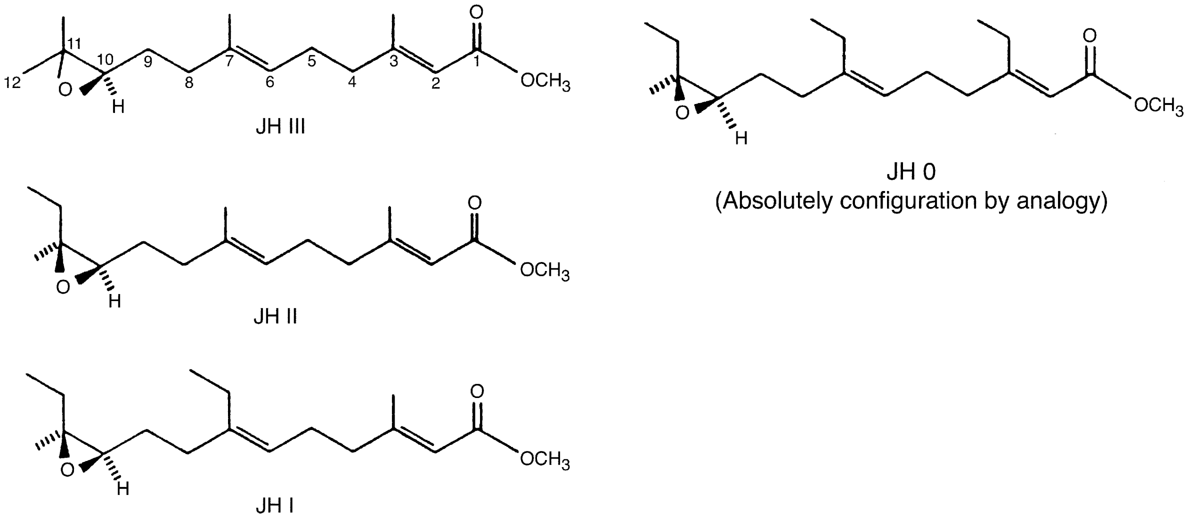

FIGURE 1 Structures of the naturally occurring known juvenile hormones.

evolutionarily conserved regions. Current conclusions remain, however, tentative, because of the limited number of sequences available.

## IV. VITELLOGENESIS

All insects make yolky eggs via the incorporation of the precursor Vg. In the majority of species, Vg synthesis is controlled by JH, and Vg uptake by the oocytes may also be under JH control. In species such as the mosquito *Aedes aegypti*, however, ecdysone appears to be one of the essential hormones that drives this process. In a few species of moths, on the other hand, such as *Bombyx mori* or *Hyalophora cecropia*, vitellogenesis appears to occur without any involvement of known hormones.

Endocrine control of vitellogenesis was first shown for the blood-sucking bug *Rhodnius prolixus* by Wigglesworth through ingeniously designed operations and manipulations based on the biology of this insect. Wigglesworth determined that the corpus allatum, located posterior to the brain, is activated and liberates a gonadotropic hormone following the essential blood meal. Subsequent to this first report, many insect species of several orders had been found to require the corpora allata for egg growth, i.e., vitellogenesis. For several decades the research centered around the brain-controlled corpus allatum activation and/or inhibition; basically, this required the surgical removal and reimplantation of the corpora allata as well as specific brain surgery and the manipulation of environmental conditions such as photoperiods, rearing temperatures or observed mating routines. Then, with the availability of the pure JH, it became feasible to begin an analysis of the details of how this hormone actually may act in the promotion of egg growth. It was shown in 1969 that JH is primarily involved in the *de novo* synthesis of the yolk precursor Vg in the cockroach *Leucophaea maderae*. All species that rely on the corpora allata for egg growth were subsequently found to produce Vg when treated with JH or JH analogues.

Vgs are synthesized on the endoplasmic reticulum (ER) of the fat body as exportable proteins. JH stimulates the incorporation of phospholipids into the ER and thus augments the quantity of ER and the number of available binding sites for the Vg polysomes in the cockroach *L. maderae*. The effect of this augmentation of polysome binding sites is intuitively seen in the increased rate of production of Vg and consequent increase in egg production. Similar observations were made in a few additional species, and it can be assumed that JH-stimulated ER proliferation is a common phenomenon in conjunction with Vg synthesis.

Vg is a *de novo*-synthesized macromolecule under control of JH. Consequently, it is possible to postulate that this hormone is involved in the control of transcription of the Vg gene in the target tissues. Vg mRNAs have been isolated from a few insect species that depend on JH. We assume that the model for transcriptional control of specific protein synthesis, developed for the steroid hormones, is applicable for JH. The search for the presumptive JH receptor has, however, not yet yielded a molecule that could unequivocally qualify for a receptor.

## V. OTHER JUVENILE HORMONE ACTIONS

### A. Stimulation of Lipophorin Synthesis

The hemolymph protein lipophorin (Lp) is a lipid carrier and functions in the shuttle of lipid reserves between storage sites and flight muscles in flying locusts. It is also known for several insect species that Lp binds lipophilic JH with high affinity, acts as a hormone carrier, and even “protects” the hormone from degradation by circulating JH esterases. The role of lipophorin in facilitation of JH uptake by the target tissues has been discussed, but concrete evidence is not available. Furthermore, JH stimulates an ongoing Lp synthesis and thus augments the titer of Lp in circulation in adult females of the cockroach *L. maderae* and presumably in other species as well. This is presumably beneficial, because more JH molecules can now be sequestered and removed from circulation and thus may serve to increase the half-life of the available JH. A small amount of Lp enters the growing oocytes (presumably nonspecifically) during vitellogenesis and thus contributes to the protein reserves in the egg.

### B. Vg Uptake by Oocytes

Throughout the past two decades, there have been anecdotal reports in the literature on JH-stimulated incorporation of Vg into oocytes. Most of these reports rely on correlative observations. Recently, however, reports on a few insect species have provided evidence that Vg uptake by oocytes occurs via receptor-mediated endocytosis that requires the presence of JH.

### C. Courtship Behavior

Reproduction in bisexual species often requires mating. Intuitively, it is assumed that courtship behavior is endocrinologically controlled. Numerous correlative lines of evidence that this might be so can be found in early literature. The first experimental evidence that JH is involved in the development of female receptivity was provided by Engelmann in 1960 for the cockroach *L. maderae*. Similar findings have been reported subsequently for other species, and it has been shown that courtship behavior of females and males may be influenced by hormones. Hormones, such as JH, may directly act on the central nervous system, resulting in modified behavior, or may stimulate the production of sex-attractant pheromones that are perceived by the opposite sex. However, it cannot be generalized that this applies to all species and it is reported that JH, for example, may not have any role in courtship behavior in a number of investigated species.

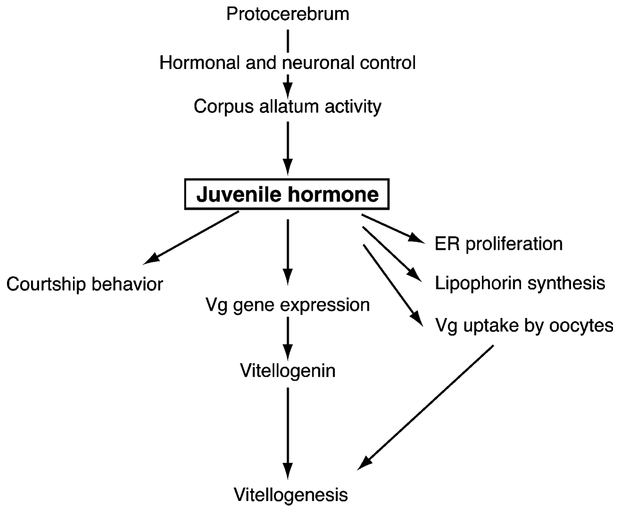

FIGURE 2 Scheme for the central role of juvenile hormone in insect vitellogenesis. ER, Endoplasmic reticulum; Vg, vitellogenin.

## VI. SYNERGISM

In species that rely on the presence of JH for vitellogenesis, it is found that this hormone participates in a multiplicity of detailed events, all of which lead synergistically to the production of a mature yolky egg (Fig. 2).

The brain ultimately controls the activity of the corpora allata via hormonal and neuronal pathways, depending on the species and environmental conditions. Control of transcription of the Vg gene is an all-or-none event. The precise details of this process are currently not fully understood. Other aspects of JH involvement in vitellogenesis mostly entail the enhancement of ongoing events.

## Glossary

**fat body** The tissue in insects that is the equivalent of the vertebrate liver.

**juvenile hormone** Sesquiterpenoid molecules that facilitate retention of juvenile characteristics in larval and nymphal insects and thus prevent metamorphosis. The identical molecules function as gonadotropic hormones in the majority of adult insects by promoting the production of the yolk protein precursor, vitellogenin.

**lipophorin** A circulating macromolecular protein with a lipid moiety of 10–30%. It functions in transport of lipids and binds and transports juvenile hormone.

**vitellin** The predominant yolk protein of the eggs. It is immunologically identical to vitellogenin [vitellus (L) = the yolk of the egg].

**vitellogenesis** Refers to the making of a yolky egg, a process that primarily involves incorporation of vitellogenin into the oocytes and results in production of the complete and mature egg.

**vitellogenin** A macromolecular protein of the hemolymph. It is the precursor to the major yolk protein vitellin.

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysteroid Action in Insect Reproduction • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development • Juvenile Hormone Biosynthesis • Juvenile Hormones, Chemistry of • Neuropeptides: Roles in Regulation of Juvenile Hormone Production**

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# Juvenile Hormone Biosynthesis

Stephanie Sen, Sara Jull, and Rebecca Whelchel
*Indiana University/Purdue University*

I. INTRODUCTION
II. CARBON SOURCES FOR JH BIOSYNTHESIS
III. ENZYMES OF THE MEVALONIC ACID PATHWAY
IV. LATER STEPS IN JH BIOSYNTHESIS
V. METABOLIC CONTROL OF JH BIOSYNTHESIS
VI. SUMMARY

**Several chemically diverse hormones have been implicated in controlling insect development. Of these, one of the most widely studied is the sesquiterpenoid juvenile hormone. The insect order Lepidoptera, which includes all moths and butterflies, is unique in that it produces several homologous juvenile hormones, and the order Diptera produces another derivative.**

## I. INTRODUCTION

Sesquiterpenoid juvenile hormones (JHs) are neuroendocrine gland products found in insects. The most common chemical form is JH III (Fig. 1); however, the insect order Lepidoptera also produces homologous JH structures, including JH 0, methyl-JH I, JH I, and JH II. An additional JH, $JHB_3$, which is the C6,7-epoxy derivative of JH III, has been found in the order Diptera. Homologous juvenile hormones are unique structures found only in insects, whereas JH III has been detected in two plant species and the corresponding desoxyderivative of JH III, methyl farnesoate, is an important hormone of crustacea.

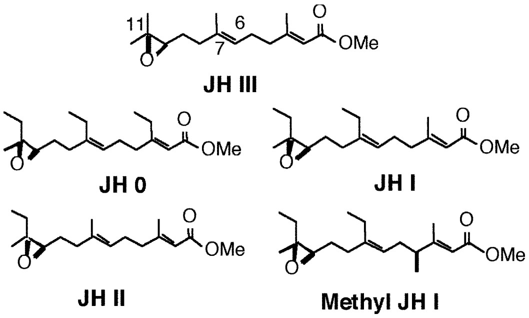

FIGURE 1 Juvenile hormone (JH) structures.

JH biosynthesis is localized within the paired neuroendocrine gland, the corpus allatum (CA). Once made, the hormone is immediately secreted into the hemolymph for delivery to target tissues. An exception to this scheme is found in the male moths *Hyalophora cecropia* and *Manduca sexta*; these animals secrete the acid form of JH from the CA and subsequently make the methyl ester within their accessory gland. Although metabolic localization should simplify the task of identifying the enzymes involved in JH biosynthesis, the corpus allatum gland is minuscule and therefore conventional enzymological techniques are usually impractical.

Both the amounts and the specific structures of JHs produced by an insect species represent a highly orchestrated event. An example of this is seen in the sphinx moth, *M. sexta*. This insect makes JH III and all known homologues, but not all at the same time—the embryonic stage makes JH 0 and methyl-JH I, the larval stages make JH I and JH II, and the adult stage makes JH II and JH III. Because of this complexity, it can be concluded that JH biosynthesis is regulated and that the enzymes involved in JH formation may have unusual substrate specificity. Despite the fact that researchers have been studying JH metabolism for over half a century, there remain significant gaps in our understanding of this important biosynthetic pathway.

## II. CARBON SOURCES FOR JH BIOSYNTHESIS

Because all juvenile hormone structures are terpenoid in nature, it is reasonable to suppose that these compounds could be derived from the mevalonic acid (MVA) pathway. Initial studies focused only on confirming this hypothesis, and establishing the existence of alternate pathways, such as methylation of JH III to generate the various JH homologues, did not occur.

A series of elegant *in vivo* and *in vitro* studies were performed utilizing the radiolabeled precursors [$^{14}$C]acetate, [$^{14}$C]propionate, [$^{14}$C]mevalonate, [$^{3}$H]homomevalonate, and [$^{3}$H]methionine. Degradative analysis of the metabolites formed indicated that JH III was appropriately labeled by acetate and mevalonate, that homomevalonate was precursor to JH homologues, and that [1-$^{14}$C]propionate was incorporated into JH I and JH II, the former being radiolabeled at both the C-7 and the C-11 positions. Importantly, L-methionine was incorporated only into the methyl ester moiety of the hormones, thereby establishing that acetyl coenzyme A (coA) and propionyl coA are the carbon sources for JH skeleton production in insects.

Acetyl coA is ubiquitous in nature and can be derived from a variety of metabolic precursors, including carbohydrates, lipids, and amino acids. Propionyl coA is likely to be derived from fewer sources, with metabolic candidates being odd-numbered fatty acid and branched-chain amino acid degradation products. The fact that insect hemolymph contains high concentrations of amino acids would support the latter being the primary source of propionate. Precursors of acetyl coA and propionyl coA have been identified in both lepidopterous and nonlepidopterous insects. Early studies showed that radioactively labeled glucose, valine, and isoleucine were all incorporated into the JH of moths. These results were elaborated on and it has been established that trehalose (a source of glucose) is sufficient to support JH biosynthesis in the cockroach, *Diploptera punctata*. Although amino acids were a poorer carbon source for JH III-producing insects, the order Lepidoptera was found to very efficiently metabolize the branched-chain amino acids isoleucine and valine. The former, which is degraded within the mitochondria to acetyl coA and propionyl coA, was sufficient to support the biosynthesis of JH in these insects. Further evaluation of insect amino acid metabolism indicated that transaminase activity is high in all tissues except the CA of nonlepidopterous insects. This fundamental difference is clearly critical to our understanding of why only lepidopteran insects make juvenile hormone homologues.

## III. ENZYMES OF THE MEVALONIC ACID PATHWAY

All animals utilize the MVA pathway for biosynthesis of sterols and terpenes, thus insects are likely to utilize similar, if not the same, enzymes for the construction of JH. Interestingly, although plants can make the mevalonate precursor isopentenyl diphosphate (IPP) from 2*C*-methyl erythritol-4-phosphate, they also make JH III exclusively from the MVA pathway.

As its name implies, the MVA pathway utilizes mevalonate (Mev) as an important biosynthetic intermediate. This compound is derived from the condensation of two molecules of acetyl coA followed by the condensation of acetoacetyl coA with another molecule of acetyl coA to produce hydroxymethylglutaryl coA (HMG coA) (Fig. 2). These reactions are catalyzed sequentially by the enzymes acetoacetyl coA thiolase and HMG coA synthase, which have been well characterized in a variety of (noninsect) organisms. Mevalonate is produced by the NADPH-dependent reduction of HMG coA by HMG coA reductase. This last step in mevalonate construction is considered to be the rate-limiting step for the MVA pathway. HMG coA reductase is regulated transcriptionally and translationally and its stability/activity are dependent on both phosphorylation and association with endogenous factors. Both sterol and nonsteroidal compounds have been implicated in regulating HMG coA reductase, including the terpenol farnesol, which is an important intermediate of JH biosynthesis.

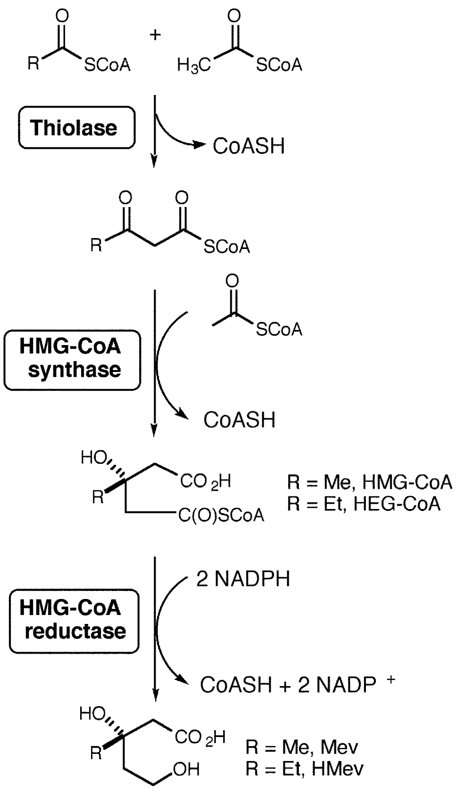

**FIGURE 2** Acetoacetyl coenzyme A thiolase, hydroxymethylglutaryl (HMG) coA synthase, and HMG coA reductase.

Both acetoacetyl coA thiolase and HMG coA synthase have been identified in the biosynthesis of JH, although only the latter has been characterized extensively. Using radioactively labeled acetyl coA as substrate and relying on sequential thiolase and synthase activities, HMG coA was produced with corpora allata homogenates of several insect species. The apparent $K_m$ for acetyl coA was 200 $\mu M$ for *D. punctata* and 400 $\mu M$ for *Locusta migratoria*. The synthase is strictly cytosolic, is activated by $Mg^{2+}$, and is inhibited by excess acetoacetyl coA.

Using acetyl coA and propionyl coA as substrates, the formation of both HMG coA and hydroxyethylglutaryl coA (HEG coA) was demonstrated. Compared to rat liver, insect CA homogenates produce more HEG coA, although the nonlepidopterous insect *Schistocerca nitens* made considerably more HEG coA than did the lepidopteran *M. sexta*. Enzyme activity did not parallel the rate of JH release in *D. punctata* and *L. migratoria*, and, for the latter, no loss of synthase activity was observed when the connection between CA and brain was severed. Although the sequence of the CA-specific synthase is not available, two HMG coA synthase (HMGS) genes have been identified in *Blattella germanica*. Both code for functional cytosolic proteins and share considerable sequence similarity with other known synthases. *Blattella germanica* HMGS-1 and HMGS-2 are expressed differentially within the insect and are coordinately regulated with HMG coA reductase in the fat body, but not in the ovaries.

HMG coA reductase (HMGR) has been studied using CA homogenates of several insect species. The enzyme, which is microsomal and requires detergent for activity, catalyzes the stereospecific reduction of (3*S*)-HMG coA to mevalonate using NADPH as cofactor and is inhibited by the anticholesteremic agent compactin. As seen with other HMGRs, activity is dependent on the enzyme's phosphorylation state, and phosphorylation of the reductase by an ATP-dependent kinase renders the enzyme inactive. As expected, the addition of ethylenediaminetetraacetic acid (EDTA) enhanced activity, whereas the addition of $MgCl_2$, ATP, or $F^-$ was inhibitory. HMG coA reductase is most active near neutral pH. An HMGR that is transcribed in the CA of the moth *Agrotis ipsilon* has been recently cloned. The predicted molecular mass of the reductase is 90 kDa and it shows high sequence homology with other animal

HMGRs, including those of *Drosophila melanogaster* and *B. germanica*.

Despite these similarities, the HMG coA reductase of insect CA is different from related enzymes. Antibodies to rat HMGR did not cross-react with the corresponding enzyme in *D. punctata*, suggesting differences in the overall structure of the two animal enzymes. The substrate specificity of HMGR was studied by comparing the reduction of HMG coA to that of HEG coA. Insect HMGR reduced more HEG coA than did rat liver enzyme, although *S. nitens* CA, which produces only JH III, was better able to reduce the homologous substrate, compared to *M. sexta*. In vertebrates, HMGR regulates flux within the mevalonate pathway and is considered the rate-determining step of cholesterol biosynthesis. In contrast, JH does not appear to be regulated by changes in HMGR. Enzyme activity was found to be high at developmental stages that produce little to no JH, and HMGR activity was not modulated by the addition of mevalonate, mevinolin, or farnesol.

The conversion of mevalonate to IPP (Fig. 3) is catalyzed by three sequential ATP-dependent enzymes: mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate (MPP) decarboxylase. In relation to JH biosynthesis, these enzymes have been poorly characterized, although studies with fluoromevalonate and the homologous substrate, homomevalonate (HMev), have demonstrated that there are distinct enzymological differences between insects and other animals. Fluoromevalonate (Fmev) functions as a selective lepidopteran JH antagonist by inhibiting the metabolism of both mevalonate and homomevalonate to JH. Although its precise mode of action is unknown, studies with yeast and rat liver MPP decarboxylase indicate that the compound is phosphorylated and that the corresponding 5-diphosphate derivative functions as the active inhibitor of MPP decarboxylation. The homologous substrate homomevalonate, which is converted to homoisopentenyl diphosphate in *Drosophila* $K_c$ cells and is incorporated into homologous JH structures in Lepidoptera, does not undergo decarboxylation in rat liver and is decarboxylated at one-eighth the rate of MPP in yeast. As seen with each of the earlier steps in JH biosynthesis, it would appear that insect MPP decarboxylase is better able to utilize homologous substrates compared to the analogous noninsect enzymes.

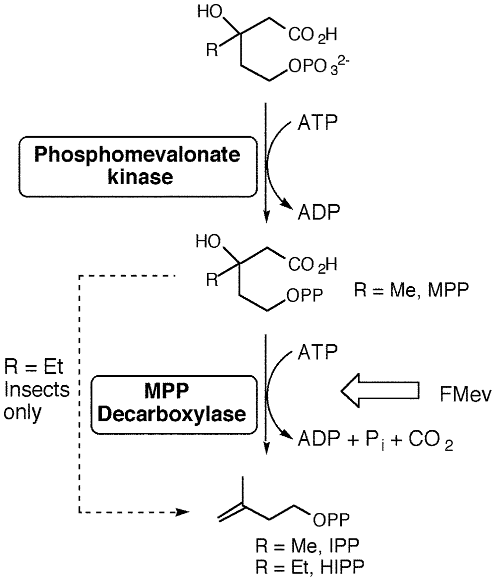

FIGURE 3 Isopentenyl diphosphate (IPP) formation from mevalonate. MPP, Mevalonate diphosphate; HIPP, homoisopentenyl diphosphate; FMev, fluoromevalonate.

Decarboxylation of MPP provides IPP, which represents the first isoprenoid intermediate of the mevalonate pathway. This compound functions as the building block for all subsequent terpenoid compounds, but to do so it must undergo double-bond isomerization to yield dimethylallyl diphosphate (DMAPP). This reaction is catalyzed by the enzyme IPP isomerase, which has been well characterized in vertebrates, bacteria, and yeast and has been identified in CA homogenates of *M. sexta* and *S. nitens*. The enzyme, which has high sequence homology between organisms, has a molecular mass of 35–40 kDa, requires divalent cations for activity, and possesses active site glutamic acid and cysteine residues that have been implicated in catalysis. The $K_m$ of IPP is $20\ \mu M$ with purified isomerase of *Bombyx mori*. Although previously considered a strictly cytosolic protein, recent studies in rat liver indicate that the enzyme may reside within the peroxisomes of eukaryotes.

In the context of homologue formation, the substrate homoisopentenyl diphosphate (HIPP) must be efficiently converted to both homodimethylallyl diphosphate (HDMAPP) and (*Z*)-3-methyl-2-pentenyl diphosphate in insects. Using IPP isomerase of pig liver (Fig. 4), (*Z*)-3-methyl-2-pentenyl diphosphate was the major product of HIPP isomerization, whereas only HDMAPP was formed when the reaction was performed with CA homogenates of adult female JH II- and JH III-producing *M. sexta* corpora allata. In the latter system, DMAPP was formed in nearly 10 times greater yield than HDMAPP. These results indicate that although IPP is the preferred substrate of insect

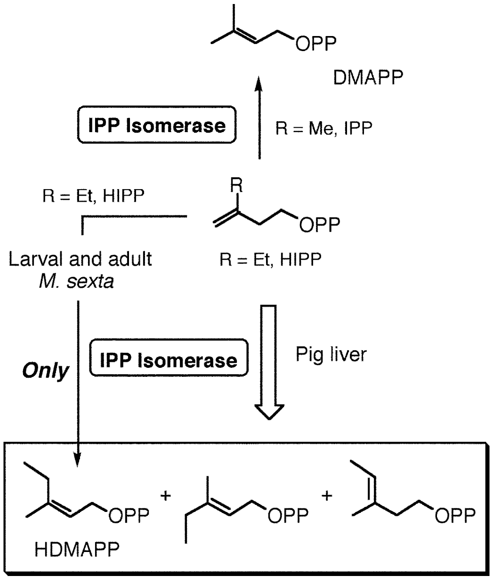

FIGURE 4 Isopentenyl diphosphate (IPP) isomerase of pig liver and *Manduca sexta*. HIPP, homoisopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; HDMAPP, homodimethylallyl diphosphate.

IPP isomerase and methyl-JH I is derived from an isomerase that is similar to that of pig liver, all other JH homologues are derived from another, structurally distinct IPP isomerase.

Once allylic and nonallylic diphosphates are present within the metabolic pool, coupling of these structures will occur to yield the corresponding JH carbon skeletons. This reaction involves the action of a prenyltransferase enzyme, which is likely to be related to farnesyl diphosphate synthase (FPPSase, or FPS). FPS catalyzes the coupling of DMAPP with two molecules of IPP to form FPP as end-product and geranyl diphosphate (GPP) as a tightly bound intermediate (Fig. 5). This enzyme has been well studied in noninsect systems, and the crystal structure of recombinant avian FPS is known. FPSs share significant sequence homology, particularly within domains 2 and 5, which have been shown to possess catalytically important aspartate residues.

Initial studies have focused on the characterization of prenyltransferase from the entire body (minus the gut) of lediopteran insects. Two purified FPSs from *Bombyx mori* are known to posses distinct enzymological properties, including substrate $K_m$, preferred divalent metal cofactor, and activity in the presence of nonionic detergent. Only one of these synthases efficiently couples homologous substrates, although this activity is not the same as that derived from *M. sexta* CA homogenates. For the latter, a strong preference for the substrate HDMAPP was seen, and both homogeranyl diphosphate (HGPP) and bishomogeranyl diphosphate (BHGGP) were excellent substrates of the prenyltransferase. The intermediacy of (*Z*)-3-methyl-2-pentenyl diphosphate in the biosynthesis of methyl-JH I was established by preparing authentic hormone using purified FPS of pig liver to couple nonallylic substrate with BHGPP.

FPSs from *D. melanogaster*, *B. mori*, and *A. ipsilon* are known. All share significant sequence homology to other FPSs; however, the latter two, which are transcribed in the CA and in other insect tissues, are unusual in that the aspartate-rich region of domain 2 has been modified from DDXXD (X representing any amino acid) to NDIME. It remains to be determined whether the moth clones code for an active prenyltransferase and whether they are involved in JH biosynthesis.

Homologue studies using FPS of pig liver and pumpkin show loose specificity for homologous substrates, and in competition experiments, all possible geranyl- and farnesyl-type structures are formed in essentially equal quantities. Because lepidopteran insects strictly control the structure of JH homologue produced, it can be concluded that FPS related to JH biosynthesis is structurally different from other prenyltransferases.

## IV. LATER STEPS IN JH BIOSYNTHESIS

Once the construction of the sesquiterpenoid diphosphate is completed, JH biosynthesis diverges from the mevalonic acid pathway. The last steps of hormone

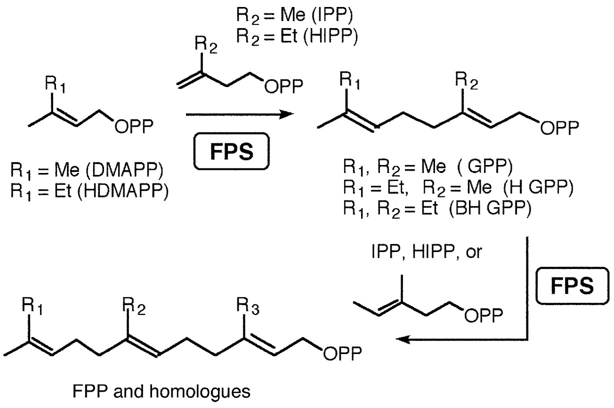

FIGURE 5 Formation of farnesyl diphosphate (FPP) by farnesyl diphosphate synthase (FPS). IPP, Isopentenyl diphosphate; HIPP, homoisopentenyl diphosphate; GPP, geranyl diphosphate; HGPP, homoGPP; BHGPP, bishomoGPP; DMAPP, dimethylallyl diphosphate; HDMAPP, homodimethylallyl diphosphate.

construction involve diphosphate hydrolysis, oxidation at C-1 to yield carboxylic acid, epoxidation of the terminal olefin, and methylation of the acid moiety. The order of methylation and epoxidation appears to be species specific. Lepidopteran insects prepare and methylate JH acid, whereas orthopteran and dictyopteran insects convert methyl farnesoate to JH.

The conversion of FPP to farnesol is mediated by the action of phosphatase. Because there is significant phosphatase activity in CA homogenates, it is likely that the enzymes that perform this conversion are nonspecific. From isoelectric focusing studies on larval CA homogenates, it appears that the bulk of the endogenous phosphatase activity has a pH close to neutral. In comparing the hydrolysis of IPP versus HIPP, both are converted to alcohol with similar efficiency, but HIPP is more slowly hydrolyzed to product, as would be expected given its hydrophobicity.

It is known that both farnesol and farnesoic acid (FA) are precursors to JH III, thus initial studies focused on determining how this oxidation occurred. Incubation of larval and adult *M. sexta* CA homogenates with radioactively labeled farnesol yielded significant quantities of farnesal (Fig. 6). Furthermore, inclusion of the redox cofactor nicotinamide adenine dinucleotide ($NAD^+$) caused a significant increase in farnesoic acid production. Although activities leading to formation of aldehyde and acid were found in mitochondrial, cytosolic, and, to a lesser extent, microsomal locations of adult CA cells, the conversion of farnesol to farnesal was localized in the cytosolic fraction of larval *M. sexta* CA homogenates. The aldehyde-forming activity, which has an apparent $K_m$ of 1 $\mu M$ for farnesol, was selectively inhibited by the active-site titrant tetranitromethane. It was not stimulated by the addition of $NAD^+$, and instead required oxygen for activity. It can be inferred from these studies that two enzymes are required for the conversion of farnesol to FA and that they are formally an alcohol oxidase and aldehyde dehydrogenase, respectively.

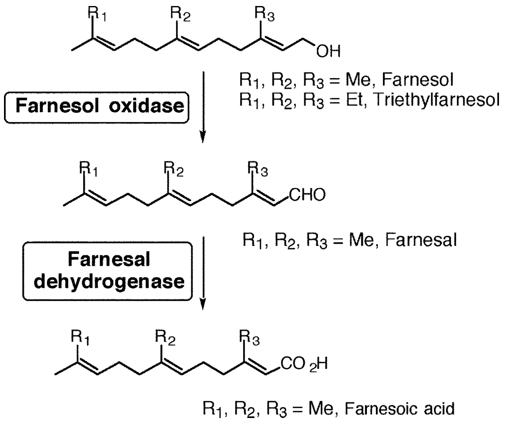
R1
R2
R3
OH
Farnesol oxidase
R1, R2, R3 = Me, Farnesol
R1, R2, R3 = Et, Triethylfarnesol
R1
R2
R3
CHO
Farnesal dehydrogenase
R1, R2, R3 = Me, Farnesal
R1
R2
R3
CO2H
R1, R2, R3 = Me, Farnesoic acid

**FIGURE 6** Farnesol oxidase and farnesal dehydrogenase.

The substrate specificity of farnesol oxidase (formally referred to in the literature as farnesol dehydrogenase) has been investigated. Using *M. sexta* adult CA homogenates, geraniol was found to be efficiently oxidized to geranial but the (2Z)-isomer of farnesol and of geraniol (i.e., nerol) was not. Further studies with *M. sexta* larval and adult CA homogenates show that the minimum substrate requirements are a trans-1° allylic alcohol that bears a hydrophobic tail. In comparing the ability of homologous farnesol structures to inhibit farnesol metabolism, it was found that substitution of methyl for ethyl at C-3 caused loss of inhibitory activity. Additionally, metabolism studies indicated that triethylfarnesol (precursor to JH 0) was not efficiently oxidized.

As previously stated, the order of the last two steps in JH biosynthesis is species dependent. In Lepidoptera, epoxidation precedes methylation; interestingly, both *H. cecropia* and *M. sexta* male moths lack JH acid *O*-methyltransferase activity in the CA. In *L. migratoria*, *Blaberus giganteus*, and *P. americana* CA homogenates, FA was found to be a poor substrate of the epoxidase and a buildup of methyl farnesoate (MF) was observed. From these studies it is inferred that it is in another biosynthetic pathway of orthopteran and dictyopteran insects that the MF is formed and subsequently epoxidized to JH III. This would also imply the presence of distinct FA *O*-methyltransferase and JH acid *O*-methyltransferase enzymes.

Both of the *O*-methyltransferases are cytosolic proteins and require *S*-adenosylmethionine (SAM) as cosubstrate. Because of its high polarity and impermeability to cell membrane, SAM must be replaced by L-methionine in whole-gland assays. Typically, high concentrations of L-methionine (200–300 $\mu M$) are needed, but *D. punctata* efficiently methylates FA with 10 times lower concentrations, indicating either a more efficient *O*-methyltransferase or methionine adenosine transferase.

In studies concerning the methylation of various carboxylic acids, differences in substrate specificity were observed for the two *O*-methyltransferases. JH acid *O*-methyltransferase of *H. cecropia* appears to be more active toward homologous substrates; thus, racemic JH I acid was more efficiently methylated than was JH II, and compared to JH I, there was only 15% incorporation of L-methionine into JH III. The enzyme also displayed enantioselectivity, whereby product analysis indicated a large preponderance of the 10R isomer (Fig. 7).

Several structure–activity relationship studies have been performed with locust FA O-methyltransferase. The enzyme shows more lax specificity than does the corresponding lepidopteran enzyme, in that a variety of FA and JH acid derivatives serve as substrates. An α,β-unsaturated carboxylic acid possessing a hydrophobic tail of at least two isoprene units appears sufficient for activity, and only electronic perturbation of the C-2, C-3 double bond is detrimental for activity. Because both enantiomers of JH III are efficiently methylated and bishomofarnesoic acid is as good a substrate as FA, the methyltransferase does not show the homologue specificity characteristic of the lepidopteran enzyme.

It appears that regulation of methyltransferase occurs late in the biosynthetic pathway. In *D. punctata*, FA O-methyltransferase activity was found to parallel JH titer levels in both the fourth larval stadium and the adult female CA glands. In Lepidoptera, methyltransferase activity disappears at the last larval stage such that only JH acid is formed before pupation. These results suggest that JH acid has a hormonal function and should be considered as an additional JH structure.

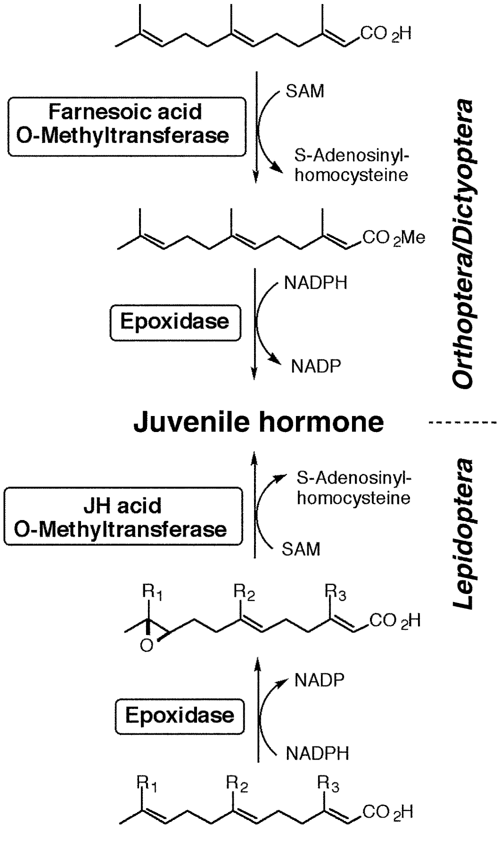

FIGURE 7 Epoxidase and O-methyltransferases.

The epoxidation of FA or MF is mediated by a microsomal NADPH-dependent oxidase. Whether two distinct enzymes exist, as is the case for O-methyltransferase, is not known. The enzyme has been characterized in both locust and cockroach CA homogenates. In both cases, MF is the preferred substrate over FA, and the 10R epoxide isomer is formed exclusively. The enzyme is inactivated by a variety of mixed-function oxidase inhibitors, including piperonyl butoxide and methylene blue, and by the cytochrome P450 inhibitor, carbon monoxide. For the latter, activity was regained on white light treatment, as would be expected if photodissociation of CO from a heme center had occurred. Using *L. migratoria* CA homogenates, the apparent $K_m$ for MF and NADPH was 7.7 and 2.6 $\mu M$, respectively. Because epoxidase activity comigrated with cytochrome *c* when separating the epoxidase from other endogenous proteins, it was proposed that the epoxidase is a P450 monooxygenase that requires cytochrome *c* (and therefore NADPH) as its redox cofactor.

The isolation of $JHB_3$ in Diptera indicates that further epoxidation of the C-6, C-7 olefin occurs; however, it is not known whether the same enzyme that is required for JH III biosynthesis is involved.

## V. METABOLIC CONTROL OF JH BIOSYNTHESIS

Excluding pathways that involve the degradation of synthesized hormone, JH biosynthesis is regulated in several ways: (1) by changes in concentration of biosynthetic intermediates, (2) by the presence (or absence) of neuropeptides produced in the brain, (3) by modulations in neurotransmitter concentrations and their corresponding receptors, and (4) by changes in cellular ion concentrations. The latter two factors appear to provide a simple on/off switch for the CA, whereas the former two factors appear to effect specific metabolic events of JH biosynthesis.

In *D. punctata*, the addition of exogenous FA caused an increase in JH biosynthesis that was greater than would be expected from its simple incorporation into the metabolic pool. This stimulatory effect, known as FEAR, only occurs at certain stages of development and may therefore represent a biologically relevant feedback enhancement of JH biosynthesis. Acetoacetyl coA was found to inhibit insect HMG coA synthase at low concentrations, and although its overall effect on JH biosynthesis is not know, it is reasonable to conclude that a buildup of this intermediate inhibits mevalonate synthesis. No other intermediates have been shown to cause a regulatory effect on JH biosynthesis, including farnesol, which is known to modulate the stability of HMGR in other organisms and is considered the nonsteroidal feedback inhibitor of the MVA pathway. Because insects lack the ability to make sterols (due to the absence of both squalene synthase and oxidosqualene cyclase), it would seem that these animals have developed an alternate regulatory mechanism for mevalonate synthesis.

The presence of allatostatin (inhibiting) and allatotropin (stimulating) neuropeptides is considered an important regulatory mechanism for JH biosynthesis. Although the mode of allatotropins is not known, the effect of allatostatins on JH biosynthesis in *D. punctata* has been elucidated. The allatostatic effect of the tridecapeptide APSGAQRLYGFGL (amide) was not due to inhibition of acetoacetyl thiolase, HMG coA synthase, or HMG coA reductase. Instead, the site of inhibition was found to be the site of transport of acetyl coA from the mitochondria to the cytosol—specifically, movement of citrate across the mitochondrial membrane and/or conversion of citrate to acetyl coA in the cytosol. Lepidopteran insects show different activity with regard to mitochondrial processing, thus it is not known whether a similar effect is seen in these insects.

## VI. SUMMARY

Insects biosynthesize JH by a unique metabolic pathway that is a hybrid between the traditional MVA pathway and a unique insect-specific pathway. JH III and all of the JH homologues are formed in the same manner, although the latter utilize a combination of propionyl coA and acetyl coA as carbon source. Because insects do not make sterols, their ability to modulate the MVA portion of the JH biosynthetic pathway by oxysterols and by farnesol is compromised. Instead, it appears that control occurs earlier and involves the movement of carbon precursors from the mitochondria to the cytosol.

## Glossary

**homologue** Compounds that have a similar core structure but have carbon skeletons that vary by one or more atoms.

**juvenile hormone** A group of structurally related sesquiterpenoids that are biosynthesized in insects and some plant species. In insects, these compounds control insect growth, development, and female fertility.

**mevalonic acid pathway** Biosynthetic pathway present in all organisms. Involves a group of enzymes responsible for the production of mevalonate, which is the universal intermediate in terpene metabolism.

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysone Action in Insect Reproduction • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormones, Chemistry of • Neuropeptides: Roles in Regulation of Juvenile Hormone Production**

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# Juvenile Hormones, Chemistry of

Noelle A. Granger
*University of North Carolina, Chapel Hill*

I. INTRODUCTION
II. DISCOVERY OF THE CHEMICAL NATURE OF JUVENILE HORMONES
III. JH BIOSYNTHESIS
IV. JH ACTIVITY
V. JH TITERS
VI. JH METABOLISM
VII. NONTRADITIONAL JHs

**The juvenile hormones are the predominant secretory products of the insect corpora allata, the two tiny glands located behind the insect brain. These versatile hormones inhibit metamorphosis during larval life and act as gonadotropic hormones in the adult, controlling ovarian development and vitellogenin synthesis. They play a role in caste determination in the social insects, in regulation of behavior in honeybee colonies, in polyphenisms of aphids and locusts, and in diapause regulation in larvae and adults.**

## I. INTRODUCTION

The juvenile hormones (JHs) are generally considered unique to insects and to a few plant species. They are neither steroid nor peptide, but rather possess an acyclic sesquiterpenoid skeleton. The different members of the JH family have methyl or ethyl groups at three branches of the carbon skeleton (C-3, C-6, and C-11) and may have two, rather than one, epoxide moieties (Fig. 1). This article provides an overview of the history of the chemical identification of the juvenile hormones, a description of the genesis of the carbon skeleton of the known members of the JH family, a discussion of the current dogma of JH activity, titers, and degradation, and an introduction to our knowledge of nontraditional JHs.

## II. DISCOVERY OF THE CHEMICAL NATURE OF JUVENILE HORMONES

The existence of a juvenilizing factor was originally demonstrated in the mid 1930s by Wigglesworth, who used the term "juvenile hormone." The history of the chemistry of the JHs began in 1956, when Carroll Williams discovered that abdominal tissues of the male *Hyalophora cecropia* moth (Cecropia) contained large quantities of lipid with JH activity, which could easily be extracted into organic solvents. This enabled the isolation and identification of the principal Cecropia JH (now called JH I) by Röller and his colleagues in 1967, through interpretation of mass and spectral data and microchemistry, with tracking of the active moiety by bioassay. The basic structure of this hormone—methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2*E*,6-tridecadienoate—was confirmed by chemical synthesis. The stereochemistry of the oxirane ring and of the double bond at C-6 was subsequently established as being cis and trans, and Meyer and Hanzmann reported in 1970 that the natural hormone was not racemic. The absolute configurations at the chiral centers (C-10 and C-11) were established in 1971 as 10R, 11S (Fig. 1).

Very shortly after the publication of the structure of JH I, Meyer and his colleagues published the structure of a second Cecropia JH, one that existed in relatively smaller amounts and to which Röller had paid little attention because of its low activity in the bioassay being used. This was JH II, which is structurally identical to JH I with the exception of having a methyl group at C-7 instead of an ethyl group (Fig. 1).

The structure of the third JH, JH III, was elucidated in a different fashion. Rather than extracting large quantities of JH for chemical characterization, Judy *et al.* maintained *in vitro* adult female corpora allata from the tobacco hornworm, *Manduca sexta*, and radiolabeled the gland secretory products by the inclusion of methyl [$^{14}$C]methionine in the culture medium. Spectral, microchemical, and chromatographic methods revealed that this JH was a

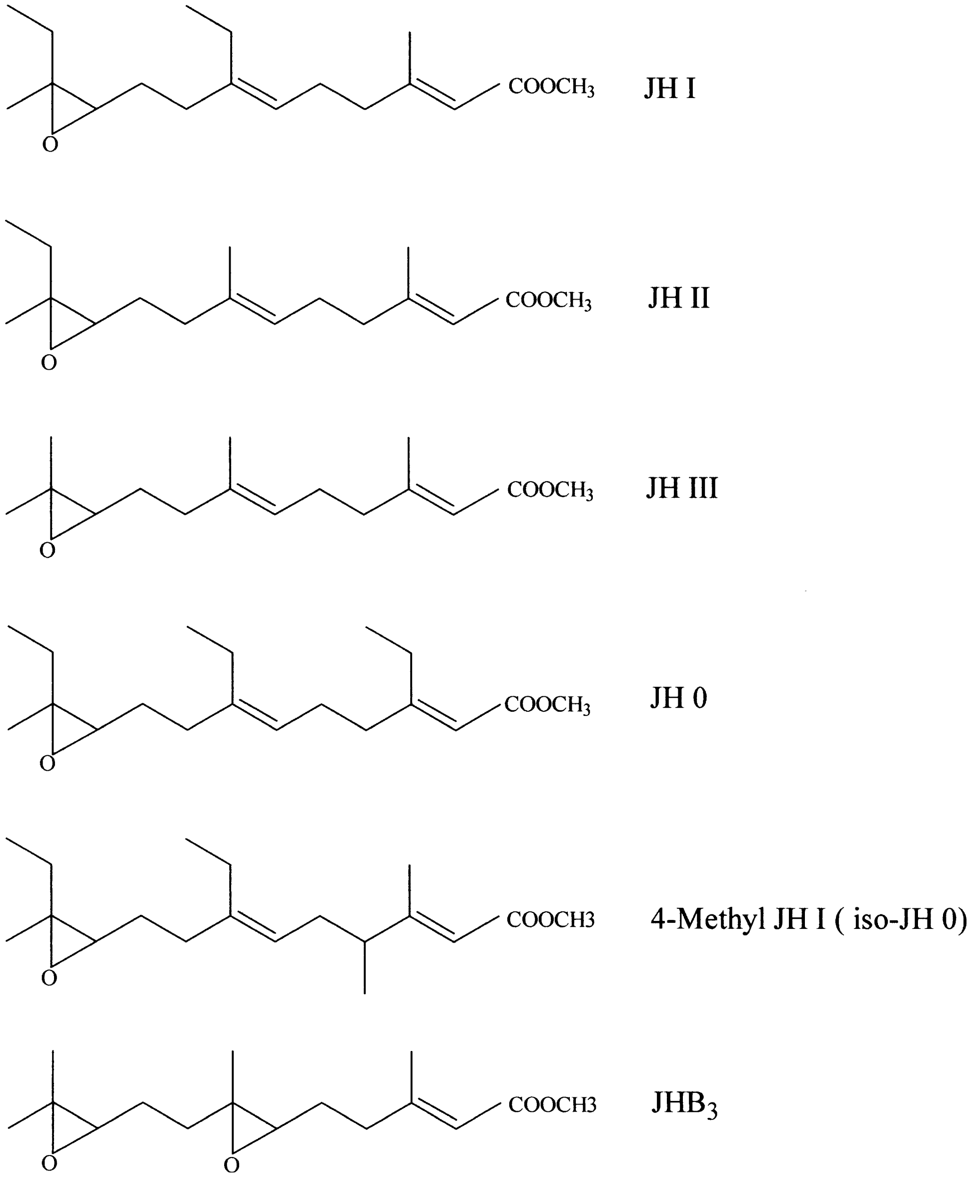

FIGURE 1 Structures of the juvenile hormones, in order of their discovery.

lower homologue of JH II, in which the three branches of the carbon skeleton at C-3, C-7, and C-11 are methyl groups (Fig. 1).

A trihomosesquiterpenoid, JH 0, and an isomer, 4-methyl JH I (iso-JH 0), were identified by Schooley and colleagues in the eggs of *Manduca*. Nothing is known of the functions of these two compounds. Most recently, an active JH bisepoxide ($JHB_3$) was isolated from incubations of larval ring glands of the fruit fly, *Drosophila melanogaster*, and was identified by chromatographic and gas chromatography/mass spectrometry (GC/MS) analysis (Fig. 1). $JHB_3$ elicits a response similar to that of JH III in a *Drosophila* bioassay and has been found in several other flies. However, because this homologue is not produced by mosquito corpora allata, $JHB_3$ may be a JH only in higher Diptera.

The JHs, as acyclic sesquiterpenoid epoxides, are highly unusual molecules. They have now been identified in approximately 100 insect species, representing at least 10 insect orders, although the existence of JHs in the most primitive insect orders (Protura and Diplura) has not yet been determined. JH III is the only, or the predominant, homologue in most of the species in which JHs have been identified; the higher homologues (iso-JH 0, JH 0, JH I, and JH II) are found only in the more advanced insect orders. In these advanced orders, e.g., the Lepidoptera, different, multiple homologues can be found in the same insect at different times during development.

Plants contain a number of relatively nonpolar compounds that possess JH-like activity in insect bioassays. None of these compounds has been found to closely resemble the JHs in chemical structure, and most of these plant-derived JH mimics affect only a narrow range of insect species. There is only one report of the identification of JH III (and methyl farnesoate, a precursor to JH III; see Fig. 2) in plants, in the sedges *Cyperus iria* and *Cyperus aromaticus*.

## III. JH BIOSYNTHESIS

JH III bears a strong structural similarity to the ubiquitous sesquiterpene farnesol, and therefore it is not surprising that the biosynthesis of JH III occurs via the normal terpenoid pathway, involving the sequential head-to-tail linkages of three five-carbon isoprenoid units. The isoprenoid units are formed from acetate via mevalonic acid (Fig. 2). The resulting farnesyl pyrophosphate undergoes esteratic cleavage to farnesol, which is then oxidized to the carboxylic acid. There is a dichotomy in the last two steps in the biosynthetic pathway, depending on the insect species. In Lepidoptera (butterflies and moths), for example, it appears that a 10,11-epoxidase converts the carboxylic acid to the epoxy acid (JH acid), which is then methylated to the methyl ester by an *O*-methyltransferase. In Orthoptera (grasshoppers, locusts, and crickets) and Dictyoptera (cockroaches) the converse appears to be true: epoxidation follows methyl transfer.

The homosesquiterpenoids JH 0, JH I, and JH I are biosynthesized from a mixture of isoprenoid (five-carbon) and homoisoprenoid (six-carbon) units (Fig. 2). Critical work by Schooley and coworkers, using cell-free homogenates of *Manduca* corpora allata, demonstrated that insects are unique in being able to synthesize homoisoprenoid units from acetate and propionate via 3′-homomevalonate (Figs. 2 and 3). Thus the synthesis of JH I would utilize one dimethylallyl pyrophosphate molecule (isoprenoid unit) and two ethylmethylallyl pyrophosphate molecules (homoisoprenoid units) for each molecule of JH I (Fig. 3). A molecule of JH II would incorporate two isoprenoid units and one homoisoprenoid unit, whereas three homoisoprenoid units would form the skeleton of JH 0 (Fig. 4).

The JHs have three unsaturated positions: $\Delta^{2,3}$, $\Delta^{6,7}$, and $\Delta^{10,11}$. The naturally occurring hormones have a 2E,6E 10-cis (JH I and JH II) configuration or a 2E,6E (JH III) configuration. The chiral centers at C-10 and C-11 in the naturally occurring hormones have been assigned a 10R,11S (JH I and JH II) or a 10R (JH III) configuration.

Specificity for the production of a particular homologue appears to be restricted to early enzymatic steps in the biosynthetic pathway. The two terminal steps have been shown to lack substrate specificity in a number of species. The best example is that of *Schistocerca gregaria* (desert locust) corpora allata, which normally synthesize only JH III *in vitro*, but will produce JH I as their sole product when incubated with dihomofarnesoic acid.

## IV. JH ACTIVITY

As previously mentioned, the corpora allata of some insects secrete only one JH, whereas others secrete a mixture of two or three. JH III is the principal form of JH found in Coleoptera (beetles), Diptera (flies), Hemiptera (bugs), and Hymenoptera (bees and wasps), although the corpora allata of some species secrete other homologues when incubated *in vitro*. The corpora allata of Lepidoptera are unique in their production of a mixture of JHs, both *in vivo* and *in vitro*. *Manduca sexta* corpora allata synthesize five homologues of JH in different amounts at different times during the life cycle of the moth, and *M. sexta* corpora allata have been shown to secrete three JH homologues *in vitro* (JH I, JH II, and JH III), in addition to their acids. Each of the JHs has a quantitatively different relative activity when bioassayed in insects of different orders, and homologues that are not present in a particular species can nevertheless elicit a typical JH response. There is no evidence to date that JH I, JH II, and JH III elicit qualitatively different physiological effects. The most lipoidal homologues, JH I and JH II, are usually more active in bioassays, compared to JH III, possibly because the greater polarity of JH III makes it more susceptible to degradation. In general, the activities of JH I and JH II are inversely related to their titers *in vivo*—that is, the most potent homologues in the bioassays are present in much lower amounts *in vivo*, compared to JH III. The significance of the mixtures of homologues in any species or at any developmental stage is unknown.

The geometrical configurations of the $\delta^{2,3}$, $\delta^{6,7}$, and $\delta^{10,11}$ positions in the JHs are important to

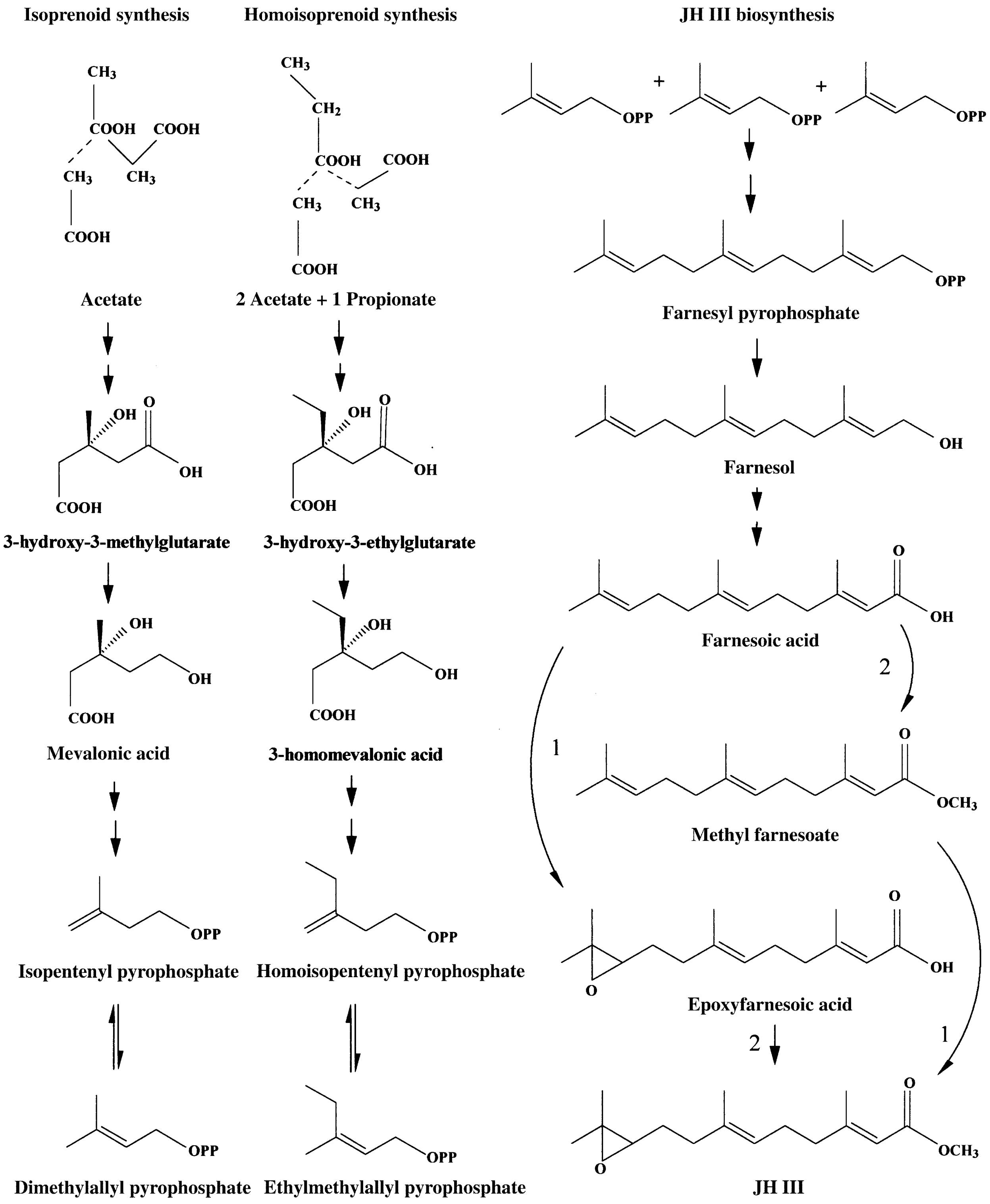
Isoprenoid synthesis
Homoisoprenoid synthesis
JH III biosynthesis
CH3
COOH
CH2
Acetate
2 Acetate + 1 Propionate
OH
O
3-hydroxy-3-methylglutarate
3-hydroxy-3-ethylglutarate
Mevalonic acid
3-homomevalonic acid
OPP
Isopentenyl pyrophosphate
Homoisopentenyl pyrophosphate
Dimethylallyl pyrophosphate
Ethylmethylallyl pyrophosphate
+
Farnesyl pyrophosphate
Farnesol
Farnesoic acid
2
1
OCH3
Methyl farnesoate
Epoxyfarnesoic acid
JH III

FIGURE 2 Juvenile hormone biosynthesis, showing differences in the sequence of the two terminal enzymatic steps, based on insect order. The production of JH III from farnesoic acid, methyl farnesoate, and epoxyfarnesoic acid involves two enzymes (1, epoxidase; 2, O-methyltransferase). It is generally accepted that the sequence 1, 2 occurs in Lepidoptera, and 2, 1 occurs in Orthoptera and Dictyoptera.

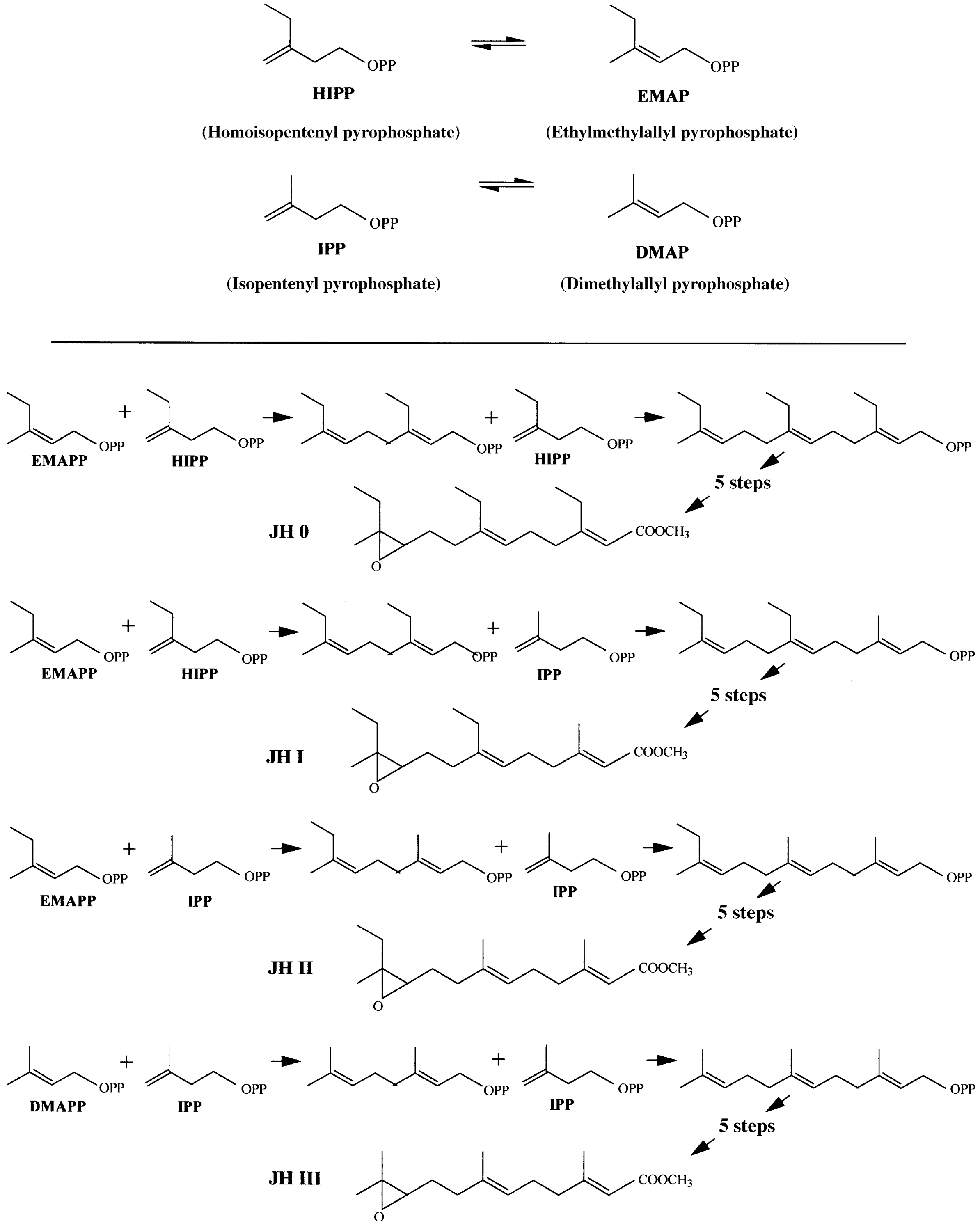
OPP
HIPP
(Homoisopentenyl pyrophosphate)
OPP
EMAP
(Ethylmethylallyl pyrophosphate)
OPP
IPP
(Isopentenyl pyrophosphate)
OPP
DMAP
(Dimethylallyl pyrophosphate)
EMAPP
HIPP
HIPP
5 steps
JH 0
COOCH3
EMAPP
HIPP
IPP
5 steps
JH I
COOCH3
EMAPP
IPP
IPP
5 steps
JH II
COOCH3
DMAPP
IPP
IPP
5 steps
JH III
COOCH3
O

FIGURE 3 Scheme for the biosynthesis of JH 0, JH I, JH II, and JH III from isoprenoid and homoisoprenoid units.

biological activity. The naturally occurring isomers of JH (2E,6E 10-cis for JH I and JH II and 2E,6E for JH III) have been shown to be the most biologically active. Other geometric isomers display varying degrees of decreased activity, with the 2Z,6Z 10-trans isomer (the complete opposite of the natural isomer) having the least activity. The enantiomeric purity of the hormones may also be important for biological activity. For example, only the natural 10R enantiomer of JH III is bound by the JH-binding protein in *Manduca* (see Section VI).

## V. JH TITERS

Hemolymph, whole-body, and tissue titers of JH have been determined in a wide variety of species. These titer measurements have shown that different insect species can have vastly different amounts of the JH homologues and that these amounts, measured principally in the hemolymph, change during the course of development. The changes in titer are linked to specific, JH-controlled developmental events. Physiological levels of JH generally range from $10^{-8}$ to less than $10^{-9}$ *M*, but higher values have been observed. For example, in the adult female cockroach, *Diploptera punctata*, as much as $10^{-5}$ *M* has been measured. The significance of differences in JH titers between species and between developmental stages, like the significance of differences in the number and mix of homologues, is unknown.

## VI. JH METABOLISM

Thus far, only two definitive pathways for the metabolism of JH have been described: (1) hydrolysis of the methyl ester of JH by hemolymph esterases, yielding JH acid, and (2) hydration of the C-10, C-11 epoxide by microsomal epoxide hydrolases (epoxidases), yielding JH diol (Fig. 4). Three classes of JH-binding proteins (JHBP) have been identified in insects, and it is assumed that most JH in insect hemolymph is bound to these binding proteins and is thus protected from metabolism by nonspecific esterases. Insect fat body is the primary source of a highly stable JH-specific esterase that either metabolizes JH that is bound to JHBP or shifts the equilibrium between JHBP-bound and free JH, increasing the amount of free JH that can be

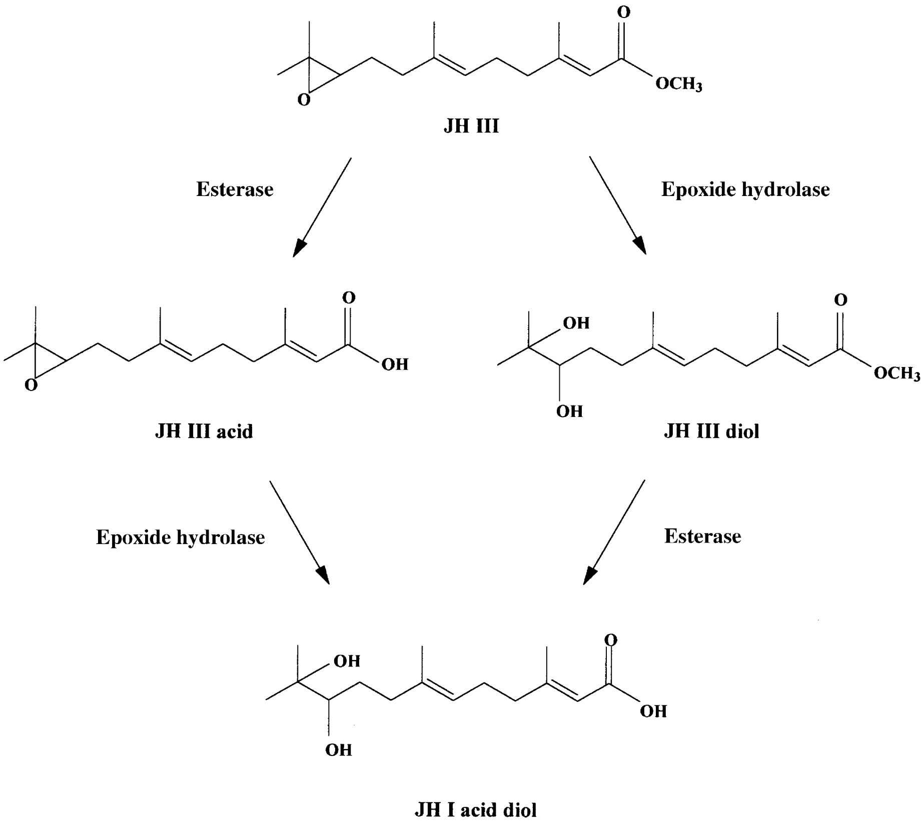

FIGURE 4 Pathways of JH metabolism.

metabolized. In either case, the circulating JH titer would decrease. Current dogma states that JH is metabolized by JH-specific esterase to JH acid, which is then hydrolyzed at the cellular level by epoxide hydrolase, yielding the JH acid diol. Although activity of JH epoxide hydrolase has thus far been assigned a secondary role, recent *in vivo* studies of the metabolism of JH acid indicate that in some species JH diol may be the primary metabolite of JH.

In addition to the primary metabolites of JH (the acid, the diol, and the acid diol), a number of highly polar JHs and/or JH metabolites have been reported, the structures of which are largely unknown. Thus, other pathways for the degradation of JH may exist.

## VII. NONTRADITIONAL JHs

JH acids (Fig. 1) have been identified as the products of adult and larval lepidopteran corpora allata. The corpora allata of adult male Cecropia lack the *O*-methyltransferase enzyme and thus synthesize and release JH acids into the hemolymph. JH acid stored in the male accessory gland can be converted to JH by an *O*-methyltransferase. In *M. sexta*, the corpora allata gradually convert from the secretion of JH to the secretion of JH acid during the first 4 days of the last larval stadium. By the time commitment to pupal development is complete, JH acid is the only product. Synthesis of JH resumes in the adult. Based on the situation in Cecropia, it is assumed that JH acid is converted to JH by peripheral tissues, and there is evidence that this occurs in the imaginal discs in *Manduca*. However, there is preliminary information that JH acid may not be a simple degradation product of JH or a precursor to JH in the biosynthetic pathway, but rather is a hormone in its own right. Treatment of Verson's gland in *Manduca* with JH acid or the acid form of a JH analogue, plus a low dose of an ecdysteroid, has been reported to induce metamorphic competency of the gland.

Methyl farnesoate (MF), an intermediary in the biosynthetic pathway of JH (Fig. 2), is released from the corpora allata of a number of insect species and has been reported to synergize the activity of JH II and JH III bisepoxide. Indeed, this molecule is suggested to be the JH of the other class of Arthropoda, the Crustacea. Crustaceans and insects have the same biosynthetic pathway for the synthesis of MF (see Fig. 2), and MF has been isolated from a number of crustacean species, most recently larvae of the barnacle. In spider crabs, from which MF was first isolated, MF has been proposed to control reproduction and to act as the "status quo" hormone, inhibiting metamorphosis. The natural isomer of crustacean MF inhibits metamorphosis in barnacles as well.

Finally, there is evidence that hormones structurally dissimilar to JH have JH-like functions in insects. For example, ovarian ecdysteroids can function as gonadotropins in mosquitoes and other Diptera, as well as in the locust. Thyroid hormones have recently been shown to mimic the action of JH on follicle cells of locust ovary, although the source of these hormones would be exogenous.

## Glossary

**chirality** Chemical nomenclature for an organic molecule, describing the right- or left-handedness of an asymmetric carbon; any carbon with four different groups attached to it is a chiral center.

**corpus allatum** A typically paired, retrocerebral endocrine gland of a single cell type, which synthesizes and secretes juvenile hormones. The corpus allatum is innervated by nerves from the brain and the subesophageal ganglion.

**corpus cardiacum** A typically paired, retrocerebral neurohemal organ for the neurosecretory cells of the brain. The corpus cardiacum contains intrinsic neurosecretory cells, which release a variety of neurohormones, as well as the axons of cerebral neuron and neurosecretory cells, which traverse this organ to reach the corpus allatum.

**ecdysteroid** Member of a family of insect and crustacean steroids responsible for the promotion of molting and growth and development; ecdysteroids are also found in plants.

**enantiomers** Different compounds with the same molecular formula (isomers) but that are mirror reflections of each other; enantiomers are designated as cis or trans, R or S, or E or Z and occur only when the isomers contain chiral molecules.

**isomers** Different compounds with the same molecular formula, but differing in the order in which their atoms are joined (structural isomers) or in the arrangement of their atoms in space (stereoisomers, diastereoisomers).

**polyphenism** The occurrence of several distinct phenotypes or forms in a given species, each of which develops facultatively in response to some cue from the internal or external environment; sequential polyphenism occurs in the metamorphosis of insects (larval, pupal, and adult forms).

**racemic** Denoting a mixture of optically active compounds (enantiomers), with the mixture itself being optically inactive because it is composed of equal amounts of all the enantiomers; a single enantiomer is optically active because it rotates the plane of a beam of plane-polarized light passing through it.

**ring gland** Endocrine gland in the higher Diptera (flies) that encircles the foregut and that is composed of fused corpora allata, corpora cardiaca (which release neuropeptides), and prothoracic glands (which secrete ecdysteroids).

**sesquiterpene** A class of hydrocarbon compounds having a 15-carbon skeleton, built from five-carbon (isoprene) units that are linked head to tail or in rings; many contain oxygen.

## See Also the Following Articles

**Ecdysone Action in Insect Development • Ecdysone Action in Insect Reproduction • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormone Biosynthesis • Neuropeptides: Roles in Regulation of Juvenile Hormone Production**

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# Knockout of Gonadotropins and Their Receptor Genes

Ch. V. Rao and Z. M. Lei
*University of Louisville Health Sciences Center*

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**Phenotypes of both gonadotropin knockout and gonadotropin receptor knockout animals have confirmed and extended the knowledge about the role of gonadotropins in ways that were not possible previously. Moreover, LH receptor knockout animals are beginning to serve as important models to further investigate the importance of nongonadal LH actions in the body and how these might be useful in better understanding LH biology and in exploring novel therapeutic applications of hCG in multiple clinical conditions. Complete characterization of knockout animal models can also serve as a guide to what symptoms to look for in patients with corresponding gene mutations.**

## I. INTRODUCTION

Hormonal ablations created by surgical, pharmacological, or immunological methods have historically provided data on complex interrelationships between gonadotropins and their roles in the hypothalamus–pituitary–gonadal axis. Cell culture systems have complemented and extended these findings. These approaches have dramatically increased the understanding of the roles gonadotropins play in pathophysiology. There are inherent limitations, however, to each of these approaches as they are nonspecific, variable, and/or incomplete. Gene-targeting technology has allowed generating mice that lack a functional gene, as well as germline passage of the mutation. These animals have provided a great deal of insight into the roles of gonadotropins in pathophysiology and also served to confirm the hypothesis generated by using other approaches. In addition, certain unforeseen phenotypes have emerged. Although both males and females are affected by knockout of the gonadotropin subunit or their receptor genes, this article focuses primarily on the female phenotype. Where applicable, the phenotypes of ligand and receptor knockouts as well as the relevant phenotypes of knockout models for other signaling systems will be contrasted and compared. Finally, the phenotypes of inactivating and activating mutations of the gonadotropin subunits and their receptor genes in humans will also be compared and discussed.

## II. FAMILIES OF GONADOTROPINS AND THEIR RECEPTORS

### A. Gonadotropins

Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG) belong to the glycoprotein hormone family that includes thyroid-stimulating hormone (TSH). LH and FSH are primarily secreted by gonadotropes in the anterior pituitary gland and hCG is primarily synthesized by syncytiotropoblasts in the human placenta. These hormones, especially hCG, are also produced in small amounts by a number of other normal and cancer tissues.

Members of these families are heterodimers of noncovalently bound α- and β-subunits. The α-subunit is identical, whereas β-subunits, which determine hormone specificity, are different among these hormones except for LH and hCG. These two hormones have similar but not identical β-subunits. The β-subunit of hCG has 30 additional amino acids at the C-terminus carrying four O-linked oligosaccharide chains, which are thought to be responsible for its longer half-life in the circulation compared with LH. Crystal structure studies revealed that LH and hCG contain a so-called cystine-knot motif that is commonly present in a family of growth factors, which include nerve growth factor, platelet-derived growth factor-β, and transforming growth factor-β (TGF-β).

The α-subunit of all the glycoprotein hormones is encoded by a single gene located on human chromosome 6q21.1–q23. A single gene encoding the FSH-β subunit is located on human chromosome 11p13. The β-subunits of LH and hCG are encoded by a gene cluster, which spans approximately 52 kb on human chromosome 19q13.32. The cluster contains one LH β-subunit gene and six hCG β-subunit genes. It appears that only three of the hCG β-subunit genes are transcriptionally active.

### B. Gonadotropin Receptors

LH and hCG share the same receptor, whereas FSH has its own receptor. Both receptors belong to the G-protein-coupled receptor superfamily. Members of this family are single-chain transmembrane glycoproteins composed of a large extracellular hormone-binding domain, seven transmembrane-spanning regions, and a relatively small intracellular region, which is coupled to G-proteins. Nucleotide sequence conservation is high in the same receptor from different species. Between the receptors, however, the sequence of the transmembrane region is highly conserved, whereas the sequence of the extracellular domain is much less conserved, which allows for hormone specificity of binding.

The LH receptor is a single-copy TATA-less gene located on human chromosome 2p21. It spans over 70 kb containing 11 exons. The first 10 exons encode the extracellular hormone-binding domain and the last exon encodes the rest of the receptor. The FSH receptor gene is located on the same chromosome, 2p21–p16, and has structural similarity except that it contains only 10 exons. The presence of multiple transcription initiation sites and alternative splicing mechanisms contributes to more than one transcript as is typically seen with these receptors. The size of full-length LH and FSH receptors is very similar, i.e., 80–90 kDa.

Binding of LH, hCG, and FSH to their cognate receptors results in the activation of adenylate cyclase, an increase in cyclic AMP (cAMP) formation, and activation of protein kinase A catalytic subunit, resulting in increased phosphorylation of $Ca^{2+}$/ cAMP-response element-binding protein and other target proteins and kinases. A single signaling mechanism may not explain all the actions of gonadotropins. Thus, protein kinase C and mitogen-activated protein kinase, as well as other kinases, and potential cross talk between them may be involved.

### C. Gonadotropins' Action

Ovaries are classical targets of LH, hCG, and FSH actions. Thus, they contain high levels of these receptors. Whereas FSH receptors are expressed only in granulosa cells, LH receptors are present in different ovarian cell types. Thus, granulosa and luteal cells contain high levels, whereas stroma, cumulus, surface epithelial cells, oocytes, and blood vessels contain low levels. LH receptors in thecal cells are constitutive, whereas those in granulosa cells are acquired during mid-to-late stages of follicular growth.

FSH actions in granulosa cells result in an increase in aromatase that converts androgens into estrogens. FSH and estrogens work in a synergistic manner to increase granulosa cell proliferation and maturation. During follicular growth, LH first acts on thecal cells to increase the synthesis of androgens, which then diffuse across the basement membrane to reach granulosa cells, where they are aromatized into estrogens. LH, together with estrogens, acts to promote oocyte maturation. LH also triggers follicular rupture and the release of the mature oocyte for capture by the fallopian tube, where fertilization takes place. LH plays an essential role in corpus luteum formation from the ruptured follicle, its subsequent growth and development, and stimulation of progesterone synthesis by small and large luteal cells, which are differentiated from thecal and granulosa cells. The actions of LH in granulosa and luteal cells result in an increased production of steroid hormones, peptide hormones, prostaglandins, and a number of growth factors, cytokines, and proteases.

Research from around the world during the past 15 years has forced a paradigm shift regarding the targets of LH and hCG actions in the body. The new targets include oocyte, early embryo/blastocyst, oviducts, uterus, cervix, placenta, fetal membranes, decidua, umbilical cord, brain, neural retina, spinal cord, skin, mammary glands, adrenal cortex–zona reticularis, urinary bladder, bone, cavernous sinus carotid rete vascular complex, and cells of the immune system. The receptors in these tissues are functional and these functions may explain a number of phenomena that were never previously explained and correct wrong attributions of phenomena to other hormones. Many of the nongonadal LH/hCG actions are beginning to lead to novel biological therapeutic uses of hCG.

Although not much work has been done, FSH may also have nongonadal sites of action. For example, FSH receptors have been found in human myometrium, fallopian tubes, and bovine uterine cervix. FSH can up-regulate LH receptors in myometrial cells just as it does in granulosa cells.

## III. LH RECEPTOR KNOCKOUT

### A. Generation of Knockout Mice

Targeted disruption of the LH receptor gene by homologous DNA recombination has recently been accomplished by our group as well as by Huhtaniemi's. We used a targeting vector in which a 4 kb DNA fragment containing a portion of the 5′-flanking region and exon 1 of the LH receptor gene were replaced with a neomycin-resistance gene cassette. Upon successful targeting in mouse 129/J embryonic stem cells, they were injected into C57BL/6J blastocysts to generate male chimeric mice carrying the disrupted gene. Some of these mice demonstrated germ-line transmission of the mutation when bred with wild-type females. Southern blot analyses of genomic DNA indicated the correct targeting of the LH receptor gene and the absence of any heterologous recombination. Reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot, and ligand binding assays demonstrated the lack of the receptor transcripts, receptor protein, and $^{125}$I-hCG binding in gonads as well as in nongonadal target tissues, indicating complete LH receptor gene inactivation.

Huhtaniemi's group replaced exon 11 and a part of intron sequences surrounding exon 11 with a neomycin-resistance gene cassette. Mutant mice contained truncated receptor transcripts but no functional receptor protein. Nevertheless, the phenotypes of the animals from both research groups were similar.

### B. Sexual Development

Inbreeding of heterozygous mice resulted in an expected Mendelian ratio of all three genotypes as well as a balanced sex ratio, indicating that LH receptor gene disruption is not lethal nor does it have any effect on sex determination. Null animals have normal genitalia except that their development was dramatically attenuated. Both adult female and male animals are sterile, indicating that the

LH receptor is critical for postnatal sexual development.

## C. Ovarian Development

The null animal ovaries were small and pale with a dramatic reduction in their wet weight. The histology is consistent with an arrest in folliculogenesis at the antral stage, which suggests that follicular growth through this stage is not LH dependent. The absence of large antral and preovulatory follicles and corpora lutea suggests that LH is indispensable for follicular growth, maturation, and ovulation.

Granulosa cells showed an increase in apoptosis and a decrease in telomerase levels, despite the presence of intact FSH signaling, which suggests that LH is an important survival factor. LH receptor inactivation had no effect on ovarian FSH and progesterone receptor (PR) mRNA levels. However, estrogen receptor (ER)-α and steroidogenic acute regulatory protein (StAR) mRNA levels decreased and ER-β mRNA levels increased, indicating that either directly or indirectly LH up-regulates ER-α and StAR and down-regulates ER-β mRNA levels. Reciprocal ER changes in LH receptor-inactivated animals are consistent with the concept that the two ERs may have different roles in regulating ovarian functions. A complete or partial anovulatory phenotype was also seen in ER-α, ER-β, PR, prolactin receptor, cyclooxygenase-2, CAAT enhancer-binding protein β, and cyclin D2 knockouts. The use of Clontech's Atlas mouse cDNA expression arrays revealed altered expression of 17 ovarian genes, approximately equal numbers of which showed a decrease or an increase.

Measurement of serum estradiol and progesterone levels indicated that ovarian steroidogenesis was decreased but not totally abolished. The continued low estradiol levels were probably due to LH-independent synthesis of thecal androgens and their aromatization under the influence of FSH in granulosa cells. The modest progesterone decrease in the face of a dramatic decrease in ovarian StAR levels suggests that an alternate mechanism in progesterone synthesis, not involving StAR, may exist. The dramatic elevation in serum LH levels could be due to a loss of estradiol negative feedback and/or loss of short-loop feedback involving LH control of hypothalamic gonadotropin-releasing hormone. A modest elevation in serum FSH levels could be due to decreased gonadal inhibin secretion.

To determine whether a decrease in steroid hormone levels was responsible for the LH receptor knockout female phenotype, 30-day-old animals were implanted with 21-day slow-release pellets containing 0.1 mg of 17β-estradiol and 5 mg progesterone. The serum hormone levels reached the physiological range and yet ovarian size increased only slightly; otherwise the ovaries remained pale and histologically similar to those of placebo-treated null animals. Injections of pregnant mare serum gonadotropin followed by hCG were unable to induce ovulation. The oocytes retrieved contained germinal vesicles and could not be fertilized *in vitro* by sperm from wild-type animals. Thus, LH signaling is required for final oocyte maturation as well as ovulation; in neither of these cases can lack of LH be compensated for by estradiol, progesterone, or FSH. Concerning the reversal of biochemical changes, only ER-α and StAR, but not ER-β, recovered, suggesting that LH action to up-regulate ovarian ER-α and StAR levels is mediated by estradiol and/or progesterone. The lack of an ER-β change suggests that, either directly or indirectly through other unknown local mediators, LH down-regulates ER-β levels.

## D. Reproductive Tract Development and Function

The reproductive tract of null animals was thin and thread-like with a greatly reduced wet weight. The thickness of all uterine layers decreased, stromal and epithelial cells showed no mitosis, and very few endometrial glands were seen.

cDNA expression array analysis revealed that approximately 230 genes showed a decrease and only 2 genes showed an increase in the null mice uterus. The changes were dramatic for some genes and less so for others. A number of genes that showed a decrease came from many gene families in which a change in expression was unexpected. Decreased gene expression after knockout suggests that, either directly or indirectly, LH increases their expression. Conversely, increased gene expression suggests that LH may normally inhibit their expression.

Hormone replacement therapy (HRT) increased uterine size. Computerized quantitative morphometric analysis and proliferating cell nuclear antigen staining showed a restoration of the thickness of all uterine layers and resumption of mitosis in stromal and epithelial cells. The number of endometrial glands, however, remained low, suggesting that LH signaling could be required for their recovery.

To explore genetic response in the uterus of null mice placed on HRT, cDNA expression arrays and semiquantitative RT-PCR were used. The results showed a reversal in both of the genes that increased (bone morphogenetic receptor protein 1A and insulin-like growth factor-binding protein-3) and in two-thirds of the genes that decreased. The genes that did not respond included those that are known to be critical for uterine morphogenesis, functional differentiation, and receptivity.

Uterine steroid hormone receptor levels responded differently to LH receptor inactivation. Whereas ER-α showed no change, ER-β decreased. The decrease in ER-β could not be reversed by HRT. Levels of both PR isoforms A (PR-A) and B were decreased with a more dramatic decrease in PR-A. HRT completely normalized PR-A but not PR-B. Androgen receptor levels were also reduced and HRT failed to completely reverse this decline. These data suggest that, either directly or indirectly through other unknown mediators, LH differentially regulates uterine steroid hormone receptor levels.

To further test uterine function in null mice following HRT, 60-day-old null mice were placed on 21-day estradiol and progesterone replacement therapy to stimulate uterine growth, followed by ovariectomy and priming with ovarian steroid hormones to make the endometrium receptive to donor blastocyst implantation. Ovariectomized wild-type mice with the same priming were used for controls. Morphological analyses demonstrated that wild-type endometrium contained numerous endometrial glands, displayed typical decidual changes such as edema, hyperemia, marked endometrial cell proliferation, and differentiation, and allowed implantation of donor blastocysts. In contrast, the null animal endometrium showed incomplete decidual changes and implantation of donor blastocysts failed. These differences between the two groups indicate that uterine LH receptors are essential for pregnancy initiation.

Null females had ambiguous vaginal openings and HRT stimulated vaginal growth and development. However, the number of leukocytes remained low, and whether LH signaling is required for their restoration remains to be determined.

## E. Mammary Gland Development

The presence of LH receptors in the mammary gland and hCG actions in the differentiation and protection of mammary gland from tumorigenesis have been well documented. LH receptor knockout resulted in complete atrophy of the mammary gland and HRT promoted ductal development and branching. However, the developmental pattern differed from that in normal wild-type siblings. These findings suggest that the actions of LH/hCG could be required for the normal branching pattern.

## F. Bone Development

Bone phenotype in LH receptor knockout animals was examined because it appears to be a direct target of LH regulation. The data revealed a decrease in femur density in 8- and 60-week-old null animals and in 60-week-old heterozygous animals compared with wild-type littermates. The femur length decreased only in 8-week-old null females. Twenty-one-day estradiol/progesterone replacement therapy in 30-day-old homozygous females and testosterone replacement in 30-day-old homozygous males did not completely reverse bone phenotype, suggesting that the actions of LH on bone may be required for the normal growth and development of bone.

## G. Metabolic Changes

The underdeveloped gonads, reproductive tract, and accessory sex organs of LH receptor knockout animals were covered by excessive fat pads. Accumulation of fat became more pronounced in 1-year-old null animals and in heterozygous animals than in wild-type siblings. This was more dramatic in a subgroup of 1-year-old heterozygous females whose body weight increased by approximately 30% compared with wild-type littermates. Histological examination of gonadal fat tissue from 1-year-old females revealed that an increase in cell size rather than an increase in cell number could be primarily responsible for increased obesity.

## H. Consequences of Partial Disruption

Inactivation of one of the alleles of the LH receptor gene had no short-term adverse consequences except for a modest increase in serum FSH levels, the reason for which is not known. It, however, has several long-term adverse consequences. For example, these animals show ovarian failure at an earlier age than wild-type animals, endometria show numerous pathological changes with an enhanced cell proliferation, onset of obesity with some animals becoming extremely obese, and bone loss. That was no apparent correlation between obesity and endometrial pathology.

## IV. FSH RECEPTOR KNOCKOUT

### A. Generation of Knockout Mice

The FSH receptor gene was disrupted by replacing the 5′-flanking region, all of exon 1, and part of intron 1 with a neomycin-resistance gene cassette, which totally silenced the gene.

### B. Postnatal Development and Reproduction

Intercrosses of heterozygous FSH receptor knockout animals produced progeny with an expected Mendelian ratio and approximately equal numbers of male and female animals. Mutant mice were acyclic and sterile.

### C. Pituitary Phenotype

Although the wet weight of pituitary gland was not affected, the anterior lobe became enlarged and contained more FSH- and TSH-positive cells, which suggests that these cells may come from the same lineage. Intermediate and posterior lobes appeared normal. Pituitary content and circulating FSH and LH levels were elevated but were normalized by a week of estradiol treatment, indicating that negative feedback regulation is intact at the pituitary level.

### D. Ovarian Development and Pathology

Ovaries of 3- to 5-month-old FSH receptor knockout mice were smaller with an arrest in folliculogenesis at the preantral stage. Ovarian stroma contained hypertrophic interstitial cells. Although serum inhibin α levels were not significantly changed, both ovarian and circulating inhibin A and B levels were diminished. Serum testosterone levels were dramatically increased; estradiol and progesterone were markedly decreased. However, ovarian aromatase mRNA and its protein levels were reported to be normal. Analyses of ovarian gene expression revealed that the LH receptor, cyclin $D_2$, c-kit, steel, ER-α, and ER-β were not changed.

More than 90% of FSH receptor knockout mice developed ovarian cysts and tumors by approximately 1 year of age. The tumors were positive for Leydig cell (3β-hydroxysteroid dehydrogenase and P450c17) and Sertoli cell (Müllerian inhibiting substance and GATA-4) markers. These findings suggest that these are sex cord-stromal-type tumors. In contrast to FSH receptor knockout mice, LH receptor knockout animals, which had elevated serum FSH and increased ovarian inhibin α mRNA levels, never developed these lesions at any age. Mice with ectopic overexpression of LH and a knockout of the inhibin α gene developed ovarian cancers, which suggests that elevation of LH levels and decreased inhibins might contribute to ovarian tumorigenesis in aging FSH receptor knockout animals.

### E. Reproductive Tract Development

Null animals have a hypoplastic and imperforate vagina. Vaginal smears showed an absence of cornified epithelial cells. Atrophic uterus contained severely reduced epithelial, stromal, and myometrial layers and less developed endometrial glands. Uterine ER-α and ER-β levels were normal, but PR-A and PR-B levels were diminished. Short-term estrogen replacement therapy demonstrated a full uterine response, suggesting that the uterine phenotype was secondary to estrogen deficiency in FSH receptor knockout mice.

### F. Skeletal Abnormalities

FSH receptor knockout females developed compressed vertebrae, resulting in a hunchback appearance. Femur weight was reduced at approximately 1 year of age. X-ray and histological analyses revealed a loss of trabecular bone. Analysis of the bone marrow revealed an accumulation of pre-B lymphocytes that is similar to that of ovariectomized animals, suggesting that skeletal abnormalities may be secondary to estrogen deficiency.

### G. Metabolic Changes

Two-month-old animals of three genotypes had similar growth and body weight. However, the weight gain was evident in homozygous and heterozygous animals by 3 to 4 months of age. All FSH receptor knockout females also showed an accumulation of abdominal fat, which was reduced by prolonged estrogen replacement therapy.

### H. Consequences of Partial Disruption

Animals with a knockout of one copy of the FSH receptor gene had irregular estrous cycles, delayed conception, reduced litter size, premature reproductive senescence, and ovarian cysts. These data suggest that both copies of a functional FSH receptor gene may be required for full reproductive capacity and that early reproductive senescence seen in certain populations of women might be associated with a copy of a dysfunctional FSH receptor gene.

## V. FSH-β SUBUNIT KNOCKOUT

### A. Generation of Knockout Mice

A standard gene targeting in embryonic stem cells was used to generate these mice. The replacement-targeting vector was constructed by deleting exons 1 and 2 and most of exon 3 of the FSH β-subunit gene. The mutant mice had no detectable FSH in the circulation.

### B. Phenotype Differences from FSH Receptor Knockout Mice

The female phenotype was similar between FSH β-subunit knockout mice and FSH receptor knockout mice. There were differences, however, in the male phenotype. For example, FSH β-subunit knockout males were completely fertile and their serum testosterone levels were normal. FSH receptor knockout males, on the other hand, were subfertile and had lower serum testosterone levels. These differences could be due to the presence of an unknown molecule(s) that might interact with the FSH receptors in FSH β-subunit knockout males. Phenotype differences between ligand and its receptor knockout were also seen in aromatase knockout and estrogen receptor knockout mice.

### C. Double Knockout of Inhibin α and FSH β-Subunit

Inhibins, members of the TGF-β superfamily, have been identified as tumor suppressors. One-month-old inhibin α knockout animals develop gonadal sex cord-stromal tumors and die from severe wasting syndrome. These animals often have elevated serum FSH, estrogens, and activin levels. In order to determine the role of FSH in ovarian tumorigenesis, a double knockout (inhibin α and FSH β-subunit genes) was made by crossing FSH β-subunit knockout males with inhibin α knockout female mice. These animals still developed ovarian tumors, but the tumors were slow-growing and less hemorrhagic with minimal cachexia-like symptoms compared with inhibin α knockout mice. Serum estradiol and activin levels and ovarian aromatase mRNA levels were lower in double knockout mice than in inhibin α knockout mice. These findings suggest that FSH is not directly involved in ovarian tumor formation, but indirectly influences gonadal tumorigenesis in inhibin α knockout mice. FSH receptor knockout mice also develop sex cord-type stromal tumors in ovaries, a finding that is inconsistent with the concept that loss of FSH signaling protects against gonadal tumors in inhibin α knockout mice. Thus, the exact role of FSH signaling in gonadal tumorigenesis requires further studies.

## VI. NATURAL KNOCKOUT OF GONADOTROPINS AND THEIR RECEPTORS IN HUMANS

### A. Mutations in LH/hCG and FSH Receptor Genes

Both activating and inactivating LH/hCG and FSH receptor gene mutations have been described in humans. All the activating mutations were missense and located in the transmembrane region (LH receptor exon 11 and FSH receptor exon 10).

Activating LH/hCG receptor mutation results in severe symptoms in males. The affected males have low to undetectable blood LH levels, elevated testosterone levels, Leydig cell hyperplasia, premature puberty, and onset of spermatogenesis as early as 3 years of age. Female carriers do not seem to have this phenotype. It is not clear why precocious puberty does not occur in females with activating LH/hCG receptor mutations.

In contrast to activating mutations, inactivating mutations are scattered throughout the entire coding regions of the gene. The types include insertion, deletion, nonsense, and missense mutations. All these mutations are autosomal recessive and identified in homozygous and compound heterozygous states. These various mutations result in partial or complete inactivation of the gonadotropin receptor genes. In many respects, the phenotypes of mice with complete inactivating gonadotropin receptor gene mutations resemble those of LH and FSH receptor knockout mice. Males with complete inactivating LH/hCG receptor mutations have abdominal testes and are sterile due to Leydig cell hypoplasia and spermatogenic failure. The external female-like genitalia (pseudo-hermaphroditism) seen in affected males were not seen in LH receptor knockout males. The affected females have normal pubertal development with primary amenorrhea and infertility, absence of preovulatory follicles and corpora lutea, small uterus, and decreased bone mass.

Only one activating FSH receptor mutation has been described thus far. Some men with inactivating FSH receptor mutations were fertile just as FSH-β and FSH receptor knockout mice were. This suggests that FSH is not essential for spermatogenesis. Complete inactivating FSH receptor mutations result in primordial to mature follicles, amenorrhea, and infertility.

### B. Mutations in the LH β- and FSH β-Subunit Genes

No germ-line mutation of the α-subunit gene has yet been reported. Targeted disruption of the α-subunit gene results in hypothyroid and hypogonadal states. Mutations in the β-subunit gene of LH and FSH in human are fairly uncommon. In general, the symptoms of affected men and women are less severe than their receptor mutations. Complete inactivation of the human LH β-subunit gene resulted in infertility, absence of Leydig cells, and arrest of spermatogenesis, but affected individuals responded normally to hCG treatment. Variable presentations among women with inactivating mutations of the LH β-subunit gene have been described. The symptoms include infertility, menstrual disturbances, and polycystic ovary syndrome. Men with inactivating FSH β-subunit gene mutations have normal puberty and mild to severe impaired spermatogenesis, but some were fertile. Women with inactivating FSH β-subunit gene mutations, on the other hand, have poor breast development, primary amenorrhea, infertility, low FSH and estrogen levels, and high LH levels.

## VII. SUMMARY AND PERSPECTIVES

One of the important findings that emerged from LH receptor knockout studies is gender difference in dependence on LH signaling. In females, it is absolute, meaning that when LH signaling is inactivated, the infertility phenotype cannot be rescued by either ovarian steroid hormones or FSH. In males, on the other hand, testosterone can rescue the infertility phenotype.

FSH is thought to be a principal factor in regulating spermatogenesis. However, FSH β-subunit and FSH receptor knockout mice and men with inactivating mutations were fertile, indicating that FSH is not essential for male fertility. Thus, the strategies to block FSH action for male contraception may not be successful. The findings from LH receptor knockout animals, on the other hand, indicate that LH is essential for spermatogenesis and fertility by increasing testosterone production by Leydig cells.

The phenotypes of heterozygous LH receptor knockout and FSH receptor knockout female mice are unexpected. Although at the age of sexual maturity these animals are indistinguishable from wild-type littermates, they developed early reproductive senescence, obesity, skeletal abnormalities, and endometrial pathology (LH receptor knockout) as they grow old. These findings suggest that both functional alleles of either LH or FSH receptor genes are required for normal reproductive and metabolic functions especially during aging. These findings indicate that certain reproductive and metabolic abnormalities in aging women could be due to allelic inactivation of gonadotropin receptor genes. This prospect holds a great deal of promise in predicting who might be at a greater risk for developing problems during aging.

Knockout of the LH β-subunit gene is not yet available. Thus, LH receptor knockout remains the only model for investigating LH biology using knockout technology. Whether LH β-subunit knockout animals would manifest any phenotypes that would be different from those of LH receptor knockout mice remains to be seen. It should not be surprising, however, if they do, as in the case of FSH-β vs FSH receptor knockout and aromatase versus ER-α and ER-β knockout mice. These differences could come from unknown ligand-activating receptors. Thus, receptor inactivation should give more definitive information than cognate hormone inactivation.

Both LH and FSH have been implicated in the development of ovarian cancer in women. However, the role of FSH has been complicated by the fact that these tumors develop even in the absence of FSH. Moreover, LH receptor knockout animals, which have intact FSH signaling, do not develop any type of ovarian tumor. LH receptor knockout animals could be useful in testing the role of LH in ovarian cancer. For example, if LH is important, then induction of ovarian cancers in LH receptor knockout animals should be very difficult when compared to wild-type or heterozygous siblings.

Double knockouts of LH and FSH receptors should provide further opportunities to test the role of each of these hormones in gonadal and nongonadal functions and tumorigenesis. Tissue-specific knockout is another powerful approach to ascertain gene function. However, this approach results in gene inactivation in only a single cell type, which could create a problem in data interpretation if the gene is expressed in multiple cell types with each contributing to the hormonal response. Despite this limitation, it is an attractive approach to gain additional insights into gonadotropin biology in normal and abnormal reproductive processes.

## Glossary

**cDNA microarray** A method for high-throughput simultaneous analysis of multiple gene expression changes.

**cystine-knot motif** The motif consists of an internal ring of two cystine bonds linking adjacent antiparallel strands of the peptide chain with the third cysteine passing through the center of the ring to complete the knot.

**genotype** Gene combination at one specific locus or any specified combination of loci.

**gonadotropins** A group of glycoprotein hormones that is capable of promoting gonadal growth, development, and function. Usually refers to luteinizing hormone, follicle-stimulating hormone, and human chorionic gonadotropin. Generally, prolactin is not included even though it also has similar effects.

**heterozygous** Having different alleles at one locus.

**homozygous** Having identical alleles at one locus.

**knockout** A genetically engineered mouse in which the endogenous gene is inactivated by homologous DNA recombination.

**Mendelian ratio** The ratio of progeny with particular phenotypes or genotypes expected accordance with Mendelian law.

**phenotype** The physical, morphological, or biochemical characteristics of an individual that are determined by the genotype and environment.

**receptor** A protein on the cell surface or inside the cell that is activated by cognate hormone binding. There is no clear-cut difference between cell surface and intracellular receptors for the same hormone. Thus, the same receptor may be found in both locations mediating different biological effects.

## See Also the Following Articles

**Apoptosis Gene Knockouts • Estrogen Receptor Biology and Lessons from Knockout Mice • Follitropin (Follicle-Stimulating Hormone) Receptor Signaling • Gonadotropin-Releasing Hormone (GnRH) • Luteinizing Hormone Receptor Signaling**

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by the placenta in pregnancy and leptin levels are high in pregnancy, although the physiologic function of this hyperleptinemia of pregnancy remains unclear. Serum leptin levels in humans peak at night several hours after the largest meal of the day and reach a nadir in the morning. Leptin production by fat cells in culture is stimulated by insulin and glucocorticoids and is inhibited by counterregulatory hormones (those that antagonize the actions of insulin-e.g., adrenaline) and their intracellular signaling mediators (Fig. 1). The regulation of leptin production by indicators of acute nutritional status such as insulin (feeding → increased insulin → increased leptin) and counterregulatory hormones (fasting → increased counterregulatory hormones → decreased leptin) makes teleological sense. The stimulation of leptin production by glucocorticoids appears counterintuitive in this light, however, because circulating glucocorticoids are important mediators of the stress and starvation responses. Indeed, although plentiful data suggest that insulin is a critical mediator of increased leptin production *in vivo*, the diurnal pattern of leptin levels and numerous other data argue against an important role for circulating glucocorticoids in the stimulation of leptin production. In contrast, adipocyte-produced autocrine- or paracrine-acting glucocorticoids could be involved (Fig. 1); the production of glucocorticoids by adipocytes increases with the accumulation of triglycerides, and mouse models with increased glucocorticoid production specifically in adipocytes have elevated leptin levels.

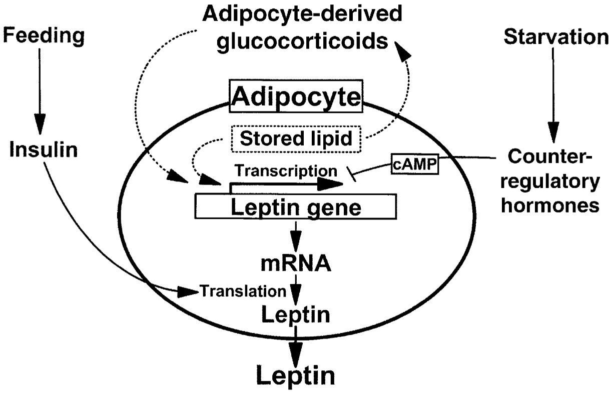

FIGURE 1 Regulation of leptin production in the adipocyte. In the adipocyte, leptin gene transcription is inhibited by the increased levels of cellular cyclic adenosine monophosphate (cAMP) produced by starvation-induced counterregulatory hormones (such as adrenaline). Insulin increases leptin protein synthesis by increasing the translation of leptin mRNA. Lipid stores in the adipocyte also regulate leptin mRNA synthesis; although the details of this process are poorly understood (dashed lines), part of this effect may be mediated by the production of autocrine-acting glucocorticoids by the adipocyte when lipid storage is increased.

A constitutive pathway mediates leptin secretion from adipocytes; leptin secretion is not altered acutely and changes in the rate of leptin secretion result primarily from regulation of leptin synthesis. Glucocorticoids increase leptin gene transcription and insulin stimulates leptin synthesis primarily by increasing the rate of translation of leptin mRNA into protein, whereas counterregulatory hormones decrease leptin gene transcription, reducing levels of leptin mRNA. Integrating this information, then, circulating leptin levels reflect adipose mass (reflecting long-term energy stores) modified by circulating levels of insulin (reflecting recent food intake) and counterregulatory hormones (reflecting lack of recent food intake).

## IV. LEPTIN RECEPTORS AND SIGNALING

### A. Leptin Receptor Isoforms

The cloning of the leptin receptor (LR) revealed at least five isoforms of the receptor (LRa–LRe) that are alternatively spliced products of the same gene. The leptin receptor is a type I cytokine receptor containing a conserved fold in the ligand-binding extracellular domain and a single transmembrane domain. Each leptin receptor isoform contains the same first 14 exons of the leptin receptor gene (encoding the majority of the extracellular domain of the receptor and the full leptin-binding region); LRe contains only these exons and is secreted. LRa–LRd contain an additional three common exons encoding a short membrane-proximal portion of the extracellular domain, a transmembrane-spanning region, and 28 common intracellular amino acids. Each of these transmembrane forms contains an alternatively spliced exon 18 that encodes intracellular tails of various lengths (LRa, 33 amino acids; LRb, 301 amino acids; LRc, 40 amino acids; and LRd, 31 amino acids).

The extracellular domains of these membrane-spanning leptin receptors mediate dimerization of the leptin receptor in the absence of ligand (leptin). Leptin binding is thought to alter the conformation of the receptor in such a way as to transmit a signal to the intracellular tail of the receptor. Like other class I cytokine receptors, the leptin receptor has no intrinsic enzymatic activity, but mediates signals via a constitutively associated protein tyrosine kinase (Jak2 in this case). Although each of the membrane-spanning LR isoforms contain a critical conserved Box1 motif required for Jak2 association and signaling, all but LRb lack other motifs that are required for effective Jak2 interaction and activation; hence, only LRb mediates effective signaling. The conformational change induced on ligand binding results in close apposition of the individual Jak2 kinase molecules associated with each monomer of the LRb dimer, leading to Jak2 transphosphorylation and activation and thence to the tyrosine phosphorylation of LRb (Fig. 2).

## B. The Long-Form Leptin Receptor, LRb

Much of what is known about LR signaling centers around the so-called long form of the leptin receptor, LRb, with its 301-amino-acid intracellular tail and full Jak2-binding region. Not only is it readily apparent that the long intracellular tail of LRb is likely to mediate downstream signals effectively, but also the original *db* mutation alters a splice site that effectively deletes LRb, replacing it with LRa. The *db/db* phenotype is similar if not identical to the phenotype of leptin deficiency that is observed in *ob/ob* mice. Thus, although the function of the other LR isoforms remains obscure, LRb mediates physiologically important leptin signals. The expression of LRb is also highly regulated; in the brain, high concentrations of LRb are found in few locations (to a great extent in hypothalamic nuclei that are known to integrate body energy status with the control of feeding and neuroendocrine function). LRb may also be expressed in a limited manner in a number of peripheral tissues, although the physiologic relevance of this expression remains unclear.

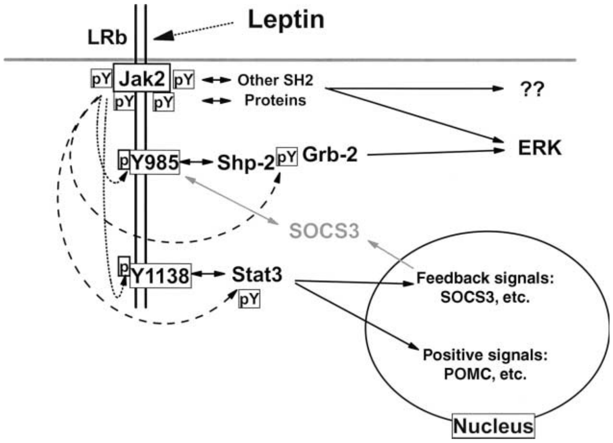

**FIGURE 2** Leptin receptor signaling. Leptin binding to the long form of the leptin receptor (LRb) results in the rapid activation and tyrosine phosphorylation of the associated Jak2 tyrosine kinase. Once activated, Jak2 then mediates the phosphorylation (dotted lines) of Tyr-985 (Y985) and Tyr-1138 (Y1138) on the intracellular tail of LRb. Each phosphotyrosine residue becomes a docking site to recruit specific downstream signaling molecules. Phosphorylated Tyr-1138 mediates the binding of the latent cytoplasmic form of the transcription factor, Stat3; Stat3 is then phosphorylated (dashed lines) by the activated Jak2, leading to nuclear translocation of Stat3 and its transcriptional activation. Once in the nucleus, Stat3 mediates transcription of leptin effectors, such as proopiomelanocortin (POMC), as well as the feedback inhibitor, SOCS3. Phosphorylated Tyr-985 mediates the binding of Shp-2, which also becomes phosphorylated by Jak2 (dashed lines), allowing the recruitment of Grb-2 and the activation of the extracellular-related kinase (ERK) signaling cascade. At long times after stimulation, when SOCS3 protein levels have increased, SOCS3 binding to phosphorylated Tyr-985 inhibits all facets of LRb signaling (shaded lines). Downstream signaling proteins likely interact with tyrosine phosphorylation sites on Jak2 as well, although these are poorly characterized.

Receptor–tyrosine kinase complexes (such as LRb–Jak2) signal by inducing the phosphorylation of tyrosine residues in the signaling complex. These phosphotyrosine residues, each in its own unique amino acid context, then become docking sites for downstream signaling proteins that contain specialized phosphotyrosine-binding domains, such as SH2 domains. Each SH2 domain recognizes phosphotyrosine in the context of specific surrounding amino acids; thus, although tyrosine phosphorylation acts as the molecular switch for SH2 domain binding, the amino acid sequence surrounding the phosphotyrosine determines which SH2 domain-containing proteins are recruited.

In addition to its complete Jak2-association domain, the intracellular domain of LRb contains two tyrosine phosphorylation sites, Tyr-985 and Tyr-1138 (Fig. 2); no other LR isoform contains intracellular tyrosine residues. During leptin stimulation, phosphorylated Tyr-1138 mediates the recruitment and activation of the SH2 domain-containing signal transduction and activation of transcription (Stat3) factor. Activated Stat3 mediates the transcription of important neuropeptide mediators of LRb action, including proopiomelanocortin (POMC) and the inhibitory suppressor of cytokine signaling (SOCS3) protein. During acute stimulation, phosphorylated Tyr-985 recruits the SH2 domain-containing tyrosine phosphatase, Shp-2, which controls the activation of the extracellular signal-regulated (ERK) serine kinase cascade. Phosphorylated Tyr-985 also mediates the binding of SOCS3 to the activated receptor, presumably mediating feedback inhibition of LRb during prolonged leptin stimulation (after LRb/Stat3-driven transcription has increased cellular

SOCS3 levels). In addition to these signals that are mediated by tyrosine phosphorylation sites on LRb proper, the activated LRb-associated Jak2 tyrosine kinase also possesses leptin-stimulated tyrosine phosphorylated residues that mediate some components of the leptin signal.

### C. The Function of Short and Secreted Forms of the Leptin Receptor

The physiologic and biochemical signaling functions of the short and secreted forms of the leptin receptor are less clear than are those of LRb. There are no known mutations in the leptin receptor gene that specifically affect one or more of the non-LRb isoforms without also perturbing LRb. Much of the phenotype of a strain of *db/db* mice that lacks all leptin receptor isoforms ($db^{3J}$) is rescued by neural expression of an LRb transgene, however, suggesting only minor roles for these other LR isoforms in the control of physiology. It is not clear, however, whether the failure of this LRb transgene to completely restore normal leptin action results from the lack of other LR isoforms in this model, or is merely due to the expression of this LRb transgene at nonphysiologic levels or locations in these mice.

The ability of the short transmembrane forms of LR (LRa, LRc, and LRd) to mediate physiologically meaningful intracellular signals remains unclear. Although each of these LR isoforms contains the critical Box1 motif required for Jak2 interaction, other sequences that are likely to be required for high-affinity interaction with Jak2 are absent from these receptors and these isoforms are devoid of potential sites of tyrosine phosphorylation. The conservation of these receptor forms across species suggests some function, however, and roles for these receptors in leptin transport, leptin clearance, or LRb inhibition have been proposed; there are few data to address definitively the validity of these proposed functions.

Circulating leptin receptor, not bound to plasma membranes, is also present in mammals. Some of this soluble receptor is the product of LRe mRNA, and some appears to result from cleavage of membrane-bound forms of the leptin receptor. This circulating receptor has a similar affinity for leptin binding as do the membrane-bound forms of the LR, and much of this circulating receptor appears to reside in complex with leptin. Circulating LR is increased in pregnancy (as are serum leptin levels). Possible roles for circulating LR include providing a circulating reservoir for leptin, stabilizing leptin, or decreasing the availability of leptin for membrane-bound forms of the leptin receptor.

## V. LEPTIN AND THE ENDOCRINE STARVATION RESPONSE

### A. Introduction

Undernutrition evokes a set of physiologic responses geared toward increasing caloric intake, redirecting metabolic fuels, and decreasing energy expenditure (the so-called starvation response). Although many of the details remain unclear, starvation inhibits hypothalamic neurons (e.g., thyrotropin-releasing hormone neurons) that would otherwise stimulate the secretion of anterior pituitary factors (e.g., thyroid-stimulating hormone), which would in turn stimulate endocrine end organs (e.g., thyroid gland) (Fig. 3). Similarly, other hypothalamic neurons (e.g., corticotropin-releasing hormone neurons) are activated, resulting in increased release of different anterior pituitary factors (e.g., adrenocorticotropic hormone) that stimulate other endocrine end organs (e.g., adrenal cortex). Other hypothalamic neurons are similarly regulated during starvation to inhibit

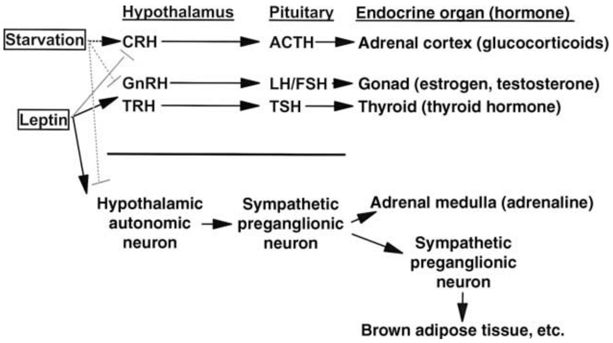
Hypothalamus
Pituitary
Endocrine organ (hormone)
Starvation
CRH
ACTH
Adrenal cortex (glucocorticoids)
GnRH
LH/FSH
Gonad (estrogen, testosterone)
Leptin
TRH
TSH
Thyroid (thyroid hormone)
Hypothalamic autonomic neuron
Sympathetic preganglionic neuron
Adrenal medulla (adrenaline)
Sympathetic preganglionic neuron
Brown adipose tissue, etc.

FIGURE 3 Leptin and the endocrine starvation response. Undernutrition, as in starvation, acts on specific sets of neurons in the hypothalamus; these neurons release factors that control endocrine function via the anterior pituitary. Starvation thereby activates the production of glucocorticoids by the adrenal and blocks the production of thyroid and gonadal hormones. Starvation also inhibits the hypothalamic neurons that activate the sympathetic nervous system, blocking the production of adrenaline by the adrenal gland and inhibiting the action of sympathetic neurons on target tissues such as brown adipose tissue. Leptin acts to oppose the starvation response at the level of these hypothalamic neurons. CRH, Corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TRH, thyrotropin-releasing hormone; TSH thyroid-stimulating hormone.

the sympathetic nervous system. Many aspects of the starvation response are mimicked by the absence of leptin action (as in *ob/ob* or *db/db* mice), and many aspects of the starvation response can be partially or completely blocked by treatment with exogenous leptin. Indeed, the central theme of leptin function is to communicate the sufficiency (or by its lack, the insufficiency) of body energy stores to the central nervous system (and potentially other tissues).

## B. Feeding, Metabolic Rate, and Fuel Distribution

Leptin regulates the intake and storage of metabolic fuels. Rodents and humans deficient in leptin or leptin action are morbidly obese. The obesity of leptin deficiency results in part from a vast increase in food intake (compared to controls with intact leptin action) in combination with decreased metabolic rate. The absence of leptin action enhances the central orexigenic (eating) response and initiates an energy-conserving alteration in metabolic rate, which is consistent with the idea that leptin represents a signal of energy repletion. In rodents, decreased metabolic rate in leptin deficiency seems to result from combined reductions in thyroid function, sympathetic activity, and locomotor activity (Figs. 3 and 4). In rodents, a great deal of this metabolic/sympathetic activity is mediated via the thermogenic brown adipose tissue, which is absent in adult humans. Depressed sympathetic function is also detectable as decreased blood pressure in the absence of leptin action. The decrease in sympathetic tone in the absence of leptin is likely exacerbated by the concomitant hypothyroidism of leptin deficiency, because thyroid hormone mediates a permissive effect on catecholamine signaling.

Defects in leptin function also increase glucocorticoid levels in rodents (Figs. 3 and 4); elevation of glucocorticoid hormones effectively redistributes energy from protein (e.g., muscle) to fat (e.g., adipose and fat deposits elsewhere). Indeed, adrenalectomy in rodents with defective leptin action greatly ameliorates the obesity syndrome, suggesting that some elements of the leptin-deficient obesity syndrome may be secondary to high glucocorticoid levels.

Each of these leptin effects (increased sympathetic and thyroid function and repression of glucocorticoids) is mediated centrally. Leptin acts in the hypothalamus to regulate signals to autonomic centers in the brain and the production of hypothalamic releasing hormones such as corticotropin-releasing hormone and thyroid hormone-releasing hormone (Figs. 3 and 4).

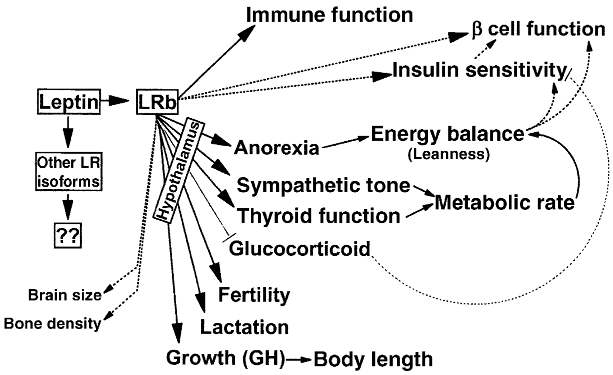

FIGURE 4 Leptin and the control of physiology. Although the actions of leptin on other leptin receptor (LR) isoforms are unclear, leptin activation of LRb controls a broad set of physiologic outputs, many via the hypothalamus. Leptin activates the growth and reproductive axes and permits lactation in postpartum females. Leptin also promotes anorexia (failure to eat). Combined with the increased metabolic rate that leptin promotes via increases in sympathetic tone and thyroid function, leptin regulates the energy balance. Leptin suppresses the stress/starvation hormones of the glucocorticoid family. Although the mechanisms are less clear (and potentially less direct; dashed lines), leptin also promotes large brain size and increased bone density. Leptin aids in controlling the storage and release of glucose by directly and indirectly controlling insulin sensitivity and insulin secretion (via the pancreatic beta cell).

Leptin, of course, is not the only controller of thyroid and glucocorticoid status. Hence, the rarity of humans lacking leptin or the leptin receptor combined with the relatively heterogeneous genetic background of humans compared to experimental rodent models complicates analysis of the endocrine/metabolic function of leptin in humans, in which the variation in so-called normal values is high and abnormal levels are generally defined by stark contrasts provided by diseases of hormone absence or unregulated hormone production. Even so, there are hints that leptin-deficient humans may be marginally hypothyroid and have mildly elevated levels of glucocorticoids.

## C. Reproduction

Reproduction requires an enormous investment of energy, especially in females, who must nourish offspring both *in utero* and postpartum (via lactation); the availability of stored energy (fat) should then critically influence fertility. Indeed, society is replete with anecdotes of impaired fertility in females with low body fat reserves (e.g., highly trained athletes or individuals with eating disorders). Leptin clearly plays a critical role as a signal of energy repletion to the reproductive system in females. Not only is leptin deficiency inextricably linked with impaired female fertility across all species studied, but also the fasting-induced decrease in fertility in rodents is blunted with leptin therapy. Male fertility is decreased by leptin deficiency, but not as much as in females, consistent with the vastly greater energetic demands of reproduction in females. In addition to the effects of leptin on postpubertal reproductive function, there has been some suggestion that leptin levels may influence the progression through puberty, although leptin is clearly not the only pubertal signal in mammals. The presence of normal numbers of quiescent follicles in leptin-deficient animals and the ability of leptin receptor-deficient gonads to function under the control of exogenous or endogenous (after transplant into normal animals) gonadotropins suggest that leptin does not control reproductive function by directly regulating the gonad. Leptin regulates reproductive function by indirectly stimulating the function of gonadotropin-releasing cells in the hypothalamus (Figs. 3–5).

The ability to treat leptin-deficient animals with leptin and so restore reproductive function has enabled the study of postfertilization reproductive function in the absence of leptin. Although there are no data from humans, leptin is clearly not required for the maintenance of pregnancy or parturition in rodents, because withdrawal of leptin postcoitus does not alter the successful completion of pregnancy or delivery of pups. Interestingly, however, the pups of these leptin-deficient mothers generally die soon after birth, without detectable milk in their stomachs. The ability of these pups to survive with wild-type foster mothers suggests a defect in lactation in leptin-deficient animals and, by extension, a role for leptin in the control of the energetically costly process of lactation (Figs. 3 and 4). Indeed, continuation of leptin therapy throughout pregnancy and postpartum permits lactation, suggesting that lactation failure in these animals is not a developmental problem, but is secondary to a hormonal signal mediated by leptin. One reasonable hypothesis is that leptin signals nutritional status to the neuroendocrine centers in the hypothalamus that control the release of prolactin by the pituitary, permitting prolactin secretion only when sufficient energy is available and leptin is present.

In humans, although it is clear that leptin regulates female reproduction (leptin- and leptin receptor-deficient human females are infertile), the specific role of leptin in puberty, parturition, and lactation has yet to be determined. It is also possible that nutritional signals other than leptin may participate in the regulation of reproductive function in humans and other mammals.

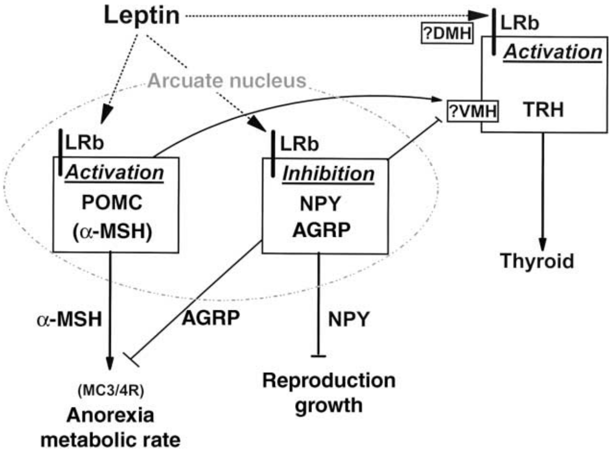

FIGURE 5 Leptin action in the hypothalamus. Leptin stimulates proopiomelanocortin (POMC)/α-melanocyte-stimulating hormone (MSH) neurons in the arcuate nucleus of the hypothalamus to activate the melanocortin receptors (MC3R, MC4R) and to promote anorexia and increased metabolic rate. At the same time, leptin acts to inhibit the arcuate neurons expressing neuropeptide Y (NPY) and agouti-related protein (AGRP). Normally, AGRP antagonizes the action of α-MSH and NPY blocks reproduction and growth; hence, leptin acts to permit reproduction and growth by inhibiting this neuron. Leptin may activate the thyroid-stimulating thyrotropin-releasing hormone (TRH) neurons in the dorsomedial hypothalamic (DMH) nucleus directly and may indirectly activate TRH neurons in the ventromedial hypothalamic nucleus (VMH) by the action of the POMC/α-MSH and NPY/AGRP neurons. These leptin-mediated increases in thyroid function likely contribute to leptin-induced increases in metabolic rate.

## D. Growth

Nutritional inputs are also critical for the generation of a large body; this is seen anecdotally in the increased height of first-generation Americans compared to their forebears, who developed under lower nutritional standards. It makes teleological sense to expend less energy generating (and maintaining) a large body in the face of undernutrition. As might be expected, leptin also plays a role in the control of linear growth (Fig. 4). Rodents devoid in leptin or leptin action are shorter than are wild-type mice, and leptin-deficient humans are shorter than would be predicted based on the heights of their parents. As for reproduction, the regulation of linear growth by leptin is mediated centrally, by the hypothalamus, although leptin may not be the unique nutritional signal to the growth axis.

The finding that animals with defects in leptin action have decreased bone density at an early age suggests that leptin may control bone mass (Fig. 4). One possibility is that leptin may indirectly regulate bone mineral content by influencing reproductive hormone and glucocorticoid levels; direct pathways (e.g., via leptin action on the bone remodeling system) as well as other indirect pathways may exist, however.

Interestingly, rodents devoid of leptin action also have decreased brain size; because a great deal of energy is required to generate and maintain a large brain, the hypothesis that leptin acts as a nutritional signal to control the expenditure of energy in the brain makes teleological sense as well. The mechanism by which leptin regulates brain size is unclear (i.e., via the hypothalamic growth axis or by some other mechanism). Although this possibility remains untested, the developmental delay noted with severe undernutrition in humans suggests that the role for leptin in regulating brain development could be conserved in humans.

### E. Integration of Neuroendocrine Function in the Hypothalamus

The mechanisms by which leptin acts in the central nervous system to control neuroendocrine function are becoming clearer. LRb is expressed in several hypothalamic nuclei, each with its own functions, as well as in a few other locations in the brain. LRb-expressing hypothalamic nuclei generally cluster in the basomedial region historically known as the "satiety center" (based on the overeating phenotype exhibited in animals following ablation of these areas). In contrast, the lateral hypothalamus (the corresponding "feeding center," the ablation of which causes anorexia) expresses no LRb. In addition to regulating feeding, the basomedial hypothalamic nuclei that express LRb regulate neuroendocrine function (including autonomic nervous system function and hormone secretion by the anterior pituitary gland).

In the basomedial hypothalamus, the arcuate nucleus expresses the highest concentrations of LRb found anywhere in the body. In this region, LRb is expressed in two sets of chemically distinct neurons (Fig. 5): (1) those that also express POMC, the precursor for the melanocortin α-melanocyte-stimulating hormone (α-MSH), and (2) those that co-express neuropeptide Y (NPY) and agouti-related protein (AGRP). Each of these neuropeptides performs distinct physiological functions. α-MSH secretion results in [via its binding to the melanocortin (MC) receptor-3 and -4 isoforms on target neurons] suppressing appetite and increasing metabolic rate. AGRP antagonizes the α-MSH/MC receptor interaction to increase appetite. NPY mediates myriad effects via several NPY receptor isoforms and suppresses the reproductive and growth axes as well as increasing appetite. Leptin activates POMC/α-MSH neurons, while hyperpolarizing and inhibiting NPY/AGRP neurons.

In addition to regulating arcuate neurons, leptin also acts on neurons in other hypothalamic nuclei: Leptin activates a subset of neurons in the dorsal medial and ventral medial hypothalamic nuclei that may also contribute to the regulation of appetite, among other things. Leptin also activates neurons in the parvocellular paraventricular nucleus, but this appears to be mediated indirectly via connections with projections from the arcuate and dorsal medial nuclei. The neurons in all of these nuclei are those that control neuroendocrine and autonomic nervous system function. Recent data suggest that α-MSH projections from the arcuate nucleus to the paraventricular nucleus contribute to the regulation of the autonomic nervous system by leptin.

Arcuate LRb neurons may regulate thyroid function (α-MSH stimulating and NPY repressing) by synapsing on hypothalamic thyrotropin-releasing hormone (TRH)-expressing neurons in the paraventricular nucleus, and leptin may directly regulate TRH-containing neurons in the dorsomedial nucleus, as well (Fig. 5). Less clear are the central mechanisms by which leptin controls adrenal cortical function, lactation, bone density, and/or brain size (Fig. 4).

## VI. LEPTIN ACTION OUTSIDE THE CNS

### A. Introduction

Because the highest concentrations of LRb are found in the central nervous system (CNS), especially in the basomedial hypothalamus, it has been widely assumed that little, if any, physiologic leptin function is mediated by direct action on sites outside of the CNS, but rather that the effects of leptin at these peripheral targets are mediated indirectly via the CNS. This initial paradigm has been challenged by a number of studies in a variety of nonneural tissues and, although not all peripheral tissues may be direct targets of leptin action, good evidence exists for direct leptin action on a number of tissues.

### B. Immune Function

It has been recognized for some time that nutritional status regulates immune function; because mounting an immune response requires the energetic investment to expand large populations of immune cells, it stands to reason that this response would be placed under some nutritional constraints. As might be expected, leptin controls at least some part of this nutritional input. Indeed, *ob/ob* and *db/db* mice display a number of immune defects, ranging from thymic atrophy and altered T helper cell ($T_H1/T_H2$) ratios to impaired wound healing. It is possible that some elements of this immune suppression of leptin deficiency are secondary to CNS or neuroendocrine outputs (such as elevation of glucocorticoid levels), but LRb-expressing immune cells have been detected and leptin directly promotes the proliferation of some lymphocyte and macrophage populations, suggesting direct leptin action on at least some immune cells (Fig. 4).

### C. Insulin Action

The high incidence of diabetes in most animal models of leptin deficiency or leptin receptor deficiency and the correlation of obesity (leptin resistance) with insulin resistance and type 2 diabetes have prompted a great deal of research on the actions of leptin in the classic targets of insulin action (liver, adipose, and muscle) and in insulin-producing pancreatic beta cells. Although leptin deficiency does not correlate perfectly with type 2 diabetes (the few identified humans deficient in leptin action are not diabetic, and the penetrance of diabetes in rodents is importantly affected by genetic background), it is clear that leptin can play an important role in the progression to insulin resistance and diabetes (Fig. 4). Rodents with genetically impaired leptin action, for instance, progress to frank diabetes at a much higher frequency than do similarly obese rodents with genetic lesions in other systems (such as the melanocortin system). Furthermore, rodents and humans with severe lipodystrophy or lipoatrophy (lack of body fat) display leptin deficiency, enormously elevated serum lipids, and severe insulin resistance and diabetes. Leptin therapy abolishes or ameliorates the insulin resistance, elevated serum lipids, and diabetes without altering the primary lack of adipose tissue.

A number of studies have suggested a potential role for leptin in the regulation of insulin secretion from pancreatic beta cells (Fig. 4). LRb mRNA has been demonstrated in the beta cell and functional data suggest that the presence of this message may be physiologically relevant. Leptin stimulates the activation of Jak2 and Stat3 in these cells. Furthermore, direct treatment of cultured beta cell lines and primary pancreatic islets with leptin results in acutely decreased insulin secretion; as in the arcuate NPY/AGRP neuron, leptin inhibits the cell membrane ATP-regulated potassium channel to hyperpolarize the cell.

Leptin also stimulates the phosphorylation of Stat3 and other receptor-proximal signals in adipose and muscle tissue. Leptin treatment of rodents stimulates gene expression in adipose tissue; this action of leptin on adipose tissue may be mediated indirectly via the central nervous system, however. Additionally, leptin activates the adenosine monophosphate (AMP)-regulated protein kinase (AMPK) in skeletal muscle; two pathways appear to mediate this action: a rapid, direct pathway and a slower pathway mediated via the central nervous system. Although leptin-stimulated AMPK activation in skeletal muscle represents a recent discovery with unknown relevance to physiology, contraction-stimulated AMPK activation in muscle increases glucose uptake into the myocyte, suggesting a potential role for this leptin signal. In the liver, little evidence exists to suggest direct action on hepatocytes, although systemic leptin administration has dramatic effects on liver physiology. Indeed, leptin administration to leptin-deficient *ob/ob* or lipodystrophic mice and lipodystrophic humans reverses the excessive buildup of hepatic triglyceride and dysregulated glucose output associated with lipodystrophy syndromes.

## VII. SUMMARY

Leptin is secreted by fat cells as a signal of body energy stores; it serves to integrate the control of energy flux and to regulate the initiation of energy-intensive processes such as growth and reproduction. Thus, leptin deficiency results in increased eating, dysregulation of body energy distribution, and elements of the endocrine starvation response (decreased growth, infertility, etc.). LRb mediates most (if not all) leptin action, and a majority of leptin effects, even in peripheral tissues, depend on leptin action in the central nervous system. We have learned a great deal about leptin function in the years since its initial description, but a great deal remains to be understood about the molecular biology and physiology of leptin.

## Glossary

**adipocyte** Specialized triglyceride-storing cells found throughout the body, commonly known as fat cells. In addition to being an important site for the storage and release of metabolic energy, adipocytes are the primary source of circulating leptin.

**autonomic nervous system** Collectively, the sympathetic and parasympathetic nervous systems. The sympathetic nervous system readies the body for action, mobilizing energy stores; actions of the parasympathetic nervous system predominate in energy uptake and storage. The sympathetic nervous system acts on some targets via direct contact of nerves with these tissues, but also mediates systemic effects by promoting release of adrenaline from the adrenal medulla. Elevation of sympathetic nervous system activity increases heart rate, blood pressure, etc.

**diabetes** Specifically, diabetes mellitus. The syndrome that results from lack of insulin action, either due to absolute lack of insulin (type 1 diabetes) or the inability to produce enough insulin to overcome insulin insensitivity (type 2 diabetes).

**endocrine system** The network of all hormones. Many endocrine organs (tissues that secrete hormones) are controlled by the action of stimulating factors released by the anterior pituitary gland. The anterior pituitary cells that secrete stimulating factors are regulated by release factors that are secreted into the portal circulation of the pituitary by hypothalamic neurons.

**glucocorticoids** Cholesterol-derived steroid hormones (e.g., corticosterone) that are the primary product of the adrenal cortex. Some glucocorticoids are produced in adipose and other tissues, as well. Glucocorticoids bind to nuclear glucocorticoid receptors to modulate transcription of target genes. Glucocorticoids are often referred to as catabolic steroids, promoting the breakdown of cellular proteins and potentiating the effects of counterregulatory hormones.

**hormone** A substance secreted by cells into the extracellular milieu to communicate with other cells. Most hormones work at long distances via the circulation, but may also act on the cell that secreted them (autocrine action) or on nearby cells (paracrine action).

**hypothalamus** The part of the diencephalon in the subconscious brain that integrates various environmental stimuli to regulate motivation, physiology, and endocrine function. The hypothalamus contains nerve centers (nuclei) that regulate the autonomic nervous system and that regulate the production of pituitary releasing factors. It lies at the base of the brain, overlying the pituitary gland, and is connected to the pituitary gland by a stalk and a specialized portal circulation that carries releasing factors to the anterior pituitary.

**melanocortins** Peptide hormones (e.g., α- and γ-melanocyte-stimulating hormones and adrenocorticotropic hormone) derived from the precursor protein proopiomelanocortin. Following their release, melanocortins mediate intercellular signaling by binding and regulating one or more of the four known melanocortin receptors on the surface of target cells.

**receptor** A molecule that binds specifically to a hormone and generates a signal to mediate the action of the hormone.

**tyrosine kinase** An intracellular signaling enzyme that functions by transferring a phosphate group from ATP to tyrosine residues in substrate proteins. Phosphorylated tyrosine residues transmit downstream signals by recruiting signaling proteins that contain specialized phosphotyrosine-binding domains.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Diabetes Type 1 • Diabetes Type 2 • Eating Disorders • Growth Hormone (GH) • Leptin Actions on the Reproductive Axis**

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# Leptin Actions on the Reproductive Axis

Paolo Magni and Marcella Motta
*Università degli Studi di Milano*

I. INTRODUCTION
II. SITES AND MECHANISMS OF ACTION OF LEPTIN
III. LEPTIN AND PUBERTAL DEVELOPMENT
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VI. LEPTIN INVOLVEMENT IN REPRODUCTIVE IMPAIRMENT IN EATING DISORDERS
VII. CONCLUSIONS

**Leptin, a peptide hormone mainly secreted by adipose tissue, represents a signal of satiety and increased energy expenditure, with important actions at the level of the hypothalamus. In addition, leptin participates in the regulation of reproductive processes, playing a role in pubertal development and adult reproductive function. Changes in plasma leptin levels may be involved in the reproductive impairment of anorexia nervosa and obesity. Therefore, leptin may be regarded as a link between modulation of energy expenditure/food intake and the reproductive function.**

## I. INTRODUCTION

The discovery of leptin, a peptide hormone mainly secreted by the adipose tissue, has provided new insights on the mechanisms controlling food intake and energy expenditure in mammals. One major function of leptin is to inform the central structures controlling energy metabolism, e.g., the hypothalamus, on the status of energy stores, represented by fat mass. Recently, the spectrum of leptin actions has been expanded, and the involvement of this hormone in the control of reproduction has been closely studied.

Leptin is now regarded as a possible link between modulation of energy expenditure/food intake and the reproductive function. In mammals, this function is dependent on energy availability. The crucial physiological observation that these two processes are reciprocally associated in experimental animals, as well as in the human species, in both physiological and pathological conditions dates back over 50 years. The existence of common mechanisms, hypothesized in the early 1970s, suggests that a threshold body fat content is required for the onset of puberty and for maintenance of normal reproductive functions in adult women. Thus, low energy stores are associated with signals leading to increased food intake and reduced energy expenditure, but also to a reversible reproductive impairment. Acute modifications of energy balance are known to modulate the hypothalamic–pituitary–gonadal axis. In several species, fasting and caloric restriction result in suppression of pulsatile luteinizing hormone (LH) secretion, via an inhibition of the gonadotropin-releasing hormone (GnRH) pulse generator, consequently causing infertility. Similarly, excessive energy storage and obesity may interfere with the correct regulation of the reproductive axis. Defective circulating leptin, due to an inactivating mutation of the leptin gene, has been described in the *ob* mouse, which has a phenotype that includes severe and early-onset obesity, extreme insulin resistance, hyperphagia, reduced energy expenditure, and infertility in both sexes. The administration of leptin to *ob* mice results in the reestablishment of fertility, with normalization of body weight and food intake. These findings clearly suggest an important role for leptin in the modulation of reproduction, along with that related to food intake and energy expenditure.

## II. SITES AND MECHANISMS OF ACTION OF LEPTIN

Leptin achieves most of its metabolic and reproductive effects by interacting with specific receptors located in the central nervous system, mainly in the hypothalamus (Fig. 1). More specifically, leptin

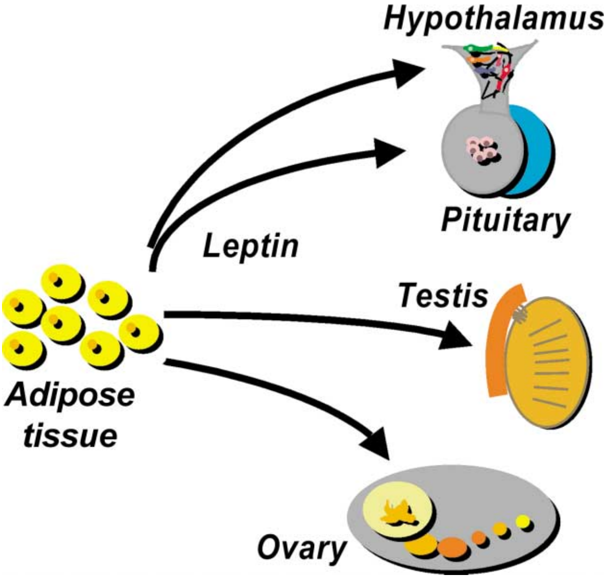

**FIGURE 1** Central and peripheral sites of action of leptin for the modulation of reproduction. Leptin, secreted by adipose tissue, acts at multiple levels of the reproductive axis, eliciting different effects on reproduction depending on its concentration in the blood.

receptors are present in four hypothalamic nuclei, i.e., medial hypothalamic, paraventricular (PVN), arcuate (ARC), ventromedial (VMN), and dorsomedial (DMN) nuclei. Leptin receptors, similar to class I cytokine receptors (gp130-like), possess a single transmembrane domain. The six identified isoforms, OB-Ra–OB-Rf, differ in the length of their intracellular domain (Fig. 2); the long isoform, OB-Rb, is abundantly expressed in the hypothalamus.

Leptin modulation of food intake, as well as of the GnRH–gonadotropin system, is believed to be transduced by neurons not only possessing leptin receptors, but also expressing different neuropeptides, i.e., corticotropin-releasing hormone, melanin-concentrating hormone, agouti-related peptide (AGRP), galanin, neurotensin, the most well-studied neuropeptide Y (NPY), and the proopiomelanocortin (POMC) derivatives, suggesting an action of leptin through the activation of these receptors. For instance, administration of leptin to rats results in a down-regulation of NPY gene expression, a reduction of food intake, and an increased energy expenditure. Because NPY is also involved in control of the reproductive function, it is possible that some actions of leptin related to this function might operate at the hypothalamic level through the NPY reproductive network that includes the ARC–median eminence–medial preoptic area. The interplay between NPY and leptin is also suggested by the observation that a cross of NPY knockout mice with *ob* mice decreases the degree of obesity present in the latter.

Inhibitory actions of leptin on food intake are also significantly mediated by signals transduced by melanocortin receptors, and particularly by the MC3 and MC4 receptor isoforms. POMC-deficient mice and humans are hyperphagic, obese, and leptin resistant. However, no major alterations of the reproductive axis or fertility are observed in MC3 receptor-, MC4 receptor-, and NPY-deficient mice: this is suggestive that either these systems do not mediate the reproductive effects of leptin, or, alternatively, that compensatory mechanisms are operative in these animals.

Leptin receptors are also localized at the pituitary level (Fig. 1), and leptin increases gonadotropin secretion and plasma levels in the rat, independently of its hypothalamic action. Messenger RNA transcripts encoding the leptin receptor are present in adult rat pituitary, human fetal pituitary, pituitary adenomas, and ewe anterior pituitary. In addition, a leptin receptor protein is expressed in 29 and 90% of the gonadotropes of the pars distalis and the pars tuberalis of ovine anterior pituitary, respectively; both OB-Rb and leptin are also expressed in normal and neoplastic human pituitary tissue. These data suggest that leptin has the potential to regulate pituitary function via endocrine, paracrine, and/or autocrine mechanisms. Moreover, high concentrations of leptin inhibit pituitary cell proliferation in human and rat pituitary cell lines. Consequently, leptin might directly and positively influence

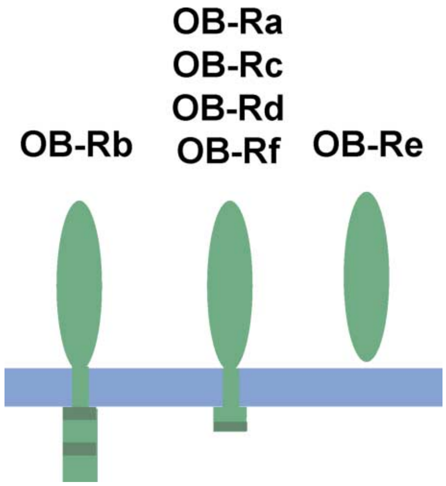

**FIGURE 2** The six leptin receptor isoforms. Due to differential RNA splicing, leptin receptors are present in different membrane and soluble (OB-Re) isoforms.

the functions of the gonadotropes by amplifying the stimulatory actions on the hypothalamus–pituitary–gonadal (HPG) axis exerted at the hypothalamic level; in addition, leptin might be involved in the regulation of the growth and differentiation of pituitary cells.

The presence of leptin receptors in peripheral structures of the reproductive system, namely, testis and ovary (Fig. 1), suggests that, in addition to the hypothalamus, leptin may exert its action also on these peripheral organs (see following sections).

## III. LEPTIN AND PUBERTAL DEVELOPMENT

The obese and leptin-defective *ob* mouse displays a marked reproductive immaturity in both sexes, with reduced gonadal and uterine weights, low follicle number, and low gonadotropin levels. All these parameters are corrected by leptin administration in male and female *ob* mice. Thus, leptin seems to act as an initiating and permissive factor for puberty, as observed also in normal mice and rats. On the other hand, it is difficult to correlate the complex mechanisms of puberty with modifications of putative single signals such as leptin. Food restriction is known to delay pubertal onset, whereas refeeding abolishes this delay. In addition, murine and human genetic models of leptin deficiency fail to enter puberty, and treatment with leptin can establish a pulsatile secretory pattern of gonadotropins that is characteristic of early puberty. On the other hand, the female transgenic skinny mouse, an *in vivo* model of chronic hyperleptinemia in the absence of adipose tissue, enters puberty precociously. Data regarding the effects of leptin administration on pubertal onset are controversial: intracerebroventricular leptin administration prevents the delay in vaginal opening induced by chronic food restriction in the rat. By contrast, artificially raised leptin levels are not sufficient to abolish the delay of pubertal onset caused by food deprivation. The occurrence of inactivating mutations of the leptin gene, as observed in the *ob* mouse, are extremely rare in humans; however, the few such cases reported confirm that leptin plays an important role linking adiposity and reproduction. In particular, adult female and male patients with the Arg105Trp replacement in the leptin gene are obese and are characterized by primary amenorrhea and prepubertal status, respectively. This observation also supports the concept that leptin is not only a permissive factor for the reproductive function in the adult, but might also represent a metabolic gate for the beginning of puberty, suggested by the peripubertal rise of plasma levels of the hormone in both sexes.

Age seems to represent an important factor that brings about a significant effect on serum leptin concentrations from prepuberty into early puberty. The sexual dimorphism in leptin concentrations becomes evident only after puberty (Fig. 3). In males, leptin levels rise throughout childhood, reach a peak in the early stages of puberty, and then decline, whereas they increase steadily during pubertal development in females. Consequently, adult leptin levels are three to four times higher in females than in males (Fig. 4). The reason for this postpubertal sexual dimorphism in leptin levels is not clear. After puberty, serum testosterone and testicular volume are inversely related to leptin levels in males, whereas in females, when adjusted for adiposity indexes, estradiol is directly correlated with leptin levels. These observations indicate that androgens and estradiol might account, at least in part, for the gender differences in circulating leptin levels. This is also supported by *in vitro* studies showing that androgens and estrogens inhibit and stimulate leptin expression and release from human adipocytes in culture, respectively. Thus, puberty represents a turning point in the sexual dimorphic relationships between the reproductive axis and leptin by determining the

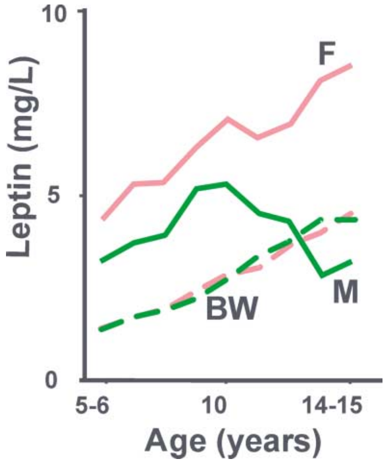

**FIGURE 3** Sexually dimorphic leptin levels in puberty. In male (M) and female (F) children, plasma leptin levels (solid lines) rise with age and body weight (BW; dashed lines), but in males there is a decrease to adult levels once testosterone production begins in the testis. Modified from Horlick M. B., *et al.*, (2000), Effect of puberty on the relationship between circulating leptin and body composition. *J. Clin. Endocrinol. Metab.* 85(7), 2509–2518, Copyright The Endocrine Society, with permission.

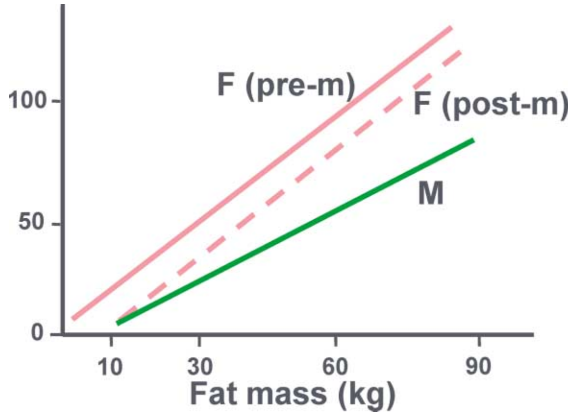

**FIGURE 4** Sexually dimorphic leptin levels in adults. Plasma leptin levels are proportional to fat mass, but are higher in premenopausal (pre-m) women than in postmenopausal (post-m) women and in men (M). Modified from Rosenbaum, M., *et al.*, (1996), Effects of gender, body composition, and menopause on plasma concentrations of leptin. *J. Clin. Endocrinol. Metab.* **81**(9), 3424–3427, Copyright The Endocrine Society, with permission.

steroid milieu that leads to a different regulation of leptin secretion in the sexes.

## IV. LEPTIN AND MALE REPRODUCTION

Leptin receptors are present in the rat and mouse testis and Leydig cells. Leptin exerts a rapid and dose-dependent inhibition of LH-stimulated testosterone production in rat Leydig cells in culture. Human chorionic gonadotropin (hCG)-stimulated testosterone suppression is accompanied by a parallel reduction of androstenedione levels and a concomitant rise in the precursor metabolites 17-OH-progesterone, progesterone and pregnenolone, compatible with a leptin-induced impairment of 17–20 lyase activity. In addition, leptin inhibits testosterone secretion from adult rat testicular slices incubated *in vitro*, but not from prepubertal testes. These observations indicate that leptin has the potential to modulate the paracrine network that controls gonadotropin-stimulated testicular steroidogenesis, which is analogous to its actions in the ovary.

A negative correlation between plasma testosterone and leptin exists also in humans. In particular, hypogonadal men display higher levels of leptin that are normalized after androgen replacement therapy. Obese subjects, as a group, show raised levels of leptin in blood and reduced androgen concentrations. In obese men, the inhibitory effect of leptin on hCG-stimulated testosterone production appears at concentrations within the range of circulating leptin levels. In addition, the degree of the androgen reduction is related to the amount of fat mass and also to leptin levels. The androgen response to hCG stimulation is impaired in obese men; it is possible that leptin excess might play an important role in the development of reduced androgen production in male obesity.

Immunohistochemical studies demonstrate that mouse testis germ cells express leptin receptors in a stage- and age-dependent manner. Furthermore, *in vitro* treatment of isolated seminiferous tubules with leptin leads to Stat3 phosphorylation, which indicates that OB-R is functional and capable of signal transduction in germ cells. These data suggest that leptin might have additional testicular effects, possibly on the proliferation and differentiation of germ cells, and that the lack of its action(s) might be locally involved in the pathogenesis of infertility observed in leptin-deficient mice.

## V. LEPTIN AND FEMALE REPRODUCTION

Leptin receptor expression is abundant in the ovary. Studies conducted on thecal and granulosa cells show that leptin has a negative effect on ovarian steroid production, both in rodent and bovine models. In particular, leptin inhibits insulin-induced progesterone and 17β-estradiol production by isolated bovine granulosa cells, it prevents insulin-induced progesterone and androstenedione secretion in bovine ovarian thecal cell, and it blocks the hormonally stimulated release of 17β-estradiol by rat granulosa cells in culture. Incubation of granulosa cells from fertile women with leptin significantly reduces the production of 17β-estradiol stimulated by follicle-stimulating hormone and insulin-like growth factor I. These observations indicate that leptin, at concentrations commonly found in obese women, has the potential to interfere with estradiol production by the dominant follicle *in vivo*. In addition, an excess of leptin might alter the ovarian response to local tropic stimuli, produced by the dominant follicle. If elevated leptin levels interfere with the development of the dominant follicle and reduce estradiol production, there would be no adequate trigger for LH secretion, which would result in anovulation. In agreement with this observation, administration of leptin in immature gonadotropin-primed rats and exposure of intact rat ovaries to leptin lead to a marked decline (three- to fourfold) in ovulation rate. All these observations might explain, in part, the high incidence of reproductive dysfunction in obese women and the improvement of anovulation after weight loss.

A putative role of leptin has been proposed in the pathophysiology of polycystic ovary syndrome (PCOS). Insulin resistance and hyperinsulinemia are frequently found in patients with PCOS, although insulin is able to stimulate leptin synthesis and secretion from adipocytes *in vitro*. However, the available data are controversial, and the reports are of increased or unchanged leptin levels in PCOS patients. To evaluate the importance of leptin in female fertility, serum and follicular fluid leptin levels have been measured in different categories of patients: women who succeeded and women who failed in becoming pregnant after assisted reproductive cycles, and patients with PCOS. It was found that lower follicular fluid leptin concentrations are a predictor of pregnancy success, both in normal women and in those with PCOS. However, plasma and follicular fluid leptin levels, after adjustment for body mass index (BMI) and age, are again not significantly higher in the PCOS group than in normal controls. These data are compatible with the hypothesis that when intraovarian concentrations of leptin are high, they might reduce estradiol production and interfere with the development of the dominant follicle and oocyte maturation, thereby contributing to the pathogenesis of female infertility frequently found in obese women.

Pregnancy represents an important challenge in terms of energy metabolism. Several observations suggest the involvement of leptin in this process. Leptin and the related receptors are expressed in the placenta; during pregnancy, especially in the second and third trimesters, serum leptin levels are elevated in both animal models and humans and drops sharply after delivery. Interestingly, the elevated leptin levels during pregnancy cannot be attributed solely to gestational weight gain and increase in BMI. Instead, this increase is mainly the result of other factors, which include the secretion of placenta-derived leptin and the soluble form of its receptor into the maternal circulation, as well as changes in the levels of hormones that might stimulate leptin secretion (e.g., insulin, estrogens, and hCG). Pregnancy-induced hyperleptinemia is not associated with a decrease of food intake or with an increase of energy expenditure, suggesting that the functional reason for elevated leptin is not yet fully understood. In addition to being a site of leptin synthesis, the placenta is a site of abundant leptin receptor expression, both the long signaling and short transporting isoforms. It is possible, therefore, that placenta-derived leptin might play a paracrine and/or autocrine role in placental-fetal physiology. Placenta-derived leptin might act as an important growth factor for the fetus and/or a signal of energy status between mother and fetus. In addition, the reduction in leptin levels seen after delivery might play a role in the reduced fertility found during the period of lactation.

## VI. LEPTIN INVOLVEMENT IN REPRODUCTIVE IMPAIRMENT IN EATING DISORDERS

Human obesity is often associated with reproductive disturbances. In this condition, the excessive fat mass might negatively affect the reproductive axis through an altered secretion of at least two known adipose products, leptin and estrogen. However, the picture observed in common human obesity seems much more complex than in several rodent models of obesity. In obese patients, plasma leptin levels are usually proportional to fat mass and thus are elevated; however, in spite of this, an excess of adipose tissue is maintained, leading to the hypothesis of a sort of leptin resistance that cannot be overcome by the high plasma levels of leptin. Leptin might have a dual effect on reproduction, and the major site of action may differ depending on its circulating levels in the blood (Fig. 5). The positive action of leptin at the hypothalamic–pituitary level might be crucial as a trigger of puberty, and might play a predominant role in conditions with abnormally low plasma concentrations, such as in subjects with very low BMI (anorexic patients) or in patients with homozygous mutations of the leptin gene. Conversely, in obesity, central leptin receptors, which are sensitive to

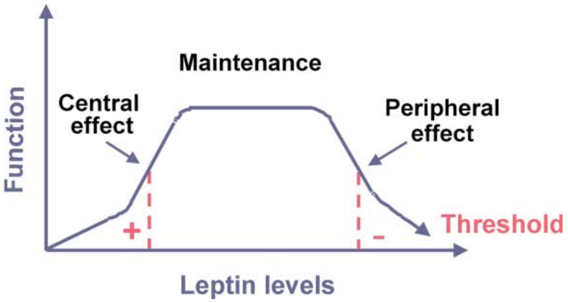

**FIGURE 5** Proposed dual action of leptin on reproductive function. Leptin concentrations above a minimal threshold are necessary in the hypothalamus to activate the reproductive axis. Leptin excess above a certain threshold, such as is found in obesity, might impair gonadal steroidogenesis and have deleterious effects on reproduction. Modified from Caprio *et al.* (2001), Copyright Elsevier Science, with permission.

extremely low hormone concentrations, are protected from hyperleptinemia by the saturable transport system of the blood–brain barrier, whereas peripheral leptin receptors are directly exposed to high ligand concentrations, with possible negative effects on gonadal steroidogenesis. It can be hypothesized that a specific and narrow range of leptin concentrations is necessary to maintain a normal reproductive function in both sexes, and that concentrations below or above these thresholds might interfere in opposing ways with the function of the reproductive axis. In any case, the interactions between leptin and the reproductive system appear to be bidirectional, because there is an effect on fertility not only by plasma leptin levels but also by gonadal function (i.e., gonadal production of sex hormones), which modulates leptin synthesis and secretion.

## VII. CONCLUSIONS

The discovery of leptin and its related receptors has provided new insights into the pathophysiology of reproduction. It is well established that leptin acts at various levels of the reproductive system, involving different tissues and multiple biochemical pathways. The effect of leptin on reproductive function seems to be determined by different thresholds of activity, depending on its site of activity. In conclusion, leptin appears to accomplish several requirements, linking the regulation of energy balance and the control of reproduction. Future studies in this field should address physiological issues, such as the complete spectrum of the pleiotropic actions of leptin, as well as pathological problems. In this regard, it might be of great interest to test whether leptin or leptinomimetic agents may be useful in correcting metabolic and reproductive dysfunctions that often are simultaneously present in a patient affected by obesity or by eating behavior disorders such as anorexia nervosa or bulimia nervosa.

## Glossary

**gonadotropin-releasing hormone** Hypothalamic peptidic hormone stimulating the secretion of gonadotropins.

**leptin** Cytokine-like peptide composed of 146 amino acids, mainly secreted by adipocytes.

**leptin receptors** Exist in several isoforms and are similar to class I cytokine receptors.

**neuropeptide Y** A 36-amino-acid neuromediator of appetite and other functions.

**ob mouse** Leptin-defective mouse model of obesity.

**polycystic ovary syndrome** Disease characterized by obesity, insulin resistance, ovarian dysfunction, and cutaneous symptoms.

## See Also the Following Articles

**Eating Disorders • Gonadotropin-Releasing Hormone (GnRH) • Insulin Resistance in PCOS (Polycystic Ovary Syndrome) • Leptin • Neuropeptide Y (NPY) • Polycystic Ovary Syndrome (PCOS)**

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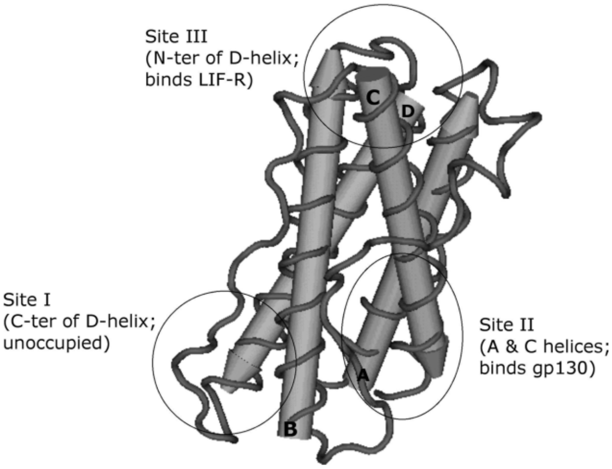

**FIGURE 1** The helix folding based on the crystal structure of leukemia inhibitory factor (LIF). The four helices are labeled on the N-termini. The receptor-binding sites as defined by mutagenesis are indicated.

the phenotype of LIF knockout mice. The mice show impairments in several systems, including reproduction, the hypothalamic–pituitary–adrenal (HPA) axis, the nervous system, and the immune system.

## II. RECEPTOR USAGE IN THE GP130 FAMILY

The gp130 cytokines assemble receptor complexes that contain multiple receptor components, including at least one molecule of gp130. The extracellular domain of gp130 includes a cytokine-binding domain (CBD), also referred to as cytokine-binding module (CBM), cytokine-binding homology region (CHR), or cytokine receptor-like domain (CRD); this domain contains the characteristic four conserved cysteines in the N-terminal fibronectin type III module and a WSXWS motif (using the single-letter amino acid code, W is tryptophan and S is serine; X is any amino acid) in the C-terminal fibronectin type III module. The CBD is sandwiched between an N-terminal immunoglobulin G (IgG)-like domain that plays a role in ligand recognition and three C-terminal fibronectin domains that are adjacent to the plasma membrane (Fig. 2).

With the exception of viral IL-6, gp130 cytokines require additional receptor components in order to activate gp130. IL-6 and IL-11 utilize the receptor specificity subunits, IL-6Rα and IL-11Rα respectively to generate high-affinity complexes with gp130. The cytoplasmic domains of specificity subunits are short or absent, suggesting that they do not contribute to signal transduction. Specificity subunits can usually function *in trans* as soluble receptors. The importance of specificity subunits is that their restricted expression determines which cell types respond.

To form high-affinity receptor complexes, LIF requires an additional transmembrane gp130-related subunit, LIF-R (sometimes referred to as LIF-Rβ). LIF-R resembles gp130 except that LIF-R contains an additional CBD on the N-terminal side of the IgG-like domain. LIF-R is detected as a 190-kDa component on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), whereas gp130 migrates more rapidly, ranging from to 130 to 150 kDa. CT-1 can also directly activate complexes of gp130 and LIF-R. A specificity subunit for CT-1 has been proposed but has not yet been cloned. At very high concentrations, CNTF can directly activate gp130/LIF-R, but at low concentrations the specificity subunit CNTFRα is required to activate the complex. CNTFRα is a glycophosphatidylinositol (GPI)-linked receptor that is primarily expressed on neurons. CNTF lacks a signal sequence and is not normally secreted. A secreted ligand for CNTFRα/gp130/LIF-R has been recently identified. CLC is secreted when bound to cytokine-like factor-1 (CLF), a soluble receptor that contains a CBD and an IgG-like domain. The CLC/CLF complex activates gp130/LIF-R in the presence of membrane-anchored CNTFRα. CLC can also be secreted when bound to CNTFRα.

In humans, OSM can activate either gp130/LIF-R complexes or gp130 in combination with another related transmembrane component, OSM-R. OSM-R resembles LIF-R except that the N-terminal CBD contains only a single fibronectin type III module. In mice, murine OSM will activate only gp130/OSM-R. If human OSM is expressed in mice, gp130/OSM-R is not activated; instead, gp130/LIF-R is activated. The large number of gp130 cytokines that activate LIF-R/gp130 complexes complicates the interpretation of the biological activities of exogenous LIF.

## III. RECEPTOR ENGAGEMENT BY LIF

LIF is active in bioassays at low concentrations, displaying $EC_{50}$ values below the picomolar level. LIF binds to the LIF-R subunit with an affinity of 1–5 n*M*. The affinity is converted to about 50 p*M* by the recruitment of gp130 into the receptor complex. The higher affinity of the complex reflects a reduction in the off rate. Size-exclusion chromatography indicates that the stoichiometry of the complex is likely to be a trimer of LIF, LIF-R, and gp130. There is a pronounced species specificity to receptor activation by LIF. Human LIF will activate both human LIF-

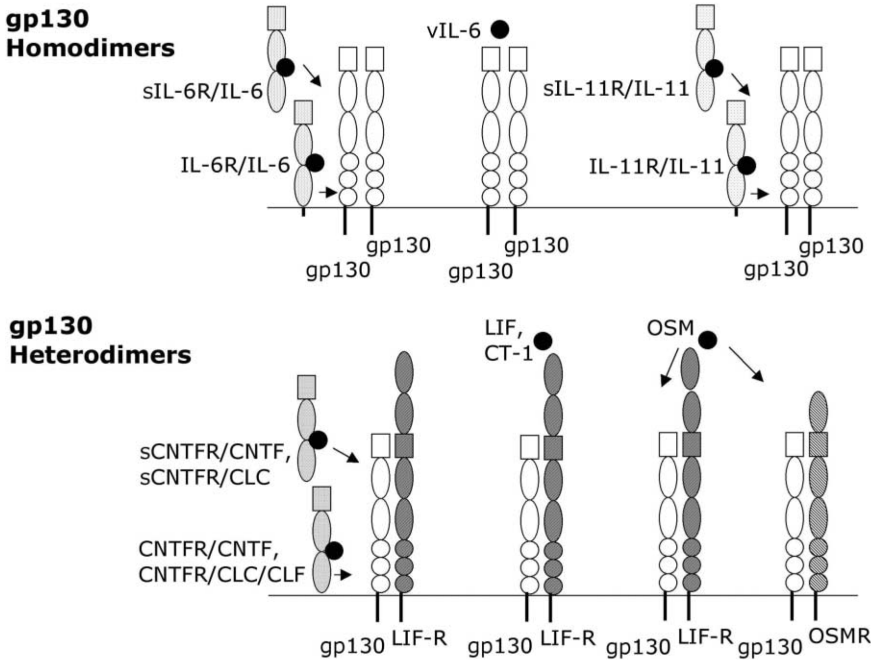

**FIGURE 2** Receptor interactions in the gp130 family. Ovals denote the fibronectin type III modules of the cytokine-binding domain, squares denote immunoglobulin G-like domains, and circles denote fibronectin type III domains. IL-6, interleukin-6; sIL-6R, soluble IL-6 receptor; vIL-6, viral IL-6; LIF, leukemia inhibitory factor; CT-1, cardiotropin-1; OSM, oncostatin M; CNTFR, ciliary neurotropic factor receptor; CLC, cardiotropin-like cytokine; CLF, cytokine-like factor-1.

R/gp130 and murine LIF-R/gp130 complexes with high affinity. In contrast, murine LIF is only a very weak agonist for human LIF-R/gp130.

The crystal structure of LIF has provided a basis for rational mutagenesis of LIF. Two receptor-binding sites have been identified. The gp130 molecule binds to a site in the middle of the A- and C-helices of LIF, referred to as site II. Site II is topologically similar to the region where the second growth hormone receptor binds in the growth hormone/growth hormone receptor complex (Fig. 1). LIF-R binds to site III at the N-terminus of the D-helix. Site III is topologically unrelated to the receptor-binding sites on growth hormone. Because the two receptor recognition sites on LIF, sites II and III, are topologically separate, disruption of site II creates a LIF-R antagonist that occupies LIF-R in nonproductive complexes that fail to recruit gp130. LIF-R antagonists constructed in this way are useful reagents for inhibiting LIF-R activation. Residues in the region equivalent to site I on growth hormone do not appear to be essential for LIF-R or gp130 binding. Site I is occupied on some of the other gp130 cytokines by the specificity subunits IL-6Rα, IL-11Rα, and CNTFRα. In terms of the receptors, particular amino acids in the CBD of gp130 and the IgG-like domain of LIF-R have been suggested to participate in LIF binding. Confirmation awaits the crystal structure of the full complex.

## IV. SIGNAL TRANSDUCTION

LIF-R and gp130 rely on intracellular Janus tyrosine kinases (JAKs) that bind to the cytoplasmic domains of the receptor components near the plasma membrane (Fig. 3). Three of the four JAKs (JAK1, JAK2, and Tyk2) can be activated by LIF, but JAK1 is the most important for LIF signaling. Receptor oligomerization by LIF results in JAK activation and receptor autophosphorylation. Phosphorylated tyrosines subsequently recruit signal transducers and activators of transcription (Stats), primarily Stat3, and to a lesser extent, Stat1. The Stats are then phosphorylated by JAKs, triggering the release of Stats from the receptors. The phosphorylated Stats dimerize and are translocated to the nucleus, where they can influence transcription. Stat3 targets include the acute-phase genes in hepatocytes, transcription factors such as Jun B and c-Fos, and the neuropeptide vasoactive intestinal peptide (VIP). The activation of Stat3 underlies the ability of LIF to synergize with

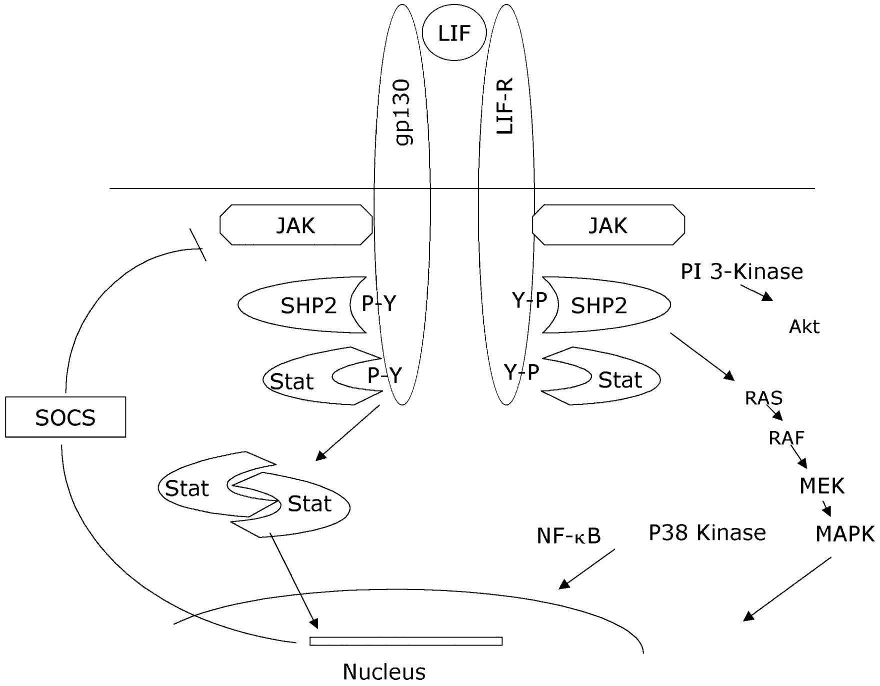

**FIGURE 3** Signal transduction pathways activated by leukemia inhibitory factor (LIF). Aside from the JAK/Stat pathway, the precise routing of signals is poorly defined in most cell types (see text for discussion).

growth factors that activate the Smad transcription factors. For example, LIF and bone morphogenetic protein-2 (BMP-2) activate synergistically the glial fibrillary acidic protein (GFAP) promoter to enhance astrocyte differentiation. One of the general targets of Stat3 is the transcription of molecules that inhibit active JAKs, such as suppressor of cytokine signaling-1 and-3 (SOCS-1 and SOCS-3).

LIF-R/gp130 autophosphorylation also results in activation of the Ras/mitogen-activated protein kinase (MAPK) pathway, possibly via SHP2 (SH2 domain-containing protein tyrosine phosphatase-2) binding to SH2-containing adapter proteins. Stat3 activation and MAPK activation appear to work together in supporting proliferation. In BAF cells transfected with LIF-R/gp130, Stat3 provides an anti-apoptotic signal and MAPK provides a mitogenic signal. LIF activation of phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), nuclear factor κB (NF-κB), and p38 kinase can also be observed in various cell types. For example, NF-κB is required for the promotion of survival of primary sensory neurons by LIF-R ligands. By utilizing chimeric receptors, it is possible to compare the signals generated by gp130/gp130, gp130/LIF-R, and gp130/OSM-R complexes. The comparisons indicate that although there is overlap in pathway activation, there is specificity as well. Hence the equivalence or nonequivalence of gp130 cytokines is assay dependent.

## V. LIF GENE ORGANIZATION AND EXPRESSION

### A. Species

LIF homologues have been sought in many species in the hope that they will maintain embryonic stem cells that can be used to create transgenic animals. LIF sequences have been identified in tissues from humans, mice, rats, hamsters, pigs, American minks, cows, muskrats, and even the marsupials, brushtail possums. Although LIF has not been identified in chickens, chickens make a cytokine called growth-promoting activity (GPA) that resembles CNTF in structure and activity, but is secreted. Frogs have been shown to have factors with sequences that resemble those of gp130 and LIF-R, but the extent of ligand conservation is not yet clear.

### B. Chromosomal Location

In humans, the LIF gene is located at 22q.12 and is near the gene for a related family member, OSM, suggesting that both products are a result of recent gene duplication. The human and mouse LIF genes are about 80% identical in amino acid sequence and both genomes show the LIF/OSM synteny.

### C. Transcripts

LIF transcripts are composed of three exons (Fig. 4). Only the last two exons contribute to the mature peptide. The first exon determines the nature of the signal sequence and therefore influences the localization of the protein. At least three different versions of the first exon can be selected by alternative splicing. In mice, one of the versions, the M-form, has been associated with the expression of LIF in the extracellular matrix, and the D-form has been associated with diffusible expression. An additional form, the T-form, which completely lacks a signal sequence and the N-terminus of the mature peptide, produces a truncated version of LIF that is cytoplasmic. Similar transcripts have been identified in human tissues, although in humans the M-form lacks an in-frame methionine and gives rise to a mixture of secreted and cytoplasmic forms of LIF. The three alternatively spliced versions of LIF are expressed in different ratios by different cell types, suggesting that the splicing is regulated. Transgenic mice overexpressing murine LIF M-form or D-form under the same promoter develop different phenotypic abnormalities, indicating that the manner of presentation may be important in shaping the biological activity.

### D. Expression

LIF expression is kept under tight temporal and spatial control. LIF transcripts have been detected in many tissues in mice, including (at least) heart, liver, pituitary, pancreas, breast, uterus, gut, germ cell, kidney, normal pigment epithelium, cartilage, lung, lymphocytes, monocytes, osteoblastic cells, bone marrow stromal cells, and thymus. However, strong expression of LIF is restricted in healthy mice to the uterine endometrial glands of pregnant females just prior to implantation. Strong LIF expression is also detected in affected tissues following infection, inflammation, and injury. For example, LIF is elevated in synovial tissues in rheumatoid arthritis, the bronchoalveolar lavage of patients with acute respiratory distress syndrome, the urine of patients undergoing acute rejection after kidney transplantation, and in the early stages of contact dermatitis. The cellular sources of LIF include cells of the immune system, such as monocytes/macrophages and T cells, as well as stromal cells, such as fibroblasts, muscle cells, keratinocytes, epithelial cells, synoviocytes, chondrocytes, osteoblasts, neurons, Schwann cells, and astrocytes. Inducers of LIF expression include bacterial products and other cytokines, such as IL-1, IL-6, IL-16, and tumour necrosis factor (TNF), as well as reproductive hormones. LIF expression is typically local; only in the event of septicemia does LIF rise substantially in serum.

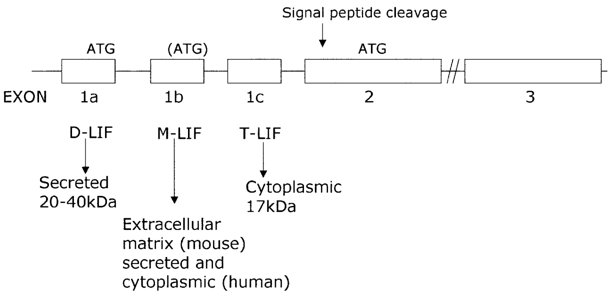

**FIGURE 4** The leukemia inhibitory factor (LIF) gene contains three exons. The mature protein is encoded within the second and third exon. The first exon determines the nature of the signal peptide and hence the cellular localization of the protein. The first exon is alternatively spliced, giving rise to three transcripts. In humans, the in-frame ATG in the second exon is missing in the M-LIF form and alternative translation initiation sites are employed.

## VI. *IN VITRO* ACTIVITIES OF LIF

Both gp130 and LIF-R are expressed by most tissues and cell lines. Hence, LIF is active in a wide variety of biological assays, evoking cellular responses that include proliferation, differentiation, protection from apoptosis, inhibition of differentiation, and gene activation. Table 2 lists a sample of *in vitro* activities in a range of cell types. Of the numerous activities, the neurotropic activities of LIF have attracted strong interest from clinicians as a way to support neurons in degenerative diseases. Provided that the appropriate receptor components are present, activities similar to those of LIF will often be observed with other gp130 cytokines. There are currently only a few examples *in vitro* for which the identities of the signal-transducing subunits (gp130, LIF-R, and OSM-R) have been shown to make a qualitative difference.

## VII. *IN VIVO* ACTIVITIES OF LIF

### A. Systemic LIF Elevation

Experiments in mice have revealed that prolonged elevation of LIF wrecks havoc on the body. Mice

TABLE 2 *In Vitro* Activities of LIF[a]

| Cell type | Activity |
|---|---|
| Embryonic stem cells | Prevents differentiation |
| M1 leukemic cell line | Differentiation |
| Macrophages | Chemotaxis |
| Hematopoietic progenitors | Proliferation of primitive and committed myeloid progenitors, megakaryoctes |
| Osteoblasts | Proliferation, survival, differentiation |
| Adipocytes | Differentiation, inhibits lipoprotein lipase |
| Fibroblasts | Increases TIMP1, proliferation |
| Synoviocytes | Increases cytokine production |
| Chondrocytes | Increases TIMP1, IL-1β |
| Endothelial cells | Increases protein S, inhibits proliferation |
| Myoblasts | Proliferation |
| Cardiac myocytes | Survival |
| Hepatocytes | Induces acute-phase proteins |
| AtT-20 (pituitary corticotrophs) | Increases POMC expression, ACTH, and growth hormone secretion |
| Lung epithelial cells | Increases cytosolic phospholipase $A_2$ |
| Keratinocytes | Increases cytokine production |
| Spinal cord precursors | Neuronal and astrocytic differentiation |
| Sensory neurons | Survival |
| Sympathetic neurons | Increases acetylcholine synthesis and decreases catecholamines synthesis |

[a] The list of *in vitro* activities for LIF presented here includes a small selection of the large number of activities observed following LIF application to different cell types and tissues.

implanted with LIF-secreting cells become cachectic and hypermotile and ultimately die after 6–8 weeks of treatment. Both subcutaneous fat and abdominal fat are completely depleted. The mice also display thymus atrophy, osteopetrosis, calcification of skeletal and heart muscles, and numerous other pathologies. Injecting LIF daily for 2 weeks results in a milder phenotype; however, at high doses, behavioral changes, loss of body fat, and thymus atrophy still occur. Megakaryocyte and platelet levels are also elevated. Most of the symptoms of the LIF-treated mice can be understood in terms of the *in vitro* activities of LIF on different cell types.

## B. LIF − /− Mice

Experiments in which LIF is overexpressed *in vivo* may offer more insight into the consequences of generalized LIF-R activation than true LIF function in which LIF expression is restricted temporally and spatially. LIF knockout mice are more informative, with the caveat that the extent (if any) to which LIF *in vivo* functions are masked by redundancy with other gp130 cytokines is unknown. So far, the double knockouts with IL-6 − /− mice and CNTF − /− mice demonstrate only very modest phenotypic synergy, but that may not be true for other gp130 cytokines.

Two different groups described LIF − /− mice in the early 1990s. Despite expectations of a lethal deficit in embryonic stem cells, the mice were viable but smaller than their wild-type littermates.

### 1. *Implantation*

LIF − /− males are fertile and LIF − /− females are infertile. The fertility defect derives from a maternal defect in implantation. LIF −/− blastocysts implant successfully when transplanted into foster wild-type females, whereas even wild-type blastocysts fail to implant into foster LIF − /− females. Introducing exogenous LIF into LIF − /− females throughout the period of normal implantation restores implantation. Additional work has carefully defined the spatial and temporal expression of LIF, LIF-R, and gp130 during the establishment of pregnancy, but the functional role of LIF in implantation is still poorly defined. Low LIF production is also associated with reduced fertility in humans, indicating that increasing LIF might aid fertility in some women.

### 2. *Hematopoiesis*

The stem cell compartment and the immune responses of LIF − /− mice are abnormal. The numbers of pluripotent stem cells in the spleen and the bone marrow of the mice are reduced, but the overall numbers of nucleated cells in the spleen and bone marrow and the numbers of circulating mature red cells and platelets are normal. This suggests a reduction in the stem cell pool rather than a reduction in the ability of hematopoietic cells to differentiate. Indeed, LIF − /− stem cells developed normally when introduced into an irradiated host, arguing that the deficiency derives from the microenvironment for supporting stem cells in the LIF − /− mice and not from the stem cells. The thymic T-cell responses to concanavalin A (Con A) and allogenic stimulation are also strongly reduced in the LIF − /− mice, indicating alterations in the thymic epithelium. Wild-type animals reconstituted with LIF − /− stem cells regain normal T-cell responses, again suggesting that the deficiency derives from the stromal microenvironment.

Subjecting the LIF − /− mice to specific models of injury and inflammation has yielded a number of differences from wild-type mice, especially at the interface of the immune system, the endocrine system, and the nervous system. A few of the conditions tested and the resulting findings are described in the following sections.

### 3. *Nerve Injury and Inflammation*

LIF is essential for neuronal and muscle repair following trauma. For muscle, the absence of LIF reduces the size of muscle fibers, probably by reducing the proliferation of myoblasts. For neurons, the absence of LIF changes their intrinsic growth status. Normally, conditioning lesions enhance the growth status of neurons. If the sciatic nerve is axotomized *in vivo* 2 weeks before dorsal root ganglion (DRG) neurons are transplanted into culture, the conditioned neurons will show increased neurite outgrowth in culture. In such a model, neurons from the LIF − /− mice fail to demonstrate conditioning. Furthermore, when regeneration is measured after a nerve crush, peripheral nerve regeneration in the LIF − /− mice is impaired. The LIF − /− sensory neurons fail to progress out of the crush site, whereas neurons from wild-type mice penetrate beyond the crush site into the distal nerve stump. The lack of regeneration is consistent with an intrinsic defect in the ability of LIF − /− neurons to extend neurites. However, an extrinsic change in the inflammatory cell infiltrate may also have shaped the behavior of the LIF − /− neurons.

In a separate study of LIF − /− mice, following nerve crush, inflammatory cells infiltrated the nerve more slowly compared to the infiltration in wild-type mice. The slowed inflammatory response was also observed when the brain cortex of LIF − /− mice was lesioned. Both microglia and astrocytes were slow to respond in the LIF knockout. Although LIF appears to be pro-inflammatory within the nervous system, that is not true everywhere. Injection of complete Freund's adjuvant (CFA) into the footpad of an adult LIF − /− mouse resulted in increased edema and cellular infiltration compared to wild-type mice. Local introduction of LIF via an injection of an adenoviral vector expressing LIF suppressed the inflammation. The disparate effects of the absence of LIF on cellular infiltration in the nerve injury model and the CFA footpad model argue that the function of LIF in the cytokine networks must be different in each of the models.

### 4. *Hypothalamic–Pituitary–Adrenal Axis*

LIF plays an important role in regulating the hypothalamic–pituitary–adrenal (HPA) axis in response to psychological stress or inflammation. When adrenocorticotropic hormone (ACTH) is measured, LIF − /− mice display lower basal levels compared to wild-type mice. Injection of CFA into the tail vein or injection of turpentine into the hindlimb muscle fails to elicit in LIF − /− mice a normal rise in ACTH and corticosterone. In these models of inflammation, in intact mice, LIF expression is highly induced in the hypothalamus and weakly induced in the pituitary. The critical LIF activity is the induction of proopiomelanocortin (POMC) expression and ACTH secretion in the pituitary.

## VIII. SUMMARY

LIF is a four-helical bundle cytokine that belongs to the gp130 family of cytokines. Along with several other members of the family, LIF activates receptors composed of the signal-transducing components, LIF-R and gp130, which activate the JAK–Stat pathway. The sharing of receptor components accounts for the redundancy in activities observed with other family members *in vitro*. Most cell types express LIF-R and gp130, but their responses to LIF are varied, including proliferation, survival, prevention of differentiation, differentiation, and gene activation. LIF is highly expressed in the endometrial glands of pregnant mice; otherwise, it is only weakly expressed except in inflammation and tissue injury. Experiments with LIF − /− mice demonstrate an essential role of LIF in implantation, generation of a normal stem cell compartment, repair of nerve injury, control of inflammation, and the regulation of the HPA axis.

## Glossary

**cytokine-binding domain** Conserved domain in the extracellular region of type I cytokine receptors; composed of two fibronectin type III modules that can function as a ligand-binding pocket.

**gp130** Signal-transducing receptor component shared by a large family of cytokines, including interleukin-6 and leukemia inhibitory factor.

**immunoglobulin-like domain** Domain of about 100 amino acids; predicted to adopt a seven-stranded immunoglobulin-like fold that in cytokine receptors has been shown to participate in ligand binding.

**Janus tyrosine kinases** Family of cytoplasmic protein tyrosine kinases; bind to the cytoplasmic domains of cytokine receptors and are activated on ligand binding.

**leukemia inhibitory factor receptor** Signal-transducing receptor component; closely related to gp130 and binds leukemia inhibitory factor with nanomolar affinity.

**signal transducers and activators of transcription (Stats)** Family of receptor-binding transcription factors that are translocated to the nucleus when phosphorylated by Janus tyrosine kinases.

**Src homology 2 domain-containing protein tyrosine phosphatase-2** Key protein in transducing signals from cell surface receptors to the nucleus; functions as an adapter as well as a phosphatase.

**suppressor of cytokine signaling (SOCS)** Family of proteins characterized by a central SH2 domain and a suppressor of cytokine signaling box that mediates negative feedback by inhibiting Janus tyrosine kinases.

## See Also the Following Articles

**Erythropoietin, Biochemistry of • Granulocyte Colony-Stimulating Factor (G-CSF) • Growth Hormone (GH) • Interleukin-6 • Leptin • Prolactin (PRL)**

## Further Reading

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# LH

**See *Luteinizing Hormone***

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# Ligand Modification to Produce Pharmacologic Agents

Eugene N. Bush
*Abbott Laboratories, Illinois*

I. GENERAL CONCEPTS OF CHEMICAL COMMUNICATION
II. RECEPTOR CLASSIFICATION
III. PHARMACODYNAMIC CONSIDERATIONS

IV. PHARMACOKINETIC CONSIDERATIONS
V. SUMMARY

**Cellular communication in biological systems is based on receptor targets for naturally produced chemical ligands. Natural ligands may have limitations with regard to efficacy and specificity. Structural modifications of natural ligands may alter activity and pharmacokinetic characteristics. Conformationally restricted compounds can show improved selectivity. Systematic chemical substitutions and random screening procedures may result in ligands with a spectrum of activity from antagonist to full agonist, as well as specificity at the receptor target.**

## I. GENERAL CONCEPTS OF CHEMICAL COMMUNICATION

Chemical communication is utilized at several levels within a multicellular organism. Certain organs or tissues (endocrine glands) contain specialized neuroendocrine cells that produce and secrete chemical agents into the extracellular fluid in response to a stimulus. These naturally produced molecules diffuse into the general circulation and reach target cells at sites distant from the site of secretion, serving as ligands to mediate communication at the target cell receptor. Intercellular communication may also be more discrete. Secreted paracrine (local mediator) factors, including peripheral and central nervous system neurotransmitters, have local effects on nearby cells; autocrine factors are secreted by and affect the same cell. Many chemical mediators are not secreted by cells—rather, they are formed within and are recognized by targets within the same cells.

A pharmacologic agent or drug is a compound that, when administered to an organism, produces an effect on a specific function or process. A drug is an exogenous agent, one that originates from outside of an organism. Many pharmacologic agents are synthetic analogues of natural substances that act on specific receptor targets. Pharmacologic agents alter a specific natural process in an organism either by mimicking the effect of an endogenous beneficial or pathological chemical or by blocking the effect of a native compound. A natural compound may be present in abnormally large amounts in a disease state. Cushing's disease, for example, results from overproduction of glucocorticoid hormones (cortisol) by the adrenal cortex, and Graves' disease is characterized by overproduction of thyroid hormones. In other circumstances, an endogenous ligand may not be consistently elevated during a disease, yet blockade of the receptor for the ligand may still be beneficial. For example, β-adrenergic blockers can decrease cardiac rate and contractility in a patient with hypertension, but the elevated blood pressure cannot be attributed to excessive β-adrenergic stimulation. In other circumstances, the endogenous ligand may be lacking or absent. This is the basis of treatment of type 1 diabetes with insulin, because pancreatic beta cells do not adequately produce and secrete insulin. Hormone replacement is also used in treatment of hypothyroidism, Addison's disease (lack of cortisol production by the adrenal cortex), diabetes insipidus (lack of antidiuretic hormone), and postmenopausal osteoporosis (lack of estrogen). Similarly, hormone replacement may be used for therapeutic effects when there is no obvious lack of endogenous ligand, such as the in the use of growth hormone to promote longitudinal growth in adolescents with short stature. Receptor over- or underexpression or altered signal transduction may also be present in a disease state, and thus the normal quantity of natural ligand is not sufficient.

## II. RECEPTOR CLASSIFICATION

The receptor concept was first proposed by Langley in 1907 to explain the means by which the natural toxin curare could selectively block contraction of skeletal muscle. Prior to the development of technology for the determination of protein and genetic sequences, the existence of many different specific receptors could be inferred and categorized from the responses of cells to specific chemical stimuli. Currently, the definitive classification of receptors is based on amino acid sequence, although posttranslational modifications such as glycosylation or sulfhydryl crossbridge formation may also regulate receptor activity and/or specificity. Receptors are grouped into superfamilies and families based on sequence or structural homologies, the endogenous ligand, the activity of selective agents that mimic or block the endogenous ligand, and the cellular second-messengers systems used by these receptors (Table 1).

Many receptors are integral plasma membrane proteins with a binding site for the ligand present on the outer cell surface. The guanosine triphosphate (GTP) binding proteins, or G-proteins, are adapters for signal transduction between receptors and many different intracellular transduction systems. Specific G-protein complexes bind to these receptors and regulate signaling proteins such as adenylate cyclase

TABLE I Receptor Superfamilies Based on Structural Similarities

| Superfamily | Structural feature | Example | Second messenger |
|---|---|---|---|
| G-protein coupled | Single chain, seven transmembrane-spanning domains | Rhodopsin, α- and β-adrenergic subtypes, angiotensin II, FSH, GnRH, MC4 | Adenylate cyclase (increase or decrease cAMP), phospholipase C (increase IP3 and diacylglycerol) |
| Ligand-gated ion channels | Multiunit oligomeric complex | Nicotinic acetylcholine, GABA | Ions ($Na^+$, $K^+$, $Ca^{2+}$) |
| Receptor tyrosine kinase | Heteromeric, activation via autophosphorylation of tyrosine | Insulin, IGFs, epidermal growth factor | Tyrosine-phosphorylated proteins |
| Receptor protein tyrosine phosphatase | Binding sites for phosphotyrosine residues | PTP1B, LAR | Dephosphorylated proteins |
| Cytokine receptor | Multiple forms families—single-transmembrane protein | GH, PRL, EPO, TNFα, IL-6, leptin | JAK (tyrosine kinase)–Stat |
| Intracellular hormone receptor | Single chain, hetero- and homodimers | Steroids, thyroid, retinoic acid, PPARγ | DNA binding and transcription |

and phospholipase C. The G-protein-coupled receptors (GCPRs) are a large family of receptors that share the common structural feature of a single protein chain with seven transmembrane-spanning domains. Knowledge of common amino acid sequences of transmembrane domains has led to identification of a number of genes that encode previously uncharacterized receptors for which neither the endogenous ligand nor physiological role was known. The inventory of known endogenous peptide and protein ligands has also been expanded through the appreciation of structural homology. Many newly described receptors may be potential therapeutic targets, and this strategy may lead to identification of promising new drug targets as the human genome is fully characterized. Furthermore, the genomic strategy based on sequence homologies to known receptors is not restricted to the GPCRs.

Many receptors are classified as hormone-responsive or ligand-gated ion channels. These receptors are not configured as a single chain like the GCPR, but are instead an oligomeric complex of proteins with at least one ligand recognition element and one ion-specific pore. Both the ligand-binding site and the ion pore contribute to selectivity. In the presence of a ligand, the pore is either opened or closed, thereby regulating ion flux, electrical potential, and other cell processes. The nicotinic receptors at the neuromuscular junction control the interaction of actin and myosin through regulation of sodium ion flux and intracellular calcium ion mobilization. There are specific receptors in pancreatic beta cells that bind sulfonylurea analogues and stimulate the secretion of insulin by closing adenosine triphosphate (ATP)-sensitive $K^+$ channels; these have been the target for oral hypoglycemic agents for decades.

Receptor tyrosine kinases directly phosphorylate tyrosine residues in their own intracellular domain, as well as several signal transduction/adapter proteins, including the insulin receptor substrates (IRS1–IRS*x*). Insulin and the insulin-like growth factors are examples of this receptor type. Receptor protein tyrosine phosphatases dephosphorylate receptors and molecules of signal transduction. Inhibition of these may lead to improved receptor tyrosine kinase signal transduction and to new agents for treatment of diabetes. Activation of these phosphatases may be useful for treatment of cancers and immune disorders.

Cytokine receptors are configured as a single protein chain with one transmembrane domain that does not possess intrinsic tyrosine kinase activity. These receptors associate with and activate the Janus kinases (JAK) and signal transducers and activators of transcription (Stats). Examples of ligands for cytokine receptors are growth hormone, prolactin, interleukin-6 (IL-6), and leptin.

There are also receptors that are present in one or several intracellular compartments in membrane-bound or soluble form. The intracellular receptor superfamily includes all of the steroid hormone (estrogen, androgen, progesterone, glucocorticoid, mineralocorticoid, and vitamin D) receptors. Many of these are not exclusively receptors for a single ligand; rather, their binding to ligand and activation may be modulated by signals from cell surface receptors as well. In this respect, the estrogen receptor is the best characterized, but other steroid receptors are similarly modulated by cell surface messengers.

Thyroid hormone and vitamin A (retinoic acid) receptors form heterodimers with the retinoic acid X receptor (RXR), which binds the 9-cis form of retinoic acid, permitting dual regulation of receptor activity by the ligands for each receptor. The intracellular receptors have DNA-binding domains and activate or inhibit the transcription of specific genes that possess a hormone response element (HRE), a site where the activated intracellular receptor can bind.

Receptors confer specificity, and it is assumed that receptors are the most favorable targets for therapeutic intervention. Although other components of signal transduction may not bind extracellular ligands directly, they specifically recognize and bind to the receptors and other specific downstream molecules of signal transduction. These transducers may also be suitable targets for new specific drug development. Examples of these targets are ligand-regulated transcription factors; mitochondrial, nuclear, and vesicular structural and functional proteins; genes (DNA); and gene transcripts (mRNA).

## III. PHARMACODYNAMIC CONSIDERATIONS

If nature has designed a ligand molecule with high affinity and an ability to activate its target, then why is there a need to alter it? First and foremost, not all situations require complete activation of a ligand-dependent response. In many situations, partial activation, partial inactivation, or a complete blockade of a receptor target will lead to a desired response.

A ligand that activates a receptor, leading to a response, is considered an agonist (Fig. 1). In most cases, there is a direct relationship between the concentration of compound and the magnitude of the response. At higher concentrations, a maximum effect is reached and further increases in concentration do not lead to a greater response. The magnitude of the maximum effect of the agonist relative to the natural ligand is the intrinsic activity of the agonist, usually expressed as a percentage. In case the natural ligand is not known, the maximum effect of another reference compound may be used. The concentration of agonist that produces 50% of the maximum response (effective concentration) is designated the $EC_{50}$. The ratio of $EC_{50}$ values for different ligands that produce activation of the receptor is an indicator of relative potencies.

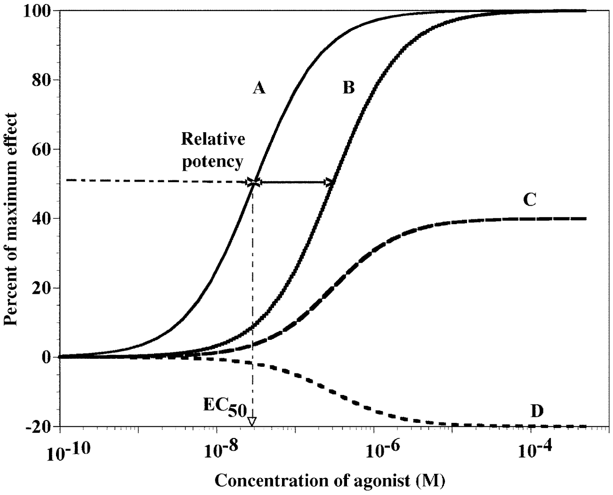

FIGURE 1 Agonist concentration–response. The curves are fit to the equation $E = ([A]IA)/([A] + EC_{50})$, where $E$ is the effect, [A] is agonist concentration, IA is the intrinsic activity of the agonist, and $EC_{50}$ is the agonist concentration associated with a half-maximum response. The $EC_{50}$ for curve A is $3 \cdot 10^{-8}$ $M$, and for B, C, and D is $3 \cdot 10^{-7}$ $M$. Curves A and B represent full agonists, where A is 10-fold more potent than B. Curve C is a partial agonist with 40% of maximum intrinsic activity. Curve D is an inverse agonist acting on a receptor with constitutive activity, resulting in a 20% decrease in activity.

Full activation and blockade are not the only events that result from a ligand–target interaction. Some agents do not possess the intrinsic activity of the natural ligand in all cells and tissues. These partial agonists will produce only a partial activation of their receptor, and in the presence of a full agonist they will behave more like antagonists. Tissue differences in intrinsic activity may be associated with a compound, producing a tissue selectivity even when the same receptor is present in multiple tissues. Another issue is that the receptors may associate with other elements of signal transduction, even in the absence of ligand. These receptor systems may possess basal constitutive activity, and the presence of an inverse agonist will decrease the basal activity mediated by the receptor.

An agent that prevents activation of the receptor by an agonist is considered an antagonist (Fig. 2). Whereas an agonist presumably results in some conformational change that promotes interaction with a second-messenger system, an antagonist binds but does not favor a productive interaction with second messenger, even though the receptor may be induced to bind to the next element of signal transduction. Endogenous antagonists for some receptors have been identified. The agouti-related protein (AGRP) is an antagonist of melanocortin receptors in the hypothalamus. Antagonist activity, like agonist activity, is not all or none. If the antagonist binds to the same binding site as the

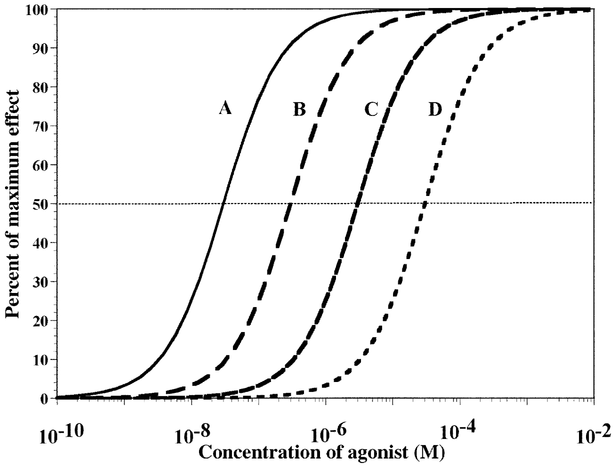

FIGURE 2 Antagonist evaluation. The response to agonist A is blocked in the presence of antagonist, requiring greater concentration of A to produce an effect. Curve A is agonist alone, B is agonist in presence of $10^{-7}\,M$ antagonist, C is agonist plus $10^{-6}\,M$ antagonist, and D is agonist plus $10^{-5}\,M$ antagonist.

agonist, they may compete with the agonist for these sites. Increasing concentrations of agonist increase agonist accessibility to the binding sites, thereby surmounting the effect of the antagonist. There is a parallel rightward shift in the concentration–response curve of the agonist in the presence of a competitive, reversible antagonist. If one calculates the ratio of $EC_{50}$ values in the presence vs absence of the antagonist, and compares these concentration ratios vs the concentration of antagonist at each ratio via linear regression, there is ideally a linear relationship between the agonist concentration ratio and the antagonist concentration (Fig. 3). By extrapolation, the concentration of antagonist that produces a twofold increase in the agonist requirement to reach a 50% response is determined. The negative logarithm of this concentration of antagonist is the $pA_2$, a measure of the affinity of an antagonist for the receptor. The $pA_2$ values for different antagonists of the receptor may be compared in order to describe relative antagonist activities quantitatively.

The interactions between a ligand and receptor are not usually covalent. Rather, the molecules associate via thermodynamically favorable hydrogen bonds, dipole and ionic interactions, and hydrophobic interactions, via functional groups on the ligand and the receptor (Table 2). The overall affinity between ligand and receptors is the product of several individual interactions between functional groups. Although each of these individual interactions is relatively weak, a high affinity and practically irreversible interaction is possible as a result of the interactions between many functional groups.

There are at least two divergent approaches to structural modification when an endogenous ligand is known. First, one can start with the native hormone structure, making structural analogues with conservative, systematic changes. Changes in structure that affect affinity or activity may help to identify important functional groups, and groups that stabilize the three-dimensional configuration of the ligand such that the receptor-interacting functional groups are favorable or optimum for interaction with the receptor. A second approach is to construct a library of compounds, and screen compounds without preselection based on structural similarity to the hormone. Hits would then be further modified to optimize activity. These two strategies are not mutually exclusive and may be used together at various stages in a cycle of structure identification and refinement.

A linear peptide hormone such as glucagon, somatostatin, or gonadotropin-releasing hormone (GnRH) may have considerable flexibility. A conservative substitution of one D-amino acid for the glycine in position 6 of GnRH results in greatly improved affinity and *in vitro* potency, due to induction of a beta turn in an otherwise extended molecule, and bringing the ends into close proximity. Covalent crossbridges between amino acid sidechains or amino- to carboxyl-terminal cyclization will result in a even more rigid structure. Octreotide and

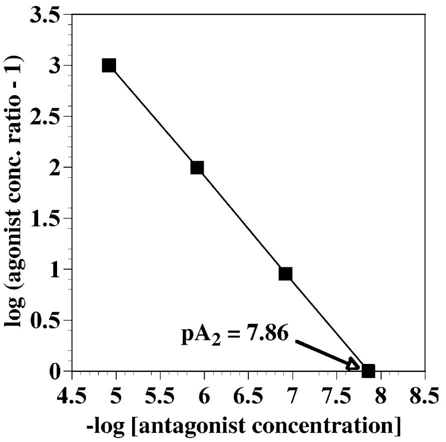

FIGURE 3 Schild plot. The ratio of concentration of agonist required to produce a 50% of maximum effect in the presence of antagonist over the $EC_{50}$ for the agonist is decremented, log transformed, and compared to the negative log of the concentration of antagonist. The $x$ intercept is the $pA_2$, the negative log of the concentration of antagonist that results in a twofold increase in agonist $EC_{50}$.

TABLE 2 Functional Groups Important for Ligand–Receptor Interactions

| Interaction | Functional group | Energy of interaction ($\Delta H$, kcal mol$^{-1}$) |
|---|---|---|
| $H^+$ bonding | Carboxyl, amino | ~1 in aqueous solution |
| Dipole | OH, ether, ester, amide | 1 |
| Hydrophobic | Aliphatic, aromatic | Entropy driven |
| Covalent | Several, including C–C | 100 |

Octreoscan are cyclic somatostatin analogues used for treatment and diagnosis of neuroendocrine cancers. Alternatively, part or all of a peptide backbone can be replaced with a rigid nonpeptide lattice in order to maintain the important functional groups in a favorable configuration for receptor interaction. This approach has led to the development of several nonpeptidic antagonists of the peptide hormone receptors (for example, the GnRH receptor).

Another reason for searching for novel, synthetic ligands is that the endogenous ligand may be nonselective. The endogenous catecholamines norepinephrine and epinephrine activate a multitude of α- and β-adrenergic receptor subtypes. A further complication is that several receptor subtypes may coexist in the same tissue, making it difficult or impossible to evaluate a specific receptor interaction. For this reason, it is desirable to express a single subtype in a cell line to avoid the confusion of multiple receptors interacting with a ligand. In order to develop a specific ligand for a single receptor subtype, it is necessary to test ligand affinities for the other related and unrelated receptors, also expressed individually in cell lines.

In addition to the systematic alteration of the ligand molecule and testing the affinities and activities at the natural receptor either in explanted tissue or expressed in host cells, it is valuable to probe the structure of the receptor. This can be accomplished with techniques such as site-directed mutagenesis and with chimeric receptors to determine the functional groups that participate in the interactions with agonist and antagonist ligands as well as those that are important for signal transduction. This approach has been used for characterization of the GnRH, melanocortin-4, and thyrotropin-releasing hormone (TRH) receptors, the β-adrenergic and α-adrenergic receptor subtypes, and many others.

There may be an issue of species-specific interactions, particularly with ligands that are very dissimilar to the native ligand. Some of the functional groups in these molecules in the vicinity of the binding site may take advantage of interactions that are not utilized by the native ligand, when there may be differences in the primary structure of the receptor. If the ultimate goal is to identify novel ligands for new drug development, it may be necessary to screen the compounds with a human receptor, because ligands may have very different effects in human vs animal species targets. It also may be difficult to assess the efficacy of a human-specific compound with *in vivo* testing in animals. The $\beta_3$-adrenergic receptor is an example of the problems associated with species specificity, in that several highly selective agents developed in rodents are not as selective in humans. However, human receptor selectivity is also a problem in the evaluation of nonpeptide GnRH antagonists.

Some targets require pulsatile rather than continuous activation in order to maintain receptor concentration as well as the postreceptor transduction system. For example, GnRH is released into the hypophyseal portal circulation as a synchronized secretion from a diffuse neural network in the median eminence. The frequency of this oscillation is approximately once per hour in humans. Idiopathic hypogonadotropic hypogonadism may result from inadequate or absent GnRH secretion. Pulsatile administration of GnRH can stimulate pituitary gonadotropin release, thereby restoring gonadal function and maturation. In contrast, the continuous administration of GnRH or a GnRH agonist will produce an initial burst of gonadotropin release from the pituitary, followed by a decrease in pituitary GnRH receptor concentration, gonadotropin biosynthesis and storage in the pituitary, and low concentration of gonadotropins in the circulation. This leads to suppressed gonadal steroid production. The chronic effect of GnRH agonist administration is essentially the same as the response to a GnRH antagonist, although the antagonist response is immediate.

The biphasic response to GnRH agonists also illustrates the need for comparison of acute vs chronic effects of administration of a ligand. Single-dose testing requires a relatively small quantity of compound and may produce an *in vivo* response. However, the response to a single dose may not predict the effect of chronic administration. Agents that produce a receptor desensitization or altered transcription may require treatment for several days or longer, and the chronic effect may not be clearly predicted by the single-dose response. An example is the activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) in adipose tissue by the thiazolidinedione rosiglitazone. In the Zucker fa/fa rat model of extreme insulin resistance, rosiglitazone leads to an increase in insulin sensitivity accompanied by decreased basal plasma insulin concentration, and an increase in adipose tissue and skeletal muscle insulin sensitivity mediated by increases in cell surface glucose transporters. The decrease in circulating insulin and improved glucose transport *in vivo* do not reach a nadir until 4 days of treatment. Agents that produce chronic weight reduction may not suppress appetite or cause a sudden weight loss on acute administration. Anorexigenic agents such as dexfenfluramine and dextroamphetamine may produce acute suppression of appetite and weight loss, but chronic treatment does not result in persistent effects despite continued administration due to development of tolerance. The tolerance may be target dependent or ligand dependent, and it is important to establish this distinction in a drug discovery research program.

There are often alternatives to development of a novel ligand for a specific target, because physiological systems are often controlled by a cascade of regulatable processes. The biosynthesis, storage, release, and removal of the endogenous ligand may be controlled by other targets. Type 2 diabetes is the result of an inadequate production of insulin and accompanying insulin resistance. The sulfonylurea oral antidiabetic agents facilitate insulin release. Insulin-sensitizing agents such as metformin or rosiglitazone affect targets downstream of the insulin receptor. Some targets may be better suited for activation or blockade with exogenous agents or may offer more specific control of a physiological process.

## IV. PHARMACOKINETIC CONSIDERATIONS

Much of the research directed toward the identification and characterization of high-affinity, selective ligands is performed with cell cultures or in subcellular fractions. In these systems, a ligand placed into a culture medium has rapid access to receptors, and the ligand concentration can be maintained relatively constant. Except for ligands that must penetrate into cells in order to reach their molecular target, there are no diffusion barriers. This situation is very different from the fate of a ligand *in vivo*. Because a ligand must reach its target at a sufficient concentration, for an adequate period of time to achieve receptor activation or blockade, it is not surprising that many compounds with great affinity for specific receptors are ineffective in whole animals. The delivery of a compound to its site of action depends on pharmacokinetic considerations.

### A. Absorption

The primary route of administration for drugs is the oral route. In order for a ligand to be absorbed from the gastrointestinal tract, it must dissolve in the aqueous intestinal contents and pass through or between the gastrointestinal epithelial cells. *In vitro* permeability models, such as flux through caco2 colonic cancer cells, can be used to predict absorption from the intestines. In general, large molecules, including peptides and proteins, are rapidly digested in the gastrointestinal lumen. Many small molecules are also unsuitable for oral administration, due to poor permeability or rapid degradation.

Injectable formulations provide the most direct route for systemic delivery of poorly absorbed ligands. An infusion pump can be used to provide either continuous delivery or, in situations in which continuous administration is not effective due to receptor desensitization, many discrete pulses. If long-term continuous delivery of ligand is required, a sustained-release formulation may be utilized. For example, long-acting neutral protamine Hagedorn (NPH) insulin and depot formulations of GnRH agonists or antagonists will release ligand for hours to months, respectively. Many steroid hormones are poorly absorbed when given orally, and in some instances this may be surmounted via structural modification. For example, 17-α-ethynyl-estradiol is orally bioavailable, whereas 17-β-estradiol is not. Formulation may be an important issue, because dissolution and solution properties can determine rate of absorption. The concentration of compound in solution may also be an important consideration, because the delivery of a concentrated solution of compound may not result in the same effect as the same dose in a more dilute solution. For example, several GnRH antagonists will aggregate or gel in aqueous solutions above approximately 1 mg/ml. This results in an uncontrolled sustained release formulation with a much longer duration of action than a dilute solution, both administered at the same dose.

## B. Distribution

Distribution of ligands into target tissues is required. An apparent volume of distribution can be calculated from the ratio of concentration of drug at time of dosing ($C_0$) determined by extrapolation and the dose, assuming that the ligand is uniformly distributed (Fig. 4). A large volume is associated with lower plasma concentrations compared to a low volume, assuming equivalent rates of elimination. The receptor may be readily accessible from the circulation, so the concentration in plasma approximates the concentration in the target tissue. Alternately, the receptor may be in a distinct compartment—for example, the majority of neurons in the brain that are separated from the circulation by a blood–brain barrier. It may be more relevant to measure the concentration of drug in the target tissue when possible, because the concentration in the plasma may be much different than the local concentration at the target. For ligands intended to reach intracellular targets, either a large distribution volume or a facilitated or active transport is required. In general, the apparent volume of distribution is directly proportional to lipophilicity. The liver will also extract highly lipophilic agents from the circulation prior to elimination. Protein binding may also influence distribution. A drug that is very highly bound to proteins in the circulation will often have a high plasma concentration and a small distribution volume. Many hormones bind to carrier proteins; only the free or unbound ligand is available to the receptors. A carrier-bound ligand can be delivered selectively to sites where the carrier is modified or degraded, leading to local release of active ligand. For example, insulin-like growth factor-I (IGF-I) is bound by several IGF-binding proteins. The binding proteins are degraded by specific proteases secreted by IGF-responsive tissues, leading to localized delivery of free IGF-I.

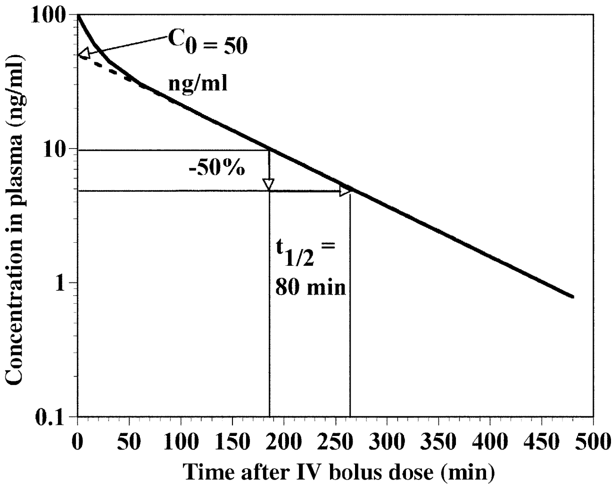

FIGURE 4 Elimination of a single intravenous bolus dose of 20 μg/kg at time zero. The log of the concentration of compound in the circulation decreases over time. Terminal plasma half-life ($t_{1/2}$) is the time required for the concentration of compound to decrease by 50% during the log-linear elimination phase. This initial rapid decline in concentration is attributed to distribution of compound. These data can be fit to a two-compartment model: $C = C_0(e^{-k-1t} + e^{-k-2t})$, where $C$ is the concentration of compound at time $t$, $C_0$ is the initial concentration determined by extrapolation, and $k-1$ and $k-2$ are the distribution and elimination rate constants, respectively. The volume of distribution ($V_D = \text{dose}/C_0$) is 400 ml/kg. The area under the curve estimated from the 0- to 480-min time course is 6777 ng $\text{ml}^{-1}$ min, and the whole-body clearance is 2.95 ml $\text{kg}^{-1}$ $\text{min}^{-1}$.

## C. Elimination

The liver and kidneys are responsible for the removal of both endogenous and exogenous ligands from the circulation by excretion and metabolism. Whether an agent is excreted intact or is biotransformed, the compound is cleared from the circulation. The time required for the plasma concentration to decrease by 50% is the plasma half-life (Fig. 4). Many endogenous ligands have short half-lives, in the order of minutes, and one major goal of structural modification is to decrease the elimination rate, prolonging the compound's presence so that convenient once or twice daily dosing is feasible. The sites of metabolic inactivation can be predicted by *in vitro* metabolism studies with liver slices and microsomal preparations. In some cases there will be active metabolites of a ligand. Under these circumstances, the metabolites may not have the same degree of specificity for the intended target as does the parent ligand, so an *in vitro* testing strategy for development of highly specific agents may be foiled by *in vivo* biotransformation.

The area under the curve (AUC) in the plot of plasma concentration vs time is a quantitative measure of the exposure to compound. Measurement and comparison of the AUC do not require compartmental modeling, as is the case for apparent volumes of distribution and half-lives. Whole-body clearance is the measure of the rate of disappearance of drug, determined from the ratio of the dose divided by the AUC. It is the volume of blood or plasma that is cleared of compound per unit time, and, like AUC, it does not require the assumption of a compartmental model. Although clearance cannot be readily attributed to physiological filtration and flow rates, they may be used for comparison. A compound with a very high clearance—for example, greater than cardiac output—is probably not a feasible drug candidate. Even a clearance greater than hepatic blood flow may be prohibitively high. The dosing interval, the mode of delivery (i.e., continuous vs intermittent), and possibly even the route of administration are dependent on the clearance. A final caution is that there are species differences in pharmacokinetics, just as there are differences in receptors. It is not always possible to make accurate predictions of pharmacokinetic characteristics in humans based on animal studies.

### D. Other Considerations

A therapeutic target may be discrete, and local rather than widespread effects may be desirable. In some cases, such as with beta agonists and glucocorticoids administered via inhalation for treatment of asthma, or local treatment of pain or inflammation with local anesthetics or glucocorticoids, the intended site of action is readily accessible. An ideal agent for local administration would not be biologically available for systemic effects. Chemotherapy for solid tumors with specific cytotoxic or cytostatic agents may affect rapidly dividing cells throughout the body, and it would be desirable to restrict the effects of treatment to the therapeutic target. Local targets may be used to both identify and treat these diseases, such as with the somatostatin analogue octreotide (Octreoscan), which is used for delivery of radionuclide scanning agents for diagnosis of neuroendocrine cancers, as well as for cytostatic treatment of diseases such as breast cancer or advanced-stage prostate cancer resistant to androgen deprivation.

*In vivo* pharmacodynamic testing may often be conducted in a species in which a model can be established. Mice are often used for *in vivo* testing, partly because the compound requirements are minimized relative to larger species. In addition, many new targets have been validated by characterization of the phenotypes associated with over- and underexpression in mice; this may lead to testing of leads in either wild-type or genetically altered mice. A problem of false negative information may arise if the pharmacokinetic characteristics of the compound in mice are not favorable or representative. For example, leuprolide acetate administration via daily subcutaneous injection of as much as 8 mg/kg/day does not suppress testosterone in male mice, although it is effective in male rats or dogs at less than 0.1 mg/kg/day. Lupron 1 Month Depot will decrease testosterone in mice at 1–2 mg/kg/day, but is effective in rats or dogs at less than 0.1 mg/kg/day. The antitumor efficacy of this agent against human prostate cancer cells injected into nude mice will be dictated by the host, rather than by the cells.

Another topic related to pharmacokinetics is the stability or shelf life of a formulation of ligand. If the ligand binds appreciably to surfaces, precipitates, forms gels or complexes, oxidizes, hydrolyzes, supports microbiological growth, or is otherwise altered, suitable formulations must be developed prior to valid evaluations either *in vivo* or *in vitro*.

## V. SUMMARY

Receptor targets for chemical ligands mediate communication in biological systems. The natural ligands may have limitations with regard to efficacy and specificity. In some cases the target is known but the endogenous ligand is not. Systematic chemical substitutions and random screening result in a spectrum of activity from antagonist to full agonist, as well as specificity at the receptor target. Current technology makes it possible to clone and express human receptors in cell culture and to study individual receptor subtypes without confusion or contamination by other subtypes. Structural modifications of the natural ligands may alter activity and pharmacokinetic characteristics. Conformationally restricted compounds may improve selectivity. Synthetic analogues may interact with functional groups on the receptor other than those that bind the natural ligand, and may result in a species-specific compound.

## Glossary

**affinity** The degree of attraction between a ligand and a specific receptor. It is based on a high degree of structural complementarity between the ligand and the binding site of the receptor.

**intrinsic activity** The maximal response caused by a drug in a tissue preparation relative to the endogenous ligand or another reference compound, if the endogenous ligand is not known. A full agonist causes a maximal response and a partial agonist causes less than a maximal response. The terms “intrinsic activity” and “efficacy” are often used interchangeably, although efficacy requires both intrinsic activity and potency.

**ligand** A molecule that is bound by another molecule, i.e., a target molecule or a receptor. The binding between the ligand and its target molecule is usually not covalent and does not usually result in a chemical alteration of the ligand. However, the interaction triggers changes in the target molecule that lead to changes in function in the target cells.

**pharmacokinetics** The study of the kinetics of drugs in humans, animals, and *in vitro* test systems, including the processes of absorption, distribution, metabolism, and excretion.

**potency** The dose or concentration of a drug required to produce an effect. The relative potencies of two or more ligands are determined from the ratio of concentrations or doses required to produce the same effect. By convention, $EC_{50}/ED_{50}$, or the concentrations/doses producing a 50% of maximum effect, are usually compared.

**receptor** A target molecule or an organized assembly of molecules. A receptor has a binding site for a ligand and usually possesses other structural and functional domains for interaction with itself, its surroundings, and other molecules of signal transduction to transfer information, leading to a change in function.

## See Also the Following Articles

**Flt3 Ligand • Gonadotropin-Releasing Hormone (GnRH) • Gonadotropin-Releasing Hormone Pharmacology: Agonists and Antagonists • GPCR (G-Protein-Coupled Receptor) Structure • Protein Kinases**

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# Lipoprotein Receptor Signaling

Daniel Laskowitz and Salvatore V. Pizzo

*Duke University*

I. THE LDL FAMILY OF RECEPTORS
II. LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN
III. SCAVENGER RECEPTORS AND LDL RECEPTOR BIOLOGY
IV. THE EXPANDING NEUROBIOLOGY OF apoE

**Since their original description by Brown and Goldstein, lipoprotein receptors have been recognized as playing an integral role in cholesterol homeostasis. This family of receptors is responsible not only for binding and internalizing lipoprotein-containing particles for use within the cell, but also for regulation of endogenous cellular cholesterol metabolism and synthesis. In addition to this role in cholesterol regulation, it is now known that several members of this family of receptors are capable of initiating signaling cascades. Convergent experimental and clinical evidence suggests that these signaling capabilities may mediate important biological processes, including normal central nervous system development and the brain response to injury, immune regulation, and atherogenesis. Increased insight into the mechanisms of lipoprotein signaling may increase our fundamental understanding of the biology of these multifunctional receptors**

**and holds the promise of novel therapeutic strategies for human disease.**

## I. THE LDL FAMILY OF RECEPTORS

The presence of the low-density lipoprotein (LDL) receptor on the surface of cells is essential in maintenance of extracellular and membrane cholesterol homeostasis. The mature LDL receptor is a highly conserved cell membrane glycoprotein consisting of five distinct domains, including a short cytoplasmic tail, a transmembrane domain, an O-linked sugar domain, an epidermal growth factor (EGF) precursor homology domain, and a ligand-binding domain.

The cytoplasmic domain of the LDL family of receptors is composed of a stretch of approximately 50 amino acid residues that are required for internalization of the receptor–ligand complex. The tetrapeptide NPXY is one of the short sequences that targets LDL receptors to coated pits on the surface of cells for subsequent internalization. Although the cytoplasmic tail of the LDL receptor family has been traditionally thought of in the context of endocytosis and lysosomal trafficking, it may actually serve a dual function. Recent evidence suggests that this domain is a key in initiating signaling events by binding a family of cytoplasmic adapter and scaffolding proteins. These adapter proteins interact with the cytoplasmic tail of the LDL receptor family proteins and initiate signal transduction pathways that involve tyrosine and mitogen-activated protein (MAP) kinase-mediated cellular events involved in cell adhesion, activation, and cytoskeletal organization. Interestingly, the binding of these adapter proteins inhibits the endocytosis pathway, suggesting a possible regulatory mechanism.

The transmembrane domain of the LDL receptor family consists of approximately 25 hydrophobic amino acids. Although this domain plays a primary role in membrane anchoring, recent evidence supports the hypothesis that this region also plays an important role in transducing the signal initiated by receptor–ligand interaction across the plasma membrane.

The O-linked sugar domain of the LDL receptor is a serine- and threonine-enriched stretch of 58 amino acid residues located just outside of the plasma membrane. A majority of the hydroxyl side chains are glycosylated in a posttranslational process whereby galactosyl and sialyl residues are added to the O-linked *N*-acetylgalactosamine moiety. Despite the fact that the structure of this domain has been clearly delineated, the function of the O-linked sugar domain remains unclear. One plausible hypothesis is that this region facilitates receptor–ligand interactions by physically extending the receptor-binding domain into the extracellular milieu.

The EGF precursor homology domain is a common feature of all of the receptors in the LDL family. This domain is composed of approximately 400 amino acids and lies immediately adjacent to the ligand-binding site. As suggested by its the name, this domain contains amino acid sequences that are similar to those present in EGF. Two of these EGF repeats are located in tandem near the amino terminus, whereas the final repeat is located at the carboxyl terminus of the EGF homology domain. The remainder of this domain is composed of five sequences, each comprising approximately 50 amino acids that contain the consensus tetrapeptide YWTD. Although the function of this domain has not been fully defined, deletion of this sequence impairs pH-dependent LDL disassociation from its receptor and subsequent recycling of the receptor.

Finally, the ligand-binding domain protrudes into the extracellular milieu and mediates the interaction between the receptor and lipoproteins containing apolipoprotein E (apoE) or apolipoprotein B-100. The amino terminus includes a stretch of 292 amino acids containing seven LDL receptor type A (LA) repeats arranged in tandem. This area is highly enriched in cysteines and folds into a structure exposing clusters of aspartic acid and glutamic acid residues on its surface. These negatively charged regions are found at the carboxyl terminus of each of the seven repeat units. The electrostatic interactions between these negatively charged patches and positively charged regions of the lipoprotein subunits apoE and apolipoprotein B-100 are believed to play an important role in facilitating receptor–lipoprotein interactions. In general, repeats two through seven are believed to act in concert to facilitate receptor binding with apolipoprotein B-100, whereas repeat five is necessary for apoE binding.

In the years following the original description of LDL receptor–ligand interactions, several related proteins have been described in the LDL receptor family. These receptors include the very-low-density lipoprotein (VLDL) receptor, the gp330/megalin receptor, the apoE receptor 2 (ER2), and the LDL receptor-related protein (discussed in detail in Section II). These receptors share structural homology to the LDL receptor, including the EGF domain, O-linked sugar domain, and cytoplasmic region, with one or more short sequences mediating receptor internalization via coated pits.

In addition to their function in cholesterol metabolism, recent attention has focused on the ability of the LDL family of receptors to initiate signaling cascades. These receptors may be particularly important in the central nervous system, and converging clinical and experimental evidence suggests that the LDL receptor family is involved both in normal brain development and response to injury. For example, although inactivation of the either the VLDL receptor gene or the ER2 gene does not result in obvious neurological abnormalities, a recent study demonstrated that simultaneous targeted disruption of the ER2 and VLDL receptor genes resulted in a distinctive neurological phenotype. During the second week of life, these double-knockout mice developed failure to thrive, progressive ataxia, and tremor. This neurological abnormality was progressive, and animals rarely survived beyond 3 to 4 weeks of age. At autopsy, the brains of these animals exhibited distinctive histological abnormalities, including the loss of the normal cellular organization in the hippocampus and cerebellum.

Surprisingly, these developmental anomalies were identical to those previously identified in mice lacking the reelin or mDab1 genes. Reelin is a large extracellular protein that regulates the neuronal migration that forms the radial glial network during development. mDab1 (so named because of similarity to the *Drosophila disabled* gene product) is a cytoplasmic adapter protein that functions downstream of reelin. mDab1 interacts with several neuronal membrane proteins, including LDL receptors, presumably through interaction with the NPXY internalization motif present in the cytoplasmic tails of this receptor. Mice deficient in mDab1 develop a neurological syndrome that is similar to reeler and VLDL receptor/ER2 double-knockouts mice, characterized by migrational abnormalities in cerebellum and cortex, resulting in progressive ataxia and hindlimb weakness. Thus, it appears that the VLDL receptor and ER2 share overlapping roles as obligatory components of the reelin/disabled pathway involved in normal neuronal migration. The exact mechanism by which this occurs remains unclear, as neither the VLDL receptor nor ER2 contains a kinase domain, although it has been speculated that members of the LDL receptor family recruit adapter proteins and kinases into the receptor complex, as has been described in other signaling pathways.

The role of the LDL receptor family in central nervous system (CNS) development is not limited to the ER2 and VLDL receptor. Recently, it was reported that mice deficient in megalin (another member of the LDL receptor family) demonstrate holoprosencephaly, another disorder of neuronal migration. Although the molecular basis for this outcome is poorly defined, these results strongly suggest that the LDL family of receptors is important for CNS development and function. Thus, more than two decades after the original classic articles describing the structure and function of the LDL receptor in the context of cholesterol metabolism, we are only beginning to learn of the diverse biological properties that this multifunctional receptor family plays in cellular communication and signal transduction.

## II. LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN

Following modification by lipoprotein lipase and the association of apolipoproteins, VLDLs become chylomicron remnants. These are cleared from blood by a receptor-mediated mechanism in the liver. Although recognized as distinct from the LDL receptor, the chylomicron remnant receptor also has a high affinity for apoE and recognizes the remnant particles via this incorporated subunit. In 1988, this remnant receptor was cloned and dubbed the LDL receptor-related protein (LRP). Subsequent analysis has demonstrated that the LRP is present in a variety of tissues, including liver, kidney, placenta, and brain. The pool of LRP receptors in the liver is critical for catabolism of a number of proteins, whereas in brain it is postulated to play an important role in normal development of the CNS and response to neurological disease.

LRP is a large receptor with a primary sequence of 4525 amino acids (molecular weight of ~600,000), and it bears many structural similarities to other members of the LDL receptor family. This receptor is posttranslationally cleaved, yielding a heavy chain of molecular weight 515,000 and a light chain of molecular weight of 85,000. The latter subunit is tethered in the membrane. Like the LDL receptor, the extracellular domain of LRP includes a cysteine-enriched ligand-binding domain and an EGF precursor homology domain that probably functions in the acid-dependent dissociation of ligand from the receptor. Unlike the LDL receptor, however, the O-linked sugar domain is not present in the extracellular portion adjacent to the membrane. As with all of the members of the LDL receptor family, LRP, through its light chain, is a transmembrane protein that is anchored by a single transmembrane segment. The cytoplasmic tail of the protein is 100 amino acids long, approximately twice as long as the LDL receptor, and contains the NPXY motif; however, unlike the LDL receptor, this sequence is not important for receptor-mediated endocytosis. Rather, endocytosis of LRP depends on the presence of a YXXL motif. The latter motif is also known as the immunoregulatory tyrosine activation motif (ITAM) and is found in receptors that participate in antigen presentation. Its potential significance in LRP will be considered next.

LRP is capable of a number of important functions, including immune regulation, atherogenesis, and the clearance of structurally diverse and toxic ligands. LRP has relatively broad binding characteristics and is considered to be a multiligand receptor. A variety of physiologically important ligands that are bound to LRP are targeted to coated pits then internalized, dissociated in endosomes, and degraded in lysosomes. LRP has multiple, independent binding sites for these ligands, and it is believed that lysosomal degradation is mediated by the EGF precursor homology domain. Binding of a ligand to a receptor-binding domain without an intact EGF homology domain may allow the bound ligand to be recycled with the receptor rather than entering the lysosomal degradation pathway. Thus, it appears as though the ultimate fate of a ligand bound to LRP is determined in part by its binding site on the receptor.

A further understanding of the complex biology of LRP was made possible when it was discovered that LRP is identical to the previously characterized $\alpha_2$-macroglobulin ($\alpha_2$M) receptor. *In vivo*, the proteinase inhibitor $\alpha_2$M circulates in blood at a very high concentration (1–5 μ*M*). On binding of proteinases to $\alpha_2$M, the molecule undergoes a conformational change, exposing a receptor recognition site present in each of its four identical subunits. This so-called activated form of $\alpha_2$M is designated $\alpha_2M^*$. $\alpha_2$M is capable of inhibiting the function of proteinases of all four mechanistic classes. *In vitro* treatment of $\alpha_2$M with ammonia or methylamine results in a similar conformation change, resulting in a form of $\alpha_2M^*$ that also binds to LRP. The fact that either a proteinase or a small nucleophile triggers similar conformational changes in $\alpha_2M^*$ is unusual; it is the result of an internal γ-glutamyl-β-cysteinyl internal thiolester bond in each of the four $\alpha_2$M subunits. This bond ruptures when proteinases bind to $\alpha_2$M or can directly react with small nucleophiles such as ammonia or methylamine, also rupturing the bond. As already noted, LRP has broad ligand specificity and it appears to function in the clearance and internalization of a number of ligands, including *Pseudomonas aeruginosa* exotoxin A. In fact, endocytosis via the LRP is requisite for *Pseudomonas* toxicity, and cell lines deficient in this receptor are resistant to *Pseudomonas* cytotoxicity. LRP also binds to tissue and urokinase-type plasminogen activator complexes with plasminogen activator inhibitor-1. The list of other LRP ligands is very long, including LDL, lactoferrin, lipoprotein lipase, tissue factor pathway inhibitor, matrix metalloproteinases, factor VIII light chain, thrombospondin, and antithrombin III–thrombin complexes. Receptor-associated protein (RAP) is an unusual "ligand" for LRP and other members of the LDL family. RAP is not found in the extracellular environment. It is believed to function as an intracellular chaperone that promotes proper folding of LRP and prevents it from binding ligands before LRP reaches the cell surface. When cloned RAP is added to the external environment of cells that have LRP on their surface, it blocks the binding of all known ligands to this receptor.

In the past several years, studies have demonstrated that LRP can target delivery of antigens to antigen-presenting cells. This subsequently results in activation of the immune system. These antigens, however, must be in complex either with $\alpha_2M^*$ or heat-shock proteins. Complexes with antigens are formed as a result of coincorporation of these proteins into $\alpha_2$M during the course of proteinase activation. Complexes with heat-shock proteins probably are produced intracellularly—for example, in tumor cells—and are shed during cell death. The occurrence of ITAM in LRP is, therefore, quite intriguing. This sequence is present in other receptors that bind and internalize antigens complexed to various carrier proteins. It appears to be essential both for endocytosis and the activation of signaling cascades critical for antigen processing.

In addition to its role in binding and clearing such structurally and functional diverse ligands, there is now abundant evidence that the LRP is capable of initiating a signaling cascade. For example, on binding of the LRP to endogenous ligands such as lactoferrin, *Pseudomonas* exotoxin A, or lipoprotein lipase, a signaling cascade is initiated, resulting in increased intracellular free $Ca^{2+}$, $[Ca^{2+}]_i$, and inositol phosphates, including inositol (1,4,5)-trisphosphate ($InsP_3$) via a pertussis-toxin sensitive G-protein. Apolipoprotein E may also serve as an endogenous ligand capable of signaling via this receptor. In fact, a recent observation suggests that both the intact apoE holoprotein and the peptides derived from the apoE receptor-binding region are capable of initiating signaling cascades that result in increased $[Ca^{2+}]_i$ and $InsP_3$ turnover in murine peritoneal macrophages. This signal is mediated by a pertussis-toxin sensitive G protein and is blocked by RAP and $Ni^{2+}$, characteristic properties of LRP-dependent ligand binding and signal transduction. These effects are likely to be biologically relevant given the known immune-modulating functions of this receptor, and there are now a number of observations demonstrating immune abnormalities in apoE-deficient mice.

There are a number of suggested mechanisms whereby LRP may contribute to the process of atherogenesis. In response to cholesterol loading, macrophages secrete large amounts of apoE, which is available to incorporate into lipoproteins at the site of developing atheromatous lesions. It has been postulated that this can trigger a secretion/recapture mechanism in which secreted apoE-enriched lipoproteins are locally cleared and internalized by LRP-expressing macrophages, facilitating development of the plaque and the transition of macrophages into lipid-laden foam cells. Independent of its role in cholesterol homeostasis, the ability of LRP to initiate signal transduction in resident macrophages may also have important biological consequences in atherogenesis. For example, there is an increasing understanding that inflammatory processes are important in the development of atheromatous plaques. LRP modulates cellular immune responses, and it has been speculated that apoE–LRP interactions may modulate atherogenesis by regulating macrophage activation and inflammation at endothelial surfaces in areas of plaque development. This is consistent with observations that the early atheromatous plaques in apoE-deficient mice are associated with up-regulation of interleukin 6 (IL-6), vascular adhesion molecules, and increased numbers of CD4 lymphocytes, which are critical for activation of immune responses. It is also consistent with observations that macrophage-specific expression of apoE reduces atherosclerosis in apoE-deficient mice independently of systemic cholesterol levels.

Thus, the ability of LRP to initiate cellular signaling mechanisms may ultimately prove to be an important factor in diverse clinical disease processes. Interestingly, the receptor-competent form of $\alpha_2M^*$ also initiates a signaling cascade in macrophages, but it is believed that this signal is not initiated via LRP. Instead, there appears to be a distinct receptor, dubbed the $\alpha_2M$ signaling receptor ($\alpha_2$MSR), which is capable of initiating signal transduction independently of ligand internalization. These two receptors may be differentiated by a variety of functional characteristics, including competition with RAP and $Ni^{2+}$. As noted above, both block ligand binding to LRP; however, neither affects $\alpha_2M^*$ binding to $\alpha_2$MSR (Table 1). The biological role of this newly described receptor remains unclear, although it has been the focus of considerable recent investigation.

## III. SCAVENGER RECEPTORS AND LDL RECEPTOR BIOLOGY

Following exposure to cholesterol, macrophages become filled with cholesterol ester and assume the morphology and functional properties of foam cells. Although the uptake of cholesterol by subendothelial macrophages has long been recognized as important to atherogenesis, it was unclear for many years how this process occurred, because there did not appear to be clear evidence that cholesterol was internalized by LDL receptors. This quandary was resolved by the demonstration of macrophage scavenger receptors that are capable of mediating the endocytosis of modified cholesterol. These scavenger receptors have a particularly high affinity for acetylated LDL and were originally dubbed acetyl-LDL receptors. However, it was subsequently recognized that they could bind a relatively broad range of ligands, including oxidized-LDL, polyribonucleotides, polysaccharides, and anionic phospholipids. In recognition of their ability to bind and clear such structurally diverse ligands, they have been renamed scavenger receptors. Although the exact structural determinants that confer recognition by the scavenger receptor have not been fully elucidated, it is known that polyribonucleotides will

**TABLE 1 Functional Characterization of Ligand Binding to LRP vs $\alpha_2$MSR**[a]

| Activity[b] | LRP | $\alpha_2$MSR |
|---|---|---|
| RAP competition | + | − |
| $Ni^{2+}$ blocks binding | + | − |
| Increase in $InsP_3$ | + | + |
| Increase in $[Ca^{2+}]_i$ | + | + |
| Pertussis toxin sensitivity of $InsP_3$ synthesis | + | − |
| $[Ca^{2+}]_i$ sensitivity to pertussis toxin | + | − |
| Up-regulated by insulin | + | +++ |
| Receptor desensitization | − | + |
| Increase in cytosolic pH | − | + |

[a] Functional differences between the low-density lipoprotein receptor-related protein (LRP) and the newly described $\alpha_2$-macroglobulin signaling receptor ($\alpha_2$MSR).

[b] RAP, Receptor-associated protein; $InsP_3$, inositol 1,4,5-trisphosphate.

bind with high affinity only if they are capable of forming a stable, four-stranded helix.

Two scavenger receptor isoforms have been described as representing alternative splicing of a common gene. Expression of both type I and type II scavenger receptors has been detected *in vivo*, primarily on resident tissue macrophages. The scavenger receptor is a homotrimeric integral membrane protein, with each monomer composed of approximately 450 amino acids. The receptor has six distinct domains, of which the first five domains are identical between the type I and type II receptors. These include a short cytoplasmic tail of approximately 50 amino acids located at the amino terminus; a single transmembrane domain; a juxtamembrane spacer region of approximately 45 amino acids; an extracellular $\alpha$-helical domain, and a collagen-like domain containing approximately 24 Gly-X-Y triplet repeats, with the Y position primarily occupied by proline or lysine residues. The primary structural difference between the type I and type II scavenger receptors occurs in the final extracellular ligand-binding domain located at the carboxyl terminus. Here, the type I receptor has an 11-residue stretch that is highly enriched in cysteine residues (SRCR domain), whereas the type II receptor has a truncated carboxyl terminus composed of approximately 6–17 amino acids. Despite this difference in the ligand-binding domain, both types of receptors have a comparably broad range of ligand-binding specificity.

As already noted, scavenger receptors facilitate the endocytosis of cholesterol by subendothelial macrophages and the subsequent formation of foam cells. Support for the role of the macrophage scavenger receptor in atherogenesis is strengthened by the fact that scavenger receptor mRNA has been colocalized with atheromatous plaques; oxidatively modified LDL is ubiquitous in atheroma; and *in vitro* expression of scavenger receptors in macrophages causes massive uptake of cholesterol and a cellular morphology similar to that seen *in vivo*.

In addition to its role in atherosclerosis, the scavenger receptors may participate in cellular adhesion. This hypothesis is based on the observation that monoclonal antibodies directed against scavenger receptors are capable of blocking the adhesion of macrophages to an artificial substrate. Such a role may explain why these receptors are more highly expressed in resident tissue macrophages as compared to circulating monocytes. A third biological role postulated for scavenger receptors is in host defense. In particular, several gram-negative bacterial endotoxins bind to both type I and type II scavenger receptors and are cleared from the circulation in this fashion. Scavenger receptors also bind lipoteichoic acid, which is expressed on the surface of many gram-positive pathogens. Given the ability of the scavenger receptors to bind constituents of both gram-positive and gram-negative bacteria, it has been suggested that scavenger receptors served as a primitive form of host defense that arose early in the evolution of the immune response by recognizing oxidatively damaged pathogens as “non-self” and facilitating their clearance.

As with LRP, recent attention has focused on the signaling properties of scavenger receptors. It appears as though different subtypes of scavenger receptors may mediate distinct pathways. Interestingly, these pathways may even occasionally have opposing biological properties. For example, oxidized-LDL binds preferentially to one class of macrophage scavenger receptors, initiating a signaling cascade characterized by pertussis-toxin-sensitive hydrolysis of phosphoinositides and elevations in $[Ca^{2+}]_i$. This has the downstream effect of suppressing a number of genes mediating the inflammatory response, including tumor necrosis factor $\alpha$ (TNF$\alpha$), interleukin-1$\alpha$ (IL-1$\alpha$), monocyte chemoattractant protein-1 (MCP-1), and nitric oxide release triggered by exposure of macrophages to lipopolysaccharide (LPS). Oxidized-LDL induces these anti-inflammatory effects in part by inhibiting the binding of the transcription factor nuclear factor $\kappa$B (NF-$\kappa$B) to its cognate binding site in the TNF$\alpha$ promoter.

In contrast, ligands such as maleylated bovine serum albumin (maleyl-BSA) and the polynucleotide poly(I:C) bind to a distinct macrophage scavenger receptor characterized by a relatively high number of receptor sites ($2 \times 10^6$/cell) and low affinity ($K_d$ of 4.9 $\mu M$). Binding of ligands to this receptor has predominantly pro-inflammatory effects, with up-regulation of genes encoding MCP-1, TNF$\alpha$, and IL-1$\beta$. Thus, maleyl-BSA and oxidized-LDL appear to bind distinct scavenger receptors and initiate discrete second-messenger pathways with opposing biological effects. Although binding of either ligand initiates hydrolysis of phosphoinositides, activation of phospholipase $A_2$, and elevation of $[Ca^{2+}]_i$, the scavenger receptor for oxidized-LDL is coupled to a pertussis-toxin-sensitive G-protein, whereas that for maleyl-BSA is not, and binding of oxidized-LDL initiates more extensive and prolonged spiking of $[Ca^{2+}]_i$ than does binding of maleyl-BSA. Although not yet well understood, such so-called oscillatory patterns trigger different cellular responses other than single spikes in $[Ca^{2+}]_i$.

Of note, not all ligand–scavenger interactions initiate a signaling cascade. For example, acetyl-LDL and malondialdehyde-LDL appear to bind a third discrete class of scavenger receptor, characterized by a low number of receptor sites (~20,000/cell) and high affinity ($K_d$ of 9.6 nM). Binding of acetyl-LDL, however, to this receptor does not initiate a signal transduction pathway. Thus, it appears that there are at least three classes of receptors that can be functionally distinguished by ligand-binding and signaling properties: high-affinity acetyl-LDL; low-affinity maleyl-BSA; and oxidized-LDL scavenger receptors. The cloning of these receptors has also revealed at least three distinct classes of scavenger receptors. The scavenger receptor A (SRA) avidly binds acetyl-LDL, maleyl-BSA, polynucleotides, and polysaccharides. Two subtypes of scavenger receptor A, SRA-1 and SRA-2, have been cloned. Both have short amino-terminal cytoplasmic tails that do not contain known signaling sequences. Scavenger receptor B (SRB) binds to acetyl-LDL but not to polysaccharides or polynucleotides. A third type of scavenger receptor (SRC) is not expressed in mammals but has been identified in *Drosophila melanogaster*. It is clear that the biological properties of scavenger receptors extend beyond their ability to clear chemically modified and oxidized ligands. Better characterization and insight into the signaling properties of these multifunctional receptors may well yield insight into their role in inflammation, cell adhesion, and atherogenesis.

## IV. THE EXPANDING NEUROBIOLOGY OF apoE

The recent observations that an apoE isoform is linked to functional outcome in a number of neurological diseases has led to a resurgence of interest into its ability to initiate a signaling cascade in macrophages and neurons. Apolipoprotein E is a 299-amino-acid protein that is a constituent of VLDLs, high-density lipoproteins (HDLs), and chylomicron remnant particles. There are three common human isoforms of apoE, designated E2, E3, and E4, which differ by single amino acid substitutions at positions 112 and 158, i.e. E3 (Cys112Arg158), E4(Arg112Arg158), and E2(Cys112Cys158). Apolipoprotein E is the primary apolipoprotein produced by brain, which is its largest extrahepatic source. Interest in the neurobiology of apoE intensified after the observation that the *APOE4* allele increased susceptibility to the development of sporadic and late-onset Alzheimer's disease (AD). Initial efforts to understand the role of apoE in AD were focused on disease-specific mechanisms, such as interactions with β-amyloid or tau. However, there is accruing evidence to suggest that presence of the *APOE4* allele may play a deleterious role in modifying outcomes not only from AD, but also from a diversity of acute and chronic neurological diseases, including intracranial hemorrhage, stroke, multiple sclerosis, and traumatic brain injury.

These observations have led to intensified efforts to understand the neurobiology of apoE in human disease. It has recently been realized that biologically relevant concentrations of apoE are capable of initiating a signaling response in neurons, presumably via the LRP. Consistent with these observations, we have recently created a family of peptides derived from the receptor-binding region of apoE (residues 130–155) and have demonstrated that a signaling response can be initiated in a CAD neuronal cell line on exposure to either human recombinant apoE or apoE peptidomimetics. This signaling response is characterized by the intracellular mobilization of calcium, $InsP_3$ turnover, and activation of protein kinase C (PKC), and appears to be specific to peptides containing residues 146–149 (Table 2). Moreover, the ability of both these peptides and the intact apoE to signal is blocked by the preincubation of cells with RAP, implicating LRP as the putative signaling receptor (Fig. 1).

Based on this evidence, it appears plausible that that the ability of apoE to initiate a signaling response in neurons may mediate an adaptive response to CNS injury. In fact, several studies have demonstrated that apoE initiates neurotrophic responses to promote

TABLE 2 Neuronal $Ca^{2+}$ Signaling Induced by apoE-Mimetic Peptides[a]

| Apolipoprotein E residue | 10 μ*M* | 100 μ*M* |
|---|---|---|
| apoE (130–149) | + | ++ |
| apoE (133–149) | – | ++ |
| apoE (130–146) | – | – |
| Scrambled apoE (133–149) | – | – |

[a] Apolipoprotein E (apoE)-mimetic peptides were taken from the sequence identified as containing the receptor recognition site, amino acids 130–149. The ability of these peptides to elevate $[Ca^{2+}]_i$ in fura-2AM-loaded differentiated, neuronal CAD cells was studied by digital imaging microscopy. Neither the scrambled peptide nor the peptide in which residues 146–149 were truncated retained the ability to initiate neuronal calcium responses.

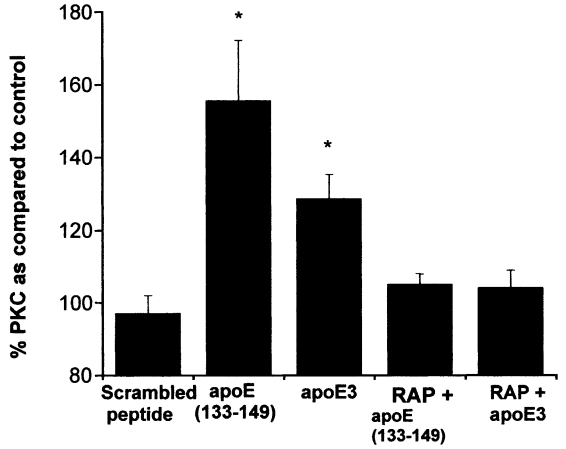

FIGURE 1 RAP blocks protein kinase C (PKC) activation by apolipoprotein E (apoE) and mimetic peptide. The CAD neuronal cell line was differentiated for 5 days in serum-free medium, then stimulated with the indicated ligand for 40 min at 37 °C. Incubation was stopped by addition of ice-cold homogenization buffer. PKC activity was assayed by phosphorylation of the PKC substrate histone IIIs, using [$\gamma$-$^{33}$P]ATP. Receptor-associated protein (RAP) was preincuabted with cells for 40 min at a concentration of 250 $\mu M$ prior to addition of ligand. Results are presented as percent change in PKC activity as compared to control (no stimulation) values in cell lysate ± standard deviation. There was a statistically significant ($^*p < 0.05$) increase in PKC activation following stimulation with either apoE3 or apoE(133–149). Preincubation with RAP blocked this response.

neurite outgrowth and survival. The exact mechanisms by which this occur remain to be defined, although both direct neurotropic effects and indirect interactions with growth factors and extracellular matrix proteins have been described. The hypothesis that apoE plays an important role in the CNS by virtue of it ability to initiate a signaling cascade is also consistent with the observations that ER2/VLDL receptor double knockouts and megalin knockout mice have developmental abnormalities resulting from impaired neuronal migration, as described previously. The nature of the isoform-specific roles that apoE3 and apoE4 play on neurons remains to be elucidated, although several recent reports have demonstrated that there may be discrete signaling pathways that are isoform specific. For example, apoE4 but not apoE3 stimulates the transcriptional activity of a cyclic AMP-response element-binding protein, resulting in the activation of an extracellular signal-regulated kinase cascade in hippocampal neurons. Such an isoform-specific novel signaling pathway may provide insight into the role of this polymorphism in human disease.

Another possible mechanism that would explain the role of apoE in modulating the CNS response to acute and chronic brain injuries is that this protein down-regulates the glial response to injury. The brain has a limited repertoire of responses to both acute and chronic injury, and the cornerstone of the brain inflammatory response is activation of microglia, the CNS resident macrophage. Glial activation results in the secretion of glutamate, proteases, and reactive oxygen species, all of which promote secondary neuronal injury in both acute and chronic diseases of the CNS. A role for apoE in modulating the function of microglia would be consistent both with the experimental evidence to date and with the clinical observations implicating apoE in neurological disease. In fact, both apoE and mimetic peptides derived from the receptor-binding region have been demonstrated to initiate a signaling cascade in macrophages and to down-regulate glial activation in cell culture models and *in vivo* paradigms of brain injury.

Thus, it is becoming increasingly clear that lipoprotein receptors play diverse roles that extend beyond cholesterol homeostasis. The ability of lipoprotein receptors to initiate signaling cascades enables them to participate in a variety of biological responses, including neuronal development and response to CNS injury, host immunity, and atherogenesis. A better understanding of these signal transduction cascades will no doubt play an important role in developing novel therapeutic intervention for a variety of human diseases.

## Glossary

**epidermal growth factor homology region** A domain common to all receptors of the low-density lipoprotein family; adjacent to the ligand-binding site.

**low-density lipoprotein** Molecule that is a mixture of lipid and protein, characterized as having a density of 1.019–1.063 g/cm$^3$ and a particle size of 180–250 Å.

**low-density lipoprotein receptor** Specific receptor for low-density lipoproteins; the first identified member of a large receptor family.

**low-density lipoprotein receptor-related protein** A receptor that binds many different ligands, including low-density lipoproteins; a member of the low-density lipoprotein receptor family.

**mitogen-activated protein kinases** Crucial enzymes in signal transduction pathways. Despite their name, they are activated by the binding of ligands to cell surface receptors. Examples of ligands are growth factors, hormones, and proteins that bind to members of the low-density lipoprotein receptor family.

**protein kinase C** Family of serine/threonine protein kinases that are activated by Ca$^{2+}$ and/or phospholipids in response to extracellular signals. Kinases phosphorylate their substrates, generally promoting activation.

## See Also the Following Articles

**Epidermal Growth Factor (EGF) Family • Interleukin-6 • Steroidogenic Acute Regulatory (StAR) Protein, Cholesterol, and Control of Steroidogenesis**

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# Luteinizing Hormone (LH)

Margaret A. Shupnik
*University of Virginia*

I. INTRODUCTION
II. STRUCTURE, SYNTHESIS, AND SECRETION
III. BIOLOGICAL ACTIONS ON TESTES
IV. BIOLOGICAL ACTIONS ON OVARIES
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VI. REGULATION BY OTHER HYPOTHALAMIC PEPTIDES
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VIII. SUMMARY

**The pituitary gonadotropins, luteinizing hormone and follicle-stimulating hormone, play a central role in mammalian reproduction, controlling the production of the sex steroids and gametogenesis. Synthesized and secreted from the same pituitary gonadotrope cells, both hormones share several structural characteristics. However, the two hormones bind to different receptors on their target organs, the ovaries and testes, have distinct biological functions, and are subjected to differential physiological regulatory mechanisms. This article focuses on the secretion of luteinizing hormone as well as its biological actions in the gonads.**

## I. INTRODUCTION

Recent gene disruption studies as well as naturally occurring biological mutations have confirmed the critical role of luteinizing hormone (LH) in stimulating steroidogeneis in the ovaries and testes. A lack of LH results in infertility in both males and females and inappropriate patterns of LH secretion may result in subfertility. The pulsatile secretion of LH is regulated by secretion of the hypothalamic peptide gonadotropin-releasing hormone (GnRH) as well as by levels of the sex steroids 17β-estradiol (E2), progesterone (P), and testosterone (T) (Fig. 1). The steroids may act indirectly to regulate LH via actions on the hypothalamus to alter the GnRH pulse generator, or directly at the level of the pituitary gonadotrope to influence gonadotropin synthesis and sensitivity to steroids and GnRH. The relative importance of each site of steroid regulation may vary between species, and in the female complex dynamic interactions between sex steroids, the hypothalamus, and the pituitary are of critical importance in orchestrating the reproductive cycle and normal fertility. The focus of this article is on synthesis of LH, biological actions of LH in males and females, and physiological regulation of LH by steroids and hypothalamic peptides.

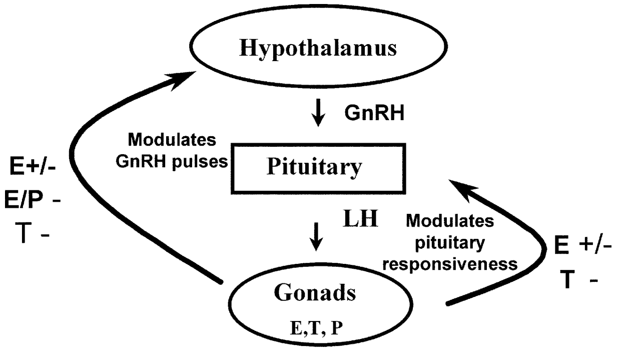

FIGURE 1 Regulatory hormonal feedback loops in the hypothalamic–pituitary–gonadal axis. Secretion of pituitary luteinizing hormone (LH) is stimulated by hypothalamic gonadotropin-releasing hormone (GnRH) pulses, which vary in frequency and amplitude with physiological state. LH secretion is favored when GnRH pulse frequency is higher, and is increased with higher amplitude pulses. LH binds to specific membrane receptors on the Leydig cells in the testes, and on the theca and granulosa cells in the ovaries, to stimulate the production of the sex steroids 17β-estradiol (E), testosterone (T), and progesterone (P). The steroids promote development of eggs and sperm in the gonads and have many other biological effects on other target tissues, including actions on the hypothalamus and pituitary in the regulation of LH secretion and subunit gene expression. Estradiol, progesterone, and testosterone bind directly to receptors in the pituitary gonadotrope. In the hypothalamus, E may act directly on GnRH neurons through the estrogen receptorβ isoform, but all steroids may act indirectly through other hypothalamic neurons that subsequently influence GnRH release. High physiological T concentrations suppress hypothalamic GnRH pulses and the pituitary response to GnRH. At the pituitary level, E increases the number of GnRH receptors and increases responsiveness of the LH genes to GnRH, but can also directly suppress LH secretion in humans. Estradiol treatment alone increases GnRH pulse frequency but decreases pulse amplitude, whereas E plus P suppresses GnRH pulses.

## II. STRUCTURE, SYNTHESIS, AND SECRETION

LH belongs to a family of glycoprotein hormones consisting of LH, follicle-stimulating hormone (FSH), the pituitary hormone thyrotropin, and placental chorionic gonadotropin (CG), which is expressed in humans and horses. In each case, the hormone consists of two noncovalently associated subunits: the α-subunit is identical in all family members and a unique β-subunit (LHβ, FSHβ, CGβ, and thyrotropinβ) provides biochemical and biological specificity to the intact hormones. The two LH subunits are encoded by separate genes located on different chromosomes, and transcription of the subunit genes for the intact hormone must be coordinated between the different transcriptional units. In the mouse, the α-subunit gene is located on chromosome 4, with the single LHβ gene on chromosome 7. In humans, the α-subunit gene is located on chromosome 21, and the human LHβ (hLHβ) gene is located in a gene cluster on chromosome 19 containing one copy of hLHβ and six copies of both bona fide and pseudogenes for hCGβ. This suggests that the hLHβ and hCGβ genes are closely related, and in fact the proteins have significant (83%) homology. The hCGβ gene coding region is believed to have evolved from the hLHβ gene through a frameshift mutation in the last exon that extends the reading frame for 29 additional amino acids at the C-terminus. The promoter regulatory regions of the gene, however, are quite different, and hCGβ gene expression is modulated very little or not at all by the changes in hypothalamic peptides and steroids that are critical in determining LH subunit gene transcription.

Both LH subunits and CGβ are glycosylated, and these modifications play a role in both hormone half-life in the serum and biological activity on the LH receptor (LH-R), which also binds CG. As secreted proteins, the glycoprotein hormone peptides are translated from their mRNAs as prohormones containing a hydrophobic sequence at their N-termini, which are cleaved as peptides are modified to add complex sugars, combine with their designated subunit partner, and are packaged into secretory granules. The mature α-subunit protein from human tissues has 92 amino acids and contains two N-linked sugars, whereas the hLHβ protein has 121 amino acids and one N-linked sugar modification. The 145-amino-acid hCGβ protein contains two N-linked glycosylation sites and, in the C-terminal extension region, four additional O-linked glycosylation sites not contained in hLHβ. This structural modification of hCGβ results in a much longer half-life of the intact hCG in serum relative to hLH, and plays an important role in the greater biological potency of hCG at the LH/CG receptor. In general, both intact hormone and some free (i.e., uncombined) α-subunit can be detected in secretory granules in the pituitary or placenta and in serum. There does not appear to be any physiological role for the free α-subunit, and the uncombined protein contains an additional O-linked sugar; it is not known whether this is a result of processing that occurs because there is additional α-subunit available or if this serves some other regulatory function.

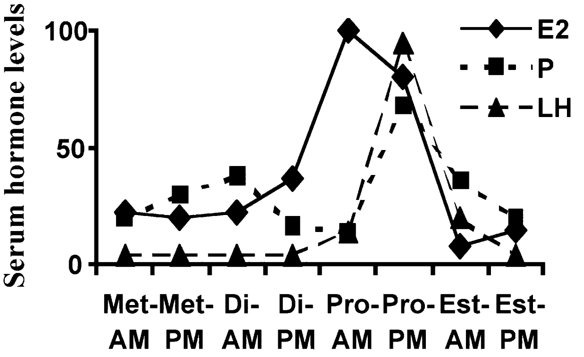

FIGURE 2 Serum levels of luteinizing hormone (LH) and steroids during the female rat reproductive cycle. Levels of LH, progesterone (P), and 17β-estradiol (E2) are plotted relative to 100% of maximal levels secreted during the 4-day rat estrous cycle, measured at 9 AM and 6 PM on each day of the cycle. Follicular development occurring during diestrus (Di) and proestrus (Pro) results in rising E2, increased GnRH receptor number, and an increased GnRH pulse frequency, leading to a surge of LH and P. After ovulation in the late afternoon of proestrus, corpora lutea formation during estrus (Est) and metestrus (Met) secretes sufficient levels of E2 and P to suppress GnRH pulses and thus LH.

In adult humans and animals, LH secretion is rhythmic and has a fairly consistent pattern of one pulse approximately every 90 min in males, but varies both in frequency and in amplitude throughout the reproductive cycle in females. LH secretion (Fig. 2) is generally lower during the early phase of the female ovarian cycle, but rises as the follicle develops and estrogen and P levels begin to increase, culminating in an LH "surge". The surge of high levels of serum LH that occurs midcycle in females is critical for ovulation. After ovulation, when the egg is released, the corpus luteum synthesizes P and E2; this stage of the ovarian cycle is correlated with a low level of LH secretion (Fig. 2). Variations in LH secretory patterns occur in response to alterations in the secretion of the hypothalamic peptide gonadotropin-releasing hormone and in levels of the sex steroids, E2, P, and T, as part of the long endocrine feedback loops (Fig. 1) regulating reproduction and fertility. GnRH is secreted episodically into the portal circulation that links the hypothalamus and pituitary gland. Stimulation of LH and FSH secretion is frequency dependent, with more rapid pulse frequencies (30- to 90-min intervals) generally favoring LH and slower pulse frequencies (120-min or longer intervals) generally favoring FSH. Steroid feedback to regulated LH levels may occur at the level of the hypothalamus, to alter GnRH pulse frequency, or at the level of the pituitary gland, to modulate either LH synthesis or the response of the gland to GnRH. In males, steroid feedback is generally negative, whereas in females both positive feedback prior to the LH surge and negative feedback during the rest of the ovulatory cycle are important for normal fertility.

## III. BIOLOGICAL ACTIONS ON TESTES

LH plays a critical role in endocrine function of the adult testes, acting on the Leydig cells to promote the production of the sex steroid testosterone, which is required for normal sperm production and fertility. In the mouse, α-subunit gene expression is detected fairly early in development, on day embryonic day 10.5 (E10.5), whereas LHβ is not expressed until day E16.5 and FSHβ is not expressed until E17.5 of the 20- to 21-day gestational period. Thus, neither LH nor FSH secretion occurs prior to the prenatal development of the testes, and normal male sexual differentiation and genital development occur in the absence of circulating pituitary gonadotropins. This point has been substantiated by gene disruption studies in mice in which the common α-subunit gene is "knocked out" by insertion of an artificial DNA sequence, and no α-subunit protein is made in either the gonadotropes or the thyrotrope cells that synthesize thyrotropin. Homozygote knockout males have normal sexual differentiation and genital development but are both hypothyroid and hypogonadal, with no circulating LH, FSH, or T, and are infertile. The mice have decreased testis size with normal epididymis and vas deferens; however, the seminal vesicles are atrophied, consistent with the lack of serum T, and there is a block in spermatogenesis at the first meiotic division. The directed disruption of the LHβ gene specifically in an animal model has not been reported. However, mutations in the gene for early growth response protein-1 (Egr-1), a transcription factor required for LHβ gene expression, can result in a functional loss of LHβ and serum LH and subsequent infertility in males under certain conditions. Male infertility results if the Egr-1 mutation is severe and targets both the N-terminus and the DNA-binding domain of the protein, but not if the Egr-1 targets the DNA-binding region alone. Because the first mutation also affects expression of the LH receptor gene, both LH synthesis and action as well as other potential unknown molecules and pathways are affected. A recent report described the phenotype of mice in which the LH-R gene was disrupted, resulting in spermatogenic arrest and severe under-development of internal and external genitalia, including abdominal testes, disarray of seminiferous tubules, diminished number of Leydig cells, and low T. Testosterone treatment resulted in descended testes and larger penis size as well as resumption of spermatogenesis, but the mice remained infertile.

In humans, a short period of high levels of gonadotropin secretion after birth in both sexes occurs during the first 3–4 months; this is followed by a period of suppressed gonadotropin levels until puberty. There are no known human gene deletions for the LHβ gene or for the LH-R. However, several inactivating mutations in these genes confirm that a functional loss of LH results in extremely low to absent T production, Leydig cell hypoplasia, absence of the development of male secondary sexual characteristics, and infertility. Rare cases of activating receptor mutations in boys result in precocious puberty and high levels of gonadotropin-independent testosterone.

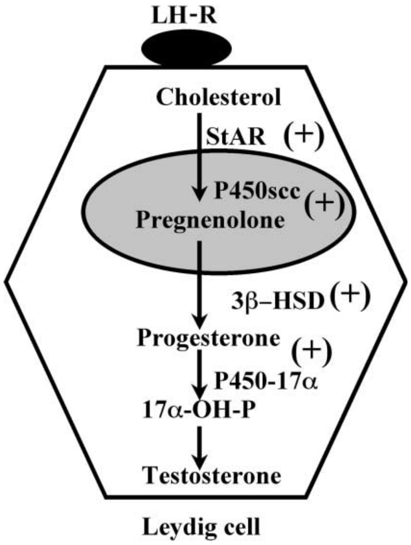

FIGURE 3 Luteinizing hormone (LH)-stimulated steroid synthesis in Leydig cells in the testes. The testes contains several cell types, including the Leydig cells, which contain LH receptors and produce testosterone, and the Sertoli cells, which synthesize a testosterone-binding protein that acts to maintain the high testosterone concentrations required for spermatogenesis. LH stimulates (+) the transport of the cholesterol precursor into the mitochondria, indicated by the gray oval, by the steroidogenic acute regulatory (StAR) protein, and several enzymatic steps in the mitchondria and endoplasmic reticulum that modify the cholesterol backbone to the final product. These steps include P450 side chain cleavage enzyme (P450scc), which converts cholesterol to pregnenolone; 3β-hydroxysteroid dehydrogenase/isomerase type I(3β-HSD), which converts pregnenolone to progesterone; and 17α-hydroxylase/17,20 lyase (P450-17α), which acts on pregnenolone to produce 17α-OH progesterone (17α-OH-P), a precursor of the androgens androstenedione and testosterone.

LH acts through a serpentine (seven-transmembrane) domain $G_s$-protein-coupled membrane receptor. Biological activity is primarily dependent on $G_s$ stimulation of adenylyl cyclase and increased intracellular cAMP, which then activates protein kinase A (PKA) and subsequently several other enzymes. In males, LH-Rs are located primarily on the Leydig cells in the testes, which produce all of the testicular T. This T production, along with the FSH-induced androgen-binding protein produced in the Sertoli cells, maintains the high androgen concentrations required for spermatogenesis (Fig. 3). The production of the sex steroid occurs by modification of precursor cholesterol esters in several steps by cytochrome P450 enzymes that are contained in the mitochondria and endoplasmic reticulum. LH stimulation of steroidogenesis occurs at several sites and includes induction of steroidogenic acute regulatory protein (StAR) synthesis and phosphorylation. Although the entire molecular mechanism of StAR activity is not yet clear, it facilitates the overall rate-limiting step in steroidogenesis, the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Cholesterol is then modified by the actions of the P450 cholesterol side chain cleavage enzyme (P450scc; CYP11A), the key and rate-limiting enzyme in steroidogenesis, which removes the six-carbon cholesterol side chain to form pregnenolone. Pregnenolone is further metabolized to P by the actions of the endoplasmic reticulum enzyme

3β-hydroxysteroid dehydrogenase/isomerase type 1 (3β-HSD1), and production of the androgens androstenedione and testosterone is further catalyzed by a cascade initiated by the P450 17α-hydroxylase/17,20 lyase enzyme (P450-17α; CYP17). In addition to its actions on StAR, LH also stimulates the production and activity of the steroidogenic enzymes P450scc, 3β-HSD1, and P450-17α, at least partly by stimulating transcription of the enzyme genes through the cyclic adenosine monophosphate (cAMP)-activated transcription factor, cAMP response element binding (CREB) protein, and related family members. Thus, LH stimulates many if not all of the steps leading to androgen synthesis. Activating mutations in the human LH-R have been found to result in precocious puberty in young boys, presumably because the LH-R increases intracellular cAMP concentrations in the absence of ligand, stimulating StAR and the steroidogenic enzymes.

## IV. BIOLOGICAL ACTIONS ON OVARIES

The ovary, the site of both steroid synthesis and egg or ovum production, is a complex tissue and its physiology will not be covered in detail here. Steroid synthesis in the ovary occurs in ovarian follicles, which contain two types of steroidogenic cells, the granulosa and the theca cells (Fig. 4); these cells lie on either side of the follicular basement membrane, whereas interstitial cells are contained between the follicles. Granulosa cells contain receptors for both FSH and LH, whereas the theca cells contain receptors for LH alone. The effective physiological production of the two main female sex steroids, P and 17β-estradiol, requires cooperation between the two cell types. LH plays a critical role in inducing the levels and activity of StAR, the main transport protein for moving cholesterol into the mitochondria for enzymatic modification, and the rate-limiting step in steroidogenesis in both granulosa cells and theca cells. FSH induces LH receptors on the granulosa cells of the follicle, and gonadotropin (either LH or FSH) treatment results in increased CYP11A activity, leading to increased P production. Progesterone cannot effectively be modified to E2 within the granulosa cells. Instead, it is transported to the theca or interstitial cells, where it can either enter the circulation or serve as a substrate for the CYP17 enzyme, which converts P to 17-OH progesterone, a precursor of the androgens androstenedione and T. Testosterone serves as the substrate for E2 via the enzyme aromatase (P450arom; CYP19), which is not expressed in theca cells. Estradiol synthesis requires transport of the testosterone from the theca to the granulosa cells, where it is converted to E2 by CYP19. The theca cells also contain StAR and the enzymatic machinery to produce P, so androgens made in the theca cell can arise from either granulosa cell- or theca cell-produced P. The vast majority if not all E2 production occurs in the granulosa cell. LH does not appear to have much influence on CYP17 and CYP19 levels, which rise in rodent models prior to the LH surge. In fact, these enzymes are critical in providing the increased serum E2 levels that are required for the LH surge.

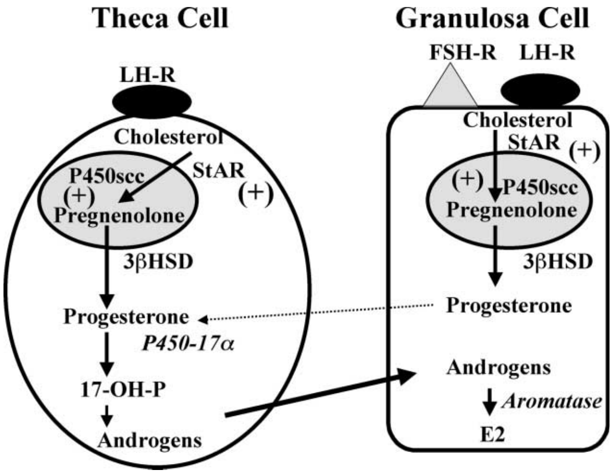

FIGURE 4 Luteinizing hormone (LH)-stimulated steroid synthesis in ovarian cells. Production of progesterone and 17β-estradiol (E2) from cholesterol precursors requires cooperation of the granulosa cells, which contain both LH and follicle-stimulating hormone (FSH) receptors (LH-R and FSH-R), and the theca cells, which contain only LH receptors. Both cells can synthesize progesterone. However, only theca cells contain the enzyme 17α-hydroxylase/17,20 lyase (P450-17α), which modifies progesterone to 17-OH progesterone (17-OH-P), a precursor of the androgens androstenedione and testosterone, and progesterone from either theca or granulosa cells may be used for this purpose. Only granulosa cells contain aromatase, which converts androgens to E2. FSH induces LH receptors as well as the side chain cleavage enzyme (P450scc;), which converts cholesterol to pregnenolone in the mitochondria. LH stimulates (+) the transport of the cholesterol precursor into the mitochondria by stimulating the steroidogenic acute regulatory (StAR) protein, as well as mitochondrial P450scc activity, in both granulosa and theca cells, but other enzyme activities in the endoplasmic reticulum are not stimulated. Enzymes unique to a specific cell type are shown in italics.

The increased level of LH-R on the mature follicle makes it highly sensitive to LH, and the LH surge signals the rupture of the follicle and ovulation. After ovulation, the granulosa cells from the ruptured follicle undergo the process of luteinization and form the corpus luteum (CL), an endocrine structure that synthesizes the E2 and P secreted during the next phase of the ovarian cycle, referred to as the luteal phase of the cycle in humans. LH is required for the development of the CL; however, this structure and steroid synthesis in the rodent ovary are maintained during pregnancy by pituitary prolactin and by uterine prolactin-related molecules. In humans, the CL contains LH-R, and LH could maintain CL steroidogenesis. However, negative steroid feedback results in lower serum LH, and the maintenance of the CL in early pregnancy occurs through hCG, which is produced by the trophoblast cells of the placenta and acts on the LH/CG-R in the CL.

The importance of LH in the ovarian cycle and fertility is underlined by several gene knockout models and naturally occurring human mutations. Female mice with disrupted $\alpha$-subunit genes are infertile, with a failure of the vaginal orifice to open, small ovaries with no antral follicles or corpora lutea, thin uteri, and severely depressed E2 levels. Introduced mutations in the mouse gene for early growth response protein-1,a transcription factor required for LH$\beta$ gene expression, result in a functional loss of LH$\beta$ and serum LH and subsequent infertility. The animals with these mutations have normal follicular development, because this occurs under the influence of FSH, but there is no ovulation or subsequent corpus luteum development. Steroid synthesis can be induced by gonadotropin (LH or hCG) administration. Disruption of the LH-R in female mice results in ambiguous vaginal opening, dramatically decreased ovarian and uterine weights, arrested follicular growth, dramatically decreased StAR levels, and decreased E2. Estradiol and progesterone replacement therapy in these animals results in normal vaginal development, but has no effect on ovarian morphology, and animals remain infertile. Thus, steroids cannot replace LH actions for ovarian morphology or overall fertility, and LH likely plays additional roles in this process. Hypersecretion of LH in female mice, as engineered in a transgenic mouse model, results in enlarged, cystic, and hemorrhagic ovaries, elevated T:E2 ratios, and infertility due primarily to anovulation.

In humans, LH-R-activating mutations identified to date do not cause precocious puberty in females, because the receptors still respond with higher activity to the high levels of serum LH required to cause ovulation. Inactivating LH-R mutations in women do not interfere with the development of primary and secondary sexual characteristics, but result in low levels of E2 and P and in the absence of ovarian corpora lutea, thus resulting in infertility. Women with polycystic ovarian syndrome (PCOS) are infertile or subfertile and are hyperandrogenic. Many PCOS women have elevated LH/FSH levels, but it is uncertain whether this helps drive the higher serum androgens or is the result of inappropriate steroid feedback at the hypothalamus and pituitary.

## V. REGULATION BY GnRH

The single most important physiological determinant of LH secretion in mammals is the hypothalamic decapeptide gonadotropin-releasing hormone, and animals or humans lacking GnRH do not secrete gonadotropins and are infertile. GnRH is secreted episodically into the vessels of the hypophyseal portal system linking the hypothalamus and the pituitary; it acts on specific seven-transmembrane G-protein-coupled GnRH receptors (GnRH-Rs) on the membrane of the pituitary gonadotrope. Pulsatile secretion of GnRH, defined by both pulse frequency and amplitude, is critically important in determining the amount and identity of the gonadotropin secreted. Rapid GnRH pulse intervals lasting 30 to 90 min favor LH secretion, and longer pulse intervals favor FSH secretion. Both LH and FSH are synthesized in the same cells, although a population of gonadotropes will contain cells that primarily contain one of the two hormones, and the mechanism by which cells sort secretory vesicles to release primarily LH or FSH is unknown.

The intermittent nature of the GnRH signal is essential in maintaining gonadotropin secretion. Elegant studies in primates and sheep, using direct physical sampling of GnRH secretion or direct administration of GnRH into the portal system, have shown that sustained exposure to high concentrations of GnRH reduces the response of the gonadotrope cells to subsequent GnRH stimulation. This process of homologous desensitization of the GnRH receptors (GnRH-R) leads to subsequent suppression of gonadotropin secretion and occurs even within the time frame of endogenous GnRH pulses. The mechanism of desensitization is not known; it may involve both reduction in receptor number and uncoupling of the GnRH-R from its regulatory G-proteins, $G_{\alpha q}$ and $G_{\alpha 11}$, and the loss of downstream signaling. GnRH binding activates phospholipase C, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) and results in increased intracellular calcium concentrations due to calcium influx through voltage-dependent L-type calcium channels and release from intracellular stores. Desensitization has been shown to diminish the GnRH response through all of these signaling cascades. The mechanism for GnRH-R uncoupling has not been defined. In other G-protein-coupled receptors, this process usually involves receptor phosphorylation in the third intracellular loop or the C-terminal tail. The GnRH-R lacks a C-terminal intracellular tail and does not have significant amino acid homology with these receptors in the third intracellular loop, suggesting that desensitization may occur at some point after receptor binding and activation.

In addition to effects on LH secretion, GnRH pulse frequency has profound effects on the transcription rates of the all of the gonadotropin subunit genes, as well as the GnRH-R, as shown in rats *in vivo* and in rodent pituitary cells in culture. Continuous GnRH or rapid pulses (one pulse every 8 or 30 min) preferentially stimulate α-subunit mRNA and gene transcription; intermediate pulse frequencies (one pulse every 30 to 60 min) preferentially stimulate the LHβ subunit gene and increase levels of GnRH-R and LHβ subunit mRNA. GnRH pulses have a less robust effect on FSHβ mRNA levels and gene transcription, and occur only at slow pulse frequencies (one pulse every 120 or 240 min). This may reflect the fact that the FSHβ gene is also regulated by gonadal and pituitary peptides such as activin, inhibin, and follistatin, which have little if any effect on LHβ and may reduce the importance of regulation by GnRH. The stimulation of the GnRH-R by GnRH is reflected in increased GnRH binding to pituitary cell membranes at times leading up to the LH surge, and this helps explain the "priming" effect of GnRH pulses on GnRH-R.

Because the gonadotrope contains only one type of GnRH-R, the mechanism by which frequency-dependent signals are decoded at the level of the receptor and sensed differentially by the gonadotropin subunit genes has been a subject of much conjecture and investigation. Possible points of GnRH regulation include preferential sensitivity to different signaling pathways recruited by varying pulse frequencies, different gene targets such as DNA sequences and transcription factors, or both. Unfortunately, there is no current cell model system that maintains the strict GnRH pulsatile dependency required *in vivo* for gene expression, and dependence on differential intracellular signaling pattern activation for subunit gene activation has been difficult to confirm. There are also differences in DNA sequences of the promoters of the LH subunit genes from various species (humans, rats, mice, and bovines), allowing for potential species-specific differences in gene regulation between model systems. GnRH-stimulated transcription of the human and rodent α-subunit gene requires both calcium influx and MAPK activity, whereas activation of the LHβ genes from several species has been reported to require preferentially either calcium influx, PKC, or various MAPK family members. However, because intermediate to high GnRH pulse frequencies stimulate the expression of the GnRH-R as well as LHβ and the α-subunit, the LH genes are maximally stimulated when GnRH-R concentration is high, and FSHβ is maximally stimulated when GnRH-R concentrations are low. These data have been verified in cells that have artificially engineered levels of GnRH-R, and this has led to the hypothesis that the higher levels of GnRH-R activate some additional signaling pathway, perhaps associated with G-protein βγ subunits that either preferentially stimulate the LH subunit genes or silence the FSHβ gene.

It is clear that the two LH subunit genes from any one species have both common and divergent gene regulatory regions, and that the divergent regions likely contribute to the differential regulation by GnRH. Both subunit genes bind transcription factors that direct gene expression to the pituitary gonadotrope, but are not required for hormonal regulation. Prominent among these common factors is steroidogenic factor-1 (SF-1), which plays a central role in the expression of many genes in reproductive and steroidogenic (gonads and adrenals) tissues. SF-1-dependent genes include those encoding the α-subunit, LHβ, and the GnRH-R; animals in which the SF-1 gene is disrupted are infertile.

GnRH regulation of the subunit genes utilizes pathways with at least some degree of species specificity. Stimulation of the human α-subunit gene requires a cyclic AMP response element (CRE), which binds several members of the CREB protein family. These transcription factors are phosphorylated posttranslationally, and thus are activated, by multiple protein kinase enzymes. The kinases are modified and activated by several signaling pathways stimulated by GnRH, including PKC, MAPK, and calcium. The rat and mouse α-subunit genes do not contain CREs, and although the DNA sequences necessary to confer a GnRH response have been identified, the proteins binding to them have not. The LHβ genes from several species require the transcription factor Egr-1, mentioned previously, for LHβ expression. Egr-1 protein synthesis is dramatically stimulated by GnRH through the PKC pathway. The protein can also be posttranslationally modified and activated by GnRH signaling cascades, resulting overall in increased binding of Egr-1 to the LHβ gene promoter and in stimulated LHβ mRNA and protein levels. The rat, but not the bovine, LHβ gene also requires gene promoter sequences that bind specificity protein-1 (Sp-1), a protein similar to Egr-1, for full stimulation, and the two regions are proposed to cooperate by binding additional co-activator proteins.

Regulation of LH subunit gene activity through specific divergent GnRH response elements and transcription factors cannot explain the pulsatile GnRH control of this process. Rhythmic transcription is at least partly due to secondary induction of enzymes or other proteins that subsequently act in opposition to GnRH. Sophisticated molecular biology techniques have identified numerous GnRH-induced genes, including those for signaling molecules, transcription factors, and adapter proteins, that could serve this function. For example, Egr-1 stimulates not only the LHβ gene, but also the gene for a MAPK phosphatase, which opposes and limits the actions of the MAPK activated by GnRH. GnRH also stimulates synthesis of Nab-1 and Nab-2, proteins that bind to Egr-1 and alter its biological activity. Thus, GnRH can induce proteins that initiate the stimulatory LH response as well as help to extinguish it.

## VI. REGULATION BY OTHER HYPOTHALAMIC PEPTIDES

Although several additional peptides have been reported to stimulate or inhibit LH secretion, or to modify the effects of GnRH on LH stimulation, none of the peptides has the central physiological role of GnRH in controlling LH serum levels. The majority of information on their biological effects is derived from animal models, particularly rats, and effects of nearly all the peptides can be either positive or negative, depending on the particular animal model or endocrine state. Galanin and oxytocin stimulate LH secretion, and hypothalamic release of both, as well as neuropeptide Y (NPY), is highest in rats during proestrus when LH secretion rises. The stimulatory effects of oxytocin and NPY are greatest in the presence of E2 and are inhibited in the presence of P. Direct effects of NPY on pituitary cells have been difficult to demonstrate, emphasizing the importance of the physiological interactions between the various hypothalamic peptides, steroids, and GnRH *in vivo*. Pituitary adenylyl cyclase-activating peptide (PACAP) stimulates LH release from isolated pituitary cells and augments the GnRH stimulatory effect. PACAP is released into portal blood and is also synthesized in anterior pituitary cells. PACAP stimulation of intracellular cAMP levels and cross talk with GnRH signaling pathways contribute to the stimulatory actions of PACAP on the gonadotrope. The gonadotrope cells contain high-affinity receptors for PACAP and oxytocin, allowing them to act at low physiological concentrations. In contrast, the pituitary contains a small number of high-affinity NPY-binding sites that are often masked by a large number of lower affinity sites. NPY treatment increases GnRH binding to rat pituitary cells, thus increasing GnRH effectiveness.

Two peptide types, substance P and the opioids, have been reported to suppress GnRH stimulation of LH secretion under specific physiological states. Substance P binding sites exist on rodent and human pituitary tissues. The number of substance P receptors varies during the rat estrous cycle and is inversely related to the number of GnRH binding sites. Substance P inhibits GnRH-stimulates LH release from cultured rat or human pituitary cells but potentiates the response in pig cells, thus demonstrating some species-specific responses. Opioid peptides, particularly β-endorphin, are secreted into portal blood in a temporal pattern inversely related to GnRH secretion. The opioids act at the hypothalamus to inhibit GnRH secretion, and this is their most important physiological effect on the hypothalamic–pituitary reproductive axis. However, β-endorphin can also suppress GnRH-stimulated LH release by acting through μ- and δ-opioid receptors on rat pituitary cells. Inhibition is not observed in E2-treated pituitary cells, but is observed in the presence of P. The opioids are also synthesized in the anterior and intermediate pituitary lobes, allowing for paracrine regulation of the gonadotropins.

## VII. REGULATION BY SEX STEROIDS

Serum LH levels are closely correlated with those of the sex steroids, E2, T, and P. The steroids may act indirectly at the level of the hypothalamus to regulate the amplitude and frequency of GnRH pulses. Alternatively, direct steroid effects can occur at the level of the pituitary to modulate LH subunit gene expression, GnRH-R levels, and the response of the pituitary gland to other peptides and steroids that modulate GnRH responses. Because T may be aromatized to E2, T may have biological actions through its own androgen receptor (AR) or through the estrogen receptor (ER). Negative feedback of the steroids with respect to LH in males and females is dramatically demonstrated after gonadectomy, when LH levels rise manyfold. Elevated LH levels are also observed in aging humans, particularly postmenopausal women with low E2 levels, and in animal models in which the ARs and ERs are mutated or disrupted. Positive steroid (E2) feedback is demonstrated in females at the time of the proestrus or midcycle surge in LH, leading up to ovulation. Steroid modulation in both cases requires actions at the hypothalamic level, but some pituitary actions of steroids are noted in both cases.

The pituitary gonadotrope contains receptors for E2, androgens, and P, and these steroids all influence LH synthesis and secretion. Estradiol treatment increases the level of GnRH-R, stimulates rat LHβ gene transcription, and stimulates the human α-subunit gene responsiveness to GnRH through effects on CREB phosphorylation. Estradiol also induces the synthesis of P receptors (PRs). All these responses contribute to positive steroid feedback in females prior to the LH surge and increase pituitary responsiveness to GnRH. In contrast, high physiological levels of T generally have suppressive effects on LH synthesis. These responses are due to direct androgen action rather than to conversion to E2, as verified by administration of aromatase inhibitors or use of the nonaromatizable androgen dihydrotestosterone (DHT). Testosterone or DHT suppresses human α-subunit gene expression directly and blunts stimulatory responses of the LH subunit genes of several species to GnRH. Testosterone also reduces the LH response to GnRH in human studies.

The sex steroids exert profound control over the GnRH pulse pattern, affecting both frequency and amplitude. Thus, they play a critical role in determining gonadotropin secretion, the female ovarian cycle, and the fertility of both sexes. In females, rising serum E2 from the developing ovarian follicle decreases the amplitude of GnRH pulses and raises the baseline secreted between pulses, with either no effect or an increase in GnRH pulse frequency; overall, in rats at proestrus, sustained GnRH release during the hormonal surge in maintained. Estradiol also induces PR expression in the hypothalamus, allowing a response to P and E2 secreted from the mature follicle and corpus luteum after ovulation. Progesterone, in the presence of E2, slows GnRH pulse frequency and overall GnRH secretion, thus suppressing LH secretion. High levels of sustained E2 or T also suppress GnRH pulses, reducing the rise in gonadotropins noted after gonadectomy in animals or humans. Sustained E2 plus P treatment suppresses the hypothalamic–pituitary axis and ovulation very efficiently, and is the basis for oral contraception.

High physiological levels of T suppress GnRH pulse amplitude in humans, based on lower LH pulses in response to GnRH, and suppress GnRH secretion in rats and sheep. However, in women with polycystic ovarian disease, the inappropriately high serum T levels are often associated with elevated serum LH. Testosterone levels in these women are lower than those found on men and women with suppressed GnRH secretion. In this case, T may interfere with P action on the hypothalamus to reduce GnRH pulse frequency. LH secretion will thus be favored. Administration of androgen antagonists restores the sensitivity of the women to estrogen plus P suppression of LH. Thus, the levels of the sex steroids relative to each other can also influence GnRH pulses.

The mechanisms by which the steroids modulate GnRH neurons have been the subject of much debate, because it has been difficult to detect steroid receptors within them. However, GnRH neurons of several species have now been found to contain ERβ, a recently identified estrogen isoform, allowing for direct estrogen actions, and PR has been detected in a small subset of these neurons in some species. Steroid effects could occur through direct effects on the GnRH neurons, or indirectly through steroid-responsive cells adjacent to the GnRH neurons, to modulate secretion of other peptides and small molecules such as nitrous oxide, opioids, serotonin, and dopamine, which subsequently regulate GnRH release. In either case, steroids can act to regulate transcription of genes for neurotransmitters or their synthetic enzymes or receptors, or may activate other intracellular signaling pathways to have nongenomic effects. For example, E2 acutely opens potassium channels and hyperpolarizes GnRH neurons, and kinase inhibitors block some of these effects. It is likely that both direct and indirect steroid mechanisms play a role in regulating GnRH pulse patterns.

## VIII. SUMMARY

LH secretion from the pituitary gonadotrope cell is controlled by interplay between the circulating levels of estradiol, progesterone, and testosterone and the specific pattern of hypothalamic-secreted GnRH pulse frequency and amplitude. Steroids regulate LH synthesis and the synthetic and secretory responses to GnRH. In females, a surge of E2 and LH is required for ovulation and fertility, whereas GnRH and gonadotropin secretion are suppressed in the presence of constant E2 and P. Luteinizing hormone plays a central role in stimulating the production of the sex steroids in the ovary and testes, through transport of the cholesterol precursor and stimulation of the steroidogenic enzymes. LH secretion and functional LH receptors on the gonads are required for normal fertility in both males and females.

## Glossary

**gonadotropin-releasing hormone** A 10-amino-acid peptide synthesized and modified from a large protein precursor and secreted in intermittent pulses from the hypothalamus; acts on specific gonadotrope G-protein-coupled receptors to stimulate gonadotropin synthesis and secretion.

**gonadotropins** Two related, but functionally distinct, peptide hormones (luteinizing hormone and follicle-stimulating hormone) synthesized and secreted from the gonadotrope cells in the mammalian pituitary. These hormones bind to receptors on the gonads, the ovaries, and testes, and individually influence either sex steroid production (primarily luteinizing hormone) or the development of eggs and sperm.

**G-protein-coupled receptors** Membrane receptors, also called seven-transmembrane, or serpentine, receptors; bind peptides or small molecules on the cell surface and bind intracellularly with small three-subunit G-proteins, the activity of which is regulated by GTP cleavage and phosphorylation. The $G_\alpha$ subunit communicates directly with and activates other intracellular signaling cascades to increase levels of cyclic AMP, phospholipids, and intracellular $Ca^{2+}$. The $G_{\beta\gamma}$ subunits were originally thought to be inert, but have recently been shown to activate protein kinase pathways distinct from that of the $G_\alpha$ subunits.

**mitogen-activated protein kinases** Dual protein kinase enzymes activated by phosphorylation of specific threonine and tyrosine residues in response to binding to G-protein-coupled and growth factor receptors. The biological activities of the kinases are opposed by phosphatases, enzymes that dephosphorylate either the threonine or tyrosine and inactivate the kinase.

**protein kinase C** Serine/threonine protein kinases that are activated by phospholipids and by increases in intracellular $Ca^{2+}$, and in response to extracellular signals from membrane-bound receptors.

**steroidogenesis** Enzymatic process of synthesizing sex steroids from cholesterol precursors, occurring in steroidogenic tissues such as the ovaries and testes. The process can occur only in cells that contain the steroidogenic acute regulatory protein, a transport protein that brings the cholesterol ester to the inner mitochondrial membrane for modification, and specific cytochrome P450 enzymes, which are located in the mitochondria and endoplasmic reticulum. The enzymes contain heme and reduce molecular oxygen, incorporating one atom specifically into the substrate as a hydroxyl group.

## See Also the Following Articles

**Corpus Luteum in Primates • Follicle-Stimulating Hormone (FSH) • Gonadotropin-Releasing Hormone (GnRH) • GPCR (G-Protein-Coupled Receptor) Structure • Luteinizing Hormone Receptor Signaling • Neuropeptide Y (NPY) • Ovulation • Polycystic Ovary Syndrome (PCOS) • Steroidogenic Acute Regulatory (StAR) Protein, Cholesterol, and Control of Steroidogenesis • Thyrotropin-Releasing Hormone (TRH)**

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# Luteinizing Hormone Receptor Signaling

Prema Narayan and David Puett
*University of Georgia*

I. INTRODUCTION
II. SIGNALING VIA THE cAMP PATHWAY
III. SIGNALING VIA THE PHOSPHOINOSITIDE PATHWAY
IV. LIGAND-INDEPENDENT SIGNALING
V. SIGNAL ATTENUATION
VI. SUMMARY

**A critical component of the reproductive axis in males and females, the luteinizing hormone receptor (LHR) is required for phenotypic male sexual differentiation of the 46,XY fetus, gonadal steroidogenesis in adult testes and ovaries, and ovulation. The major signaling pathway of this receptor in response to the gonadotropins, human luteinizing hormone and human chorionic gonadotropin, is believed to be that of intracellular cAMP production, although recent findings suggest the involvement of other signal transduction pathways in certain target cells. This article reviews the intracellular signaling pathways associated with LHR in hormone-dependent and hormone-independent activation, the latter, for example, arising from mutations of the LHR gene that promote constitutive receptor activation. The cellular mechanisms for signal attenuation at the level of LHR are also discussed.**

## I. INTRODUCTION

The luteinizing hormone receptor is a member of the glycoprotein hormone receptor subfamily, which includes the follicle-stimulating hormone receptor and the thyroid-stimulating hormone receptor. This small subfamily belongs to the larger family of rhodopsin-like seven-transmembrane G-protein-coupled receptors, which are defined by an N-terminal extracellular domain, seven transmembrane helices linked by three intracellular and three extracellular loops, and a C-terminal cytoplasmic tail. A defining characteristic of the glycoprotein hormone receptors is the presence of a large, glycosylated extracellular domain that is composed of leucine-rich repeats; this extracellular region serves as a high-affinity ligand-binding domain.

The luteinizing hormone receptor (LHR) binds the two homologous and structurally similar gonadotropins, pituitary-derived luteinizing hormone (LH) and placentally secreted human chorionic gonadotropin (hCG). These two hormones are glycosylated heterodimers that share a common $\alpha$-subunit and contain a hormone-specific $\beta$-subunit. In early development, LHR activity is essential for the maturation of fetal Leydig cells and the production of testosterone during male sexual differentiation. In adults, LHR function is required for male and female reproductive physiology. For example, in males, LHR regulates the production and secretion of androgens from the testis in response to LH. In females, LHR associated with the theca and granulosa cells of the ovary promotes follicular development and regulates the production of sex steroids in response to LH. Moreover, the action of high levels of LH produced during the midcycle LH surge triggers ovulation. Following ovulation, LHR associated with the luteal cells in the corpus luteum is responsible for the production of progesterone, a process also under the control of LH. In the event of fertilization, LHR in the corpus luteum is activated by hCG secreted by the syncytiotrophoblast cells of the placenta, resulting in the production of progesterone, which are essential for the maintenance of pregnancy. Therefore, LHR occupies a central role in the fundamental processes of development and reproduction, and precise regulation of the signaling pathways activated by LHR is vital to these processes.

## II. SIGNALING VIA THE cAMP PATHWAY

Binding of LH or hCG to LHR on the cell surface activates a cascade of intracellular events that are ultimately responsible for the modulation of steroidogenesis in the gonads. The predominant intracellular signal transduction pathway that mediates the tropic effects of LH and hCG, thus regulating steroidogenesis in gonadal cells, is the cyclic adenosine monophosphate (cAMP) pathway (Fig. 1A). This is also the predominant pathway that is activated in heterologous cells (e.g., HEK293, COS-7, and CHO) expressing LHR. On ligand binding, LHR interacts with the stimulatory $G_{\alpha s}$, resulting in activation of the membrane-bound adenylyl cyclase, leading to an elevation in the levels of the intracellular second messenger, cAMP. cAMP activates protein kinase A (PKA), which subsequently activates transcription factors by phosphorylation. The most well-studied factors are the cAMP response element binding protein (CREB) and the coregulatory CREB-binding protein (CBP). Transcription of many of the steroidogenic enzymes, when activated, results in the production of testosterone in males and estrogen and progesterone in females.

Although this signaling pathway has been well established, it may not be the only pathway that mediates all the actions of LHR. Recently, an increasing body of evidence has accumulated suggesting that additional signal transduction pathways radiating from this linear cAMP pathway may be responsible for the proliferation and differentiation of cells in the gonads. For example, PKA and the G-protein βγ-subunits can activate the mitogen-activated protein kinases (MAPKs), key regulatory enzymes that are implicated in a number of cellular functions, including proliferation, differentiation, control of cellular morphology, and apoptosis. Additionally, it has been demonstrated that in L cells expressing LHR, the G-protein βγ-subunits can activate other signaling cascades by activating phospholipase C-β (PLC-β; see later).

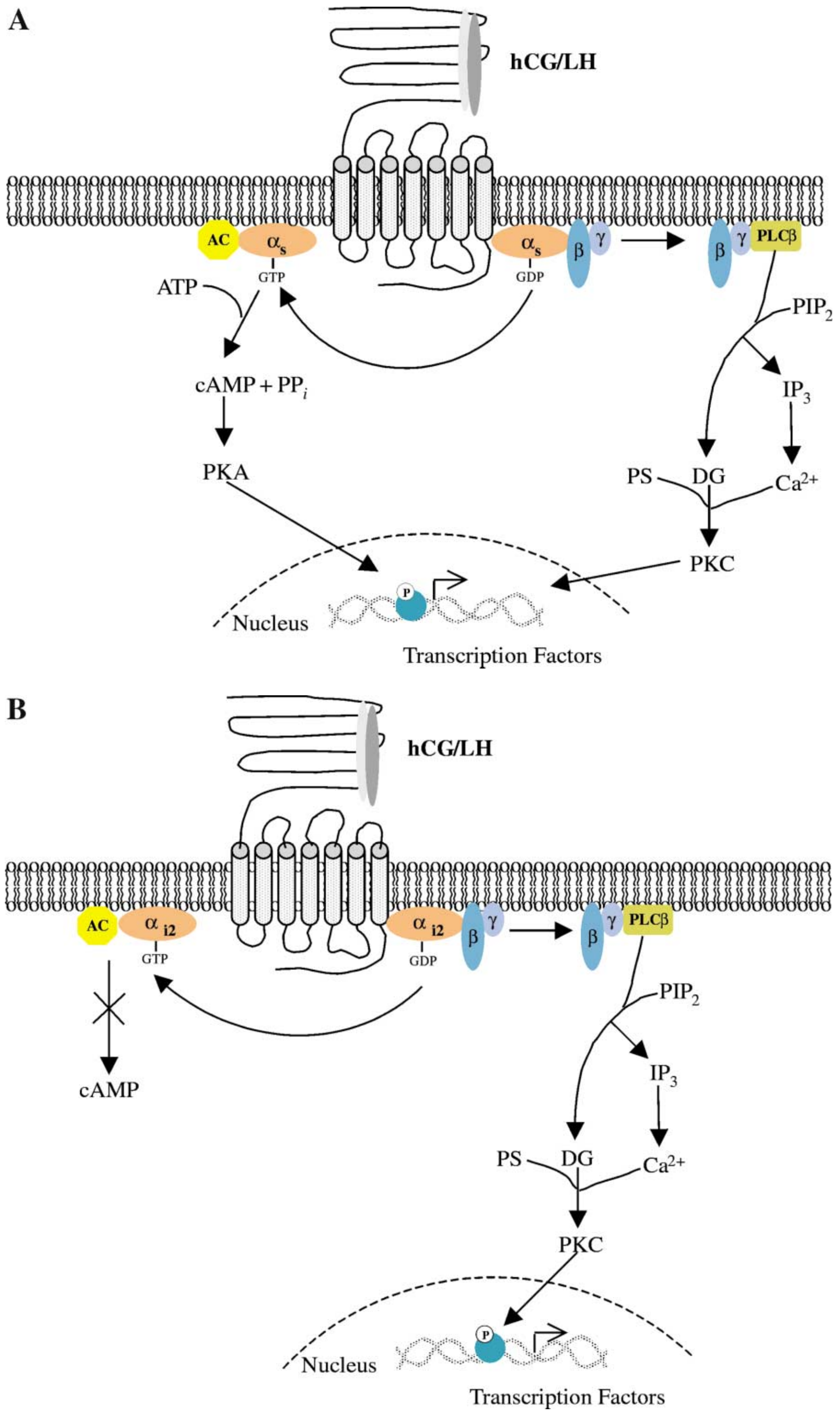
In addition to the well-established activation of the steroidogenic enzymes by LHR, ovulation triggered by the binding of LH to the large numbers of LHRs present in the granulosa cells of the preovulatory follicle initiates diverse signaling pathways radiating from the cAMP pathway. Although the details of these pathways remain to be elucidated, the end result is induction of gene expression of many immediate-early transcription factors and expression of a number of genes that mediate ovulation and differentiation of the granulosa cells of the ovulatory follicle to the luteal cells of the corpus luteum.

## III. SIGNALING VIA THE PHOSPHOINOSITIDE PATHWAY

At high hormone concentrations and high receptor densities, LHR can activate PLC-β and protein kinase C in addition to the adenylyl cyclase/protein kinase A pathway. LHR couples to $G_{\alpha i2}$, and the βγ-subunits of G-proteins released from either $G_{\alpha s}$ or $G_{\alpha i2}$ can activate PLC-β (Fig. 1B). PLC-β, in turn, cleaves the inositol 1,4,5-trisphosphate ($InsP_3$) moiety from phosphatidylinositol 4,5-bisphosphate ($PIP_2$) with the retention of 1,2-diacylglycerol (DAG) in the membrane. $InsP_3$ enters the cytoplasm and binds to receptors on the endoplasmic reticulum, resulting in the mobilization of intracellular calcium stores. DAG, in concert with phosphatidylserine and calcium, activates protein kinase C. Increases in intracellular $InsP_3$ and calcium have been observed in ovaries and in nongonadal cells (L cells, COS-7 cells) transiently or stably transfected with LHR. The extent to which LHR can activate the PLC pathway likely depends on receptor density and the levels of G-proteins present in the different cells. At present, the extent to which the PLC pathway is responsible for mediating LHR activation of steroidogenesis in the ovary and testis is unclear. Perhaps it operates at certain stages during the ovarian cycle when LH levels are greatly elevated.

## IV. LIGAND-INDEPENDENT SIGNALING

Several mutations identified in the LHR gene produce receptor proteins with altered amino acid sequences. Many of these amino acid substitutions render LHR in a constitutively activated state so that the signaling pathways are functional in the absence of hormone, whereas other mutations render LHR inactive such that the receptor is unable to signal even in the presence of hormone. Activating mutations were first identified in young boys with familial or sporadic gonadotropin-independent precocious puberty, characterized by increased testosterone and Leydig cell hyperplasia, with no obvious phenotype in females. The phenotypes of the inactivating mutations in males range from incomplete to complete pseudohermaphroditism with low testosterone levels and Leydig cell hypoplasia. In females, a milder phenotype of primary amenorrhea is observed.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Transcription Factors

**FIGURE 1** Signaling pathways mediated by the luteinizing hormone receptor (LHR). (A) The cyclic adenosine monophosphate (cAMP) pathway. Binding of human chorionic gonadotropin (hCG) or luteinizing hormone (LH) to the membrane-bound LHR converts the inactive guanosine diphosphate (GDP)-bound $G_{\alpha s}$-subunit to the active guanosine triphosphate (GTP)-bound form. This is followed by the activation of adenylyl cyclase (AC), an increase in intracellular levels of cAMP, and activation of protein kinase A (PKA); the G-protein βγ-subunits can potentially activate protein kinase C (PKC). Phosphorylation of nuclear transcription factors by PKA or PKC results in the transcription of

The activating mutations are present in transmembrane helices 1, 2, 3, 5, and 6, with the majority present in helix 6. The signaling properties of these mutant receptors have been examined by *in vitro* transfection experiments. These studies demonstrated that the mutations activate the cAMP pathway in the absence of hCG or LH. Some of the mutant receptors were able to respond to additional ligand with a further accumulation of cAMP whereas others were maximally activated and refractory to additional hormone. Many of the activating mutations also stimulate the phosphoinositide pathway, resulting in an increase in the basal level of $InsP_3$. Presently, it is unknown if additional pathways are activated by the mutant receptors and the effects, if any, of these pathways on the manifestation of the mutant phenotypes have not been elucidated.

## V. SIGNAL ATTENUATION

Attenuation of LHR receptor signaling is accomplished by a combination of two processes, receptor desensitization and receptor internalization or sequestration. Much of our current knowledge of these processes is derived from studies on the prototypical G-protein-coupled receptor (GPCR), the $\beta_2$-adrenergic receptor. Desensitization of this receptor involves the rapid (minutes after receptor activation) phosphorylation of the cytoplasmic C-terminal portion of the receptor by a specific GPCR kinase. This phosphorylation event promotes the binding of arrestin proteins that thereby facilitate the uncoupling of the receptor from the G-protein, terminating the signal. Arrestin-bound GPCR is internalized into vesicles via clathrin-coated pits. Most GPCRs then undergo dephosphorylation and are recycled to the plasma membrane, where they can again interact with their ligand (resensitization). Alternatively, GPCRs are degraded in lysosomes, resulting in a decrease in the number of cell surface receptors. This process of down-regulation occurs after prolonged exposure to ligand and occurs more slowly than rapid internalization.

Many of the molecular details of signal attenuation of LHR are similar to those described for the $\beta_2$-adrenergic receptor, although there are also many important differences. The C-terminal intracellular portion of LHR contains four serine residues that are major sites of phosphorylation. The role of phosphorylation in LHR signal attenuation, however, appears less important. Studies performed in porcine ovarian follicular membranes found no evidence that phosphorylation mediates desensitization. On the other hand, studies performed with rat LHR in transfected HEK293 cells showed that inhibition of agonist-induced phosphorylation delays the time course of LHR uncoupling but does not prevent it. Internalization of the hCG–LHR complex is mediated via clathrin-coated pits by a pathway that requires nonvisual arrestins and dynamin. In contrast to most GPCRs, arrestin binding to LHR is not dependent on phosphorylation. In addition, the internalized rat LHR is not recycled, but the hCG–LHR complex is delivered to the lysosomes, wherein both hormone and receptor are degraded. An exception to this rule is the hCG–hLHR complex in which the majority of the receptor is recycled rather than degraded by the lysosomal pathway. Therefore, the processes of internalization and lysosomal degradation of the agonist–LHR complex are important in the attenuation of hormone-mediated cAMP accumulation and down-regulation of LHR.

## VI. SUMMARY

LHR expressed primarily in the testis and ovary has a vital role in male and female reproduction by regulating cell proliferation, cell differentiation, ovulation, and steroid hormone production. The large N-terminal extracellular domain of LHR is the high-affinity binding site for LH and hCG. Agonist binding initiates a signaling cascade beginning with an accumulation of intracellular cAMP, which mediates the activation of PKA, followed by activation of transcription factors, culminating in changes in gene expression and synthesis of steroid hormones. Activation of the phosphoinositide pathway also occurs at high hormone concentration. Attenuation of LHR-mediated signaling is achieved by a combination of receptor uncoupling and down-regulation.

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specific genes. (B) The phosphoinositide pathway. LHR also couples with $G_{\alpha i2}$ to inhibit cAMP production indicated by X. The $\beta\gamma$-subunits activate membrane-bound phospholipase C-β (PLC-β) which hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG). Note that $PIP_2$, DAG, and phosphatidylserine (PS) are membrane bound and are shown here as intracellular merely for ease of representation. $InsP_3$ can increase intracellular $Ca^{2+}$, and DAG-activated PKC can regulate gene transcription.

There is much that is not known about LHR activation and signaling. The precise nature of the interaction between LHR and the G-proteins is not understood. Although it is clear that cAMP is important in LHR-mediated signaling, there is increasing evidence that additional pathways may be involved. For example, recent studies suggest that LHR may stimulate the MAPK pathway and this activation may be important in the modulation of LH-induced steroidogenesis. Furthermore, it is becoming increasingly apparent that signaling cascades are not linear but form a network of interacting pathways. cAMP coordinates multiple and divergent pathways, and the integration and control of these pathways are essential in the regulation of diverse functions such as cell proliferation and cell differentiation. The challenge is in unraveling the LHR-mediated pathways in the normal functioning of the ovary and testis, as well as in pathophysiological conditions.

## Glossary

**adenylyl cyclase** A membrane-associated enzyme responsible for converting adenosine triphosphate to adenosine-3′,5′-cyclic monophosphate. The enzyme is regulated by several $G_\alpha$ proteins.

**cyclic adenosine monophosphate** A major intracellular signaling molecule that is regulated by a number of extracellular effectors. It is synthesized from adenosine triphosphate via adenylyl cyclase and is metabolized to adenosine monophosphate by cyclic nucleotide phosphodiesterase.

**G-proteins** A family of heterotrimeric (α, β, and γ) guanosine triphosphate (GTP)-binding proteins, the α-subunit of which exhibits GTPase activity. On activation by G-protein-coupled receptors, the guanosine diphosphate (GDP)-associated α-subunit exchanges GDP for GTP and dissociates from βγ. The $G_\alpha$–GTP complex regulates the activity of membrane-associated enzymes, e.g., adenylyl cyclase and phospholipase C.

**phosphoinositides** A family of lipid molecules important in intracellular signaling. The minor membrane component, phosphatidylinositol 4,5-bisphosphate, is hydrolyzed by phospholipase C to yield inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. The former compound is responsible for release of intracellular calcium stores, and the latter component functions with phosphatidylserine and often calcium to activate protein kinase C.

**phospholipase C** A member of the family of phospholipases responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol. Enzymatic activity of the phospholipase C-β isoform is regulated by subunits of the G-proteins.

**protein kinase A** A serine/threonine protein kinase, also denoted as cyclic adenosine monophosphate (cAMP)-dependent protein kinase, containing two regulatory (R) and two catalytic (C) subunits. On binding of cAMP by the R subunits, the C subunits dissociate and phosphorylate a variety of intracellular proteins containing the consensus sequence Arg-Arg-X-Ser/Thr-Y, where X is generally a small amino acid residue and Y is a large hydrophobic residue.

**protein kinase C** A family of serine/threonine protein kinases that are activated by phospholipids and often calcium. The form involved in intracellular signaling is regulated by phosphatidylserine, diacylglycerol, and calcium.

## See Also the Following Articles

**Follicle Stimulating Hormone (FSH) • Follitropin (Follicle-Stimulating Hormone) Receptor Signaling • Luteinizing Hormone (LH) • Multiple G-Protein Coupling Systems • Protein Kinases • Thyroid Stimulating Hormone (TSH)**

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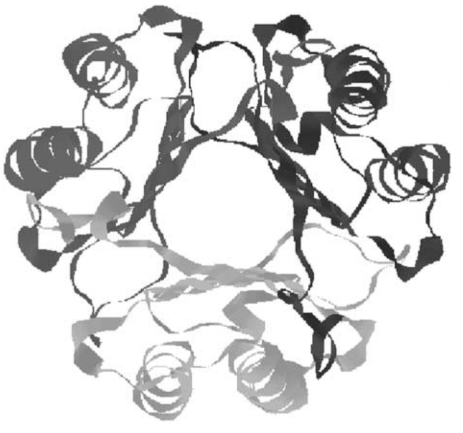

**FIGURE 1** Three-dimensional structure of human MIF, viewed down its threefold axis of symmetry.

known proteins that display any three-dimensional structural similarity to MIF are the mammalian enzyme dopachrome tautomerase and the bacterial enzymes 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI). A dopachrome tautomerase activity for MIF was discovered serendipitously during an investigation of intermediary steps in melanin biosynthesis, which involves the conversion of 2-carboxy-2,3-dihydroindole-5,6-quinone (L-dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid. While using D-dopachrome (a nonphysiologically relevant stereoisomer) as a negative control in this reaction, a novel enzyme that could convert D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid was purified. This protein was established to be MIF. MIF and D-dopachrome tautomerase share only 27% sequence identity; however, MIF shares structural similarities with D-dopachrome tautomerase, 4-OT, and CHMI, in that each protein has an N-terminal proline with an unusually low $pK_a$ that can act as a catalytic base to effect tautomerization. These structural studies have provided much impetus for the hypothesis that MIF is an enzyme; however, a physiologically relevant substrate for MIF has not been convincingly identified nor has site-directed replacement of the catalytically active Pro-1 with other residues consistently eliminated MIF's pro-inflammatory activities.

## III. IMMUNE CELL PHYSIOLOGY

Although MIF was described first as a T-lymphocyte-derived factor and then as a pituitary secreted protein, other cell types are also important sources of MIF. An investigation of hypophysectomized mice, which lack a pituitary source of MIF, revealed significant levels of circulating MIF during the acute phase of the host response to endotoxin. This led to the identification of the macrophage, which is exquisitely sensitive to endotoxin, as an important producer of MIF *in vivo*. Monocytes/macrophages, like the corticotropic cells within the pituitary gland, contain significant quantities of preformed MIF that can be rapidly released on stimulation. This is in contrast to other macrophage-derived, pro-inflammatory cytokines such as IL-1β and tumor necrosis factor α (TNFα) that require *de novo* mRNA synthesis and protein production before secretion occurs. MIF secretion from macrophages not only is rapid; it occurs in response to endotoxin concentrations that are 10- to 100-fold lower than those required to induce TNFα production. MIF protein also is produced by T lymphocytes, eosinophils, and activated endothelial cells.

Current views favor a dominant role for MIF in the innate immune response. Gram-negative endotoxin, gram-positive exotoxins, hemozoin (malaria pigment), and the pro-inflammatory cytokines TNFα and IFN-γ induce the release of MIF from monocytes/macrophages. Once released, MIF exerts potent autocrine and paracrine effects, promoting macrophage and T-cell activation and pro-inflammatory cytokine release. MIF is also a potent inducer of the cyclooxygenase 2 (COX-2) pathway [arachidonic acid, COX-2, prostaglandin E2 (PGE2)] and of the expression of matrix metalloproteinase-2. Thus, it is not surprising that the blockade of MIF by the use of monoclonal antibodies inhibits the development in experimental animals of several inflammatory diseases such as arthritis, glomerulonephritis, and septic shock.

MIF administration exacerbates endotoxin-induced toxicity, whereas immunoneutralization of MIF rescues mice from lethal endotoxic shock and reduces circulating TNFα levels by up to 50%. The pivotal role played by MIF in the pathogenesis of endotoxic shock has been confirmed by experiments carried out in $MIF^{-/-}$ mice in which the degree of resistance to endotoxin lethality observed was similar to that obtained by MIF immunoneutralization. Recent studies also have provided clinical evidence for systemically elevated MIF levels during sepsis and other generalized inflammatory conditions. The time course of MIF expression in plasma in septic shock patients parallels that of cortisol, and a significant correlation has been noted between elevated MIF levels on hospital admission and occurrence of death.

MIF also is an important regulator of adaptive immunity. Stimulation of primary T cells with anti-CD3 antibody or superantigen induces MIF mRNA expression and protein secretion. Neutralization of

T-cell-derived MIF with specific anti-MIF antibodies inhibits both anti-CD3 and superantigen-induced IL-2 secretion and reduces T-cell proliferation by 40–60%. *In vivo*, treatment of mice with anti-MIF antibodies inhibits antigen-driven T-cell proliferation and reduces the expression of antigen-specific IgG production. A role for MIF in the shaping of the adaptive immune response also is supported by expression studies using $T_H1$ and $T_H2$ T-cell subsets. In parallel to effects observed *in vivo*, it was found that, although both subsets of T cells express MIF, secretion is predominantly increased in activated $T_H2$ clones. Furthermore, studies using MIF knockout mice ($MIF^{-/-}$) have revealed that antigen-stimulated lymph node cells from $MIF^{-/-}$ mice produce higher levels of IL-4 and IFN-γ than those from wild-type mice. These data would favor a role for MIF in the development of $T_H2$-driven antibody production, at least in some settings.

In an unexpected twist, MIF was found to be specifically released from immune/inflammatory cells as a consequence of glucocorticoid stimulation. Glucocorticoids normally inhibit pro-inflammatory cytokine expression, but secreted MIF functions in a regulatory capacity to “override” or counterregulate the immunosuppressive effects of glucocorticoids on immune/inflammatory cell activation and pro-inflammatory cytokine release. Inhibition of the anti-inflammatory and immunosuppressive properties of glucocorticoids may largely explain MIF’s global pro-inflammatory effects, and an emerging body of data indicates that MIF’s position within the cytokine cascade is to act in concert with glucocorticoids to control the setpoint of the immune and inflammatory response. It has been considered that MIF neutralization may offer a powerful therapeutic strategy for the treatment of inflammatory and autoimmune diseases, particularly those that are characterized by resistance to steroid therapy or by steroid dependence. By removing the counterregulatory agent of glucocorticoid action (i.e., MIF), MIF neutralization could decrease the steroid requirement for a number of diseases and even allow the host’s natural glucocorticoid response to more effectively control an overactive inflammatory response.

## IV. HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

Immunocytochemical and immunohistochemistry techniques have shown that MIF is contained within secretory vesicles in corticotropic cells of the anterior pituitary gland. MIF protein accounts for approximately 0.05% of total pituitary protein, a level comparable to that of the classical pituitary hormones ACTH and prolactin (0.2 and 0.08%, respectively). Within corticotropic cells, MIF resides in specific granules, both alone and co-localized with ACTH. The precise contribution of the pituitary to total plasma MIF levels is unclear, but when mice receive an injection of endotoxin as a model of infective stress, there is a significant decrease in the pituitary content of MIF protein, a concomitant rise in plasma MIF levels, and a slower, time-dependent increase in pituitary expression of MIF mRNA. Other cell types also express MIF protein, however, so ambient plasma levels may reflect a collection of MIF secretions from different tissues rather than from a single, dominant organ source.

The release of MIF from the pituitary together with ACTH is somewhat paradoxical, as ACTH stimulates glucocorticoid release from in the adrenal cortex, which then acts to down-regulate the inflammatory response. The glucocorticoid-antagonistic activity of MIF likely represents a mechanism by which the host maintains a functioning immune response during situations of high endogenous glucocorticoid production such as severe trauma, stress, or life-threatening infection. Increased levels of glucocorticoids act to maintain glucose and electrolyte homeostasis during the so-called “flight or fight” response, but these glucocorticoids also can act deleteriously to suppress the immune response. MIF that is released systemically from the pituitary or by activated cells at the site of infection would counterbalance the anti-inflammatory and immunosuppressive effects of glucocorticoids. The precise outcome of this response—resolution and tissue repair versus excessive inflammation and tissue damage—would be the result of an interaction between MIF and glucocorticoid action on target cells.

MIF secretion from corticotropic pituitary cells can be induced by stimulation with corticotropin-releasing factor (CRF) and requires lower concentrations of CRF than those required to induce ACTH secretion. Interestingly, CRF also has been shown to be a potent inducer of MIF gene transcription in murine pituitary cells. A functional analysis of the MIF gene-promoter region using rodent pituitary cells has demonstrated that CRF-induced MIF expression is dependent on a cyclic AMP-responsive element-binding protein.

MIF protein and mRNA also are expressed in the cortex of the adrenal gland. As in the pituitary, the adrenal MIF content decreases following systemic administration of endotoxin and this is followed by MIF resynthesis. This type of response—decreased tissue content followed by resynthesis and induction of MIF mRNA—is not unique to the pituitary or the adrenal, but can be observed after endotoxin challenge in other MIF-expressing tissues including the spleen, the lung, the kidney, and the liver.

## V. GLUCOSE HOMEOSTASIS AND ENERGY METABOLISM

Energy metabolism is regulated by a complex network of endocrine, paracrine, and autocrine mediators acting between the liver, pancreas, muscle, adipose tissue, and brain. This network is subject to dramatic changes during stress, infection, and illness. A hypermetabolic response that is characterized by profound alterations in carbohydrate metabolism frequently develops in the host as a consequence of severe infection or trauma. If not counteracted, over time this response leads to metabolic wasting and death. These metabolic effects are believed to be mediated by stress-induced changes in circulating hormones and by the expression of inflammatory cytokines produced by immune cell activation. MIF also plays a prominent regulatory role in the metabolic features of the host response to stress.

Immunohistochemical staining of the pancreas has localized MIF to the cytoplasm of the insulin-producing beta cells within the pancreatic islets, and an increase in glucose concentration can induce the release of MIF protein *in vitro*. Immunoneutralization of MIF inhibits the first and second phases of insulin secretion by 39 and 31%, respectively, in an isolated rat islet perfusion system, whereas MIF addition increases glucose-induced insulin secretion by 140%. These data indicate that MIF is a positively acting, autocrine/paracrine regulator of glucose-induced insulin release. Stress-induced elevations of circulating glucocorticoid levels are known to induce a hyperglycemic state that increases the need for insulin so as to ensure appropriate glucose homeostasis. High circulating MIF levels may be beneficial in this context by augmenting pancreatic islet insulin production, thereby counterbalancing the hyperglycemic effect of glucocorticoids.

TNFα was once called "cachectin" for its ability to induce muscle catabolism and wasting. Treatment of myotubes with TNFα results in a depletion of MIF from intracellular pools, the release of MIF into the supernatant, and an increase in MIF mRNA transcription. Stimulation of cultured myotubes with MIF initiates an increase in the production of fructose-2,6-bisphosphate, the most potent allosteric regulator of glycolysis that acts by activating phosphofructokinase-1. An increase in the cellular secretion of lactate, the end-product of anaerobic glycolysis, also occurs. Both of these properties were originally ascribed to cachectin/TNFα. Anti-MIF inhibits these effects of TNFα *in vitro* and *in vivo*, indicating that autocrine-acting MIF is a downstream effector of the catabolic actions of TNFα.

MIF also is expressed constitutively in adipocytes and its expression appears to be dependent on glucose and insulin levels *in vitro*.

## VI. REPRODUCTIVE TISSUES

A systematic analysis of tissue MIF protein and mRNA levels has demonstrated MIF expression in the reproductive organs. In the testis, MIF expression is localized to the androgen-producing Leydig cells. The testicular interstitial fluid also contains significant amounts of MIF. The addition of MIF to Leydig cells results in a dose-dependent suppression of inhibin production by seminiferous epithelium, suggesting a paracrine role for MIF in Sertoli cell regulation. Interestingly, Leydig cell ablation does not lead to a loss of MIF in the testis, but to a switch of the site of MIF production from Leydig cells to Sertoli cells and spermatogonia. In the epididymis, MIF expression also is observed in the epithelial cells of the epididymal caput. MIF is present in seminal plasma in high concentrations of 1–10 μg/ml and likely arises from the prostate gland where the protein has been observed to be expressed within the epithelial cells. $\mathrm{MIF}^{-/-}$ male mice do not display any apparent dysfunction in their reproductive capacity, suggesting that the contribution of MIF to male reproduction either is not crucial or can be compensated for by other molecules.

MIF mRNA has been found in the oviduct, the uterus, and the granulosa cells of the ovary. Uterine mRNA levels change during pregnancy, suggesting an influence by the endocrine changes of pregnancy. $\mathrm{MIF}^{-/-}$ female mice also appear to reproduce normally.

## VII. MOLECULAR MECHANISMS OF ACTION

MIF is directly pro-inflammatory by activating or promoting cytokine expression (TNFα, IL-1β, IL-2, IL-6, IL-8, IL-12, IFN-γ), nitric oxide release, and matrix metalloproteinase-2 expression, and by inducing the COX-2 pathway. Interestingly, much of the COX-2-inducing activity of IL-1β on synovial cells can be inhibited by anti-MIF, indicating that MIF is a downstream effector of IL-1β action in these cells. MIF also plays an important role in regulating both the setpoint and the direction of the inflammatory response by counteracting the anti-inflammatory and immunosuppressive effects of glucocorticoids. Specifically, MIF counteracts the glucocorticoid-induced inhibition of inflammatory cytokine secretion in macrophages, T cells, and synovial fibroblasts. Although there are substantial data to support roles for MIF as an inflammatory cytokine and an endocrine hormone, the precise role of many of MIF's specific "inflammatory" versus "endocrine" activities in the pathophysiology of different inflammatory or autoimmune diseases remains to be established.

Several studies suggest that an interaction between MIF and a receptor on the surface of target cells is essential for MIF's activities. Perhaps the strongest of these data are those that have shown that exogenously added MIF, as well as endogenously released MIF, induces the proliferation of quiescent fibroblasts. This response is associated with sustained activation of the p44/p42 [extracellular signal-related kinase variants 1 and 2 (ERK1/2)] subfamily of mitogen-activated protein (MAP) kinases and is dependent on the activity of protein kinase A. Neither the p38 family nor the JUN N-terminal kinase (JNK)/stress-activated protein kinase family of MAP kinases is stimulated by MIF. The ERK1/2 MAP kinase signaling cascade results ultimately in the phosphorylation and activation of cytoplasmic phospholipase $A_2$ ($cPLA_2$). $cPLA_2$ is a critical mediator of inflammatory responses and its product, arachidonic acid, is the precursor for the synthesis of prostaglandins and leukotrienes. Interestingly, $cPLA_2$ also is a target for the anti-inflammatory action of glucocorticoids, and the ability of MIF to activate $cPLA_2$ is one mechanism by which MIF regulates the immunosuppressive effect of glucocorticoids on cells. In support of this hypothesis, MIF is able to counterregulate glucocorticoid inhibition of $cPLA_2$ activation and arachidonic acid release from cells. $cPLA_2$ is an important point of interaction between MIF and glucocorticoids, as the product of $cPLA_2$, arachidonic acid, is required for the activation of JNK and efficient translation of TNFα mRNA.

Another mechanism by which MIF may counterregulate the effects of glucocorticoids is via the transcription factor nuclear factor κB (NF-κB). NF-κB is a critical regulator of the inflammatory response and regulates the expression of over 60 genes for pro-inflammatory cytokines and cell adhesion molecules. Glucocorticoids have been reported to enhance the synthesis of inhibitor κB (IκB), a cytoplasmic binding protein that inhibits the translocation of NF-κB to the nucleus. Recent studies have shown that MIF inhibits the ability of glucocorticoids to induce IκB synthesis in human peripheral blood mononuclear cells. Thus, by blocking glucocorticoid-induced IκB activity, MIF promotes the translocation of NF-κB into the nucleus and enables the expression of a cellular pro-inflammatory response.

The innate immune response also is kept in check by specialized counterregulatory mechanisms such as apoptosis or programmed cell death. Defective apoptosis of macrophages may lead to an exaggerated inflammatory response, and increased T-cell apoptosis has been considered to contribute to clinical immunosuppression in the late phase of septic shock. Recent data have linked the action of MIF to the inhibition of p53, an important tumor suppressor gene and mediator of apoptosis. MIF can sustain macrophage survival and pro-inflammatory function by suppressing activation-induced, p53-dependent apoptosis. Thus, an important action for MIF in this regard would be to prolong the life span and the activity of monocytes/macrophages during inflammation. This particular mechanism could explain the important defect in macrophage responses and innate immunity that has been reported for $MIF^{-/-}$ mice.

The lack of information regarding the molecular identity of a cell surface receptor for MIF has led several investigators to consider alternative or "nonclassical" modes of action for this extracellular mediator. It has been shown recently that MIF may directly affect the transcriptional activity of activator protein-1 (AP-1)-responsive genes via an interaction with the intracellular protein, Jab-1. AP-1 is a DNA-binding transcription factor that forms a heteromeric complex with the Fos and Jun oncoproteins. It has been proposed that Jab-1 stabilizes the binding of AP-1/c-jun complexes to AP-1 promoter sites. Jab-1 also binds to, and promotes the degradation of, $p27^{Kip1}$, a protein that halts the cell division cycle. A biologically significant interaction between MIF and Jab-1 was demonstrated *in vivo* and shown to result in the inhibition of Jab-1 binding to c-jun, thus destabilizing the formation of activated AP-1 complexes. The binding of MIF to Jab-1 resulted in reduced degradation of $p27^{Kip1}$ and an inhibition of the growth-promoting properties of Jab-1 in fibroblast cells. As AP-1 has been shown to be an important regulator of several pro-inflammatory genes, this mechanism appears to contradict the pro-inflammatory and mitogenic activities described for MIF. However, a characteristic feature of MIF action is its bell-shaped dose–response curve with respect to several biological phenomena, suggesting that low versus high MIF levels may have distinct regulatory mechanisms.

## VIII. SUMMARY

The protein mediator known as MIF has emerged as a critical mediator of various inflammatory, endocrine, and metabolic responses. MIF is expressed almost ubiquitously by tissues and regulates cell activation, proliferation, and apoptosis. The cellular release of MIF is regulated by a variety of specific stimuli, and systemic levels of MIF vary as a consequence of different infective or inflammatory stressors.

## Glossary

**cytokine** A protein that is secreted in a regulated fashion by cells of the inflammatory and immune system and that delivers signals to other cell types.

**mitogen-activated protein kinases** A functional family of kinases that mediate cellular responses to extracellular signals.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Corticotropin-Releasing Hormone (CRH) • Cytokines and Anterior Pituitary Function • Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) • Interleukin-4**

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# Male Hormonal Contraception

C. Hay and F. C. W. Wu
*University of Manchester, United Kingdom*

I. INTRODUCTION
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III. ANDROGEN-ONLY PREPARATIONS
IV. ALTERNATIVE TESTOSTERONE PREPARATIONS
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**The goals of hormonal male contraception should include (1) reversible and consistent suppression of spermatogenesis to a level compatible with temporary infertility and (2) maintenance of physiological androgen-dependent sexual and metabolic functions. This article discusses different types of male hormonal contraceptive preparations, including androgen-only preparations, alternative testosterone preparations, GnRH agonists and antagonists, and combinations of progestogen and androgen. Their efficacy and safety are also examined.**

## I. INTRODUCTION

The world population reached 6 billion people for the first time in 1999, representing an increase in population of 4.4 billion since 1900. If the current average birth rate of 3.3 children per woman is maintained, the world population will approach 19 billion people by 2100. This degree of overpopulation is likely to outstrip the world's resources, leading to poverty, famine, and pollution. Provision of effective methods of contraception can slow the current rate of population expansion, thereby avoiding some of these potential consequences. Birth control has also been desirable to individuals for centuries. Female barrier methods have included fruit, paper discs, and brandy-soaked sponges used by French women in the 18th century. The 18th century also saw the first use of condoms made from animal intestine. Other widely used methods have included periodic abstinence and coitus interruptus. A revolution in contraception occurred in the 1960s with the advent of the female oral contraceptive pill (OCP). This hormonal method is highly efficacious, with a first-year failure rate of 1.8% compared to 12% for condoms. Condoms do have the advantage of protecting against sexually transmitted diseases (STDs). However, their efficacy (both in protecting against STDs and in contraception) is hampered by incorrect usage. Vasectomy is highly effective but is essentially an irreversible method. The female OCP is highly efficacious but may be contraindicated or poorly tolerated in some women. Effective family planning requires adequate education of contraceptive users. Consideration of the individual couple's circumstances and requirements is crucial to selecting the most suitable methods. There are limitations with all the existing methods. The development of a male hormonal contraceptive should therefore provide an important additional reversible method for men in stable relationships to share the risks of contraception more equitably between both partners. Interest in using a new male hormonal contraceptive has indeed been shown in 50–70% of men surveyed in Edinburgh (Scotland), Cape Town (South Africa), and Hong Kong.

## II. BASIC PRINCIPLE OF HORMONAL SUPPRESSION OF SPERMATOGENESIS

Spermatogenesis is a complex process that begins with the mitotic division of a self-renewing stem cell population to form diploid germ cells or spermatogonia. These spermatogonia periodically differentiate into primary preleptotene spermatocytes, which then initiate meiosis. The primary spermatocytes result in spermatids. Round spermatids are mitotically inactive but go through a complex transformation (spermiogenesis) to differentiate into elongated spermatids and spermatozoa. Spermiogenesis involves DNA condensation, replacement of nucleosomal histones by protamines, and loss of virtually all cytoplasm with development of the proteolytic enzyme-rich acrosome cap and a flagellum capable of propelling beating movements connected by a mitochondria-rich midpiece. Mature elongated spermatids, embedded in Sertoli cell cytoplasm, are eventually released (spermiation) into the tubular lumen as spermatozoa. The entire sequence and duration of each step encompassed in the formation of spermatozoa from primary spermatogonia are species-specific biological constants; in humans, it takes approximately 74 days.

A sufficient testosterone (T) concentration in the seminiferous tubules is critical to the maintenance of normal spermatogenesis. The intratesticular T concentration is normally 50–100 times higher than that in the systemic circulation. The drive for testosterone production is provided by luteinizing hormone (LH) released from the anterior pituitary. LH acts on Leydig cells in the testis to stimulate androgen synthesis from cholesterol via a series of steroidogenic enzymes. In addition to testosterone, follicle-stimulating hormone (FSH) is required for the initiation and maintenance of quantitatively normal spermatogenesis. Secretion of both LH and FSH is stimulated by pulsatile gonadotropin-releasing hormone (GnRH) from the hypothalamus. This intermittent GnRH signal avoids desensitization of the pituitary gonadotrophs and maintains physiological episodic gonadotropin secretion. Negative feedback of testosterone at both the pituitary and the hypothalamic levels suppresses LH and FSH, whereas inhibin B and follistatin regulate FSH secretion (Fig. 1). Potential inhibitors of this system include testosterone, progestogens, gonadotropin-releasing hormone agonists (GnRH agonists), and GnRH antagonists, all of which (via different mechanisms) suppress both LH and FSH. Inhibition of gonadotropins will therefore abrogate both testosterone production and spermatogenesis simultaneously. When agents other than testosterone are used to suppress spermatogenesis, there will be a requirement for androgen replacement to prevent a deficient state. Thus, hormonal male contraception based on suppression of gonadotropins creates the need for exogenous testosterone replacement to maintain extratesticular secondary sexual functions, e.g., libido. In principle, exogenous testosterone on its own appears to be the ideal hormonal contraceptive. However, as discussed below, the supraphysiological doses required to suppress spermatogenesis consistently may be undesirable clinically. Current thinking deems that non-androgenic anti-gonadotropic agents combined with physiological androgen replacement is the preferred approach to effect hormonal male contraception even though potentially more complex regimens are required.

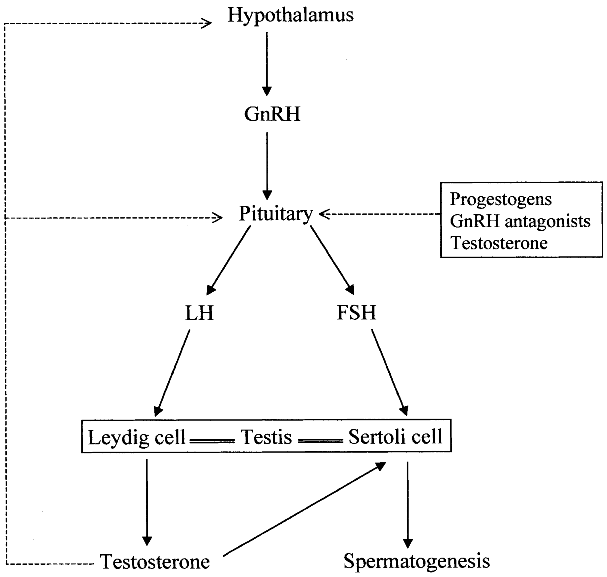

**FIGURE 1** The hypothalamic–pituitary–gonadal axis. Solid arrows show the pathway of spermatogenesis promotion and the dashed arrows show inhibitory influences. Endogenous testosterone acts via negative feedback pathways to inhibit both GnRH release from the hypothalamus and gonadotropin release from the pituitary. Exogenous sex steroids and GnRH analogues reversibly manipulate this physiological response.

## III. ANDROGEN-ONLY PREPARATIONS

The principal androgen in men is testosterone, which is 5α-reduced to the more potent dihydrotestosterone (DHT) and aromatized to estradiol. Testosterone is rapidly degraded by first-pass metabolism in the liver following oral or systemic administration. Chemical modification of androgen preparations is therefore necessary to reduce hepatic breakdown and prolong the duration of therapeutic action. Oral preparations currently available have a relatively short half-life and frequent dosing is required. Prolongation of the half-life is achieved by formulation of T esters in lipid vehicles that are administered by intramuscular injection.

In the 1970s, studies using intramuscular testosterone enanthate (TE) confirmed that supraphysiological doses of testosterone induced azoospermia in 40–70% of Caucasian males. These studies demonstrated a relationship between the extent of spermatogenesis suppression (sperm concentration) and contraceptive failure (pregnancy) rate. The pregnancy rate for azoospermia was 0.8 per 100 person years (95% confidence interval of 0.02–4.5) comparable to female injectables, increasing to 8.1 (2.2–20.7 per 100 person years) for oligozoospermia (0.1 to 3 million/ml). The overall efficacy Pearl Index was 1.4 (0.4–3.7) pregnancies per 100 person years, which is comparable to the female oral contraceptive pill and is better than the typical first-year failure rate of condoms (12%). These studies therefore offered proof of the principle that hormonal suppression of spermatogenesis can be an efficacious and reversible contraceptive for men. These studies also showed that acceptable targets of suppression to ensure effective contraceptive protection are azoospermia and severe oligozoospermia (<1 million sperm/ml).

The androgen-only approach had two drawbacks: (1) unsatisfactory pharmacokinetics of the injectable T esters currently available, which required weekly intramuscular injections and resulted in widely fluctuating levels of testosterone, and (2) high doses (causing supraphysiological T levels) of T were required to induce and maintain adequate suppression of spermatogenesis. The androgen-related side effects encountered included acne, weight gain, behavioral changes, lowered high-density lipoprotein cholesterol (HDL-C), and increased hematocrit. For these reasons, alternative strategies have been sought.

## IV. ALTERNATIVE TESTOSTERONE PREPARATIONS

### A. Testosterone Buciclate

Testosterone buciclate is a long-acting ester developed specifically for male contraception by the World Health Organisation (WHO) and the National Institute for Child Health and Human Development (United States). To date, only one dose has been assessed, in which 1200 mg produced azoospermia in 3/8 volunteers. Further studies are currently in abeyance due to formulation problems and concerns of genotoxicity with the buciclate side chain.

### B. 19-Nortestosterone

19-Nortestosterone (19-NT or nandrolone) is a long-acting ester with potent androgenic effects and a high progestational activity (10 times that of T) but it is not aromatized. 19-NT has been shown to suppress LH and FSH effectively with full maintenance of androgen-dependent functions. With the combination of both androgenic and progestational activity, the potential for 19-NT to provide contraception as a single agent has been considered. Small trials with 19-NT alone or in combination with depot medroxyprogesterone acetate (DMPA, see below) have confirmed that azoospermia is induced without any symptoms of androgen deficiency (despite low T levels).

### C. Testosterone Microspheres

Testosterone microspheres are biodegradable polyactide-glycolide spheres containing testosterone and exhibit first-order absorption kinetics. A single dose of 315 mg administered by intramuscular (im) injection to hypogonadal men can maintain T levels in the normal range for 10–11 weeks. Recently, reformulation by another company has allowed subcutaneous administration. The pharmacokinetics in this small study were similar to those of the im microsphere preparation. Additional study of this novel mode of testosterone delivery is needed to further assess both efficacy and safety.

### D. Testosterone Implants

Testosterone implants are small cylindrical pellets containing 100 or 200 mg of crystalline T. Insertion of T pellets into the subcutaneous tissues of the anterior abdominal wall requires a minor surgical procedure under local anesthesia. The potential advantage of this delivery system is the near zero-order kinetics, which result in stable, dose-dependent testosterone levels without the repeated supraphysiological peak levels of T produced by TE and other similar injectable esters. A single administration of 1200 or 800 mg every 3 months in eugonadal men produced spermatogenic suppression equal to TE 200 mg weekly but fewer metabolic side effects were documented. Recent studies have combined T implants with progestogen (DMPA, oral and implantable desogestrel) and confirmed the synergistic actions between the two steroids in spermatogenic suppression, achieving azoospermia rates equivalent to or better than those achieved with TE alone. This suggests that lower doses of T combined with progestogens may be the optimal approach, which not only achieves maximal suppression of spermatogenesis but also minimizes potential adverse effects. Although the superior pharmacokinetics of T implants are undoubtedly beneficial, the requirement for an uncomfortable and repeated surgical procedure every 3–4 months and a nontrivial implant extrusion rate of 5–10% make this method unsuitable for male contraception either on its own or in combination regimens.

### E. Testosterone Patches

Testosterone patches provide noninvasive transdermal self-administered delivery of testosterone when applied daily to the body or scrotal skin. The main limitations include a high incidence (40–60% of subjects) of skin irritation related to the alcoholic excipients employed, the frequent shaving of the scrotum, and poor adhesiveness, particularly in hot weather. Two studies have used a body patch in combination with a progestogen (oral levonorgestrel and desogestrel) and will be discussed below.

### F. Testosterone Undecanoate

Testosterone undecanoate (TU) is an unsaturated, aliphatic, fatty acid ester of T that is partially absorbed from the gut lymphatics following oral administration. The currently available oral TU preparation (Restandol) formulated in oleic oil has low bioavailability and variable absorption and requires twice or thrice daily dosing. These problems were highlighted by a study of oral TU and cyproterone acetate (CPA) (see below). The findings suggested that oral TU is currently a poor choice for contraception. Reformulation of oral TU is under way and may produce more reliable pharmacokinetics in the future.

TU has recently been formulated for intramuscular administration in tea seed oil (in China) and subsequently in castor oil (in Germany). This promising new and as yet unlicensed preparation provides a long-acting testosterone depot (half-life of 33.9 +/− 4.9 days in castor oil) with favorable pharmacokinetic properties. In hypogonadal men, 1000 mg of TU can maintain stable plasma T levels for 12 weeks. TU alone was found to induce azoospermia in 96% of Chinese men (23/24, tea seed oil formulation, 500 or 1000 mg every 4 weeks) and in 57% of Caucasian men (8/14, castor oil formulation, 1000 mg every 6 weeks). Currently, phase III contraceptive efficacy trials are in progress in China using TU alone at 1000 mg every 8 weeks. The combination of TU with a progestogen has also been studied in Caucasian subjects (see below).

### G. 7α-Methyl-19-Nortestosterone

7α-Methyl-19-nortestosterone (MENT) is a highly androgenic α-methylated derivative of 19-NT that is not 5α-reduced to DHT. Prostate growth and skin effects (including acne and balding) are dependent on DHT action. MENT may therefore have beneficial androgenic actions without the unwanted effects on the prostate and skin. However, it is currently unclear whether the aromatized metabolite of MENT confers a physiological level and spectrum of estrogenic actions *in vivo*. The potential advantages of this synthetic selective androgen receptor modulator (SARM) are therefore 2-fold: (1) tissue specificity—in nonhuman primates, 19-NT relative potency compared to testosterone is 2-fold in the prostate but 10-fold in suppression of pituitary gonadotropin secretion and (2) the high but differential potency allows lower doses of MENT to suppress pituitary gonadotropins while potentially sparing any prostate stimulation. In theory, the lack of 5α-reduction may also be beneficial in maximizing suppression of spermatogenesis to azoospermia by lowering intratesticular DHT.

MENT has an extremely short half-life. Preliminary studies of small numbers of healthy men using subdermal MENT acetate biodegradable implants confirmed effective gonadotropin suppression and induction of azoospermia in 70% of subjects. There was also maintenance of secondary sexual function. Whether MENT, at doses that can effectively suppress spermatogenesis to the target level, can also prevent or reverse any stimulation of prostate size and maintain bone mass in man remains unproven.

## V. GnRH AGONISTS AND ANTAGONISTS

GnRH agonists initially cause an increase in gonadotropin secretion that is followed 2–3 weeks later by marked inhibition. This action is due to the development of desensitization of the gonadotroph GnRH receptor, resulting in the suppression of LH and FSH secretion. Studies in the 1980s and 1990s assessed GnRH agonists alone or in combination with testosterone enanthate and yielded disappointing results. Overall, only approximately one-quarter of men achieved azoospermia with an additional one-third achieving oligozoospermia. The poor suppression is believed to be due to rebound FSH secretion that is presumably independent of GnRH stimulation. Consequently, further study of GnRH agonists as potential candidates for male contraception has been abandoned.

GnRH antagonists are competitive GnRH receptor antagonists that cause rapid suppression of gonadotropins within hours without the initial stimulation seen with GnRH agonists. When combined with T, suppression of LH and FSH is highly effective and sustained. Studies of this combination have shown spermatogenic suppression at least equal to that of TE alone. Furthermore, when the GnRH antagonist Nal-Glu was used to initiate suppression, oligozoospermia (<1 million sperm/ml) could then be maintained with TE at 100 mg once weekly alone. A similar regime of a GnRH antagonist (cetrorelix) and 19-NT was combined and azoospermia was induced within 3 months. However, suppression was not maintained with 19-NT alone. Although these synthetic peptide compounds clearly have contraceptive potential, the disadvantages are their expense and short half-life, requiring daily subcutaneous injection. Side effects encountered have included local skin reactions and pruritus at the site of injection due to histamine release. A new generation of long-acting depots of potent GnRH antagonist preparations (e.g., Abarelix, Ganarelix, and Degarelix) being developed for prostate cancer treatment may have a place in male contraception where rapid induction of spermatogenic suppression can subsequently be maintained by testosterone alone.

## VI. PROGESTOGEN/ANDROGEN COMBINATIONS

Exogenous progestogens can inhibit gonadotropin secretion, reduce systemic testosterone levels, and suppress spermatogenesis in men. Combining a progestogen with testosterone exploits the synergistic actions of the two steroids that can be used at lower doses for spermatogenesis suppression. Moreover, some synthetic progestogens may have direct anti-spermatogenic actions in the testis (e.g., androgen receptor antagonism, inhibition of Leydig cell steroidogenesis, and 5-α-reductase), whereas physiological amounts of T will safely rescue normal androgen-dependent functions.

Four groups of synthetic progestogen are used clinically in women: (1) 19-nortestosterone derivatives (levonorgestrel/LNG, desogestrel/DSG, and dienogest/DNG); (2) 17-hydroxyprogesterone derivatives (medroxyprogesterone/MPA); (3) 19-norprogesterone derivatives (nomegestrel/NOMA); and (4) 17-hydroxyprogesterone or 19-nortestosterone derivatives with anti-androgenic actions (CPA and DNG, respectively). Structural differences between groups account for the different degrees of androgenic and gestogenic actions (Table 1). The different progestogen/androgen combinations studied to date are discussed below (Table 2).

## A. Depot Medroxyprogesterone Acetate

Depot medroxyprogesterone acetate (DMPA) has been combined with 19-NT, TE, and T implants. Azoospermia rates in Caucasians were 67 and 59% for 19-NT and TE, respectively. Side effects included weight gain and a decrease in HDL-C. Trials with 19-NT in combination with DMPA in Indonesian men found azoospermia rates of 98% vs 96% for TE alone. The combination of DMPA with T implants achieved azoospermia rates exceeding that of TE alone. There was no significant weight gain or metabolic effects (e.g., on lipid parameters). The main limitation with DMPA use is the prolonged period (up to 6 months) that is often necessary for sperm recovery following cessation of treatment.

## B. CPA

CPA has been combined with im TE and oral TU. The anti-androgenic properties of CPA are effected by blockade of T and DHT at the receptor level. This blockade will occur both at the testicular level and systemically. There is also gonadotropin inhibition at the pituitary. The combination of two oral preparations in a true male pill was assessed with oral CPA and TU. Unfortunately, azoospermia occurred in less than 20% of subjects. The reasons for this are most likely related to the inadequate androgen replacement provided by oral TU (see above). CPA at a higher dose (25–100 mg) with TE did result in azoospermia in 100% of men. Furthermore, the time to azoospermia was half that of the TE-alone group (49 days vs 98 days). Though encouraging, a dose-dependent decrease in hemoglobin and body weight was observed in those receiving CPA. These side effects are very likely anti-androgen related. These effects may limit future application of this combination.

## C. LNG

LNG has been extensively studied with a variety of androgens. Initially a dose of 500 μg daily with TE confirmed greater sperm suppression than with TE alone (azoospermia rate of 67% vs 33%). Further study with lower doses of LNG (250 and 125 μg) did not compromise sperm suppression rates. Adverse effects were dose dependent and included lowered HDL-C and weight gain. Recently, a TU/LNG combination was compared to TU alone. There was no difference in suppression to azoospermia (50%). Both groups had significant decreases in HDL-C from baseline. A testosterone patch was studied with LNG (250 μg daily) but resulted in only 2 of 11 men suppressing to azoospermia. Most recently, a T patch was combined with long-acting LNG implants, once again with relatively poor sperm suppression (severe oligozoospermia in <60% of men). A likely reason for the relatively disappointing results compared to

**TABLE 1 Pharmacodynamic Properties of Gestogens**

| Compound | Progestational activity | Androgenic activity | Estrogenic activity | Anti-androgenic activity |
|---|---|---|---|---|
| Progesterone | + | − | − | − |
| *19-Nortestosterone derivatives* | | | | |
| Levonorgestrel | + | + | − | − |
| Desogestrel | + | − | − | − |
| Dienogest | + | − | − | + |
| Norethisterone | + | + | + | − |
| *17-Hydroxyprogesterone derivatives* | | | | |
| Medroxyprogesterone acetate | + | − | − | − |
| Cyproterone acetate | + | − | − | + |

TABLE 2 **Progestogen/Androgen Combinations for Male Hormonal Contraception**

| Reference | Duration of treatment | Progestogen | Androgen | Azoospermia | Severe oligozoospermia |
|---|---|---|---|---|---|
| Handelsman *et al.* (1996) | 1 year | DMPA 300 mg | T implants 800 mg | 9/10 (90%) | 10/10 (100%)[a] |
| Meriggiola *et al.* (1996) | 16 weeks | CPA 50 mg | TE 100 mg | 5/5 (100%) | 5/5 (100%) |
| | | CPA 100 mg | TE 100 mg | 5/5 (100%) | 5/5 (100%) |
| Meriggiola *et al.* (1997) | 16 weeks | CPA 12.5 mg | TU 80 mg bid | 1/8 (13%) | 3/8 (38%) |
| Meriggiola *et al.* (1998) | 16 weeks | CPA 12.5 mg | TE 100 mg | 3/5 (60%) | |
| | | CPA 25 mg | TE 100 mg | 5/5 (100%)[b] | 5/5 (100%) |
| Bebb *et al.* (1996) | 24 weeks | LNG 500 μg | TE 100 mg | 12/18 (67%) | 14/18 (78%) |
| Anawalt *et al.* (1999) | 24 weeks | LNG 250 μg | TE 100 mg | 14/18 (78%) | 16/18 (89%) |
| | | LNG 125 μg | TE 100 mg | 11/18 (61%) | 16/18 (89%) |
| Kamischke *et al.* (2000) | 24 weeks | LNG 250 μg | TU 1000 mg | 7/14 (50%) | 13/14 (92%) |
| Buchter *et al.* (1999) | 24 weeks | LNG 250–500 μg[c] | T patch 5 mg | 2/11 (18%) | 5/11 (45%)[a] |
| Wu *et al.* (1999) | 24 weeks | DSG 300 μg | TE 100 mg | 6/8 (75%) | 6/8 (75%) |
| | | DSG 150 μg | TE 100 mg | 4/7 (57%)[d] | 7/7 (100%) |
| | | DSG 300 μg | TE 50 mg | 8/8 (100%) | 8/8 (100%) |
| Anawalt *et al.* (2000) | 24 weeks | DSG 150 μg | TE 50 mg | 4/7 (57%)[e] | 6/9 (67%) |
| | | DSG 150 μg | TE 100 mg | 7/7 (100%) | 7/7 (100%) |
| | | DSG 300 μg | TE 100 mg | 7/8 (88%) | 7/8 (88%) |
| Hair *et al.* (2001) | 24 weeks | DSG 75 μg | T patch 5 mg | 0/4 (0%) | 1/4 (25%) |
| | | DSG 150 μg | T patch 5 mg | 3/6 (50%) | 3/6 (50%) |
| | | DSG 300 μg | T patch 5 mg | 4/7 (57%) | 5/7 (71%) |
| Kamischke *et al.* (2000) | 24 weeks | NETE 200 mg | TU 1000 mg | 13/14 (93%) | |

*Note.* DMPA (depot medroxyprogesterone acetate) was administered by a single im injection. CPA (cyproterone acetate) was administered orally once daily. TE (testosterone enanthate) at 100 mg was administered by im injection once weekly. LNG (levonorgestrel) is administered orally once daily. TU (testosterone undecanoate) was administered either orally (80 mg) twice daily or im (1000 mg) every 6 weeks. T patch (testosterone patch) delivers 5 mg/24 h. DSG (desogestrel) is administered orally once daily. NETE (norethisterone enanthate) was administered im every 6 weeks.

[a] Severe oligozoospermia in this paper was defined as $<3$ million sperm/ml.

[b] One subject was azoospermic at week 12 but had a count of 0.1 million/ml at week 16.

[c] Subjects were commenced on 250 μg daily until week 12. If they had not suppressed to azoospermia at week 12, the dose was increased to 500 μg daily.

[d] One subject dropped out of the trial.

[e] Two subjects dropped out of the trial for personal reasons.

injectable or implantable preparations of T may relate to the unreliable administration or absorption of T so that circulating levels in only the low normal range can be achieved. This highlights the critical role of testosterone in achieving efficient spermatogenesis suppression: a sufficient but not supraphysiological amount of T conveniently delivered with kinetics that can sustain stable plasma levels is required. Currently available preparations fall short of these requirements.

## D. DSG

DSG is an oral third-generation progestogen with potent progestational activity and lower androgenicity (see Table 1). These potentially favorable properties led to the study of DSG in combination with TE. DSG has been widely used in the combined female oral contraceptive pill (Marvelon, NV Organon) where no decrease in HDL-C is observed in the women treated. Oral DSG and TE has been shown to produce 100% azoospermia (see Table 2) with certain dose combinations. These results demonstrate the synergistic action of this steroid combination. A 20–25% decrease in HDL-C levels was observed in these studies. One study has assessed doses of DSG (75, 150, and 300 μg) in combination with 300 mg T implants. The treatment phase was short but demonstrated a dose-dependent reduction in sperm counts. The median sperm counts at week 8 (end of treatment) were 24 million, 10 million, and 0.1 million sperm per milliliter in the 75, 150, and 300 μg dose groups, respectively. No effect on HDL-C was evident. Long-term studies would be needed to further assess this approach. DSG has been combined with a T transdermal patch but similar to the LNG/patch study, sperm suppression was inferior to that seen with other regimens.

### E. NETE

Norethisterone enanthate (NETE) is a depot progestogen administered by intramuscular injection converting to the metabolically active norethisterone (NET, Table 1). NET is both aromatized (to ethinylestradiol) and 5α-reduced (to 5α-NET), with androgen receptor binding being 10% that of testosterone binding. NETE and TU have been combined in a 6-week regimen. The potential for reducing the frequency of doses (im injections) is important if a male hormonal contraceptive is to be widely accepted. The results have been very encouraging (see Table 2), with azoospermia in 93% of men. Adverse effects of the TU/NETE combination have included moderate increases in hemoglobin (within the normal range) and decreases in HDL-C.

## VII. RESPONDERS AND NONRESPONDERS

There are clear differences in the rates of azoospermia achieved in Asian (Chinese and Indonesian) and Caucasian populations. In the WHO studies, azoospermia was achieved in 90% of Asian men vs 74.3% of European or American men. Greater suppression in Asian men was also seen in the im TU study discussed above (96% vs 57%). The possible explanations for these differences include genetic and environmental influences that are not yet fully understood. Asian men are known to be more susceptible to suppression of both gonadotropins and sperm counts when given a T infusion. Differences in sperm production rates have also been shown in testicular biopsies at autopsy, with Asian men having a lower rate than White or Hispanic men.

Intraethnic variation is seen within Caucasian populations with suppression to azoospermia in 40–70% of men on the WHO trials of TE alone. No correlation was found with physical size, body mass, or baseline biochemical, endocrine, or semen parameters. Differences in 5α-reductase activity were found between oligozoospermic and azoospermic volunteers. The increased 5α-reductase activity found in the oligozoospermic subjects may reflect increased testicular DHT availability. This could be sufficient to provide the stimulus to maintain a residual degree of spermatogenesis in a hypogonadotropic state. Tissue sensitivity to androgen action and androgen metabolism could be relevant in the response to hormone-induced suppression of spermatogenesis. A recent study evaluated the CYP3A4 gene (instrumental to hepatic testosterone metabolism) and the androgen receptor gene in 75 men who had participated in contraceptive trials. No significant difference in genetic polymorphisms for either gene was identified. Further studies with sufficiently large numbers of subjects examining the pharmacogenomics of spermatogenesis may allow the prediction of response and guide the development of hormonal contraceptives of maximal efficacy.

## VIII. SAFETY AND ACCEPTABILITY

In a WHO efficacy study, over three-quarters of the couples who participated did so because of dissatisfaction with their current contraceptive method. Furthermore, only 1.5% of volunteers withdrew from the study due to dissatisfaction with the protocol. A large cross-cultural study found that women feel that the responsibility of contraception too frequently falls to them. Over 70% of the women surveyed felt that a male contraceptive pill was a good idea. These facts would suggest that there is an interest and a need for an alternative contraceptive method.

An important aspect of any male hormonal method is that there is a time lag from the start of treatment until sperm suppression is sufficient for contraceptive efficacy. This is because sperm production takes 2–3 months and therefore following gonadotropin suppression a similar period of time is necessary to reach severe oligozoospermia. Vasectomy is an irreversible male method, is commonly used, and has a lag time similar to that of contraceptive efficacy (2–3 months). However, a hormonal method may be preferable to some couples to allow reversibility in the future. A time lag of 1 month applies to the female contraceptive pill and this is also widely accepted by couples in common clinical practice. An important point is that hormonal methods for either the male or the female should be suggested as the sole method of contraception only when the user is in a stable relationship. In this situation, the choice of contraceptive is often planned and a lag time is unlikely to deter users.

Trials to date have treated men with progestogen/androgen combinations for up to 1 year. The data have shown that these regimens are safe over this time period in healthy young men. The main side effects have been weight gain and a decrease in HDL-C. Weight gain appears to be an androgenic effect and is associated with an increase in lean body mass. The cardiovascular significance of lowered HDL-C is not currently known. It is also likely that these effects would be patient specific as is seen with the female OCP and hypercoaguability, for example. The potential risk of prostate disease when androgen is administered to healthy men is also an area of concern. Essentially, long-term studies are needed to assess this risk. Data from short-term studies to date have demonstrated no association with prostate disease, prostate volumes, or changes in serum prostate-specific antigen. While these risks will continue to be evaluated, in the meantime regimes are chosen to try and minimize androgenic effects. As discussed, the choice of progestogen and the dose of androgen are therefore relevant.

## IX. SUMMARY AND CONCLUSIONS

Further understanding of the precise mechanisms of spermatogenesis may provide additional nonhormonal targets for male contraception. The postmeiotic germ cell differentiation and epididymal sperm maturation processes are both potential areas to consider. Public-funded research is at a stage where realization of male hormonal contraception is within our reach. The beginning of participation by the pharmaceutical industry is encouraging and a necessary step leading to product development. A progestogen/androgen combination is most likely to be the first product to be marketed. Further development of long-acting depot formulations or oral and nonsteroidal compounds will provide a variety of contraceptive formulations that will allow men to have a wider choice and encourage acceptability and usage.

## Glossary

**azoospermia** The absence of sperm in the ejaculate.

**gonadotropins** The pituitary hormones luteinizing hormone and follicle-stimulating hormone.

**oligozoospermia** A count of $<3$ million sperm/ml in the ejaculate.

**severe oligozoospermia** A count of $\leq 1$ million sperm/ml in the ejaculate.

## See Also the Following Articles

**Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Follicle-Stimulating Hormone (FSH) • Gonadotropin-Releasing Hormone Pharmacology: Agonists and Antagonists • Luteinizing Hormone (LH) • Spermatogenesis, Hormonal Control of**

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# Melatonin in Humans[1]

ALFRED J. LEWY AND LAURIE HURTADO VESSELY
*Oregon Health & Science University, Portland*

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**Melatonin, a hormone produced by the pineal gland, is secreted in a nocturnal circadian pattern under the control of the endogenous circadian pacemaker. Light exposure and exogenous melatonin can influence the endogenous pacemaker and melatonin secretion; thus, studies in this area may lead to improved therapeutic approaches to sleep-related disorders.**

## I. INTRODUCTION

Melatonin is the primary product of the pineal gland. The nocturnal circadian pattern of melatonin secretion is controlled by the endogenous circadian pacemaker (ECP) in the suprachiasmatic nucleus (SCN) of the hypothalamus. The ECP is entrained by photic stimuli from the 24-h light/dark cycle. The secretory pattern of melatonin can be used to assess the phase of the ECP. Both bright light exposure and exogenous melatonin can influence the phase of the ECP, which can be put to therapeutic use in a number of clinical situations. Totally blind people often have free-running circadian rhythms that are not entrained to the 24-h day/night cycle. In these blind free-runners (BFRs), the circadian phase drifts each day, causing recurrent insomnia. Properly administered melatonin can advance the circadian rhythms of BFRs, and most of them can be entrained to the 24-h day/night cycle. Sighted people can develop circadian rhythm disturbances as well. Both light and melatonin can be used to advance or delay circadian rhythms that are out of phase due to advanced and delayed sleep phase syndromes, jet travel, shift work, and winter depression (seasonal affective disorder).

## II. MELATONIN AS A HORMONE

Melatonin is an indoleamine synthesized from serotonin in two steps (Fig. 1). The conversion of serotonin to *N*-acetylserotonin is the rate-limiting step. Melatonin was first isolated from bovine pineal tissue in 1959 by Lerner and co-workers. It is lipophilic, a property that allows it to pass through cellular and nuclear membranes. The main site of melatonin secretion in humans is the pineal gland, although it may also be synthesized in the retina, lacrimal gland, and gut. Melatonin circulates via the cerebrospinal fluid (CSF) and the bloodstream and then binds to specific seven-transmembrane, G-protein-coupled receptors at various sites, of which the SCN of the hypothalamus is the best characterized. A nuclear receptor for melatonin has also been identified. Melatonin is found diffusely in nature. Although its function in vertebrates is the most clearly defined, it is also found in numerous plants and in lower phyla.

## III. CIRCADIAN PHYSIOLOGY

Melatonin's participation in the endogenous circadian system is its most clearly defined role in humans. In all vertebrates that have been studied, the pineal gland is involved in the rhythmic production of melatonin, with active production occurring only at night. In humans, the pineal gland measures between 5 and 7 mm, is shaped like a pine cone, and lies (outside the blood–brain barrier) in the center of the brain, attached by a stalk to the roof of the third ventricle. Melatonin secretion is thought to represent the hormonal signal indicating nighttime darkness. In some nonmammalian vertebrates (including birds, reptiles, amphibians, and fish), the pineal gland contains photoreceptors involved in the regulation of melatonin secretion. This is in contrast to mammals, in which retinal, rather than pineal,

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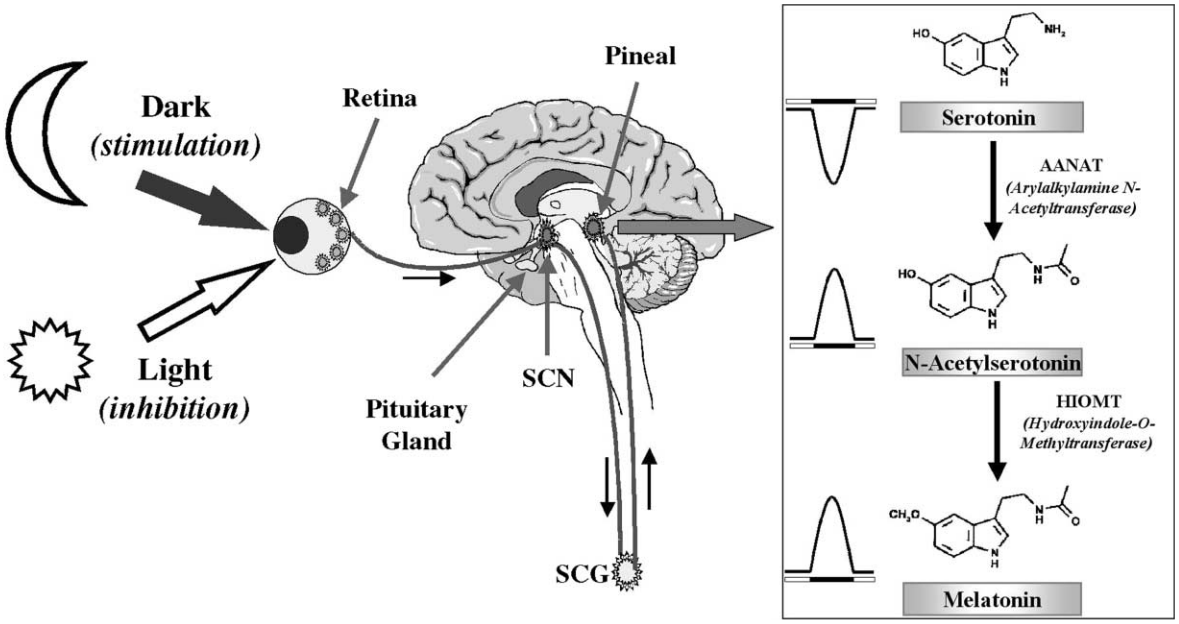

**FIGURE 1** Regulation of melatonin synthesis. Melatonin is synthesized in the retina and in the pineal gland. In the pineal gland, melatonin synthesis follows a rhythm driven by the suprachiasmatic nucleus (SCN), the master biological clock, following a multisynaptic path, including, among others, the superior cervical ganglia (SCG). Norepinephrine released from postganglionic fibers activates $\alpha_1$- and $\beta_1$-adrenoceptors in the pinealocyte, leading to increases in second messengers (i.e., cAMP, inositol trisphosphate, diacylglycerol) and in the activity of AANAT, the rate-limiting step in melatonin synthesis. The system is dramatically inhibited by light, the external cue that allows entrainment to the environmental light/dark cycle. The photic signal received by the retina is transmitted to the SCN via the retino-hypothalamic tract, which originates in a subset of retinal ganglion cells. The pineal melatonin thus serves as the internal signal that relays day length, allowing regulation of seasonal changes in reproductive physiology and behavior. The pars tuberalis of the pituitary gland interprets this rhythmic signal and generates a precise cycle of expression of circadian genes. Melatonin synthesis in the photoreceptors of the retina follows a similar daily rhythm that is generated by local oscillators.

the demonstration that melatonin inhibited dopamine release from retina through activation of presynaptic heteroreceptors, and the design and synthesis of the first competitive melatonin receptor antagonist luzindole (luz: Spanish for light; indole: basic chemical structure of melatonin). Luzindole (2-benzyl *N*-acetyltryptamine) was designed to mimic light signals by blocking the effects of melatonin, the hormone of darkness.

The first cDNA encoding a melatonin receptor, currently known as $\mathrm{Mel}_{1c}$, was cloned by expression of purified mRNA from *X. laevis* melanophores into mammalian cells in culture. This landmark discovery led to the cloning of the mammalian melatonin receptors $\mathrm{Mel}_{1a}$ and $\mathrm{Mel}_{1b}$, belonging to a novel subfamily of seven-transmembrane-domain G-protein-coupled receptors. These mammalian melatonin receptors, currently referred as $\mathrm{MT}_1$ and $\mathrm{MT}_2$, respectively, exhibit distinct molecular and pharmacological characteristics and are localized to different chromosomes ($\mathrm{MT}_1$, 4q35.1; $\mathrm{MT}_2$, 11q21–q22).

A third mammalian melatonin receptor, $MT_3$ (previously referred to as $\mathrm{ML}_2$), was pharmacologically characterized but is yet to be cloned. The $MT_3$ melatonin receptor can be activated by both melatonin and its precursor *N*-acetylserotonin and has a pharmacological profile distinct from any other known mammalian melatonin receptor. A protein (quinone reductase II: QR2) purified from hamster kidney was found to have a pharmacological profile identical to that of the $MT_3$ binding site of hamster brain. Whether the $MT_3$ and QR2 sites are the same or distinct proteins needs to be further investigated.

The synthesis of new molecules with various degrees of selectivity for $\mathrm{MT}_1$, $\mathrm{MT}_2$, and/or $MT_3$ melatonin receptors has allowed identification of specific melatonin receptors in native tissues. The recombinant $\mathrm{MT}_1$ and $\mathrm{MT}_2$ melatonin receptors expressed in mammalian cells show picomolar affinity for the radioligands 2-[$^{125}$I]iodomelatonin and [$^3$H]melatonin, whereas in native tissues the $MT_3$

melatonin receptor binds melatonin with nanomolar affinity. The MT$_1$ receptor is pharmacologically characterized by a higher affinity for 2-iodomelatonin and a lower affinity for 6-chloromelatonin than for melatonin. The MT$_2$ receptor shows similar affinity for melatonin, 2-iodomelatonin, and 6-chloromelatonin. Ligands with higher selectivity for the MT$_2$ receptor than for the MT$_1$ melatonin receptor include luzindole and the nonindolic 4-phenyl acetamidotetraline (4P-ADOT) and 4-phenyl propionamidotetraline (4P-PDOT). The affinity of the MT$_1$ and MT$_2$ melatonin receptors for *N*-acetylserotonin is approximately 1000 times lower than that for melatonin. By contrast, the *MT$_3$* melatonin receptor is characterized by similar affinity for *N*-acetylserotonin and melatonin, and shows high selectivity for the agonist 5-methoxycarbonylamino-*N*-acetyltryptamine (5-MCA-NAT).

One important caution in classifying melatonin ligands is that their intrinsic efficacy at initiating (agonist, partial agonist), reducing (inverse agonist), or blocking (antagonist, partial agonist) a signaling response depends on the cellular milieu in each target tissue or cell, the proportion and levels of the different receptor types expressed, the types of G-proteins and scaffolding molecules, and the pharmacological specificity and selectivity for each receptor. For example, luzindole, the first competitive melatonin receptor antagonist discovered, as well as the MT$_2$ selective ligands 4P-PDOT and 4P-ADOT, are antagonists in the mammalian retina, suprachiasmatic nucleus, and arterial vessels. However, 4P-PDOT acts as a partial agonist in the rat peripheral microcirculation, in that it directly inhibits leukocyte rolling, and in human MT$_2$ melatonin receptors expressed in mammalian cells, in that it inhibits stimulated cAMP formation. Additionally, both luzindole and 4P-PDOT show inverse agonist efficacy in mammalian arteries and cell lines expressing constitutively active MT$_1$ melatonin receptors.

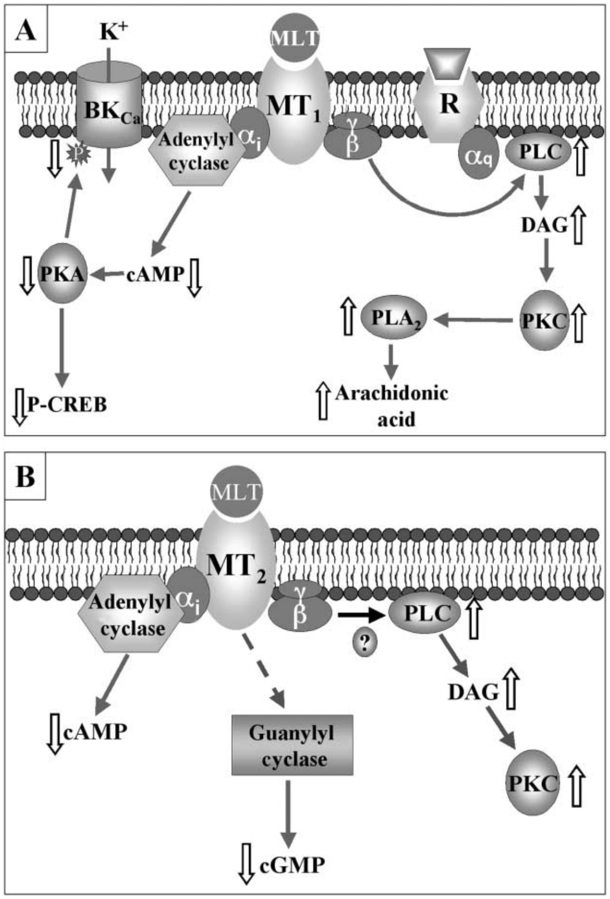

FIGURE 2 Signaling events mediated by activation of MT$_1$ and MT$_2$ melatonin receptors. (A) Melatonin (MLT) signals through activation of the MT$_1$ melatonin receptor via two parallel pathways mediated by the α-subunit [i.e., inhibition of cyclic adenosine monophosphate (cAMP) formation] and the βγ-subunits [i.e., potentiation of phosphoinositide turnover stimulated by a G$_q$-coupled receptor (R)] of G$_i$. (B) Signaling pathways coupled to MT$_2$ melatonin receptor activation. Melatonin-mediated phase shifts of circadian rhythms through MT$_2$ receptors are mediated by protein kinase C (PKC) activation (the mechanism leading to PKC activation remains putative, however). DAG, Diacylglycerol; PKA, protein kinase A; CREB, cAMP-responsive element binding protein; BK$_{Ca}$, calcium-activated potassium channel; cGMP, cyclic guanosine monophosphate; R, G$_q$-coupled receptor (i.e., prostaglandin PGF2α receptor FP and purinergic receptor P2Y).

## B. Melatonin Receptor Signaling

### *1. MT$_1$ Receptors*

Activation of MT$_1$ or MT$_2$ melatonin receptors promotes coupling to heterotrimeric G$_i$-proteins formed by α, β, and γ subunits and to their dissociation into α and βγ dimers (Fig. 2A). The G$_{\alpha i}$ and G$_{\beta\gamma}$ dimers interact with effector molecules involved in the transmission of cell signaling. Our current understanding is that the mammalian MT$_1$ melatonin receptor signals through the activation of two parallel pertussis toxin-sensitive pathways involving inhibition of adenylate cyclase via coupling to G$_{\alpha i}$ proteins and potentiation of phospholipase stimulation via G$_{\beta\gamma}$ proteins. Activation of the MT$_1$-melatonin receptor through the G$_{\alpha i}$ proteins (G$_{\alpha i2}$ and G$_{\alpha i3}$) inhibits forskolin-stimulated cAMP formation, protein kinase A (PKA) activity, and phosphorylation of the cAMP-responsive element binding (CREB) protein. On the other hand $MT_1$ melatonin receptor activation via the released $\beta\gamma$-subunit potentiates phospholipase C stimulation induced by prostaglandin PGF2α or adenosine triphosphate (ATP) activation of their receptors (FP and P2Y, respectively). Heterologous potentiation of phospholipase C activation through $MT_1$-generated $\beta\gamma$ leads to subsequent stimulation of protein kinase C (PKC) and inositol 1,4,5-trisphosphate ($InsP_3$)-mediated intracellular calcium increase. Activation of $MT_1$ melatonin receptors potentiates α-adrenergic mediated vasoconstriction by blocking calcium-activated potassium channels ($BK_{Ca}$) in smooth muscle. This blockade appears to result from decreases in cAMP formation and $BK_{Ca}$ channel phosphorylation. $MT_1$ melatonin receptor activation also directly vasoconstricts cerebral arteries through blockade of $BK_{Ca}$ channels.

In the SCN, melatonin increases potassium conductance by activation of an inward rectifier potassium channel (GIRK) Kir3, through a mechanism that may involve activation by the $\beta\gamma$-subunits of the $G_i$-protein coupled to the $MT_1$ melatonin receptors. Activation of an $MT_1$ receptor appears to be involved in melatonin-mediated hyperpolarization of neonatal pituitary cells, as well as in the inhibition of calcium influx through voltage-activated L calcium channels in neonatal rat gonadotrophs. The $MT_1$ melatonin receptor also couples to pertussin toxin (PTX)-insensitive Qq/11 proteins, leading to the mobilization of intracellular calcium in pars tuberalis cells in culture.

### 2. *$MT_2$ Receptors*

Activation of the $MT_2$ melatonin receptor modulates cAMP and guanosine 3′,5′-monophosphate (cGMP) formation (Fig. 2B). Activation of recombinant $MT_2$ melatonin receptors expressed in mammalian cells inhibits forskolin-stimulated cAMP formation and directly inhibits cGMP formation through a currently unknown mechanism. In the SCN, melatonin phase shifts circadian rhythms of neuronal firing rate, through activation of a receptor coupled to a pertussis toxin-sensitive G-protein. This response is completely antagonized by selective $MT_2$ antagonist 4P-PDOT and inhibitors of the protein kinase C. Although the exact mechanism through which $MT_2$ melatonin receptor activation phase shifts circadian rhythms is not known, it appears to involve phospholipase C (PLC) stimulation, increases in diacylglycerol (DAG) production, and PKC activation.

### 3. *Melatonin Receptor-Mediated Physiological Responses*

Melatonin plays a key role in a number of physiological, neuroendocrine, and behavioral responses, including regulation of circadian rhythms and sleep; reproduction in seasonally breeding mammals; and visual, cardiovascular, and neuroimmunological functions (Fig. 3). These effects of melatonin are mediated through the activation of high-affinity melatonin receptors identified using radioligand binding, *in situ* hybridization with specific riboprobes or oligonucleotide probes, and immunohistochemistry using receptor-selective antibodies. Functional responses mediated through activation of $MT_1$ or $MT_2$ melatonin receptors in target tissues were identified using melatonin receptor-selective and specific ligands, and mice with deletion of the $MT_1$ receptor.

Melatonin receptors in the neural retina and superior colliculi are targets for the regulation of visual function. Melatonin released locally from retinal photoreceptors or other cells modulate a number of retinal functions, including calcium-dependent dopamine release, pigment aggregation, disk shedding, and phagocytosis. At picomolar concentrations, melatonin inhibits dopamine release through activation of an $MT_2$ presynaptic heteroreceptor. In the mammalian retina, dopamine mimics light and melatonin mimics the effect of darkness, exerting reciprocal modulation on light- and dark-mediated processes.

In the mammalian SCN, activation of melatonin receptors mediates two distinct functional responses: acute inhibition of neuronal firing by the $MT_1$ receptor and phase shifts of circadian rhythms by the $MT_2$ receptor. Both the acute inhibition of neuronal firing and the phase shift of circadian rhythms of activity occur at temporally sensitive times of the day. Neurons in the SCN show the highest sensitivity to inhibition of neuronal firing by melatonin at dusk, suggesting that the $MT_1$ receptor may play a role in altering the SCN state of excitability as the clock shifts from day to night. Thus, this property of melatonin may underlie the mechanism by which it promotes sleep, possibly by inhibiting neuronal activity in the SCN and/or other areas of the limbic system. By contrast, activation of the $MT_2$ receptor phase shifts the clock at both dusk and dawn, which coincides with the times of rise and fall of melatonin production. In the fall, as night length increases, the duration of melatonin production also lengthens. It is therefore possible that the $MT_2$ melatonin receptor

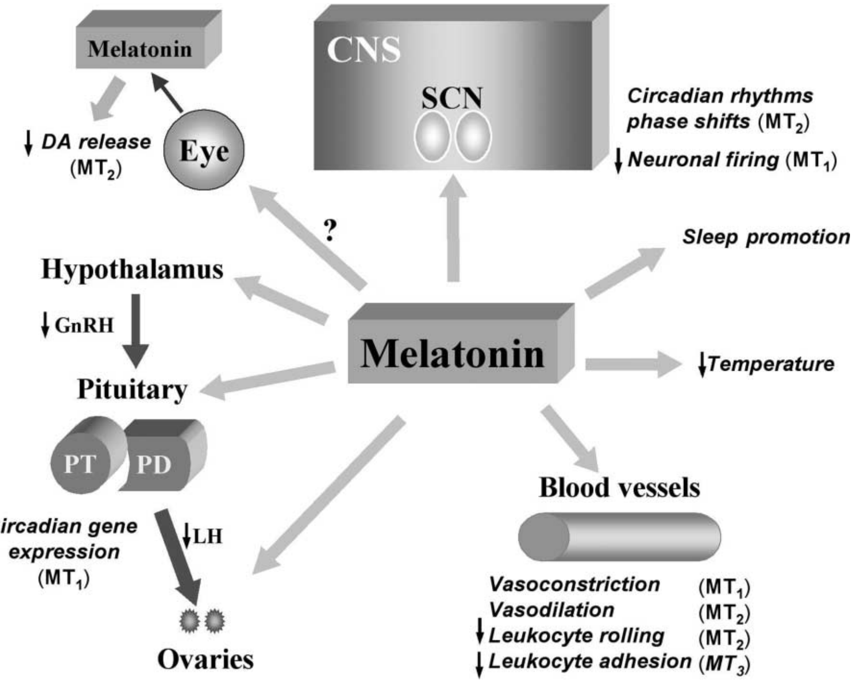
Melatonin
CNS
SCN
Circadian rhythms phase shifts (MT$_2$)
↓Neuronal firing (MT$_1$)
↓DA release (MT$_2$)
Eye
?
Sleep promotion
Hypothalamus
↓GnRH
Melatonin
↓Temperature
Pituitary
PT
PD
Circadian gene expression (MT$_1$)
↓LH
Blood vessels
Vasoconstriction (MT$_1$)
Vasodilation (MT$_2$)
↓Leukocyte rolling (MT$_2$)
↓Leukocyte adhesion (MT$_3$)
Ovaries

FIGURE 3 Melatonin-mediated physiological responses. Schematic representation of some of the most well-recognized functions of circulating melatonin: circadian behavior, sleep, reproduction, immune system effects, and vasculature effects. Melatonin synthesized in the retinal photoreceptors is secreted locally and is known to inhibit dopamine (DA) release. Melatonin and dopamine can act as signals for dark and light, respectively, mutually inhibiting each other and functioning as major players in the dark/light adaptation process. MT, Melatonin receptor; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; CNS, central nervous system; SCN, suprachiasmatic nucleus; PD, pars distalis; PT, pars tuberalis.

provides photoperiodic information to the clock to maintain synchrony of photoperiodic responses. In summary, activation of MT$_1$ and MT$_2$ melatonin receptors may provide important integrated control of distinct aspects of SCN physiology by melatonin.

Nonneuronal melatonin receptors in vascular beds regulate cardiovascular function and temperature. Melatonin, through activation of an MT$_1$ receptor, potentiates adrenergic constriction in some peripheral arteries, whereas it shows a direct vasoconstrictor effect in cerebral arteries. Further, activation of the MT$_2$ receptor mediates a vasodilator response in arterial beds. Inflammatory responses follow a diurnal rhythm that appears to be generated by melatonin. Melatonin appears to reduce the acute inflammatory process by inhibiting leukocyte rolling in the microvasculature, through activation of MT$_2$ receptors, and leukotriene B$_4$-induced leukocyte adhesion to endothelial cells, through a melatonin receptor with the pharmacological characteristics of the $MT_3$ site.

Melatonin regulates reproduction in seasonally breeding animals and the dynamic physiological adaptations that occur in response to changes in day length. As the duration of the dark period changes, so does the duration of the melatonin acrophase, which then serves as the link between the circadian clock and peripheral tissues. In the pars tuberalis of the pituitary gland, the nocturnal secretion of pineal melatonin suppresses expression of the clock gene *Per1* by inhibiting the c-AMP-dependent pathway (receptor–cAMP–CREB phosphorylation–*Per1* gene expression) and simultaneously sensitizes the cAMP signaling pathway. As the levels of circulating melatonin decrease at dawn, the pars tuberalis is released from transcriptional repression, which facilitates the induction of *Per1* gene expression by adenosine, a transmitter that stimulates cAMP production via the A$_{2b}$ adenosine receptor. This may be a general mechanism by which the hormone melatonin regulates gene expression, thus linking the central circadian pacemaker and peripheral tissues, resulting in modulation of circadian and seasonal rhythms.

Studies of the sites and mechanisms by which melatonin regulates reproduction have focused on the hypothalamus and pituitary as target tissues. Melatonin receptor activation regulates gonadotropin-releasing hormone (GnRH) secretion from hypothalamic neurons, which in turn control the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which alter reproductive function at the level of the gonads. In pituitary gonadotrophs, possibly through activation of an $MT_1$ receptor, melatonin inhibits GnRH-induced calcium increase and LH secretion. Additionally, the presence of melatonin in ovarian follicular fluid suggests the possibility for a direct effect of the pineal hormone in ovarian function as well.

The $MT_2$ melatonin receptor appears to modulate responses in various nonneuronal targets. Activation of this receptor was shown to enhance cell-mediated and humoral immunity, to inhibit proliferation of human choriocarcinoma JAr cells (probably by delay of the cell cycle transition from $G_1$ to S phase), and to decrease the expression of the glucose transporter Glut4 and glucose uptake in human brown adipocytes.

## III. MELATONIN RECEPTORS AS THERAPEUTIC TARGETS

Melatonin-mediated activation of the $MT_1$ receptor in the SCN and/or other limbic system areas may mediate somnogenic effects, and activation of the $MT_2$ subtype may be involved in the regulation of circadian rhythms. Thus, it follows that $MT_2$-selective melatonin receptor agonists and/or antagonists may be used to treat disorders involving alterations in the phase of the circadian clock, as observed in depression, blindness, and delayed sleep phase syndrome, or following a rapid change in the light dark/cycle, such as during jet travel and shift work. The use of specific and selective $MT_1$ and $MT_2$ melatonin receptor antagonists will help to elucidate the functional role of melatonin in mammals and may prompt development of type-selective analogues for the treatment of insomnia and circadian sleep/mood disorders involving alterations in phase.

## Acknowledgments

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## Glossary

**agonist** Ligand with positive intrinsic efficacy that induces a change in receptor conformation, leading to a functional response.

**circadian rhythm** Endogenous adaptations of physiology and behavior made by organisms in response to day and night cycles [(L) circa = about; dies = day].

**constitutive activity** The ability of a receptor protein to adopt an active conformation and to signal in the absence of ligand.

**G-protein-coupled receptors** Seven-transmembrane-spanning receptors, coupled to signal transduction systems utilizing heterotrimeric ($\alpha\beta\gamma$) guanosine triphosphate-binding proteins (G-proteins).

**intrinsic efficacy** Property of a molecule that changes the conformation of a receptor, leading to a functional response.

**inverse agonist** Ligand with negative intrinsic efficacy; induces a functional response that is opposite to that of an agonist and decreases basal or constitutive receptor activity.

**neutral antagonist** Ligand with no intrinsic efficacy; binds to the receptor without inducing a functional response on its own and blocks functional responses mediated by agonists.

**partial agonist** Ligand with intrinsic efficacy lower than that of the agonist. Partial agonists either activate the receptor, inducing a response, or block the response induced by an agonist.

**pertussis toxin** Bacterial toxin produced by *Bordetella pertussis*; catalyzes ADP-ribosylation of the α-inhibitory subunit of $G_i$-protein and abolishes responsiveness of adenylyl cyclase to inhibitory ligands.

**photoperiod** Annual variation of daily changes in a physiological response that results from exposure of an organism to a natural light/dark cycle. Long photoperiodic responses are associated with longer days and shorter duration of the melatonin peak, because light inhibits melatonin synthesis.

**pineal gland** Endocrine gland (also called the pineal body) located in the center of the brain; main site of production of the hormone melatonin.

**seasonal rhythm** Endogenous rhythm synchronized to the time of the year by the natural change in the length of the daily light phase.

**suprachiasmatic nucleus** Hypothalamic nuclei; major endogenous pacemaker of circadian rhythms in mammals, i.e., the endogenous circadian clock.

## See Also the Following Articles

**Endocrine Rhythms: Generation, Regulation, and Integration • GPCR (G-Protein-Coupled Receptor) Structure • Melatonin in Humans • Membrane Receptor Signaling in Health and Disease**

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# Membrane Receptor Signaling in Health and Disease

ALFREDO ULLOA-AGUIRRE,
GUADALUPE MAYA-NÚÑEZ, AND CARLOS TIMOSSI
*Instituto Mexicano del Seguro Social, Mexico*

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**The primary function of cell surface receptors is to recognize a specific signal or ligand from among an immense number of chemically diverse substances and to act as signal transducers and amplifiers of the stimulus or message carried by external, systemic, or local stimuli. Extracellular signal-evoked receptor activation triggers a cascade of intracellular events; this usually culminates in a highly specific biological response, either stimulatory or inhibitory, of a given cell function.**

## I. INTRODUCTION

The three main classes or superfamilies of cell surface receptor proteins are defined according to the way in which they evoke intracellular signaling: (1) by G-protein coupling, (2) by enzyme coupling, and (3) by ion channel linkage. G-Protein-coupled receptors are seven-transmembrane-helix protein molecules that mediate their intracellular actions through the activation of one or more members of the guanine-nucleotide-binding signal-transducing proteins (G-proteins), which carry the information received by the receptor molecule to cellular effectors such as enzymes and ion channels. Activated effectors modify, in turn, levels of particular second messengers that regulate a wide variety of cellular processes. Enzyme-coupled receptors function directly as enzymes or through associated enzymes. Most have a single transmembrane (TM) segment with a ligand-binding site outside the cell and a catalytic site inside the cell. A majority of these receptors are protein kinases or are associated with protein kinases that trigger a cascade of protein–protein interactions and phosphorylations on activation by their cognate ligands. Finally, ion-channel-linked receptors or transmitter-gated ion channels are multisubunit ionotropic receptor molecules that rapidly mediate signals carried by a small number of neurotransmitters across the synapse of the central and peripheral nervous systems. In these particular receptors, ligand binding leads to opening of an intrinsic pore, through which ions can flow down their electrochemical gradients, transforming a chemical signal into charge flux across the membrane. The activity of a given family or type of membrane receptor can be influenced, positively or negatively, by signaling pathways from other receptors (i.e., receptor cross-talking) in a variety of ways, generating the functional flexibility required by complex systems.

This article focuses on how membrane receptors and their coupled signal transducers evoke intracellular signals in normal and abnormal conditions. Special emphasis is given to the G-protein-coupled receptor (GPCR)–G-protein system, for which nearly 2000 receptors have been cloned since initial cloning of bovine opsin, the photoreceptor of the rod cell.

## II. G-PROTEIN-COUPLED RECEPTORS AND G-PROTEINS IN HEALTH

### A. GPCRs

G-protein-coupled receptors are a large and functionally diverse superfamily of membrane receptors; they consist of a single polypeptide chain of variable length that traverses the lipid bilayer seven times, forming characteristic transmembrane helices connected by alternating extracellular and intracellular sequences or loops (Fig. 1). Many signaling cascades use this class of receptor to convert external (e.g., photons and odorants) and internal (e.g., neurotransmitters, peptides, and glycoproteins) stimuli to intracellular responses. G-Protein-linked receptors characteristically bind large G-proteins, which in turn act as mediators of receptor-evoked effector activation. The nature of the second-messenger pathways activated in response to ligand binding to a given GPCR is essentially determined by the type of G-protein(s) coupled to the receptor (see later). The regulation of receptor–G-protein signal selectivity and specificity is highly complex and involves the activation of a network of mechanisms and pathways that eventually lead to biological responses. Based on nucleotide and amino acid sequence similarities, the GPCRs can be grouped into three main families: (1) family A, the rhodopsin/$\beta_2$-adrenergic receptor-like family, which comprises receptors that respond to a large variety of stimuli, including photons, odorants, neurotransmitters, and glycoprotein hormones; (2) family B, the glucagon/vasoactive intestinal peptide (VIP)/calcitonin receptor-related family, which includes receptors for a variety of peptide hormones and neuropeptides, including parathyroid hormone, corticotropin-releasing hormone, VIP, calcitonin, glucagon, growth hormone-releasing hormone, and secretin; and (3) family C, the metabotropic glutamate/calcium-related family, which includes the taste receptors, the metabotropic glutamate and calcium receptors, the putative pheromone receptors, and the γ-aminobutyric acid (GABA) receptors. Other receptors belonging to the GPCR superfamily are grouped in family D (pheromone-like receptors) and family E (cyclic AMP receptors).

Agonist binding to GPCRs provokes changes in the conformation of the receptor molecule involving particularly the relative positions of the seven-TM domains; the molecular perturbations of the membrane-embedded helices (Fig. 1) are thought to cause changes in the cytoplasmic (i.e., the intracellular loops) face of the receptor, promoting G-protein coupling and activation. Most of the primary sequence homology among the different groups of these types of receptors is contained within the hydrophobic TM domain (particularly for families A and B) or the intracellular loops (for the metabotropic neurotransmitter/calcium receptors). Receptors for small ligands (e.g., molecules that bind to some receptors belonging to family A, such as the retinal chromophore and biogenic amines) characteristically bind the ligand through a pocket or crevice, involving highly conserved residues located in the middle and extracellular third of hydrophobic TM helices. Receptors for small peptides bind their ligands through regions comprising either the extracellular loops or both the TM domains and the extracellular loops, whereas for moderate-sized peptides, binding usually occurs in both the extracellular loops and the amino-terminal segment. For larger ligands, such as the glycoprotein hormone receptors, the binding site usually resides within a large extracellular amino-terminal sequence alone. Similar to peptide receptors belonging to family A, binding of peptide ligands to receptors in family B involves the extracellular loops. In the calcium-sensing and the metabotropic glutamate receptors, the principal determinants for both ligand binding and signal specificity reside in the large amino terminus; receptor

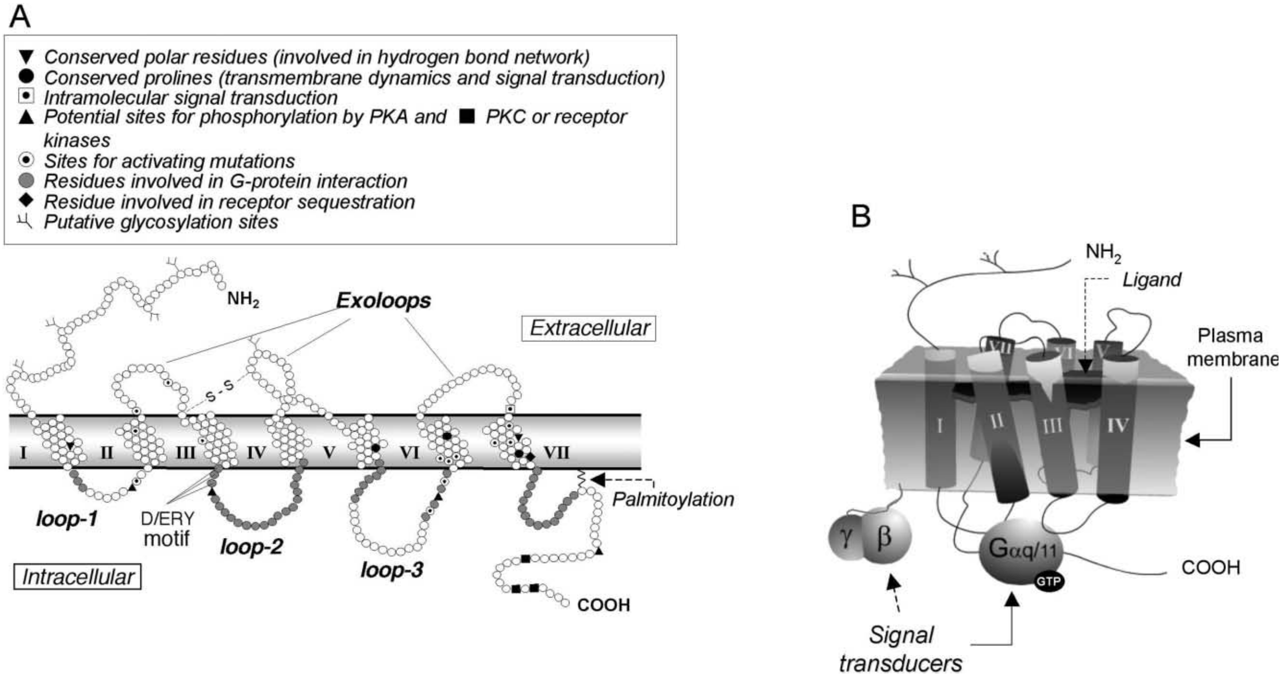
A
▼ Conserved polar residues (involved in hydrogen bond network)
● Conserved prolines (transmembrane dynamics and signal transduction)
⊡ Intramolecular signal transduction
▲ Potential sites for phosphorylation by PKA and ■ PKC or receptor kinases
⊙ Sites for activating mutations
● Residues involved in G-protein interaction
◆ Residue involved in receptor sequestration
Putative glycosylation sites
NH₂
Exoloops
Extracellular
S-S
I
II
III
IV
V
VI
VII
Palmitoylation
loop-1
D/ERY motif
loop-2
loop-3
Intracellular
COOH
B
NH₂
Ligand
Plasma membrane
VII
I
II
III
IV
γ
β
Gαq/11
GTP
COOH
Signal transducers

**FIGURE 1** (A) Model of the proposed seven-transmembrane-spanning domains of a prototypic GPCR belonging to family A. Some structural characteristics of these particular receptors are shown. The structure also shows the location of some mutations that lead to constitutive activation of the receptor (e.g., glycoprotein hormone, rhodopsin, and adrenergic receptors). Reproduced from Ulloa-Aguirre *et al.* (1999), with permission. (B) Counterclockwise orientation of GPCRs from TM domains I–VII. The closed loop structure is representative of receptors for peptide ligands. In this arrangement, the core is composed mainly of TM domains II, III, V, and VI, whereas domains I and IV are peripherally sequestered. Proximity between helix II and helix VII is characteristic of this family of GPCRs. G-Proteins are closely associated with the intracellular domains.

activation requires, however, interactions of the liganded amino-terminal domain with membrane-associated domains.

In these particular membrane receptors, the G-protein-coupling domains lie within the divergent sequences of their intracytoplasmic domains and/or in residues located in the cytoplasmic face of the TM domains. In fact, mutations in residues located in the intracellular loops or in the TM helices may lead to loss of function or gain of function of the altered receptor (see later). Activation of the receptor and, consequently, of the G-proteins requires not only disruption of intra- and interhelical interactions and the occurrence of TM movements, but also disruption of interactions between specific residues located in the intracellular loops [e.g., in family A receptors, the highly conserved aspartic acid/glutamic acid–arginine–tyrosine (D/ERY) sequence located at the boundary of TM-III and the loop-2 intracellular sequence (Fig. 1)] and residues forming a pocket through interhelical interactions. Thus, agonist binding provokes a series of conformational changes in the receptor molecule that eventually lead to an enhanced accessibility of the G-proteins to the receptor regions involved in G-protein activation.

Several mechanisms regulate the functional level and activity of GPCRs. Continuous or prolonged stimulation of a cell generally results in progressively attenuated responses to subsequent stimulation by the same agonist. This decrease in cellular responsiveness to further stimulation, or desensitization, protects the cell from excessive stimulation. Early desensitization occurs rapidly and involves phosphorylation of residues located in the intracellular domains by second-messenger-dependent activated kinases or by a special class of serine/threonine-specific kinases called G-protein-coupled receptor kinases. The subsequent binding of a group of soluble inhibitory proteins, the arrestins, amplifies the desensitization process and turns off the receptor by impeding its coupling to G-proteins. Arrestins have also been shown to couple GPCRs to the activation of particular types of kinases, the Src-like kinases, and to facilitate the formation of multimolecular complexes, including components of the mitogen-activated protein kinase (MAPK) and JUN N-terminal kinase (JNK) pathways (see later). In this vein, some adapter proteins bearing PDZ [a 90-amino acid repeat initially recognized in the postsynaptic density (PSD)-95 proteins] or SH2 or SH3 (Src homology) domains (all involved in downstream protein–protein interactions) provide the molecular basis for direct, heterotrimeric G-protein-independent interactions between GPCRs and several intracellular signaling molecules, such as the small G-protein Ras (see later). A more profound receptor deactivation process, long-term desensitization, may be observed after a prolonged time of agonist exposure; long-term desensitization, which involves a decrease in the net complement of receptors specific for a particular agonist, is subserved by several biochemically distinct mechanisms, including receptor down-regulation (i.e., loss in total cellular content of functional receptors) and internalization.

## B. Heterotrimeric G-Proteins (Large GTPases)

Extracellular signals received by GPCRs are coupled by G-proteins to the regulation of effector enzymes that provoke generation of second messengers. G-Proteins are signal-transducing molecules belonging to a superfamily of proteins regulated by guanine nucleotides. These heterotrimeric proteins have α-, β-, and γ-subunits that are encoded by distinct genes.

G-Proteins are defined by their α-subunits; $G_{\alpha}$-proteins can be divided into four main classes ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$), grouped on the basis of amino acid identity and effector regulation. The $G_{\alpha}$ subunits are highly diverse and their tissue distribution varies, being either ubiquitous (or nearly ubiquitous) or expressed in selected tissues. The $G_{\alpha s}$ class includes $G_{\alpha s}$ and $G_{\alpha olf}$ and is involved in the activation of the various types of the enzyme adenylyl cyclase to enhance the synthesis of the second messenger, cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A; the $G_{\alpha s}$ subunit may also participate in the regulation of $Ca^{2+}$ and $Na^{+}$ channels. The $G_{\alpha q}$ class includes $G_{\alpha q}$, $G_{\alpha 11}$, and $G_{\alpha 14-16}$; proteins of this class are predominantly associated with activation of the enzyme phospholipase $C\beta_{1-4}$, which catalyzes hydrolysis of the lipid phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to form two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). Proteins of the $G_{\alpha q}$ class also activate protein kinase C (PKC). The $G_{\alpha i}$ class includes $G_{\alpha gust}$, $G_{\alpha t}$, $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha z}$, which mediate a variety of effects, such as inhibition of adenylyl cyclase, activation of $K^{+}$ channels, $Ca^{2+}$ channel closure, and inhibition of inositol phosphate turnover. Finally, the $G_{\alpha 12}$ class is defined by $G_{\alpha 12}$ and $G_{\alpha 13}$; signaling pathways regulated by these G-proteins include modulation of the sodium–proton exchanger NHE1 as well as regulation of cell growth and differentiation.

The β- and γ-subunits of G-proteins bind tightly to each other in diverse ways; although this diversity may theoretically yield 30 or more different βγ complexes, βγ dimerization is highly specific, allowing for differential effector regulation by unique $G_{\beta\gamma}$ complexes. Both the $G_{\alpha}$ subunits and the $G_{\beta\gamma}$ dimer play a major role in intracellular signal transduction, and the presence of all components of the heterotrimer is required for a receptor to trigger intracellular signaling.

The G-protein-mediated signaling is initiated on receptor activation (Fig. 2). In the inactive ("off") state, the α-subunit of the heterotrimer is bound to a molecule of guanosine diphosphate (GDP); the GDP-bound $G_{\alpha}$ subunit can interact with receptors, an association that is greatly enhanced by the $G_{\beta\gamma}$ complex. Activation of a GPCR is followed by activation of the trimeric $G_{\alpha/\beta\gamma}$-protein complex by guanine nucleotide. Receptor-promoted and $Mg^{2+}$-dependent GDP → guanosine 5′-triphosphate (GTP)

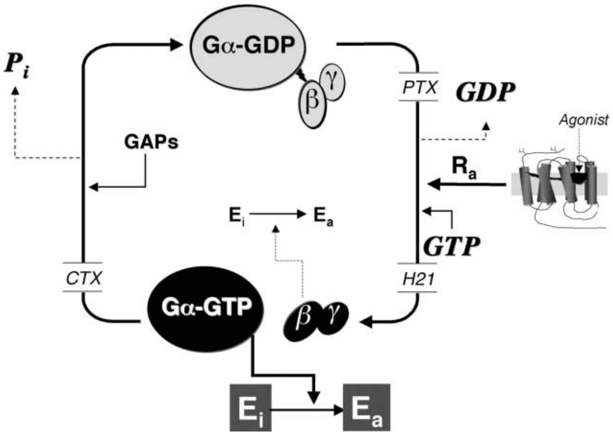

FIGURE 2 Regulatory cycle of a trimeric G-protein. The unoccupied receptor interacts with a specific agonist, leading to the activation of the receptor. Activated receptor ($R_a$) interacts with the trimeric G-protein, promoting $Mg^{2+}$-dependent GDP–GTP exchange and subunit dissociation, allowing interaction with effectors (E). The sites of action of pertussis toxin (PTX) and cholera toxin (CTX) are shown (see text for details). The H21 mutation on $G_{\alpha s}$ blocks signal transduction by preventing GTP activation. Inorganic phosphate ion ($P_i$) is released from GTP. GAPs, GTPase-activating proteins; $E_i$, inactive effector; $E_a$, activated effector.

exchange within the $G_\alpha$ guanine nucleotide-binding site leads to a conformational change of the subunit that causes G-protein activation and dissociation of the trimeric complex into $G_\alpha$-GTP and $G_{\beta\gamma}$ ("on" state). Both of these complexes can then activate or inhibit signaling pathways by engaging in interactions with effectors (enzymes or ion channels); $G_{\beta\gamma}$ activation results solely as a consequence of its release from the $G_{\alpha/\beta\gamma}$ complex. Termination of signaling depends on the intrinsic GTPase activity of the α-subunit, which leads to hydrolysis of GTP to GDP, promoting dissociation of the $G_\alpha$ subunit from effector and reassociation with the βγ dimer, switching the G-protein complex to the "off" membrane-associated state. GTP hydrolysis by $G_\alpha$ is regulated by GTPase-activating proteins (GAPs) specific for a given member of the heterotrimeric G-protein family. Some effectors in G-protein-mediated signaling pathways may act as GAPs on cognate $G_\alpha$ subunits; other proteins, known as *r*egulators of *G*-protein *s*ignaling (RGS proteins), also act as GAPs that selectively and potently deactivate $G_\alpha$ subunits by accelerating the rate of intrinsic GTPase activity.

In addition to involvement in acute signaling functions, GPCRs also induce longer term effects on gene expression and cell proliferation. Signaling pathways involved in cell proliferation are stimulated by several extracellular ligands that transmit signals from the plasma membrane, through the cytoplasmic space, and finally to the nucleus. Similar to tyrosine kinase-linked receptors (see later), GPCRs may activate intracellular signaling in relationship with mitogenic effects. These GPCR-triggered proliferative effects are mediated by $G_\alpha$-proteins of the $G_{\alpha q}$, $G_{\alpha i/Go}$, $G_{\alpha s}$, or $G_{\alpha 12}/G_{\alpha 13}$ class as well as by the corresponding βγ dimers. Growth-promoting GPCRs activate the mitogen-activated protein kinase cascade and ultimately regulate the expression of genes essential for proliferation. This occurs through mechanisms that may or may not involve the small GTP-binding G-protein Ras. In addition, a family of enzymes closely related to MAPK, the JUN kinases (stress-activated protein kinases), selectively phosphorylate and regulate the activity of the c-JUN protein (a transcription factor that induces the expression of genes linked to growth responses) under the influence of certain GPCRs, an effect mediated by the Ras-related small GTP-binding proteins Rac1 and Cdc42.

From the previous discussion, it is clear that structural alterations in key residues of the receptor molecules or the G-proteins may lead to altered function of the GPCR–G-protein system. Thus, mutations in sites involved in ligand binding usually result in altered receptors that are unable to recognize the signaling molecule and to become activated (loss-of-function mutations), whereas mutations in sites involved in receptor activation or G-protein coupling may lead either to loss of function or to constitutive activation (activation in the absence of ligand; gain-of-function mutations) of the receptor molecule. Other mutations may cause improper folding, altered intracellular trafficking, and reduced membrane expression of the receptor molecule. On the other hand, G-protein function may be altered in a number of disease states as a result of both adaptive and maladaptive mechanisms, including mutations in genes encoding G-protein subunits, changes in expression levels of G-protein subunit mRNAs or proteins, or posttranslational modifications of G-proteins. Both somatic and germline mutations in genes encoding GPCRs and G-proteins have been found to cause human disease. Examples of some of these mutations and their impact on intracellular signaling and cell function are discussed in the following sections.

## III. G-PROTEIN-COUPLED RECEPTORS IN DISEASE

Structural alterations in GPCRs lead to abnormal function of the receptor molecule. Diseases caused by such alterations are shown in Table 1.

### A. Rhodopsin/Retinitis Pigmentosa

Rhodopsin is the photoreceptor in rod cells; it mediates vision in dim light and is coupled to the retinal G-protein transducin ($G_t$). Rhodopsin-activated $G_t$ allows light to excite neurons by freeing them from neurotransmitter inhibition. Mutations in rhodopsin lead to misfolding and abnormal trafficking of the nascent receptor protein, causing retention of the mutant protein in the endoplasmic reticulum and death of the rod cells. This causes retinitis pigmentosa, a disease encompassing a clinically variable and genetically heterogeneous group of inherited retinopathies, in which mutated rhodopsin leads to loss of night vision and of the peripheral visual field. Retinal pigmentary changes are characteristic of this disease and result from the release of pigment by degenerating rod cells in the retinal pigment epithelium. Numerous inactivating mutations have been linked to retinitis pigmentosa; mutations in the TM domain, in cysteine residues forming disulfide bonds, or in the carboxyl-terminal domain of the receptor may cause severe or

**TABLE 1 Examples of Diseases Caused by Mutations in G-Protein-Coupled Receptors**

| Disease | Receptor involved[a] | Function[b] |
|---|---|---|
| Retinitis pigmentosa | Rhodopsin | ↓ or ↑ |
| Color blindness | Red/green opsins | ↓ |
| Idiopatic hypogonadotropic hypogonadism | GnRH | ↓ |
| Altered gonadal function | | |
| Precocious puberty | LH | ↑ |
| Leydig cell hypoplasia | LH | ↓ |
| Ovarian dysgenesis | FSH | ↓ |
| Altered thyroid function | | |
| Resistance to TSH | TSH | ↓ |
| Hyperfunctioning thyroid adenoma | TSH | ↑ |
| Multinodular goiter | TSH | ↑ |
| Congenital hyperthyroidism | TSH | ↑ |
| Nephrogenic diabetes insipidus | Vasopressin | ↓ |
| Altered parathyroid function | | |
| Familial (benign) hypocalciuric hypercalcemia | Calcium sensing | ↓ |
| Neonatal severe hyperparathyroidism | Calcium sensing | ↓ |
| Autosomal dominant hypocalcemia | Calcium sensing | ↑ |
| Isolated glucocorticoid deficiency | ACTH | ↓ |
| Hirschsprung's disease | Endothelin-B | ↓ |
| Jansen-type metaphyseal chondrodysplasia | PTH–PTHrP | ↑ |
| Blomstrand disease | PTH–PTHrP | ↓ |
| Dwarfism [little (lit) mouselike] | GHRH | ↓ |

[a] GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; PTH, parathyroid hormone; PTHrP, PTH-related protein; GHRH, growth hormone-releasing hormone.

[b] ↓, Loss of function; ↑, gain of function.

mild forms of the disease, depending on the location of the mutation. Families with a severe phenotype of retinitis pigmentosa carry a mutation at the site (Lys-296) of chromophore (11-*cis*-retinal) attachment; this alteration disrupts the inactive conformation of opsin, resulting in a constitutively active receptor that activates transducin in the absence of external stimulus (light) and the covalently linked chromophore.

## B. Vasopressin Receptor

The function of arginine vasopressin (AVP), a hormone secreted by the posterior pituitary, is to control blood osmolality. AVP controls the reabsorption or water by regulating the number of water channels present in the lumenal surface of the distal tubule and in the collecting duct of the nephron. The antidiuretic effect of AVP is exerted through the vasopressin-2 receptor (V2R), which is linked to the effector enzyme adenylyl cyclase. Inactivating mutations in the V2R lead to nephrogenic diabetes insipidus (NDI), a disease characterized by the inability of the kidney to retain water and concentrate urine. Individuals affected with the X-linked type of NDI bear mutations that alter the structure or expression of the V2R. Vasopressin-2 receptor mutations may be located in either region of the receptor and may interfere with receptor synthesis or with proper AVP binding or efficient coupling to the $G_s$-protein. This latter alteration usually leads to expression of a partial phenotype of NDI.

## C. Gonadotropin-Releasing Hormone Receptor

The receptor for the hypothalamic releasing peptide gonadotropin-releasing hormone (GnRH) is located in the cell surface of the pituitary cells that synthesize the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This receptor is preferentially coupled to the $G_{q/11}$-protein and its activation by GnRH agonists stimulates synthesis and secretion of LH and FSH. Resistance to GnRH by inactivating mutations leads to distinct forms of inherited (autosomal recessive) hypogonadotropin hypogonadism, a disorder characterized by delayed puberty, absence of secondary sexual characteristics, and low gonadotropin and sex-steroid levels. Although inactivating mutations in the GnRH receptor may be distributed along the entire coding sequence of the receptor, they have been mainly localized within the third to fifth TM domains. These mutations affect ligand binding, signal transduction, and/or processing of the receptor, leading to altered intracellular trafficking and reduced cell surface expression. Mutations of the GnRH receptor may result in a wide spectrum of phenotypes, from partial to complete hypogonadism.

## D. Adrenocorticotropic Hormone Receptor

Adrenocorticotropic hormone (ACTH) is an anterior pituitary hormone that, acting via its specific receptor, regulates adrenocortical growth and controls corticosteroid production and secretion. The ACTH receptor (ACTHR) is related to the small subfamily of melanocortin receptors and couples to the $G_s$–adenylyl cyclase pathway. Hereditary isolated glucocorticoid deficiency, a rare autosomal recessive inherited disease, may result from ACTH receptor unresponsiveness to ACTH. Patients with ACTH resistance, either homozygous or compound heterozygous for inactivating ACTHR mutations, typically exhibit deficient production of cortisol and adrenal androgens in the presence of markedly elevated endogenous plasma ACTH levels. Aldosterone levels are usually normal and respond appropriately to maneuvers that activate the renin–angiotensin axis. Inactivating mutations of the ACTHR leading to familial glucocorticoid deficiency may be scattered throughout the ACTH receptor molecule, affecting receptor structure, membrane expression, ligand affinity, and/or signal transduction. The etiology of familial glucocorticoid deficiency might be heterogeneous and genes other than that of the ACTHR might be involved and may cause the same phenotype.

## E. Glycoprotein Hormone Receptors

Glycoprotein hormone receptors have large extracellular domains (300–400 amino acids in length) and bind structurally complex ligands. From the functional point of view, these receptors comprise two halves: an extracellular amino-terminal half (exodomain) and a membrane-associated carboxyl-terminal half (endodomain). Although the exodomain alone is capable of high-affinity ligand binding, interaction with the endodomain [which comprises the seven-TM domains, the three extra- and intracellular loops, the carboxyl-terminal tail, and a short extracellular extension of the first TM domain (Fig. 1)] is necessary to generate and transmit an intracellular signal. In humans, the thyrotropin [thyroid-stimulating hormone (TSH)] receptor and LH receptor are coupled to both the $G_s$–adenylyl cyclase–cAMP pathway and the $G_q$–phospholipase Cβ–phosphoinositide/diacylglycerol pathway, whereas the FSH receptor is predominantly coupled to the $G_s$-protein. All glycoprotein hormone receptors, particularly the TSH and LH receptors, are sensitive to naturally occurring mutations, leading to cell hypo- or hyperfunction.

Thyrotropin is the pituitary glycoprotein hormone that stimulates thyroid development, growth, and function. Thyrotropin receptor gene mutations leading to loss of function of the receptor molecule have been associated with inherited hypothyroidism with TSH resistance; mutations may localize to the amino-terminal region and/or the fourth TM helix of the receptor. Mutations in the amino-terminus usually affect ligand binding, whereas alterations in TM domains may potentially impair receptor membrane expression. Whereas most of the patients with resistance to TSH due to TSH receptor (TSHR) mutations exhibit “compensated hypothyroidism” (i.e., normal serum concentrations of thyroid hormones in the presence of hyperthyrotropinemia), profound hypothyroidism with thyroid hypoplasia may also rarely occur. Reciprocally, mutations leading to constitutive activation of the TSHR and clinical hyperthyroidism may be found at both the somatic and the germ-line levels; the former is usually associated with autonomously functioning toxic (hyperfunctioning) thyroid adenomas, whereas the latter is associated with familial (autosomal dominant) nonimmune hyperthyroidism, a very rare disorder. Both conditions lead to activation of adenylyl cyclase, via $G_s$, increased cAMP production, and eventually to thyroid hyperplasia or expansion of the adenoma. Spontaneous mutations in the TSHR gene leading to hyperfunctioning thyroid adenomas may be located in the TM domains, the extracellular loops, the third intracellular loop, or, rarely, in the amino-terminus (e.g., Arg310Cys). Exceptionally, activating mutations of the TSHR may be found in multinodular goiter.

The target glands for LH are the gonads, where LH stimulates androgen production by the Leydig cells of the testes and the theca cells of the ovarian follicle. This gonadotropin also stimulates estrogen and progesterone production by the corpus luteum. In the inactivating mutations of the LH receptor (LHR) gene, the altered receptor interferes with the intrauterine development of the male external genitalia, causing a wide spectrum of phenotypic alterations, ranging from extreme forms, in which the patients present as 46, XY females (severe Leydig cell hypoplasia), to milder forms associated with hypergonadotropic hypogonadism, microphallus, and hypoplastic male external genitalia. Inactivating LHR mutations may be localized in the amino-terminus, the TM domains, or the third intracellular loop. In women, inactivating mutations in the LHR gene may lead to hypergonadotropic hypogonadism, menstrual disorders, enlarged cystic ovaries, and infertility. In the case of activating LHR mutations, the autonomous activation of the receptor causes sporadic or familial pseudo-precocious puberty, a phenotype observed only in males. Somatic activating mutations of the LHR have been found only in Leydig cell adenomas. Activating mutations of the LHR are usually located in the TM domains or in the third intracellular loop of the receptor.

Follicle-stimulating hormone is involved in the regulation and maintenance of essential reproductive processes, such as gametogenesis and follicular development. The target cells of FSH are the Sertoli cells of the testes and the granulosa cells of the ovary. Inactivating mutations in the FSHR gene have been described in selected populations and in some sporadic cases. In females, inactivating mutations in the FSHR cause gonadal dysgenesis and/or premature ovarian failure, whereas in men mutations may provoke poor quality sperm. Inactivating mutations in the FSHR have been identified mainly in the amino-terminal domain, and less frequently in the third intra- and extracellular loops of the receptor. The five mutations reported so far in the extracellular domains altered binding capacity and signal transduction by disturbing the trafficking of the receptor to the membrane, whereas the intracellular loop-3 mutation altered signal transduction but not membrane receptor expression. Only one naturally occurring activating mutation (located in intracellular loop 3) in the FSHR has been identified so far. Nevertheless, other sites for mutations (artificially-induced; not yet described to occur naturally) that may provoke constitutive activation of the FSHR include a leucine in position 460 in the TM-III residue (a residue highly conserved in $\geq 70\%$ of family A GPCRs) and a leucine in position 477 of intracellular loop 2 (a residue present in all human glycoprotein hormone receptors).

## F. Parathyroid Hormone/Parathyroid Hormone-Related Peptide Receptor

Parathyroid hormone (PTH) plays a critical role in the regulation of calcium homeostasis in kidney and bone. Its actions are mediated by two closely related GPCRs, the PTH1 receptor, which responds equally to PTH and PTH-related peptide (PTHrP), and the PTH2 receptor, which responds to PTH. Agonist occupancy of these receptors leads to activation of both the $G_s$–adenylyl cyclase and the $G_q$–phospholipase C signal transduction pathways. Mutations in the cytoplasmic ends of TM domains II and VI may induce constitutive activity of the PTH/PTHrPR–$G_s$ protein system and thus lead to a rare type of short-limbed dwarfism (called Jansen-type metaphyseal chondrodysplasia) resulting from decelerated chondrocyte differentiation and associated with marked hypercalcemia and hypophosphatemia in the presence of normal serum concentrations of PTH and PTHrP. Parathyroid hormone and PTHrP receptors are also important during fetal skeletal development; signaling through these receptors controls the differentiation of growth plate chondrocytes into hypertrophic cells. Thus, mutations that completely inactivate the PTH/PTHrP receptor may be lethal *in utero*; in fact, mutations resulting in impaired PTH/PTHrP binding (e.g., in the highly conserved Pro-132 amino acid residue of the amino-terminal domain or in the sequence of the fifth TM domain) and in severe receptor dysfunction lead to Blomstrand chondrodysplasia, an autosomal recessive inherited disease characterized by a striking increase in bone density and markedly accelerated skeletal maturation.

## G. Growth Hormone-Releasing Hormone

Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide involved in the regulation of the synthesis and secretion of growth hormone, a pituitary hormone that is responsible of regulating growth. Growth hormone deficiency causes short stature and metabolic derangements. The GHRH receptor (GHRHR) is coupled to the $G_s$–adenylyl cyclase pathway and mutations in the receptor molecule may lead, in humans, to growth failure, analogous to that showed by rodent experimental models bearing a GHRHR gene missense mutation that impairs receptor expression.

## H. Calcium-Sensing Receptor

Extracellular calcium is essential for a large array of vital processes and its concentration in extracellular fluids is under strict control by a homeostatic system that includes the kidney, bones, intestines, and the parathyroid and thyroid glands. Extracellular $Ca^{2+}$ sensing occurs through a receptor coupled to the $G_q$–phospholipase C signaling pathway. Several tissues express this receptor; in the parathyroid gland, the calcium-sensing receptor (CaR) plays a central role in the regulation of PTH secretion. Loss-of-function mutations in the CaR result in two inherited diseases, familial (benign) hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism; the former results from mutations in only one allele of the CaR gene, whereas the latter arises from mutations in both alleles. Mutations in the benign form of the disease are mainly localized either in the first 300 amino acid residues of the amino-terminal domain or in proximity to TM-I of the receptor. These mutations may cause decreased CaR sensitivity for extracellular calcium or may exert dominant negative actions on the normal receptor, leading to a more severe syndrome.

## IV. DEFECTS IN G-PROTEIN-COUPLED SIGNAL TRANSDUCTION

Abnormalities in G-protein function may be caused by: (1) posttranslational modifications of G-proteins by bacterial toxins, (2) mutations (either loss- or gain-of-function mutations) in genes encoding G-proteins, and (3) changes in expression levels of G-protein subunit mRNA or functional protein. Examples of structural alterations in G-proteins leading to abnormal signal transduction are shown in Table 2.

### A. Posttranslational Modifications—Cholera Toxin and Pertussis Toxin

Cholera toxin causes adenosine diphosphate (ADP) ribosylation of an arginine residue in position 201 within the GTP-binding domain of $G_{\alpha s}$, markedly reducing the intrinsic GTPase activity of the subunit (Fig. 2), leading to constitutive activity of the protein and increased levels of cAMP independent of the normal extracellular signal. Infection by *Vibrio cholerae* affects the intestinal tract, causing excess fluid secretion by the epithelial cells and massive diarrhea. Pertussis toxin, produced by *Bordetella pertussis*, covalently modifies several subunits of the $G_{\alpha i}$ class by ADP-ribosylation on the fourth cysteine residue from the carboxyl-terminus of the protein, leading to uncoupling of the modified G-protein from the receptor and disruption of signal transduction; this is the mechanism whereby exposure to pertussis toxin causes hypoglycemia and histamine sensitivity.

### B. Mutations in the $G_{\alpha}$-Protein

Germ-line and somatic mutations of the human *GNAS1* gene, located on chromosome 20q13.11, which encodes the $G_{\alpha s}$ protein, have been implicated in abnormal signal transduction and in several clinical disorders. Activating (oncogenic) mutations in this G-protein subunit (specifically in Arg-201 or Gln-227, which lead to inhibition of GTPase intrinsic activity) have been identified as the cause of several disorders, including subsets of growth hormone-secreting pituitary tumors, testicular and ovarian stromal Leydig cell tumors, toxic thyroid adenomas, and the McCune–Albright syndrome, a sporadic disease characterized by increased hormone production and/or cellular proliferation in a number of tissues. Less frequently, $G_{\alpha s}$ mutations may lead to nonfunctioning pituitary and thyroid adenomas, ACTH-secreting adenomas, parathyroid neoplasms, and differentiated thyroid carcinomas. Screening studies of different types of human tumors for mutations in $G_{\alpha i(2)}$ have revealed similar amino acid substitutions in a proportion of ovarian, adrenal, and nonfunctioning pituitary tumors. Conversely, heterozygous germ-line *GNAS1* gene mutations that decrease expression or function of $G_{\alpha s}$ cause Albright hereditary osteodystrophy, a disorder associated with a constellation of developmental defects (including obesity, short stature, bony abnormalities, and mild mental retardation), as well as reduced responsiveness to multiple hormones (including PTH, TSH, and glucagon). Altered expression and/or function of G-proteins may be found in a number of other abnormal conditions, including neuropsychiatric disorders, alcoholism, hypertension, and diabetes mellitus. Defects in G-proteins in such disorders may also be secondary to other alterations in cell function.

## V. ENZYME-LINKED RECEPTORS IN HEALTH AND DISEASE

More than 50 receptors belong to the superfamily of enzyme-linked receptors, including the receptors for growth hormone, insulin, cytokines, and growth factors. The enzyme-coupled receptor superfamily can be separated into the following main families: (1) tyrosine kinase receptors, (2) tyrosine kinase-associated receptors, (3) tyrosine phosphatase receptors, (4) guanylyl cyclase receptors, and (5) serine/threonine kinase receptors.

### A. Tyrosine Kinase and Tyrosine Kinase-Associated Receptors

Tyrosine kinase receptors are membrane-spanning proteins with large amino-terminal extracellular domains bearing the ligand binding site, a juxtamembrane domain, a protein kinase catalytic domain, and

TABLE 2 Examples of Diseases Caused by Mutations in $G_\alpha$-Proteins

| Disease | G-protein involved/(mutation) | Function[a] |
|---|---|---|
| *Vibrio cholerae* (cholera toxin) | $G_{\alpha s}$ ADP-ribosylation | ↑ |
| *Bordetella pertussis* (pertussis toxin) | $G_{\alpha i}$ ADP-ribosylation | ↓ |
| Albright hereditary osteodystrophy, pseudohypoparathyroidism type 1a | $G_{\alpha s}$ | ↓ |
| Nonfunctioning pituitary adenoma, adrenal and sex cord tumors | $G_{\alpha i2}$ (Arg179Cys/His or Gln205Arg) | ↑ |
| McCune–Albright syndrome | $G_{\alpha s}$ (Arg201His/Cys) | ↑ |
| Growth hormone-secreting tumors | $G_{\alpha s}$ (Arg201His/Cys or Gln227Leu/Arg) | ↑ |
| Nonfunctioning pituitary adenomas, ACTH-secreting adenoma, toxic thyroid adenoma, papillary thyroid carcinoma | $G_{\alpha s}$ (Arg201His/Cys or Gln227Leu/Arg) | ↑ |
| Pseudohypoparathyroidism type 1a and testotoxicosis | $G_{\alpha s}$ (Ala366Ser) | ↓/↑ |
| Nonfunctioning pituitary adenoma, adrenal and sex cord tumors | $G_{\alpha i2}$ | ↑ |

[a] ↓, Loss of function; ↑, gain of function.

a carboxyl-terminal tail. Based on the structure of their extracellular domains, members of this class of membrane receptor can be grouped into 16 subfamilies, which include the platelet-derived growth factor receptor, the fibroblast growth factor (FGF) receptor, the epidermal growth factor (EGF) receptor, the insulin receptor, the nerve growth factor receptor, the hepatocyte growth factor (HGF) receptor, and the vascular endothelial growth factor receptor subfamilies. Receptors belonging to this superfamily dimerize on ligand binding; dimerization is then followed by auto- or transphosphorylation of the receptor, which then interacts with associated adapter proteins or effector enzymes bearing SH2 domains, leading to the activation of a variety of signaling pathways and nuclear factors (Fig. 3). Protein kinase-associated receptors, such as the growth hormone and prolactin receptors, associate with tyrosine kinases belonging to the JAK kinase (for Janus kinase) family. Members of this family phosphorylate target proteins on ligand binding and receptor dimerization, eventually leading to gene transcription; target proteins include the Stat (signal transduction and activation of transcription) proteins. Abnormal or ligand-independent activation of the signaling cascades mediated by tyrosine kinase or tyrosine kinase-associated receptors may lead to altered cell proliferation and tumorigenesis (e.g., some mutated forms of the EGF and HGF receptors) or to severe skeletal dysplasias due to activation of cell cycle inhibitors (e.g., those induced by mutations in the FGF receptor), whereas inactivating mutations may cause abnormal embryogenesis (e.g., the *patch* mutation that affects neural crest development in mice) or to a variety of disorders, such as severe insulin resistance (e.g., leprechaunism and the Rabson–Mendenhall syndrome) and dwarfism (e.g., growth hormone receptor mutations), depending on the particular receptor involved.

## B. Tyrosine Phosphatase Receptors

Protein tyrosine phosphorylation and dephosphorylation are mechanisms crucial for the regulation of numerous cellular events. Receptor-like protein tyrosine phosphatases (PTPs) are ligand-regulated phosphatases that participate in intracellular signal transduction by countering the activities of receptors with tyrosine kinase activity. Receptor-like PTPs are subdivided into five types based on common features exhibited by their extracellular domains: (1) type I receptor-like PTPs, represented by the hematopoietic cell-restricted CD45 family (with its corresponding isoforms); (2) type II molecules, such as the LAR-like PTPs [e.g., LAR, PTPσ, and PTPδ in mammals, preferentially expressed (with the exception of LAR) in neurons and implicated in neuronal development], which contain tandem repeats of immunoglobulin-like and fibronectin type III-like domains resembling neural cell adhesion molecules; (3) type III molecules, which exhibit fibronectin type III repeats; (4) PTPα and PTPε, which have a small extracellular domain; and (5) PTPξ and PTPγ, which exhibit amino-terminal carbonic anhydrase-like domains. Many PTPs may be involved in growth defects and diseases. For example, CD45, a PTP receptor present in lymphoid cells, has been shown to be necessary for multiple signaling events in both B and T cells; loss of CD45 in mice has profound consequences for

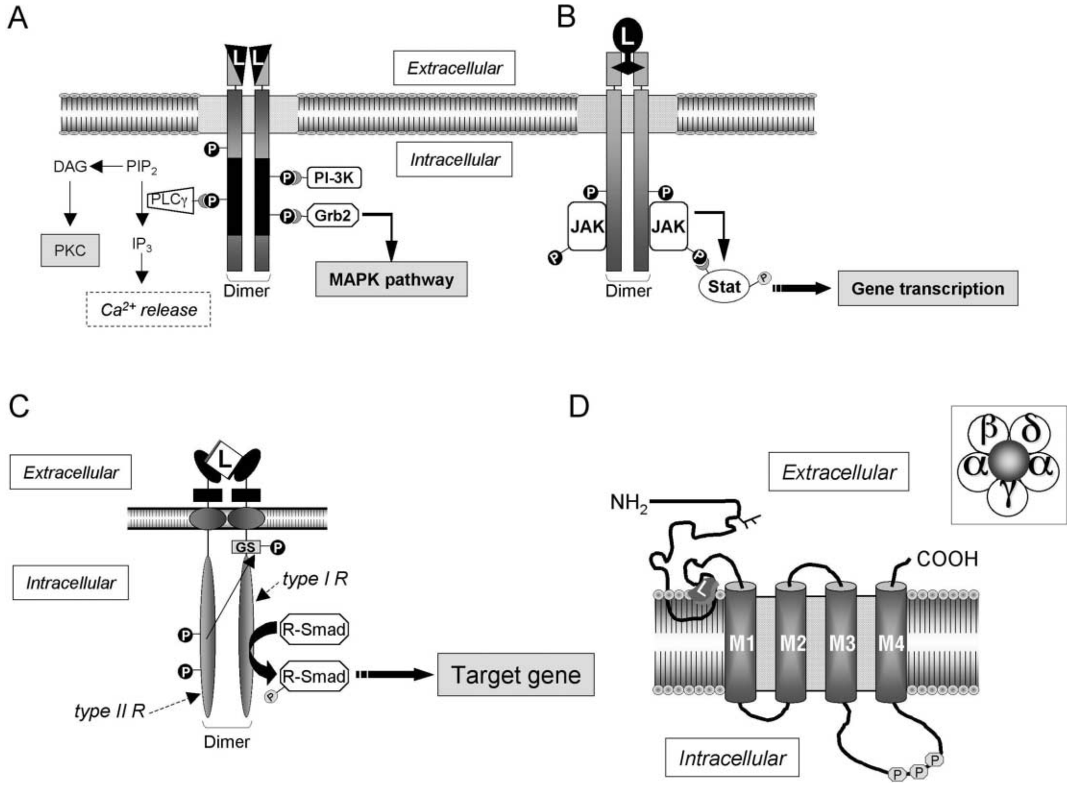

**FIGURE 3** (A) Signaling through tyrosine kinase receptors. Ligand (L) (e.g., epidermal growth factor) binding provokes dimerization of the liganded receptor; this is followed by activation of the receptor's intrinsic tyrosine kinase, leading to transphosphorylation of the receptors, docking of SH2 domain-bearing proteins [including Grb2 (growth factor receptor-binding protein 2), phopholipase Cγ (PLCγ), and phosphatidylinositol 3-OH kinase (PI-3K)], and activation of multiple signaling pathways. (B) Activation of intracellular signaling by tyrosine kinase-associated receptors. Ligand (e.g., growth hormone) binding provokes receptor dimerization, recruitment of JAK tyrosine kinase, and subsequently tyrosine phosphorylation of JAK, the receptor, and the Stat (for signal transduction and activation of transcription) proteins; Stats then dimerize and translocate to the nucleus to induce transcription of specific target genes. (C) Signaling through serine/threonine kinase receptors [e.g., the transforming growth factor-β receptor (TGF-βR)]. Binding of TGF-β to its type II receptor in concert with a type I receptor (R) leads to formation of a receptor complex and phosphorylation of the type I receptor, which subsequently phosphorylates a receptor-regulated SMAD (R-Smad) protein. The SMAD, complexed with Smad4, moves into the nucleus and activates TGF-β target genes. (D) Putative transmembrane organization of a ligand-gated ion channel [e.g., the nicotinic acetylcholine receptor (AChR)], showing the four-transmembrane model for this receptor. M, Transmembrane domain; P, potential phosphorylation sites. Inset*:* Front view of the model of peripheral (muscle) AChR with a pore at the center of the pentamer.

lymphocyte development and signal transduction. Targeted deletion of a LAR-like PTP in mice leads to abnormal neonatal death rates, and targeted homozygous disruption of the *Ptprs* gene (which encodes PTPσ) causes multiple growth defects, including stunted growth, developmental delay, and several neurological disorders.

## C. Guanylyl Cyclase Receptors

The single-transmembrane-domain-signaling guanylyl cyclase (GC) receptors have cytoplasmic domains bearing guanylyl cyclase activity; on activation by their cognate ligands, the receptors promote the production of the second messenger cyclic guanosine monophosphate (GMP), which in turn binds and activates a cyclic GMP-dependent protein kinase known as G-kinase, subsequently triggering serine or threonine phosphorylation on specific proteins. The guanylyl cyclase A and B receptors bind natriuretic peptides (involved in the regulation of cardiovascular and renal function), whereas the GC C receptor binds heat-stable enterotoxins. The orphan GC D, E, and F receptors are expressed in sensory tissues. Nearly 29 genes encoding putative guanylyl cyclases have been discovered in the nematode *Caenorhabditis elegans*, which suggests that numerous guanylyl cyclase receptors still remain to be discovered in mammals. Salt-independent chronic elevation of blood pressure, which corresponds to the phenotype observed in nearly 50% of subjects with essential hypertension, is found in GC A gene-disrupted mice. On the other hand, mice missing the GC C receptor are resistant to Sta, a heat-stable enterotoxin from *Escherichia coli* that is thought to be responsible for diarrhea in adults and infants.

## D. Serine/Threonine Kinase Receptors

Transmembrane receptors with serine and threonine kinase activity are distinct molecules that bind members of the transforming growth factor-β (TFG-β) superfamily of related polypeptide growth factors (including TFG-$\beta_{1-3}$, bone morphogenetic proteins, anti-Müllerian hormone, and activins) that are involved in a large array of cellular processes, such as growth, proliferation, differentiation, lineage determination, apoptosis, adhesion, and motility. The TFG-β receptor family consists of two subfamilies, type I receptors (formerly known as activin receptor-like kinases) and type II receptors. The TFG-β receptor-mediated intracellular signaling is initiated when the TFG-β type II receptor, which is basally phosphorylated in a ligand-independent manner, binds its ligand and activates via phosphorylation at serine and threonine residues (present within the GS domain, a highly conserved 30-amino acid region) an associated TFG-β type I receptor (Fig. 3). The activated TFG-β receptor complex then phosphorylates and activates proteins of the SMAD family, which in a complex form move into the nucleus and activate TGF-β-responsive genes. Down-regulation or loss of serine/threonine kinase functional receptors, aberrant signal–signal transduction pathways due to Smad alterations, mutations in ligand, or loss of functional genes that control the transcription and translation of TGF-β and TGF-β-related peptides may contribute to the development of several diseases. Because the effects of TGF-β on target cells include negative regulation of cell proliferation, disruption of TGF-β signaling could therefore predispose or even cause cancer (e.g., gastrointestinal cancer, head and neck carcinomas, ovarian cancer, and T-cell lymphoma). Mutations in the anti-Müllerian hormone or its receptor lead to persistent Müllerian duct syndrome, whereas mutations in Gdf5/Cdmp1 (a ligand belonging to the growth and differentiation factor subfamily of TGF-β) result in hereditary chondrodysplasia. Likewise, mutations in Alk1 (a type I receptor) may lead to hereditary hemorrhagic telangiectasia. Finally, SMAD mutations are associated with colon cancer (Smad2 and Smad4), pancreatic cancer (Smad4), and other cancers.

## VI. ION-CHANNEL-LINKED RECEPTORS IN HEALTH AND DISEASE

Channels are pores in the cell membrane; ions flow through these pores across the membrane, depolarizing or hyperpolarizing the cell. The ligand-gated ion channels are grouped into two major functional families: (1) the nicotinic acetylcholine (ACh), serotonin, and glutamate-gated ion channels, which allow passage of cations ($Ca^{2+}$) at excitatory synapses, and (2) the GABA A and GABA C receptor (GABA B receptors are GPCRs) and the glycine-gated channels, which permit passage of anions at inhibitory synapses. At the amino acid sequence level, the nicotinic ACh, serotonin, GABA, and glycine-gated channel subunits are homologous; these receptors assemble as heteropentamers of different gene products and/or splice variants. The glutamate-gated channels differ from the other amino-acid-gated channels in that they are composed of four subunits, each containing three (instead of four) TM segments; these channel receptors may be subdivided according to agonist selectivity into the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainate subtypes. The TM segments of these multisubunit receptors are arranged around a large, poorly selective central pore, the properties of which are essentially determined by the amino acid residues forming the second TM segment. In these receptors, ligand binding provokes a transient open-channel state, during which passive flux occurs. The nicotinic ACh receptor is the model for structure–function relationship studies on the superfamily of ligand-gated ion channel receptors and is the best known from the structural and functional points of view. This receptor has an $\alpha_2,\beta(\delta\gamma)$ or $(\epsilon\delta)$ pentameric composition; each subunit has a large extracellular amino-terminal domain, four TM domains, a large intracellular loop between the third and fourth TM domains, and an extracellular carboxyl-terminal domain (Fig. 3). The functional events associated with the ACh receptor include (1) binding of the neurotransmitter, (1) opening of the channel via rotation of two pore-lining α-helices, which moves the helices apart, (3) conduction across the pore, and (4) desensitization.

Ligand-gated ion channels in the neuronal plasma membrane function as effective, cell surface signal transducers via interactions with a number of extracellularly or intracellularly stimulated protein kinases. Channel phosphorylation may gate the channel, may facilitate interaction with other regulatory PDZ domain-bearing proteins (linking the receptor channel to the cytoskeleton and to appropriate intracellular signal transduction pathways, which may eventually lead to gene expression), and/or may regulate receptor desensitization and clustering. Current evidence demonstrates that ligand-gated cation channels may be additionally located presynaptically on nerve terminals in the peripheral and central nervous systems, where they function to modulate neurotransmitter release. Alterations in the primary sequence leading to modification in gating kinetics may associate with pathological processes or channelopathies. For example, mutation of the extracellular charged arginine ring in the glycine receptor can impair channel function by decreasing the sensitivity of glycine activation, reducing channel conductance, shifting the normal multisubconductance states to lower values, and decoupling ligand binding from channel gating. Mutations in the $\alpha_1$-subunit of the glycine receptor lead to hyperekplexia (excess startle response), whereas structural alterations in the Ach receptor may cause frontal lobe nocturnal epilepsy (as in those mutations involving the $\alpha_4$-subunit, which may eventually lead to different alterations in the properties of the ACh-gated channel) or myasthenic syndromes (e.g., slow-channel syndrome). The latter abnormalities arise from delayed closure of the ion channel, ACh receptor deficiency, and short channel open time; these are kinetic abnormalities that lead to high conductance or abnormal interactions of the ligand with its receptor.

## VII. SUMMARY

Cell surface receptors are signal transducers for water-soluble extracellular signals. There are three main classes of cell surface receptors: G-protein-coupled receptors, enzyme-coupled receptors, and ion-channel-linked receptors. Each class of receptor mediates the signals carried by extracellular ligands through particular mechanisms, most of them involving the intracellular activation of particular protein–protein interaction cascades. G-Protein–coupled receptors mediate their intracellular actions through the activation of G-proteins and specific effector enzymes, whereas enzyme-linked receptors function directly as enzymes or through associated enzymes that, on activation, autophosphorylate or phosphorylate the receptor molecule, subsequently recruiting associated proteins that act as intracellular signal transducers. Ion-channel-linked receptors are protein molecules that convert chemical signals into a charge flux across the cell membrane by opening an intrinsic pore, through which ions flow. Membrane receptors and their associated signal transducers may be altered in a number of diseases as a result of adaptive or maladaptive mechanisms, including mutations in their encoding genes.

## Glossary

**domain** Portion of a protein that has a tertiary structure of its own. In large proteins, each domain is connected to other domains by flexible regions of polypeptide.

**effector** Molecule that performs an action in response to a stimulus.

**guanosine 5′-triphosphate** Nucleoside triphosphate used in RNA synthesis and in some energy-transfer reactions. It also plays a special role in protein synthesis, cell signaling, and microtubule assembly.

**ligand** Any molecule that binds to a specific site on another molecule.

**neurotransmitter** Small signaling molecule secreted by the presynaptic nerve cell at a chemical synapse; relays a signal to the postsynaptic cell. A neurotransmitter can elicit either excitatory or inhibitory responses on the target synapse.

**protein kinase** Enzyme that transfers the terminal phosphate group of adenosine triphosphate to a specific amino acid of a target protein.

**protein phosphatase** Enzyme that removes a phosphate group from a protein by hydrolysis.

**receptor** Protein that binds a specific extracellular signaling molecule and initiates or blocks a response in the cell.

**second messenger** Small molecule that is formed in or released into the cytosol in response to an extracellular signal; helps to relay the signal to the interior of the cell.

**signaling molecule** Extracellular or intracellular molecule that cues the response of a cell to the stimulus of other cells.

**signal transduction** Process whereby a cell converts an extracellular signal into a biochemical response.

## See Also the Following Articles

**Heterotrimeric G-Proteins • Membrane Steroid Receptors • Multiple G-Protein Coupling Systems • Protein Kinases**

**• Receptor-Mediated Interlinked Systems, Mathematical Modeling of • Receptor–Receptor Interactions • Signaling Pathways, Interaction of**

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# Membrane Steroid Receptors

Clara M. Szego*, Richard J. Pietras*, and Ilka Nemere†

**University of California, Los Angeles,* • †*Utah State University*

I. INTRODUCTION
II. SUPRAMOLECULAR ORGANIZATION OF THE SURFACE MEMBRANE AND OCCURRENCE OF STEROID RECEPTORS
III. SPECIFIC BINDING OF STEROID HORMONES TO SURFACE MEMBRANES OF RESPONSIVE CELLS
IV. CONSEQUENCES OF RECEPTOR OCCUPANCY: ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS
V. MEMBRANE SIGNALING AND THE CELLULAR RESPONSE TO STEROID HORMONES
VI. SUMMARY

**Mutual recognition between a responsive cell and a hormone in the extracellular fluid takes place at their dynamic boundary, the cell surface membrane. This fundamental process, applicable to agonists of diverse structure, lipid as well as peptide, leads to a chain of secondary mechanisms that amplify the impact of selective interception of hormone by receptor. It is now possible to integrate this primary step in the coordinated events that constitute the cellular response.**

## I. INTRODUCTION

It seems axiomatic that mutual recognition between an agonist in the extracellular fluid and a responsive cell must take place at the cell surface membrane. As first envisioned in the immunologic context by Paul Ehrlich (Fig. 1), extracellular hormones of varied structure, lipid as well as peptide, are now understood to interact with receptors at the outer cell membrane. Examples of the lines of evidence that support this concept, and the criteria for identifying the selectivity, specificity, and affinity of such interaction between the several steroid classes and the specialized protein components of the target cell surface, are presented in this article.

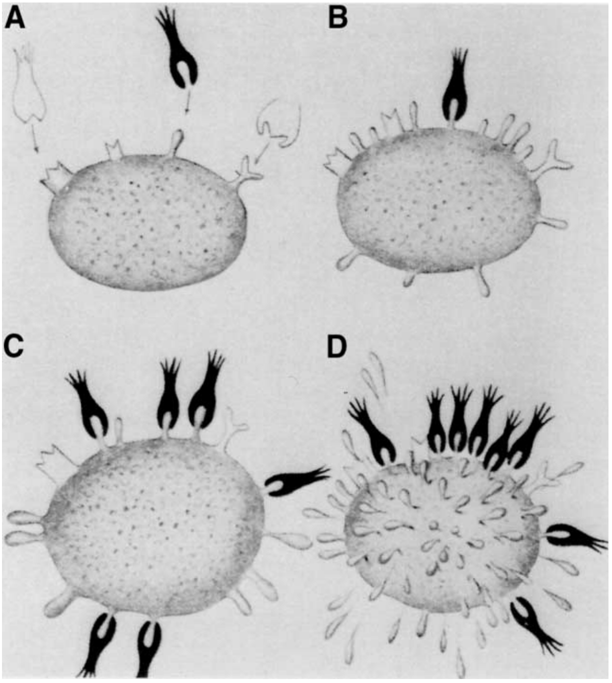

FIGURE 1 Diagrammatic representation of the "side chain" theory to illustrate Ehrlich's concept of specific recognition sites at the cell surface. (A) Complementarity of agonist and receptor. (B) Specific and reversible binding of agonist only to its own receptor. (C) The bound form of receptor is unavailable for providing negative feedback toward its own biosynthesis. (D) This results in overcorrections by regeneration. Reprinted with permission from Ehrlich (1957), with minor paraphrasing of the caption, from the Croonian Lecture, delivered to the Royal Society on 22 March, 1900.

Although the cellular actions of steroid hormones were once postulated to be regulated exclusively by receptors in the cell nucleus, thus permitting selective transcription after ligand binding, this genomic mechanism generally requires hours or days before the effects of hormone exposure are evident. In addition to the latter pathway, steroids also elicit rapid cell responses within seconds of administration. The time course of these acute events lends support to the conclusion that they do not require new gene transcription. Rather, many rapid effects of steroids, termed "nongenomic," appear to be due to specific recognition of hormone at the cell membrane. Hormone–receptor interactions at the surface membrane can initiate a cascade of signaling events that may regulate many cellular functions, both acute and prolonged.

Entry of a steroid hormone into its target cell can be astonishingly swift and requires special strategies to demonstrate its temporal dissociation from binding proper. In some cases, receptor-mediated entry appears to be closely followed by partition into the several intracellular compartments. The available means of such ultrarapid penetration and dissemination are outlined in the following discussions, as is the potential significance of sequential translocation in the overall cellular response. Accordingly, the proportion of total receptor, localized at a given moment in any cellular compartment, whether plasmalemmal, cytostructural, or nuclear, reflects the metabolic history of the receptive cell.

Finally, it is the purpose of this article to survey the transduction mechanisms available to a receptive cell for amplifying and extending the impact of initial surface perturbation by hormone capture. Through such means of communication of the primary hormonal signal, the resultant structural and functional modulations of the several intracellular compartments, including the nuclear compartment, can be coordinated into the totality of the cellular response.

## II. SUPRAMOLECULAR ORGANIZATION OF THE SURFACE MEMBRANE AND OCCURRENCE OF STEROID RECEPTORS

Steroid uptake in cells may occur by passive or facilitated diffusion across the plasma membrane or by one of several endocytotic mechanisms. Biophysical studies demonstrate that most steroid hormones are lipophilic molecules that partition deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of receptor proteins. However, steroid hormone agonists also appear to enter target cells by a membrane-mediated process that is saturable and temperature dependent.

### A. Membrane Models: from Fluid Mosaic to Lipid Rafts and Signaling Platforms

To understand the nature of steroid receptor association with cell membranes, it is important to consider current concepts of supramolecular organization of the membrane (Fig. 2). The present view of the lateral organization of plasma membrane constituents has been revised significantly from the original fluid mosaic model, wherein membrane proteins were considered to diffuse freely in a sea of lipid, above a critical temperature of 15 °C. With the wide array of molecules known to interact rapidly in receptor signaling, it is difficult to imagine how specific signal transduction could occur if components moved randomly in the lipid bilayer. Rather, new findings suggest the existence of membrane macro- and

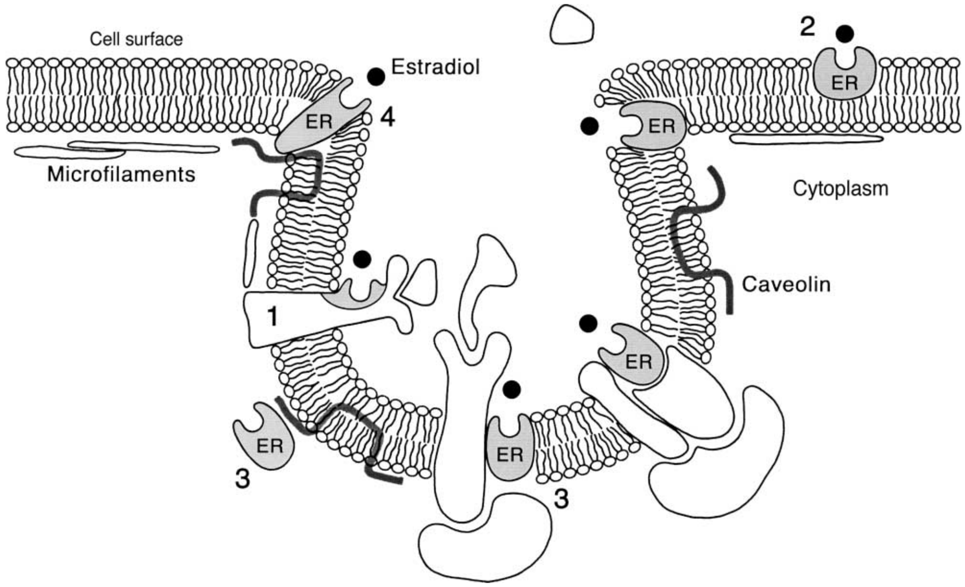

**FIGURE 2** Supramolecular organization of plasma membrane and occurrence of estrogen receptors. A model of the surface membrane from an estrogen-responsive cell in the region of a caveolar structure is depicted. Estradiol may interact with one of several different forms of membrane-associated estrogen receptors (ERs). The precise physical and full structural characterization of these molecules remains to be established. The molecules may be known membrane components, such as enzymes, G-proteins, ion channels, or receptors for nonsteroid ligands, with previously unrecognized binding sites for steroids (1); new isoforms of steroid hormone receptors (2); "classical" receptors complexed with other membrane-associated proteins (3); or novel membrane proteins (4). Similar to the ER, the androgen receptor colocalizes with caveolin-rich membrane fractions from target cells, and the androgen receptor directly interacts with caveolin-1 in an androgen-dependent process, providing evidence for a potential physiological role of this interaction. Of note, alternatively spliced transcripts of several steroid receptors occur, and these variant receptors give rise to proteins of different molecular size and, possibly, to modified properties. Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions. ER-α, for example, contains several hydrophobic regions, but it is unknown whether these are sufficient for disposition as an integral membrane protein. Posttranslational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation, and/or addition of lipid anchors or other alterations, such as palmitoylation or myristolation.

microdomains that serve to concentrate key signaling molecules for efficient coupling to effectors. The concept of a "signaling platform" has been advanced to characterize a structure in which many different membrane-associated components are assembled in a coordinated fashion.

Evidence now indicates that plasma membrane microdomains, termed "lipid rafts," arise from the phase behavior of lipid components. In the fluid bilayer of the membrane, different lipid species are asymmetrically distributed over exoplasmic and cytoplasmic leaflets of the membrane. In particular, long, saturated acyl chains of sphingolipids cluster in the presence of cholesterol to form a liquid-ordered phase, resistant to detergent solubilization. Saturated acyl chains of glycosylphosphatidylinositol (GPI)-anchored proteins, as well as transmembrane proteins and certain tyrosine kinases, can also occur within these lipid domains. Raft association may concentrate receptors for interaction with ligands and effectors on either side of the membrane, thus facilitating binding during signaling and suppressing inappropriate cross talk between otherwise conflicting signal transduction pathways.

## B. Endocytotic Adaptations

Caveolae, literally "little caves," are more specialized raft microdomains that also concentrate and assemble components of several signal transduction pathways (Fig. 2). These membrane structures can be invaginated, flat within the plane of the membrane, detached vesicles, or may be fused together to form grapelike structures and tubules (Fig. 3). Like lipid rafts, caveolae are rich in cholesterol and sphingolipids, but, unlike rafts, they are lined intracellularly with clusters of caveolin protein, a cholesterol-binding molecule that contributes to membrane lipid

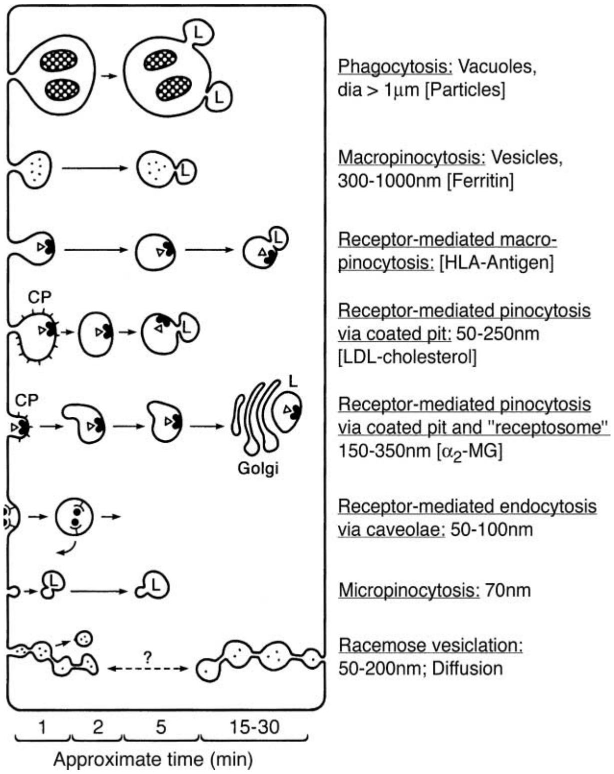
L
Phagocytosis: Vacuoles, dia > 1μm [Particles]
Macropinocytosis: Vesicles, 300-1000nm [Ferritin]
Receptor-mediated macro-pinocytosis: [HLA-Antigen]
CP
Receptor-mediated pinocytosis via coated pit: 50-250nm [LDL-cholesterol]
CP
Golgi
Receptor-mediated pinocytosis via coated pit and "receptosome" 150-350nm [α2-MG]
Receptor-mediated endocytosis via caveolae: 50-100nm
Micropinocytosis: 70nm
?
Racemose vesiclation: 50-200nm; Diffusion
1
2
5
15-30
Approximate time (min)

**FIGURE 3** Schematic representation of pathways for the internalization of extracellular agonists. Revised from Szego and Pietras (1984), with permission.

organization. The growing list of caveolae-associated molecules constitutes a "who's who" of cell signaling, including receptor tyrosine kinases, G-protein-coupled receptors, protein kinase C, components of the mitogen-activated protein (MAP) kinase pathway, and endothelial nitric oxide synthase (eNOS). In one such example, subpopulations of estrogen receptors are localized to caveolae in endothelial cells, and, in plasma membrane caveolae isolated from these cells, estradiol directly stimulates its receptors, which are coupled to eNOS in a functional signaling module to regulate the local calcium environment and blood vessel contractility.

Clathrin-coated pits are independent membrane invaginations, decorated intracellularly with the protein clathrin. They function in endocytosis of nutrients and certain receptors, such as in receptor-mediated uptake of low-density lipoprotein–cholesterol complexes, and also play an important role in signal transduction. Some agonists may be internalized via either clathrin-coated pits or caveolae, with one pathway apparently providing a default entry mechanism for the other under certain conditions.

Raft-dependent signaling is often coupled with endocytotic uptake mechanisms involving rafts as well as caveolae. Also important in this scheme is the actin cytoskeleton, considered to provide constraints for lateral mobility of lipid microdomains and to function in endocytotic trafficking. Endocytosis is a diverse set of processes that promote internalization of specialized regions of the plasma membrane as well as small amounts of extracellular fluid (Fig. 3). The best understood form of endocytosis occurs at clathrin-coated pits and involves clathrin and the dynamin GTPase, which promotes pinching-off of the endocytotic vesicle. Caveolae also play an important role in potocytosis, a mechanism for uptake of small molecules across the plasma membrane. Finally, some cell types can internalize larger amounts of fluid by macropinocytosis or can take in particulates by phagocytosis (Fig. 3). In most cells, internalized materials are first delivered to early sorting endosomes, which may mature into or be transferred to late endosomes, and, ultimately, to lysosomes. The potential role of the ubiquitin–proteosome pathway in this process remains to be determined.

## C. Steroid Receptor Variability

The precise nature of the association of steroid receptors with plasma membranes remains elusive, primarily because full structural characterization of these molecules is incomplete. The task of identifying these membrane-associated steroid receptors is made more challenging by the recent detection of multiple transcript variants of classical "intracellular" steroid receptors, and, in the case of estrogen receptor-α (ER-α), by discovery of a structurally related estrogen receptor form, estrogen receptor-β (ER-β), which is the product of a different gene. Both ER-α and ER-β gene products are expressed in membranes, and both receptors are capable of activating acute and late phases of cellular responses through activation of signal transduction cascades.

Estrogen receptor from target cell plasma membranes is a protein species with high-affinity, saturable binding specific for estradiol. In addition, antibodies to nuclear ER-α recognize surface sites, suggesting that membrane ER has antigenic homology with nuclear ER. Indeed, recent work reveals that membrane and nuclear ERs may be derived from a single transcript. Likewise, properties of membrane glucocorticoid receptors closely resemble those of the intracellular receptor. On the other hand, properties of the aldosterone receptor, as well as those of the plasma membrane receptor for 1,25$(OH)_2$vitamin $D_3$, suggest that membrane receptors for these steroids may be distinct from their "classical" intracellular counterparts. Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies or variants of nuclear receptors, and, in other instances, are products apparently unrelated to these.

Steroid receptors in membranes may also be contained in multimeric complexes with other transmembrane molecules coupled to specific signaling cascades (Fig. 2). In the case of retinoic acid, there may be binding to known membrane proteins, such as mannose 6-phosphate/insulin-like growth factor-II (IGF-II) receptors. Likewise, progesterone congeners bind with moderate affinity to γ-aminobutyric acid type A ($GABA_A$) receptors that comprise ligand-gated ion channel complexes, and pharmacologic levels of estradiol bind with regulatory subunits of independent ion channels in membranes, thus supporting the view that some effects of steroid hormones, at least at high concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites. Finally, despite subtotal ER-α gene knockout, some rapid actions of estradiol still prevail. As with the mixed steroid hormone-binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of protein receptors for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Nevertheless, available evidence suggests that a finite portion of cellular steroid receptors is associated with signaling platforms in specialized microdomains of the plasma membrane.

## III. SPECIFIC BINDING OF STEROID HORMONES TO SURFACE MEMBRANES OF RESPONSIVE CELLS

As postulated by Ehrlich in the Croonian Lecture to the Royal Society more than a century ago, the outer surface of a responsive cell is equipped with specialized components, which exhibit exquisite discriminatory capacity toward potential agonist when molecular conformations are mutually complementary (cf. Fig. 1). Indeed, in evolutionary terms, steroid recognition at the surface membrane appears to have been the primary response pathway of the primitive cell. In plant cells, the only known response pathway to steroids is via a membrane-associated receptor that regulates numerous functions in the intracellular economy, including growth and development. In the case of steroid hormones that influence the functions of eukaryotic cells, the fact that such receptor molecules are poised to extract agonist from its plasma protein carrier is directly attributable to primary evidence for noncovalent, and thus reversible, steroid–protein interaction. This property forms the basis for competitive displacement of ligand by excess, or by conformationally competent congeners.

The concept of specific membrane-associated binding sites for steroid hormones has been supported by rigorously controlled observations from many independent laboratories. Evidence is now available for the extended steroid family, which includes the retinoids, thyroid hormone, and digitalis-like steroids (cf. Table 1). The methodologic approaches have also been broad. Representative examples of several of these approaches for estrogen are presented in Figs. 4–6. However, comparable observations are available for other members of the steroid family, especially adrenocortical steroids and vitamin D metabolites (Table 2). Thus, from physical, ultrastructural, immunologic, and molecular probes, as well as direct kinetic analyses of specific binding of isotopically labeled steroid to the surfaces of isolated target cells or to their purified plasma membrane fractions, a large body of evidence now supports this view. Such membrane proteins constitute a fraction of total receptor molecules available at any given moment in the cellular target and have occasionally been overlooked when methods of sufficient sensitivity were not utilized and when signal-to-noise ratio was not taken into account. Especially instructive data are now available for pinpointing the surface orientation of specific receptor proteins for given steroid hormones at their cellular targets (Figs. 4–6). Recent ultrastructural studies have revealed extranuclear immunoreactivity for ER-α associated with membrane sites along dendritic spines and axon terminals of neurons (Fig. 6). Moreover, Fig. 5 reveals incipient receptor-mediated endocytosis in Hep G2 cells. These modern findings confirm the observations of Williams and Baba in 1967, at which time they reported, using electron microscopy and admitted excess of labeled steroids, that $[^3H]$aldosterone and $[^3H]$cortisol associated with plasma membranes of their respective target cells. It is uncanny that report of abrupt stimulation of membrane-associated adenylate

TABLE 1 General, Receptor-Mediated Functions of the Steroid Hormone Superfamily

| Hormone | Function |
|---|---|
| Estrogen | Growth and development of reproductive targets, including breast, bone, liver, and cardiovascular system |
| Androgen | Reproductive tract functions, patterns of hair growth, and influences on brain and libido in both sexes |
| Progesterone | Components of reproductive function and behavior, meiosis in oocytes, and acrosome reaction in sperm |
| Glucocorticoids | Maintenance of integrity of cell membranes; metabolic functions in protein mobilization and gluconeogenesis; neurone signaling; immune and inflammatory reactions, and apoptosis |
| Aldosterone | Promotion of reabsorption of sodium and excretion of potassium in kidney, colon, and urinary bladder; acute effects on cardiac function and on sodium transport in smooth muscle |
| Digitalis-like | Inotropic and chronotropic effects on heart; inhibition of $Na^+$, $K^+$-ATPase in this and many other tissues |
| Vitamin D | Regulation of $Ca^{2+}$ and phosphate homeostasis; promotion of differentiation of many cell types |
| Retinoids | Control of cell growth during embryonic development; antioxidant function promotes integrity of epithelial and many other tissues |
| Thyroid hormone | Energy expenditure; embryonic development and postnatal maturation of various tissues, including bone and brain |

cyclase activity by physiological levels of estrogen appeared in the same year, but these data, as fine red wine, required many years of aging before appealing to the taste of the wider scientific community.

Presently, there has been intensely renewed interest in documenting specific steroid binding to target cell membranes, and current extensions of these data are ongoing. One salient fact emerges from the combined observations, namely, that there is a striking parallel between the initial encounter of steroid, as well as peptide, agonist with the surface of its responsive cell. Such function, critical to unfolding of an orderly sequence of succeeding events through receptor-mediated coupling to further metabolic signals (see below), is also shared by many other regulatory agents, including those that promote growth and development of their target cells, such as the phytohemagglutinins in transformation of small lymphocytes, and, indeed, cytokines generally.

It is important to note emerging data, which suggest that different structural conformations of a given steroid hormone may act as specific agonists for selected cellular response pathways. For example, it is suggested that $1\alpha,25(OH)_2$ vitamin $D_3$ produces biologic responses through two distinct receptors, one predominant in the surface membrane and the other predominant in the cell nucleus, which are able to recognize different shapes of the conformationally flexible molecule. Accordingly, the functional significance of agonist–receptor interactions at the target cell surface lies in the potential for selective pathway engagement for propagation of this primary signal.

## IV. CONSEQUENCES OF RECEPTOR OCCUPANCY: ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS

Repercussions of receptor activation on the cell surface may be communicated to the farthest reaches of cell structure and function, including the transcriptional events that will eventually unfold in the nucleus. Manifold activities that are amplified over the relatively prolonged intervening period, from receptor binding to transcription, have been studied and documented for decades. In the case of estrogen, which has received the most attention among the steroid hormones in this regard, the time course of such events encompasses several orders of magnitude, leading to its general description as a continuum (Figs. 7 and 8). A similar temporal distribution pattern prevails for responses to glucocorticoids and vitamin D metabolites (Table 2).

Propagation of the minimal information, from the moment of primary capture of hormone at the cell surface, through an orderly cascade of intermediary reactions in other compartments, to the ultimate differentiation or division of the cell so mobilized, begins through recruitment of virtually instantaneous and closely-linked processes within the affected membrane and in its immediate subplasmalemmal environment. Receptor-mediated signal transduction

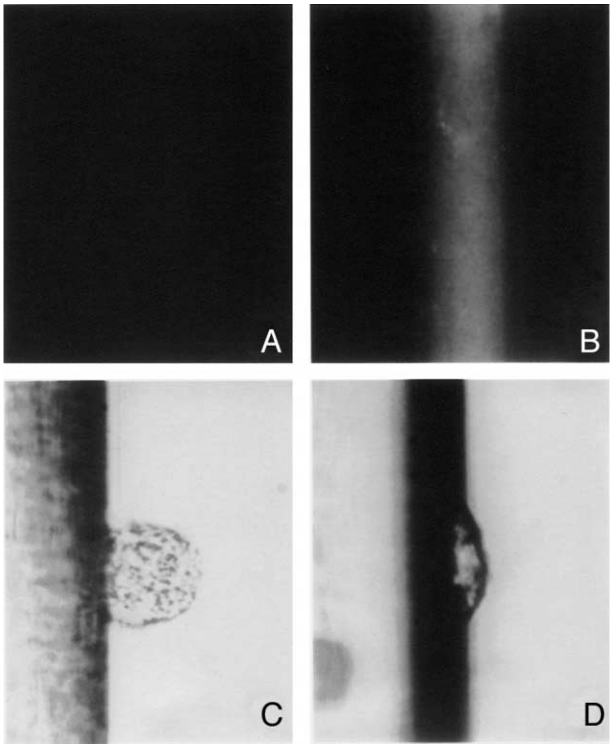

**FIGURE 4** Binding of fluorescein isothiocyanate (FITC)-labeled estradiol antiserum and isolated liver cells to estradiol immobilized by covalent linkage to albumin-derivatized nylon fibers. Incubation was conducted at 22 °C with (A) FITC-labeled nonimmune serum or (B) estrogen antiserum, the latter demonstrating availability of the steroid at the fiber surface, as shown in darkfield-UV fluorescence micrographs (original magnification × 100). In independent experiments (C and D), cells derived from liver were incubated with the derivatized fibers in $Ca^{2+}/Mg^{2+}$-free Ringer solution. Washed fibers with bound cells were photographed with an immersion lens. Some cells appear fairly rounded, whereas others tend to flatten out at the fiber surface ( × 850). Reprinted from Pietras and Szego (1979), with permission, *Journal of Cellular Physiology* © 1979.

responses have been identified for essentially all the steroid hormones (Table 3).

## A. An Orderly Cascade

It is significant to note the time course of the cellular activities, beginning with the earliest indications of membrane perturbation, which occur within seconds or less, as is seen in the nucleotide cyclase reactions. Here, again, is a significant example of a mechanism shared by steroid and peptide agonists that is particularly well illustrated in neural responses. Acute alterations in $Ca^{2+}$ and in $Na^+/K^+$ flux are likewise rapid and occur within a framework of wide differences in agonist and end organ. Abrupt changes in phosphorylation mechanisms, some of which are $Ca^{2+}$ dependent, are also recruited. Many of these changes in the cytoplasmic microenvironment, in turn, have profound effects on enzymatic reactions and on cytologic structure, with special reference to protein folding. Thus, amplification of primary hormonal signal is achieved with great conservation of energy and without further input of mass, through a limited number of receptor-mediated transduction mechanisms, linked, in part, through heterotrimeric G-proteins that are integral to the plasma membrane. These remarkably conserved features of hormone action are covered in depth elsewhere within this volume.

In the case of some hormonal responses, interaction at the surface membrane may be sufficient to elicit an alteration in cell function. For example, estradiol can directly stimulate protein kinase C activity in membranes isolated from chondrocytes, and the steroid also modulates calcium-dependent

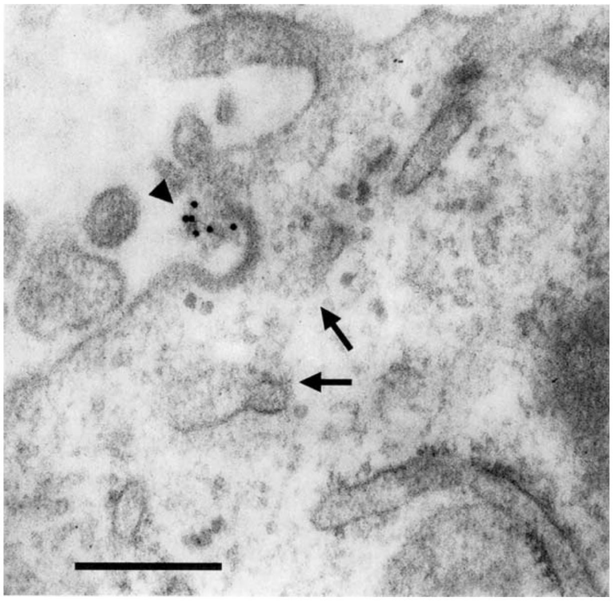

**FIGURE 5** Electron microscopic visualization of receptor-mediated specific binding and internalization of 17β-estradiol-17-hemisuccinate:bovine serum albumin (BSA) that had been adsorbed to colloidal gold (E17 BSA:Au) at surfaces of human hepatoblastoma cells. Note binding of ligand to the plasma membrane directly over a potential clathrin-coated endocytotic pit (arrowhead) and intracellular tubulovesicular structures beneath it (small arrows). In control preparations with BSA:Au (lacking derivatization with estrogen; not shown), there is minimal internalization, despite the presence of BSA:Au in abundant extracellular concentrations. Scale bar, 0.250 μm. Reproduced from Moats and Ramirez (2000), with permission of the Society for Endocrinology.

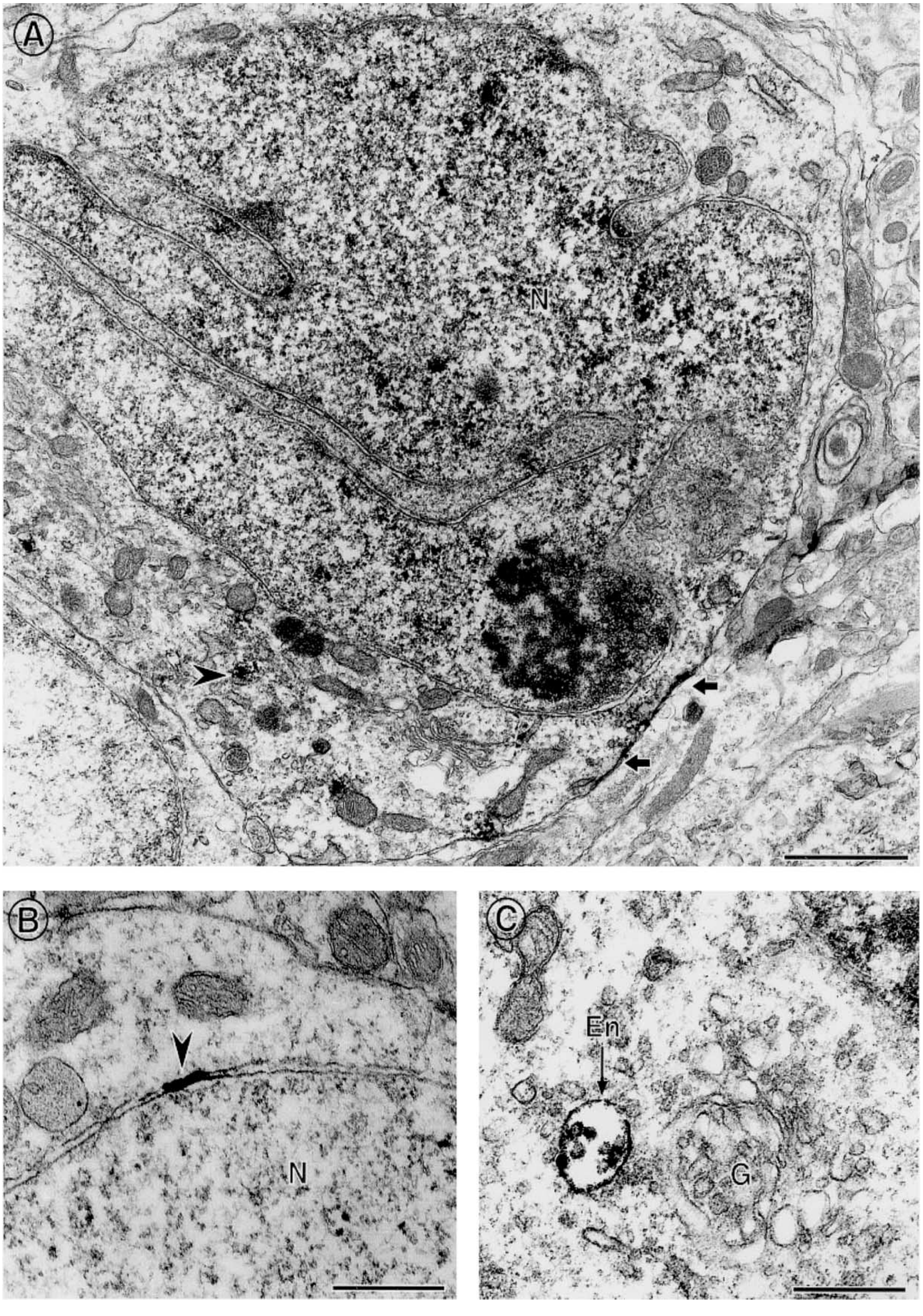
A
N
B
N
C
En
G

TABLE 2 **Parallels in Membrane-Initiated Phenomena Induced by Glucocorticoids and Vitamin D Metabolites**

| Time | Glucocorticoid | $1,25(OH)_2D_3$ |
|---|---|---|
| Seconds | Binding to surface receptor | Binding to surface receptor |
| | Electrophysiological effects | $Ca^{2+}$ channel activation |
| | Binding to intracellular receptors | Membrane: receptor internalization |
| Minutes | PKC activation/translocion[a] | PKC activation/translocation; PKA activation |
| | Capping of membrane receptors[b] | Vesicular loading of $P_i$, $Ca^{2+}$ |
| | Decreased $P_i$ uptake[a] | Increased $P_i$, $Ca^{2+}$ |
| Transport[c] | — | Secretion of calbindin, cathepsin B[c]; phosphorylation of osteopontin[d] |
| Hours | Enzyme synthesis[e] | Synthesis of $Ca^{2+}$-binding proteins; synthesis of α-tubulin[c]; proliferation of lysosomes[c] |
| | Apoptosis[b] | Cell differentiation/migration |

[a] In kidney.
[b] In lymphocytes.
[c] In intestine.
[d] In bone.
[e] In liver.

eNOS activity associated with its receptor in isolated plasma membranes from endothelial cells. Moreover, estrogens may enhance growth of mammary tumor cells, largely independent of estrogen-responsive element (ERE)-dependent transcription, by stimulating membrane-associated mitogen-activated protein (MAP) kinase pathways. Ligand-independent activation of steroid hormone receptors also occurs and may represent a more primitive response pathway, whereby cross-communication with peptide signaling systems in the cell can directly modulate the activity of steroid hormone receptors. For example, estrogen receptor can be activated in the absence of estradiol through phosphorylation by epidermal growth factor (EGF)-stimulated MAP kinase. Any comprehensive model of steroid hormone action must account for these important cellular interactions.

## B. Transitory Alterations in Cellular Architecture and Translocation of Receptor

Among the numerous, acute responses to estrogen recognition in uterine preparations are brief, transitory alterations in cellular architecture, beyond the clear evidence of regional perturbation (cf. Fig. 7); these include incipient vesiculation within the membrane (cf. Fig. 5). These cytoplasmic responses occur within seconds or less, and comprise striking transitory reduction of arrays of microtubules and microfilaments. Indeed, there is considerable evidence that microtubules and the actin cytoskeleton of the cell play an important role in endocytotic trafficking and concomitant signal transduction. In some cases, such remarkable early modifications of target cell structure may play a key role in signal propagation by serving to modulate the relative viscosity of the medium in which the hormone:receptor [H:R] complex is translocated toward, and into, the nuclear compartment.

The microtubular apparatus, with its arboreal array spanning the subplasmalemma and perinuclear/Golgi regions, has been implicated even more directly in the translocation mechanism for the vitamin $D_3$ receptor in mouse osteoblasts, as well as in the case of cellular targets to glucocorticoids. There are now clear indications that, at least for some

**FIGURE 6** Electron microscopic demonstration of localization of immunoreactivity to peroxidase-labeled receptor for alpha isoform of estrogen receptor (ERα) in the hippocampal formation of proestrous rats. Both genomic and nongenomic functions are implicit in the distribution of immunoreactivity. (A) Label is seen throughout the nucleus (N) of a neuron in the hilus of the dentate gyrus, as well as a few patches in the cytoplasm (arrowhead), and also at the plasmalemma (small arrows). (B) In another cell, a dense patch of immunoreactivity is seen in the nuclear envelope; (C) an intensely labeled endosome (En) occurs in the perinuclear cytoplasm near the Golgi apparatus (G). Additional ERα labeling was affiliated with the perikaryal plasmalemma and is apparent in dense patches of reaction product adjacent to several cytoplasmic organelles (B and C). Extranuclear sites revealed with the present methods had not been identified previously by light microscopy. Scale bars, 0.5 μm. Reprinted from Milner *et al.* (2001), by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons.

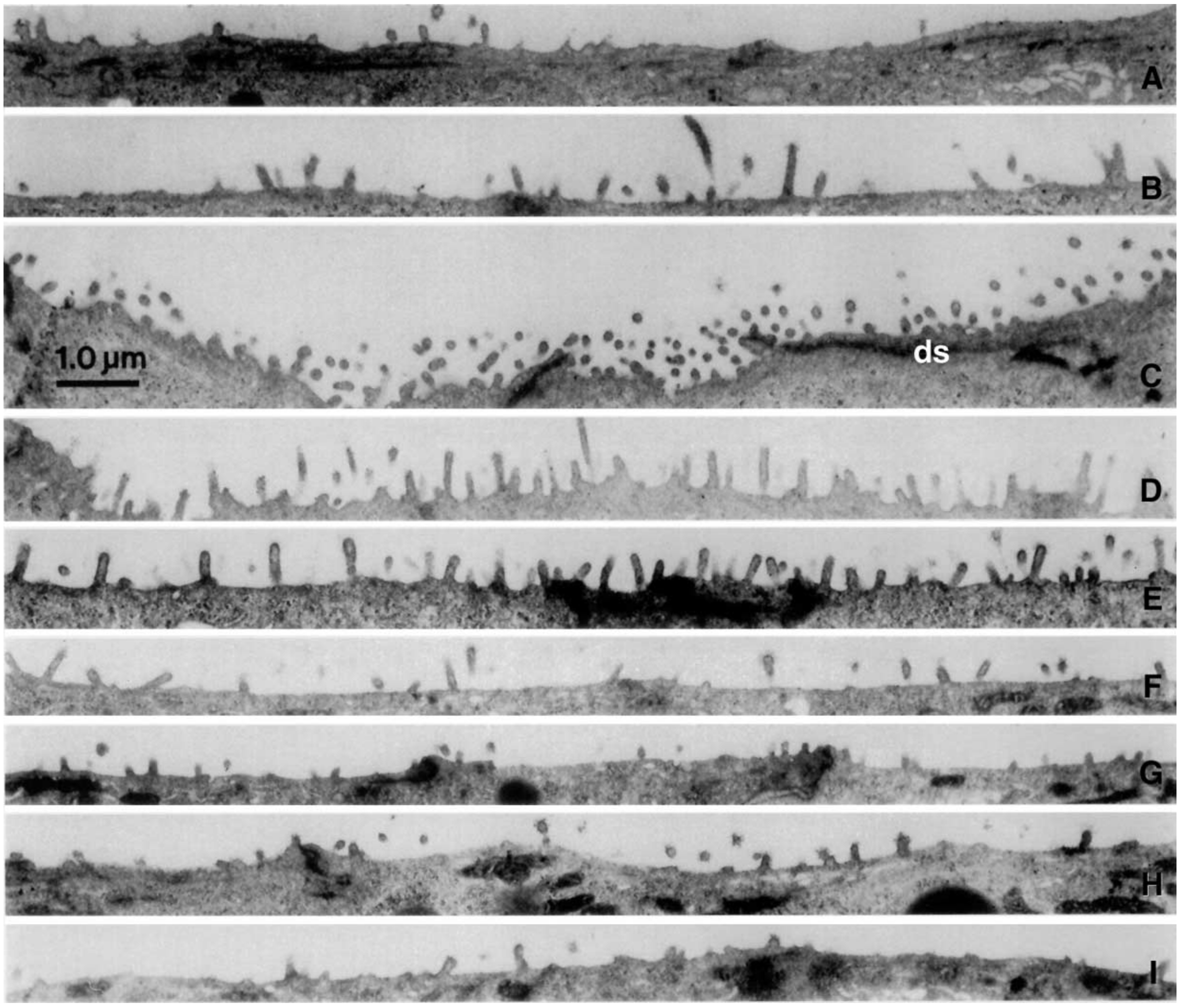

FIGURE 7 Low-magnification electron micrographic views of lumenal surfaces of uterine epithelial cells of ovariectomized rats at brief intervals after iv administration of control vehicle (A) or $E_2\beta$, 0.5 µg/100 g body wt (B–I). The relative paucity of microvilli in a control preparation is in contrast to the striking onset and progressive enhancement of these structures at 35 (B), 45 (C), 80 (D), and 120 (E) s after exposure to hormone *in vivo*. (F–I) Cell surfaces at 5, 10, 15, and 30 min, sequentially, reveal the remarkable subsidence of the mivrovillus activity. Thus, by 30 min after estrogen (I), the degree of lumenal surface investment with microvilli closely resembles the relatively quiescent control state (A). ds, Desmosomes. Reprinted from Szego *et al.* (1988), with permission.

steroid hormones, a significant portion of the hormone:receptor complex occurs in vesicular form (cf. Figs. 5–7), with the potential for fusion with other organelles.

## C. Sequential Distribution of Hormone

Because of the extreme speed of entry, the temporal association of steroid hormone with a surface receptor and its ensuing distribution in target cells have been difficult to demonstrate without appropriate precautions to eliminate nonspecific membrane-perturbing influences. These precautions include strict omission of serum and phenol red from media; use of incubation temperatures at 23 °C rather than the customary 37 °C, but not below 15 °C, when lipid components of membranes assume a rigid conformation; and, above all, sampling at very short intervals. Indeed, because of lack of appreciation by many investigators of these precautions, cumulative evidence of such association had been overlooked by some for decades.

An especially telling analysis of the $[^3H]$estradiol-17β translocation mechanism is available, using analytical cell fractionation at progressive time periods, beginning within 10 s of exposure.

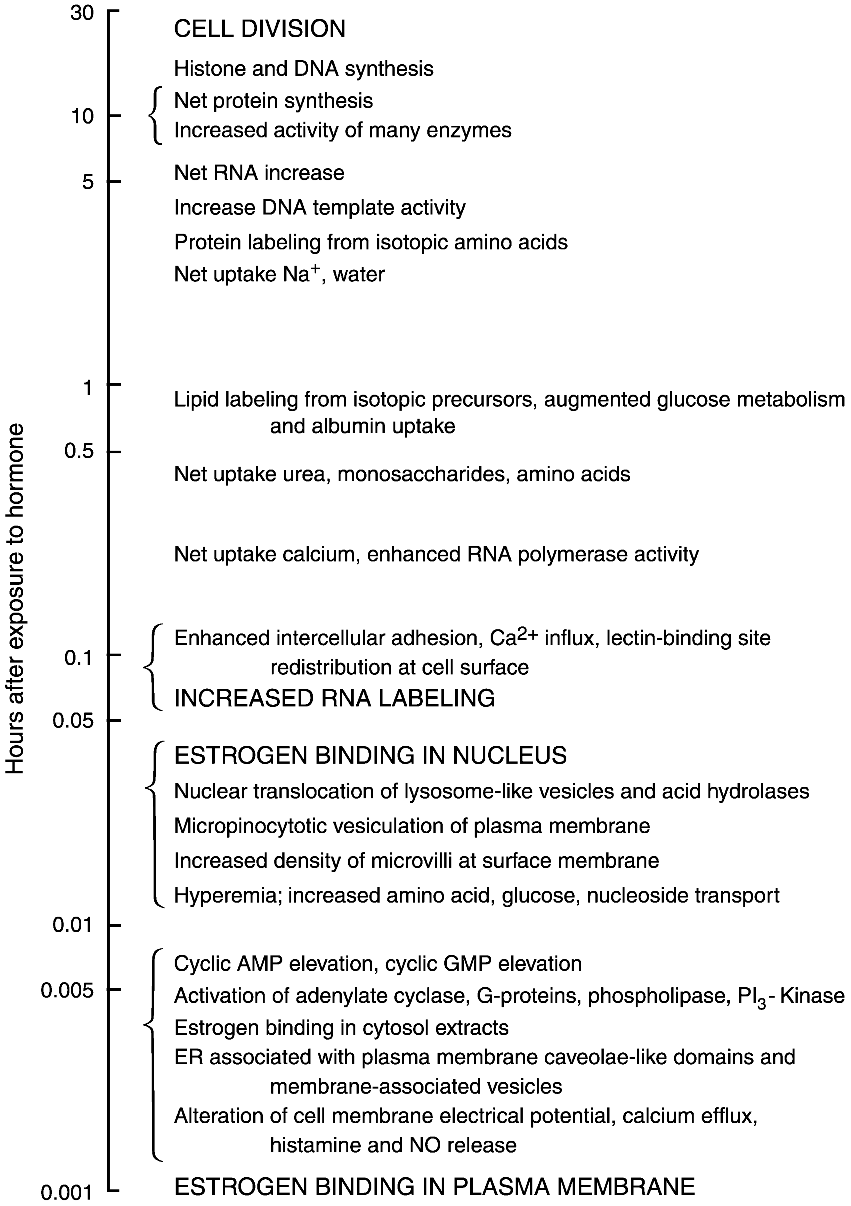
Hours after exposure to hormone
30
10
5
1
0.5
0.1
0.05
0.01
0.005
0.001
CELL DIVISION
Histone and DNA synthesis
Net protein synthesis
Increased activity of many enzymes
Net RNA increase
Increase DNA template activity
Protein labeling from isotopic amino acids
Net uptake Na+, water
Lipid labeling from isotopic precursors, augmented glucose metabolism and albumin uptake
Net uptake urea, monosaccharides, amino acids
Net uptake calcium, enhanced RNA polymerase activity
Enhanced intercellular adhesion, Ca2+ influx, lectin-binding site redistribution at cell surface
INCREASED RNA LABELING
ESTROGEN BINDING IN NUCLEUS
Nuclear translocation of lysosome-like vesicles and acid hydrolases
Micropinocytotic vesiculation of plasma membrane
Increased density of microvilli at surface membrane
Hyperemia; increased amino acid, glucose, nucleoside transport
Cyclic AMP elevation, cyclic GMP elevation
Activation of adenylate cyclase, G-proteins, phospholipase, PI3- Kinase
Estrogen binding in cytosol extracts
ER associated with plasma membrane caveolae-like domains and membrane-associated vesicles
Alteration of cell membrane electrical potential, calcium efflux, histamine and NO release
ESTROGEN BINDING IN PLASMA MEMBRANE

**FIGURE 8** Schematic representation of time course of responses of uterus to estradiol-17β. Times shown on the logarithmic scale refer to onset of unequivocal change from baseline values. Thus, times indicated are dependent in part on sensitivities of the various analytic methods applied and on the somewhat arbitrary selection of initial time points for observation in the several experimental protocols. Revised from Szego and Pietras (1984), with permission.

Estradiol-17β interacts specifically with membrane proteins in uterine cells and undergoes rapid internalization in nanometer-sized endocytotic vesicles, resulting in delivery of a portion of the steroid hormone and its associated receptor protein to the cell nucleus and nuclear protein matrix. Quantitative analyses of the postnuclear supernatant prepared from uterine cell homogenates incubated under the strictest estrogen-free conditions indicate that a significant portion of specific estrogen-binding sites is internalized from plasma membranes in vesicular form. Concomitant with a decline in plasmalemmal and presumptive endosomal fractions, a significant amount of labeled hormone occurs in Golgi and lysosomal compartments before the peak in nuclear accumulation. These observations demand further

**TABLE 3** **Examples of Acute, Receptor-Mediated Signals of Plasma Membrane Perturbation**[a]

| |
|---|
| Alterations in $Na^+$, $K^+$-ATPase activity |
| Rapid shifts in availability of cyclic nucleotides |
| Fluxes in $Ca^{2+}$ and other ions, with potential for modulation of neural activities and numerous enzymatic and mechanoeffector systems |
| Activation of the phosphoinositide cascade |
| Release of endogenous amines and nitric oxide, with influence on microcirculation |
| Structural reorganization of the cell surface, with potential for intracytoplasmic communication; formation of endosomes |
| Accentuated delivery, in microquanta, of components of lysosomes to the cell surface and interior |

[a] Properties shared, to various degrees, by steroid and peptide hormones, as well as by many other effectors, including neurotransmitters, lectins, and toxins.

pursuit with due regard for the scrupulous techniques required.

## V. MEMBRANE SIGNALING AND THE CELLULAR RESPONSE TO STEROID HORMONES

### A. Compartmentation in the Cellular Economy

Without some form of communication between the events at the cell surface and the relatively remote nucleus, separated as it is from all else in the cell by a double membrane, the coordinated response of growth or differentiation could not be achieved. Indeed, there is rapidly growing evidence that there is close synergism between the receptor-mediated, virtually instantaneous activities at the plasma membrane and their considerably delayed effects within the nucleus. Clearly, mechanisms exist for transfer of information, as well as matériel, between the two major cell compartments.

Separation of potential reactants by structural barriers of variable degree of penetrability is a primitive yet thermodynamically efficient means of maintaining a poised system. Such a system is capable of rapid responses to changes in the environment if specialized surface components can detect and capture minute amounts of specific regulatory agents. In the fullest sense, the steroid-hormone target cell is just such a system.

The initial stages of the primary response may constitute physicochemical alterations in conformation that promote propagation of signal, with the speed of the phase changes reminiscent of the child's game of cat's cradle. The information gap between the cell surface and the boundaries of the other cellular organelles, most notably, the nucleus, is then closed, with variable rates of speed, by a chain of ordered secondary reactions originating from the coupling of liganded receptor to other cell signaling proteins (cf. Figs. 8 and 9).

Now, under certain conditions, these transduced responses, in a closely coordinated system of interdependent pathways, forward the expanded signal toward the nucleus and the enhanced genomic activities to come. There have been significant advances in demonstration of hormone and/or receptor in vesicular form, in close perinuclear array at very early times after surface binding and before substantial concentrations occur within the nucleus. The specific means by which the formidable nuclear barrier is crossed have not yet been identified in the hormonal context. However, there are strong indications of organellar intervention and membrane fusion in hormone:receptor complex transport and, in specific cases, delivery through compound lysosomal pathways. At the same time, the ionic, enzymatic, and energy-generating functions, recruited in coupled fashion in the cytoplasm, prepare the responsive cell for its expanding metabolic requirements.

The genomic hypothesis of steroid hormone action has generally prevailed as the exclusive mechanism since 1961, the year in which the seminal concepts of Jacob and Monod electrified the scientific community. As is presented in other articles in this volume, in the interval between 1961 and the present, extraordinary accomplishments by a broad array of molecular biologists have extended and clarified the details of these concepts for understanding the late nuclear repercussions of a number of steroid hormones at their cellular targets, while unfortunately overlooking the well-documented responses attributable to signal at the cell surface. It was inevitable that the emphasis on the critical and novel activities triggered at the nuclear level would overshadow the parallel observations being made on receptor-mediated signals emanating from the primary recognition site, the cell membrane.

Recent advances now permit greater focus on the acute signals and their systematic transduction. This renewed outlook restores the necessary balance to our understanding of steroid hormone action, and integrates the contribution of each set of functions into a more complete whole (Fig. 9). Moreover, in the case of some hormone responses, the primary interaction at the surface membrane may be sufficient of itself to elicit a cascade of intracellular signals to specifically alter cell function.

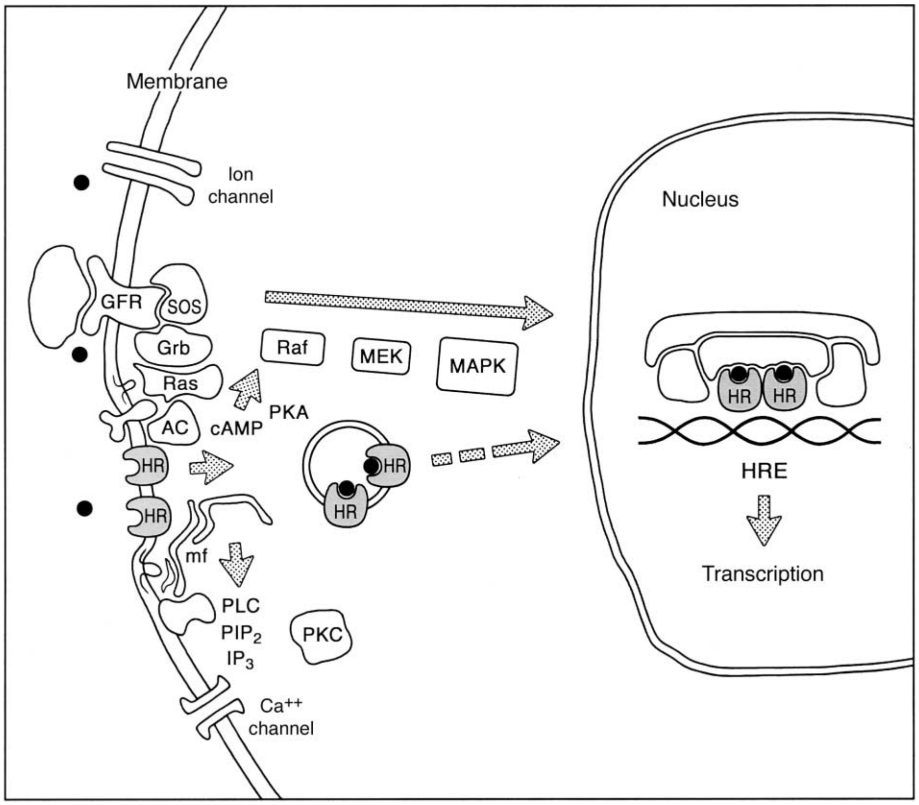

**FIGURE 9** The response as continuum: signal transduction mechanisms leading to the full sequence of receptor-mediated responses of the target cell to steroid hormone. Postulated mechanism of action of a steroid hormone (●) in target cells with a steroid hormone receptor (HR) is shown. The steroid ligands first bind to membrane-associated receptors (cf. Fig. 2). The liganded membrane receptor may affect one or more of several pathways, including phospholipase C (PLC) or protein kinase C (PKC) signaling, leading to modulation of ion channels and enhanced flux of ions, notably $Ca^{2+}$; interaction with peptides or growth factor membrane receptors (GFR) and their immediate signaling partners (SOS, Grb, Ras); or activation of MAP kinase cascades (Raf–MEK–MAPK) or G-proteins and nucleotide cyclases (AC), with generation of cyclic nucleotides (cAMP) and modulation of protein kinases (PKA). These primary membrane interactions may promote physical alteration of the steroid receptor, such as phosphorylation, via steroid-induced or ligand-independent pathways. In some cases, steroid receptors then associate with vesicular structures and microtubule–microfilament (mf) elements in the cell interior and thus gain access to other subcellular compartments. Liganded steroid receptor in the nucleus may promote association of the receptor with co-activator proteins and with specific hormone-responsive elements (HRE) in DNA, leading, in turn, to initiation of selective gene transcription. The wide array of cell responses to steroid hormones may occur as a consequence of synergistic feed-forward circuits, whereby steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of specific receptors in the nucleus.

## B. Direct, Membrane-Initiated Responses Seemingly Uncoupled from the Cytoplasmic Cascade: The Dual Functions of Surface Receptor Activation

What is not yet clear, except under the special circumstances noted below, is the question of the inexorability of the full sequence of transduction steps from cell surface recognition to genomic activation, and, thereby, to growth or differentiation. Is there a briefer, less extensive pathway—essentially only an abbreviated sequence—that leads to altered cell functions, including those related to the increase in number of osmotically-active particles at a very early stage of structural changes in membrane "permeability"? As already noted briefly, one such example that comes immediately to mind is the localized liberation of nitric oxide, which is secondary to an instantaneous surge of $Ca^{2+}$ and which occurs in response of endothelia to estrogen; these coupled events result in rapid vasodilation, thus clearly bypassing the hours-long, metabolically expensive transduction pathway leading to nuclear arousal. Such a truncated pathway may parallel only one or two early steps of the full sequential transduction route. The local effects of estrogen on electrophysiological activities of neurons are another obvious case in point. In the instances noted, there is distinct evolutionary advantage to such a shortcut. In fact, there are circumstances currently being identified, indicating that the two response-sequence stages, full and partial, coexist side by side, thus supporting acute, as well as delayed, responses to a surface signal, independently and in parallel.

Accordingly, the functions of the surface receptor are twofold. Both lead to coordination of the activities of more distal organelles. One such function is *complementary* to the more remote and time-delayed events at the genome, through communication of information, both signals and matériel, from the extracellular environment. The second function *supplements* the more delayed and metabolically demanding activities at the genome, through shortcut of the latter. Instead, signals transduced from receptor engagement of steroid ligand at the external cell surface are converted, independently of genomic activities, to sharply immediate and readily reversible stimuli, such as those eliciting changes in nervous activities and vasomotor functions—these being of evolutionary significance for survival. These dual capacities of surface receptor activation underlie perfect adaptation of the receptive cell to the processing of information from its external environment on two independent/interdependent tracks: acute and more prolonged (Fig. 9).

## VI. SUMMARY

Rigorously controlled experimental data, originating from the work of investigators dispersed worldwide, demonstrate that steroid hormones are first intercepted by specialized proteins associated with the surface membrane. Recognition occurs by features of mutual structural conformity, as predicted from principles of physics and chemistry applied to cell biology. For estrogen and glucocorticoid, such receptor molecules at the cell surface have been found to share homology with the nuclear forms, whereas this is not the case for vitamin D metabolites, aldosterone, or thyroid hormone in the limited numbers of tissues examined. Such information is generally lacking for other members of the steroid superfamily.

Capture of steroid agonist from the extracellular fluid is attributable to the competitive advantage of the cellular receptor, because its affinity for ligand is several orders of magnitude higher than that of the carrier proteins in the circulation. This demonstrates that agonal:receptor interaction is reversible, and conforms to the laws of mass action.

Once effective concentrations of hormone are so bound, the cell surface undergoes virtually instantaneous but transitory structural reorganization. These primary interactions may trigger a cascade of specific cellular responses. Thereafter, a portion of the hormone:receptor complex is internalized, generally within seconds or less, through one or more endosomal mechanisms.

Communication and coordination among the several specialized cellular organelles of the targeted cell are achieved by signal transduction processes that propel the hormone:receptor complex or other specific membrane-associated signaling partners toward and into the nucleus. These combined activities are succeeded by the late stages of the response continuum at the genomic level. The outcome is the totality of response in the context of the whole cell, through synergic functions of its organellar constituents.

## Acknowledgments

We dedicate this work to the memory of our friend and colleague, Dr. James A. Roberts (1946–2001), Professor of Gynecologic Oncology at Stanford University. We thank M. Kowalczyk for expert assistance with illustrations and C. Freeny for technical services in preparation of the manuscript. [Grant support from NIH, NSF, USDA, and the U.S. Army (DAMD17-99-19099; 17-00-10177), California BCRP (5JB-0105), the UCLA Academic Senate (1287), Susan G. Komen Breast Cancer Foundation (99-3305), and Stiles Program in Integrative Oncology.]

## Glossary

**genomic** A process related to gene transcription and its regulation.

**nongenomic** A process independent of RNA transcription.

**organelle** An intracellular, membrane-bounded compartment (e.g., mitochondrion, Golgi, lysosome, endoplasmic reticulum with membrane-bound ribosomes, nucleus) with specialized functions, reflecting division of labor within cells.

**receptor-mediated endocytosis** Cellular entry of agonist via a specialized region of the cell where receptor molecules, capable of specifically binding hormones, are localized. Such a region may also be rich in specialized proteins, such as caveolin. Induced invaginations may be pinched off from the outer membrane, becoming endosomes—vesicular channels for signal transduction.

**signal transduction** A signal is a message relayed from one site to another, in the molecular language of the cell.

The primary signal, in the hormonal context, originates from binding of the agonist (active agent, the hormone) to receptor protein at the surface of the target cell. Signal transduction involves message conversion (translation) from one molecular “language” to another, to be “read” elsewhere in the cell—e.g., surface interactions subsequent to hormonal impact lead to abruptly altered intracellular levels of substances with catalytic activities, such as $Ca^{2+}$, cyclic nucleotides, and phosphokinases (which shuttle phosphate between critical proteins, altering their structure and behavior). Thus, signal transduction, like a molecular relay, advances the hormonal message, both temporally and spatially, among the cell organelles, like a lighted fuse, progressing toward output at the terminal.

**steroid** A family of lipid structures related to the parent substance, cholesterol, which is modified by enzymes in certain tissues that synthesize highly active products with hormonal functions, such as estrogen and progesterone in ovary, testosterone in testis, and cortisol in the adrenal cortex (see Table 1).

## See Also the Following Articles

**Membrane Receptor Signaling in Health and Disease • Receptor–Receptor Interactions • Signaling Pathways, Interaction of • Steroid Hormone Receptor Family: Mechanisms of Action • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

## Further Reading

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# Menstrual Cycle

See *Corpus Luteum; Folliculogeneis; Ovulation*

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# Mineralocorticoid Biosynthesis

Eduardo N. Cozza and Laura B. Matkovic
*Universidad de Buenos Aires*

I. INTRODUCTION
II. THE $\Delta^4$ PATHWAY
III. ALTERNATIVE PATHWAYS
IV. STRUCTURE OF ALDOSTERONE
V. 18-HYDROXYCORTICOSTERONE TO ALDOSTERONE
VI. 18-OXOCORTISOL AS MINERALOCORTICOID
VII. THE $\Delta^5$ PATHWAY
VIII. EXTRA-ADRENAL BIOSYNTHESIS OF ALDOSTERONE
IX. REGULATION OF ALDOSTERONE BIOSYNTHESIS

**Biosynthesis of adrenal gland steroid hormones takes place in the adrenal cortex. Adrenal steroid hormones are grouped into three main types, mineralocorticoids, glucocorticoids, and androgens (sex steroid hormones). Mineralocorticoids are synthesized in the zona**

**glomerulosa, whereas glucocorticoids and sex steroid hormones are synthesized in the zona fasciculata and zona reticularis, respectively. The biosynthetic pathway that leads to production of adrenal steroids begins in all cases with cholesterol.**

## I. INTRODUCTION

The primary mineralocorticoid in all animal species is aldosterone; the main glucocorticoid, however, depends on the species. In effect, cortisol plays the role of primary glucocorticoid in humans and in porcine and bovine species; the main glucocorticoid in mice and rats is corticosterone. Because cortisol is a 17α-hydroxylated steroid, this glucocorticoid is produced only by species expressing 17α-hydroxylase in the zona fasciculata.

In all cases the biosynthesis of adrenal steroids starts with cholesterol, which is incorporated into adrenal cells through binding of cholesterol-enriched lipoproteins to their specific cellular membrane receptors. These occupied receptors are then internalized. In lipoproteins, cholesterol is largely esterified and is then intracellularly deesterified in lysosomes. Free cholesterol is available for immediate use in steroid biosynthesis or is reesterified and stored in intracellular lipid droplets. Cholesterol can also be synthesized in the adrenal cells from acetate, although this source does not seem to be relevant for steroid biosynthesis.

The biosynthesis of aldosterone in adrenal zona glomerulosa cells is usually divided into early and late pathways. The early pathway reactions wholly transform cholesterol into 11-deoxycorticosterone (DOC); in the late pathway reactions, mitochondria transform DOC into aldosterone.

Free cholesterol, obtained by the action of specific esterases, is incorporated into adrenal mitochondria and is cleaved by mitochondrial (inner membrane facing the matrix) cytochrome P450 side chain cleavage (SCC) via two hydroxylations at positions C-20 and C-22 of the 27-carbon cholesterol molecule; this is followed by excision of a fragment, C-22 to C-26. The product, pregnenolone, is a 21-carbon steroid, the precursor from which all $C_{18}$, $C_{19}$, and $C_{21}$ steroids are produced. The numbering of the carbon positions in $C_{21}$ steroids is shown in Fig. 1.

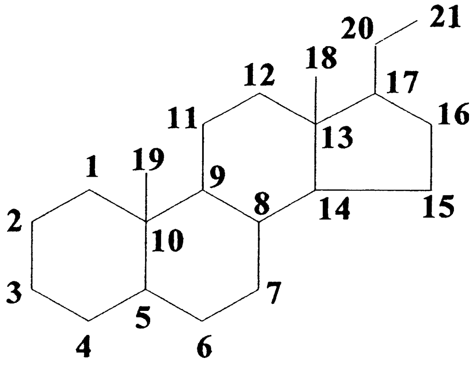


FIGURE 1 Carbon numbering in $C_{21}$ steroids.

Two kinds of reactions, hydroxylations and dehydrogenations, occur in the metabolic pathways that produce mineralocorticoid hormones from cholesterol. Dehydrogenases catalyze the oxidation of the cholesterol molecule through loss of two hydrogen atoms, using nicotinamide adenine dinucleotide (NAD) as cofactor; dehydrogenases are mainly found in the microsomal subcellular fractions of adrenal cells. Enzymes of the cytochrome P450 family mediate hydroxylations. Some of these P450 cytochromes (P450 SCC, 11,18-hydroxylase/aldosterone synthase, CYP11B1, and CYP11B2) are localized in inner mitochondrial membranes and others (21-hydroxylase, 17α-hydroxylase) are in microsomes.

Cytochrome P450 enzyme systems are protein complexes consisting of an electron transfer chain that takes reduction equivalents from the reduced form of NAD phosphate (NADPH), which is thus converted to NADP, provoking reduction of intermediate specific proteins. These proteins finally pass the electrons to the heme group of cytochrome P450. Trivalent iron in cytochrome P450 molecules is thereby transformed into the ferrous form, which has high affinity for molecular oxygen; divalent iron is then oxidized to the ferric form and iron-bound oxygen is reduced. These steps yield water and a hydroxyl group that is incorporated into the steroid molecule, provoking its oxidation. This last step takes place at the active site of the enzyme (cytochrome P450 protein).

Although this general mechanism is adapted to both microsomal and mitochondrial cytochrome P450-mediated hydroxylations, the intermediate proteins between NADPH and the cytochrome P450 molecule are different. The electron transfer chain for mitochondrial cytochrome P450 contains adrenodoxin reductase, which uses the flavin adenine dinucleotide ($FADH_2/FAD^+$) couple as cofactors and the sulfur protein adrenodoxin; in microsomes only one protein is present. The mitochondrial hydroxylating cycle is shown in Fig. 2.

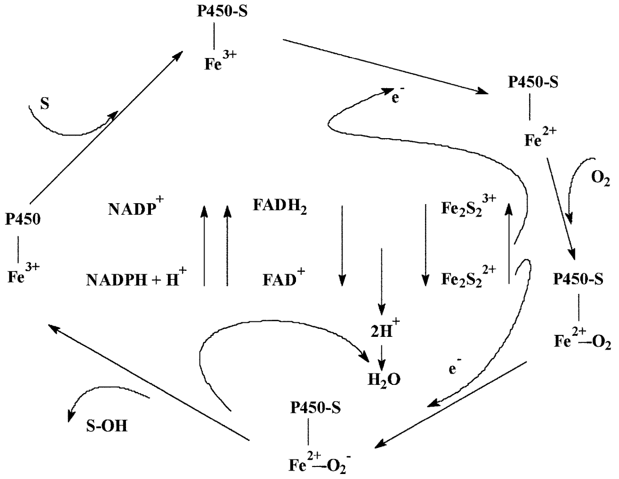


FIGURE 2 Mitochondrial hydroxylating chain. S, Steroid; P450, cytochrome P450.

Cytochrome P450 was so named because the large peak at 420 nm (Soret band due to heme absorbance) of the cytochrome P450 absorption spectrum is shifted to 450 nm in the presence of CO, which binds to reduced heme and competes with oxygen for binding to the iron. As stated before, steroidogenic P450 cytochromes are bound to inner mitochondrial membranes, facing the matrix, or to microsomal membranes. The former can be solubilized in water after treatment with appropriate detergents, whereas microsomal P450 cytochromes are difficult to remove and to obtain in aqueous solution.

## II. THE $\Delta^4$ PATHWAY

The biosynthetic pathway from pregnenolone to aldosterone is able to occur through 3β-ol-$\Delta^5$ or 3-keto-$\Delta^4$ pathways, the last one being considered as the normal or main pathway. In the $\Delta^4$ pathway, pregnenolone leaves the mitochondria and is converted to progesterone. The conversion of pregnenolone to progesterone is catalyzed by the enzyme 3β-ol-dehydrogenase $\Delta^5/\Delta^4$ isomerase; the process consists of dehydrogenation at the hydroxyl group of C-3 of pregnenolone, yielding a keto group, with subsequent migration of the double bond from C-5–C-6 to C-4–C-5 (product, progesterone). 3β-ol-Dehydrogenase $\Delta^5/\Delta^4$ isomerase is the key enzyme for switching from the $\Delta^5$ to the $\Delta^4$ pathway (see below).

As shown in Fig. 3, progesterone is then metabolized to 11-deoxycorticosterone; this is accomplished by microsomal cytochrome P450 21, which introduces a hydroxyl group at position C-21 of the substrate. DOC behaves as a mineralocorticoid, but is not as potent as aldosterone.

The last step of aldosterone production is mitochondrial and involves the conversion of DOC to aldosterone by means of three hydroxylations, one on C-11 and two subsequent hydroxylations on C-18. The overall result of these three hydroxylations is inclusion of a hydroxyl group at C-11, followed by transformation of the methyl group at C-18 to an aldehyde group (aldosterone).

The enzyme involved in the conversion of DOC to aldosterone is species dependent. Bovine and porcine adrenal glands express the cytochrome P450 11β/18-hydroxylase/aldosterone synthase, whereas human, rat, and mouse adrenal glands express cytochrome P450 CYP11B2. Both enzymes catalyze the three hydroxylation steps in glomerulosa cells of each species, but the difference is that, apparently, cytochrome P450 11β/18-hydroxylase/aldosterone synthase is also expressed in bovine and porcine adrenal fasciculata cells, but without producing aldosterone. In other words, aldosterone synthase activity of this enzyme seems to be inhibited in the fasciculata. In contrast, species expressing CYP11B2 in glomerulosa cells produce a different cytochrome P450, CYP11B1, for 11β- and 18-hydroxylations of DOC in adrenal fasciculata cells.

DOC is thus transformed into corticosterone by 11β-hydroxylation and is further hydroxylated at C-18 to yield 18-hydroxycorticosterone, which is finally 18-hydroxylated once again to produce aldosterone. The mechanism by which aldosterone is obtained in this last step deserves special attention. The hypothetical product after one hydroxylation at C-11 and two 18-hydroxylations is 18,18-dihydroxycorticosterone, which has the same oxidation state as aldosterone. The more recognized hypothesis is that this last steroid is largely unstable, suffering loss of water between both hydroxyl groups at C-18, yielding the C-18 aldehyde group of aldosterone.

## III. ALTERNATIVE PATHWAYS

Other authors have postulated that the above-stated sequence of hydroxylations, at 11β, C-18, and C-18, followed by dehydration, is flexible, and that there are alternative pathways. One of the possibilities is that DOC is first 18-hydroxylated, yielding 18-hydroxy-11-deoxycorticosterone, followed by hydroxylation at C-11 and C-18, and finally by dehydration. Another likely sequence (Fig. 4) is hydroxylations at 11β and C-18, followed by dehydration, and finally hydroxylation at C-18. In this case, the product after

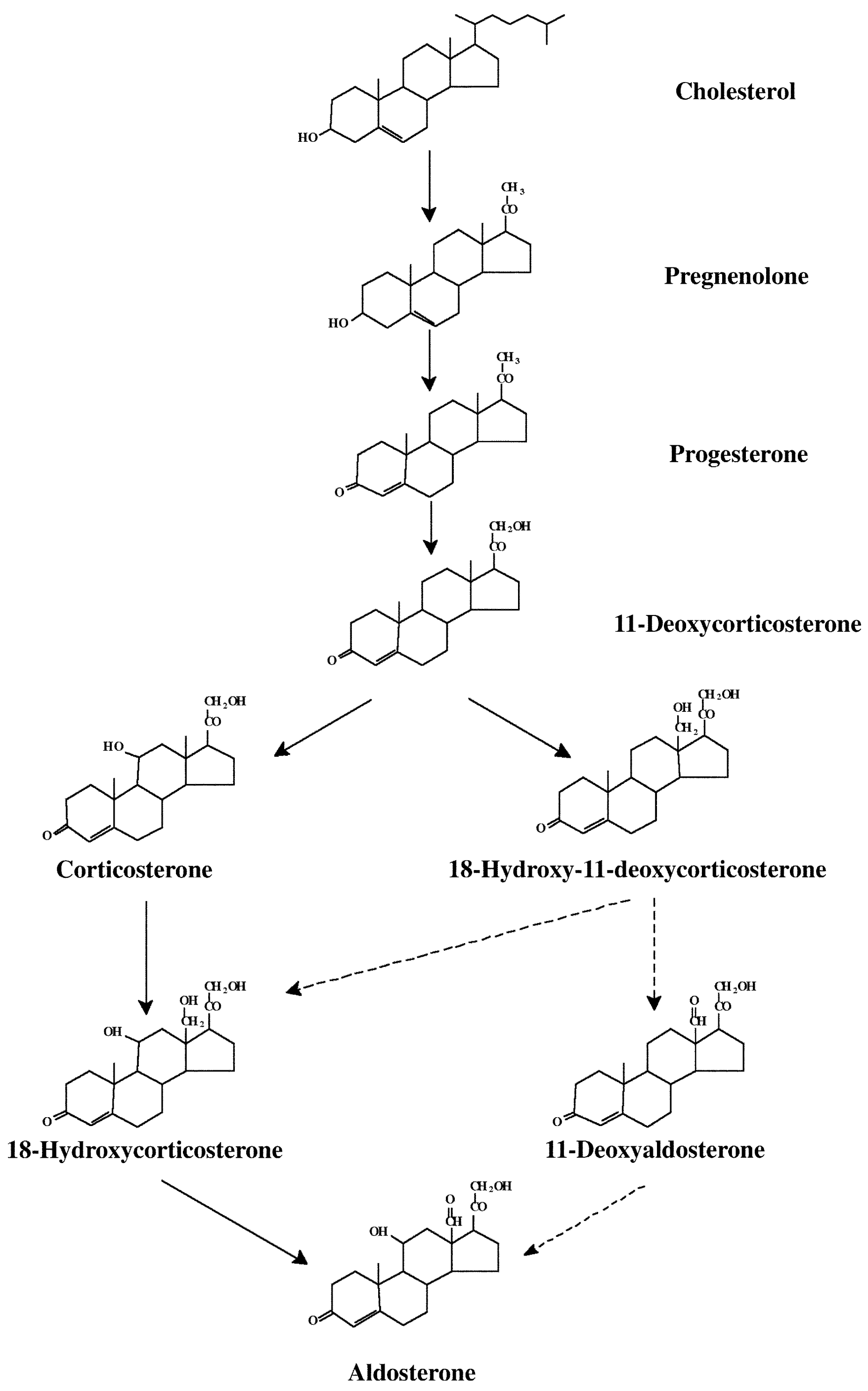
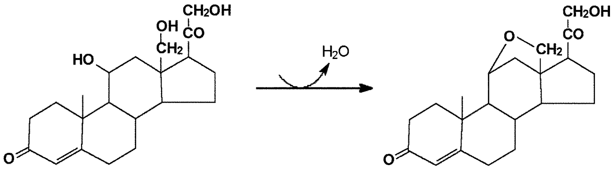

Cholesterol
Pregnenolone
Progesterone
11-Deoxycorticosterone
Corticosterone
18-Hydroxy-11-deoxycorticosterone
18-Hydroxycorticosterone
11-Deoxyaldosterone
Aldosterone

FIGURE 3 Main biosynthetic pathways to aldosterone.

the dehydration step is 11β,18-epoxycorticosterone, also named 18-deoxyaldosterone, which is the substrate for the final 18-hydroxylation. In addition, loss of water occurs by dehydration of hydroxyl groups at C-11 and C-18, rather than of the two hydroxyl groups at C-18.

## IV. STRUCTURE OF ALDOSTERONE

Structural analysis of aldosterone, either synthetic or natural, i.e., isolated from incubation media or blood, presents practically no evidence of the presence of a free aldehyde group at C-18. Further, has been



**18-Hydroxycorticosterone**

**18-Deoxyaldosterone**

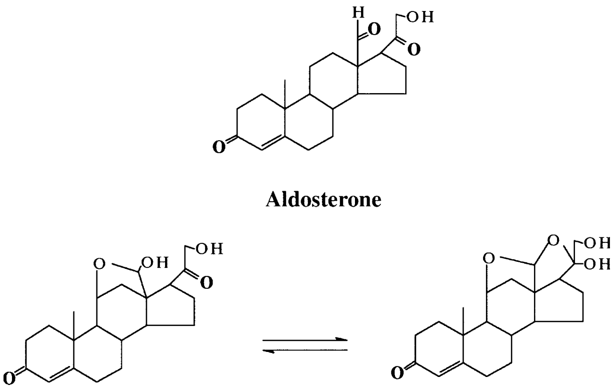
**11,18-Epoxyaldosterone**

FIGURE 4 Transformation (dehydration) of 18-hydroxycorticosterone.

established by gas/liquid chromatography and mass spectrometry (GLC–MS) and nuclear magnetic resonance (NMR) ($^1$H and $^{13}$C) that aldosterone exists in cyclic forms (Fig. 5).

The monocyclic form of aldosterone is obtained by the attack of the negatively charged oxygen atom of the hydroxyl group at C-11 on the positively charged carbon atom of the carbonyl group of the aldehyde at C-18. As a consequence of this reaction 11,18-oxygen-bridged monocyclic aldosterone is obtained, with a hydroxyl group at C-18. It is important to remark that this form of aldosterone could be considered the 18-hydroxylated derivative of 11,18-epoxycorticosterone (see above). In other words, the monocyclic form of aldosterone may be obtained directly, without passing through free-aldehyde stage, by hydroxylation at C-18 of 18-deoxyaldosterone. Within this framework it is possible to hypothesize a relationship between the second alternative biosynthetic pathway mentioned above and the monocyclic form of aldosterone.

The bicyclic form of aldosterone is then obtained when, similar to the previously described process, the oxygen of the hydroxyl group at C-18 of the monocyclic structure of aldosterone attacks the carbon of the carbonyl group at C-20. This reaction produces a bicyclic form consisting of a C-11–oxygen–C-18 ring, a C-19–oxygen–C-20 ring, and a hydroxyl group at C-20. This characteristic formation of cyclic structures is not exclusive to aldosterone; other steroids, such as 18-hydroxycorticosterone (a precursor of aldosterone in the main pathway), have the ability to form a C-18-oxygen-C-20 cyclic form, via attack of the hydroxyl group at C-18 on the carbonyl group at C-20, also introducing a hydroxyl group in the cyclic product. Open forms of 18-hydroxycorticosterone are practically nonexistent in ordinary solutions, including biological fluids.



**Aldosterone**

FIGURE 5 Open (upper structure), monocyclic (lower left structure), and bicyclic (lower right structure) forms of aldosterone.

Of particular interest is the behavior of these steroids in acidic media. Aldosterone and 18-hydroxycorticosterone yield dimeric structures when placed in acidic solutions. 18-Hydroxycorticosterone is also converted to 11,18-epoxycorticosterone under the same conditions. All of these products present much higher hydrophobicity, compared to their mother compounds. The relationship between these responses to acidic media and the biosynthesis of mineralocorticoids (aldosterone) is still unclear, but it is possible to speculate that less polar forms of these steroids favor their transport across cellular membranes (plasmatic, microsomal, and mitochondrial). Acidic conditions *in vivo* may exist in particular regions near proton pumps and/or in some organelles. In any case, it is also likely that specific enzymes catalyze the reactions obtained *in vitro* with acidic media.

## V. 18-HYDROXYCORTICOSTERONE TO ALDOSTERONE

The conversion of 18-hydroxycorticosterone to aldosterone, i.e., the last step of the main biosynthetic pathway, can be freshly analyzed with respect to the structural characteristics described in the previous section.

18-Hydroxycorticosterone, the immediate precursor of aldosterone, needs a C-18 hydroxylation and dehydration, in order to be converted to the mineralocorticoid. Two questions arise: Do these reactions take place on open or on cyclic forms of the steroids? What is the biosynthetic sequence: C-18 hydroxylation followed by dehydration, or loss of water first, and then hydroxylation at C-18?

The more abundant structure of 18-hydroxycorticosterone is its C-18-oxygen-C-20 cyclic form (Fig. 6), whereas aldosterone and 11,18-epoxycorticosterone have C-11-oxygen-C-18 cyclic structures.

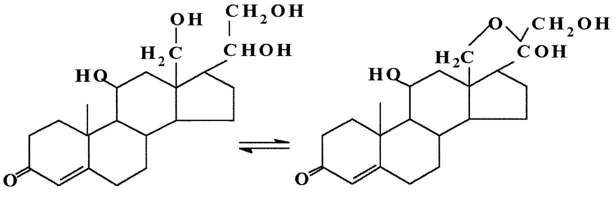


FIGURE 6 Open (I) and monocyclic (II) forms of 18-hydroxycorticosterone.

The previously mentioned alternative pathway described the transformation of 18-hydroxycorticosterone to 11,18-epoxycorticosterone (an acid-catalyzed dehydration) and, finally, aldosterone (C-18 hydroxylation). This alternative pathway is promoted when the 11,18-epoxy form is isolated from incubation media and by the presence of the same oxygen-bridged structures in 11,18-epoxycorticosterone and aldosterone.

## VI. 18-OXOCORTISOL AS MINERALOCORTICOID

As a mineralocorticoid, aldosterone is involved in renal sodium reabsorption, as opposed to atrial natriuretic peptide (ANP), which regulates sodium plasma levels. Thus, aldosteronism is a classic cause of hypertension. Glucocorticoid-suppressible aldosteronism (GSA), also known as glucocorticoid-remediable aldosteronism, is characterized by large decreases in aldosterone plasma levels and blood pressure after administration of glucocorticoids (dexamethasone).

18-Oxocortisol isolated from the urine of a subgroup of patients with GSA was found to be responsible for hypertension through its mineralocorticoid activity. The structure and pathway for the biosynthesis of 18-oxocortisol are still unclear. Structure analysis of 18-oxocortisol in comparison with cortisol, corticosterone, and aldosterone eliminated the idea that cortisol, in its conversion to 18-oxocortisol, requires the same transformation pathway that yields aldosterone from corticosterone, i.e., the conversion of the methyl group at C-18 to a C-18 aldehyde (see Fig. 7).

The normal enzymatic group of 17-hydroxylating species regulates adrenal cortex biosynthesis of cortisol in the zona fasciculata and of aldosterone in the zona glomerulosa. Within this framework, the possibility of interaction between cortisol and aldosterone synthase is not obvious. One hypothesis addressing this is based on the existence of a transitional zone between both zonae, in which fasciculata and glomerulosa cells are found together. The larger the transitional zone, the higher the plasma 18-oxocortisol level. This hypothesis is supported by the fact that aldosterone synthase very efficiently transforms cortisol (used as substrate) into 18-oxocortisol in *in vitro* incubations. 18-Hydroxycortisol, a putative intermediate between cortisol and 18-oxocortisol (similar to 18- hydroxycorticosterone in the transformation of corticosterone into aldosterone; see above), was also isolated from those incubations, and exhibited a high degree of mineralocorticoid activity.

A second and more recent and accepted hypothesis is based on the existence of an "abnormal" enzyme in the zona fasciculata; it produces 18-oxocortisol from cortisol, catalyzing the same kind of reactions seen with aldosterone synthase and corticosterone. This enzyme would be the product of a chimeric gene formed by the crossover of genetic material containing the promoter and some

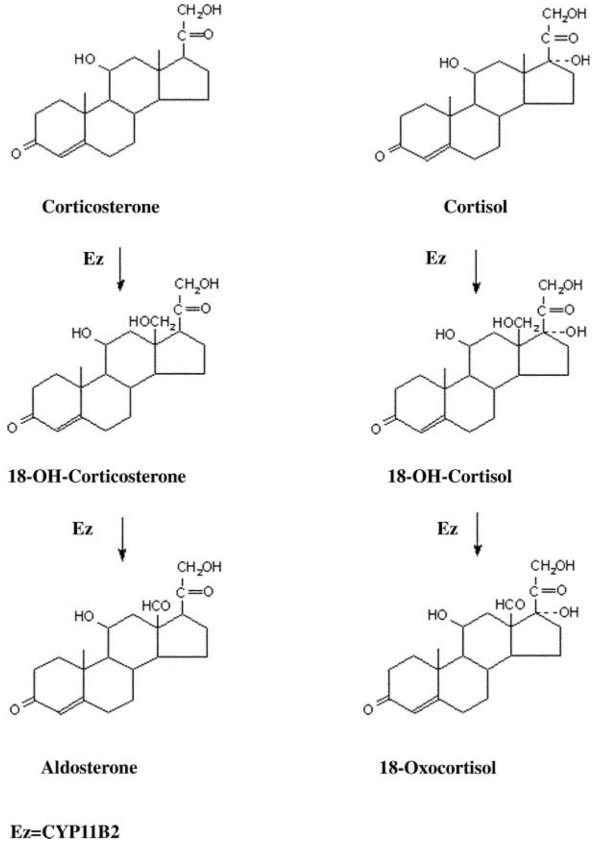


FIGURE 7 Transformation of corticosterone and cortisol to aldosterone and 18-oxocortisol, respectively.

of the first exons of the CYP11B2 gene and some of the last exons of the CYP11B1 gene. As a result of this polymorphic gene, the enzyme obtained contains the catalytic properties of CYP11B2 and the adrenocorticotropic hormone (ACTH)-responsiveregulation of CYP11B1. However, this abnormal enzyme was found only in some patients carrying GSA, and not in all of them. This field is still open to future studies.

## VII. THE $\Delta^5$ PATHWAY

In the toad *Bufo arenarum* it has been well demonstrated that aldosterone biosynthesis takes place via a complete 5-ene pathway rather than via the 4-ene pathway mentioned before. The model for this toad species postulates that the main pathway for aldosterone biosynthesis involves the following sequence: cholesterol, pregnenolone, 21-hydroxypregnenolone, 11,21-dihydroxypregnenolone, $\Delta^5$-aldosterone, and aldosterone. This sequence indicates that the main difference between the 4-ene and 5-ene pathways is that, in the 4-ene scheme, the conversion of the 3β-hydroxy-5-ene group to the corresponding 3-oxo-4-ene derivative (catalyzed by the enzyme 3β-hydroxysteroid dehydrogenase/isomerase) takes place at the level of, or close to, pregnenolone, whereas in *B. arenarum* this transformation is the last step in aldosterone biosynthesis. The enzymatic machinery for aldosterone biosynthesis in this amphibian has not yet been elucidated.

## VIII. EXTRA-ADRENAL BIOSYNTHESIS OF ALDOSTERONE

The adrenal zona glomerulosa is the main locus for the biosynthesis of aldosterone. However, this mineralocorticoid is also synthesized in other tissues, i.e., in the central nervous system (mainly hippocampus, cerebellum, and brain cortex), heart, kidney, and vascular system. Because the quantities of aldosterone biosynthesized in these tissues are around 100 times lower than in adrenal cortex, it is postulated that the mode of action of the mineralocorticoid in nonadrenal organs is autocrine (acting on the same cell) or paracrine (acting on the closest cells), rather than systemic (being secreted to blood). As an example, aldosterone produced by vascular smooth muscle cells mediates, in an autocrine manner, angiotensin II-stimulated cell proliferation. Independently of the particular role of aldosterone in each case, the general overview of aldosterone biosynthesis is that it is believed to be focused on cardiovascular homeostasis.

The biosynthesis of aldosterone in extra-adrenal tissues has been demonstrated to be carried out by the same enzymatic machinery found in the adrenal zona glomerulosa. Expression and activity of CYP11B2 in the nervous system, heart, kidney, and vascular system have been revealed by different experimental approaches in many animal species.

## IX. REGULATION OF ALDOSTERONE BIOSYNTHESIS

Although the main goal of this article is to describe the biosynthesis of aldosterone, a brief discussion of regulation of its formation is in order. The function of aldosterone is to stimulate sodium resorption from the distal renal tubule; water is passively resorbed with the sodium. The major stimuli for aldosterone secretion are hypovolemia (low blood volume), hyponatremia (low serum sodium levels), and low renal perfusion pressure. However, none of these stimuli directly affects the zona glomerulosa; instead, they are channeled through the kidney, because it has the most to lose if blood pressure falls too much. Under this last situation, the kidney is not able to remove wastes from the body.

Juxtaglomerular cells (JCs) possess a sensor for sodium concentration and renal perfusion pressure. Blood volume, however, is monitored by the sympathetic nervous system, which stimulates JCs. Any one of the three parameters sensed by JCs stimulates the secretion of renin from the same cells. Renin is a proteolytic enzyme that converts angiotensinogen, an $\alpha_2$-globulin made in the liver, to angiotensin I, an inert decapeptide. Then, in the lung, angiotensin-converting enzyme (ACE) removes two amino acids from angiotensin I to form the active angiotensin II. Angiotensin II is a potent vasoconstrictor and stimulator of aldosterone secretion from glomerulosa cells. Thus, low blood pressure, hyponatremia, and hypovolemia are corrected. Angiotensin II may also be converted to angiotensin III by a specific protease at the site of binding to angiotensin II receptors, in adrenal zona glomerulosa cells. Angiotensin III is also active. It is important to remark that intracellular renin/angiotensinogen/ACE/angiotensin II systems (renin/angiotensin system for short) have been reported in several cell systems, including glomerulosa cells.

The basic mechanism of action of angiotensin II in glomerulosa cells involves increased $Ca^{2+}$ influx, and activation of phospholipase C. This last enzyme in turn hydrolyzes phosphoinositol, which is converted to active inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ releases $Ca^{2+}$ from intracellular stores, and DAG activates the $Ca^{2+}$ and phospholipid-dependent protein kinase [also named protein kinase C (PKC)]. Although high $Ca^{2+}$ concentrations are accepted as mediators of angiotensin II-stimulated aldosterone secretion, the involvement of PKC is controversial.

When glomerulosa cell cultures are exposed to high concentrations of potassium (6–15 m*M*), the secretion of the mineralocorticoid is significantly augmented. In this case, it is believed that the intracellular renin/angiotensin system in glomerulosa cells is responsible for potassium-induced augmentation of aldosterone secretion. The initial event produced by potassium is probably related to membrane potential, which is mainly dependent on the concentration of sodium and potassium and the transport of ion through the cell membrane. In this respect, $Na^+$,$K^+$-ATPase maintains extracellular high concentration of sodium and intracellular high concentration of potassium, and inhibition of this enzyme, for example, by ouabain, provokes depolarization of cell membranes and subsequent activation of voltage-dependent calcium channels, and stimulation of aldosterone secretion. Angiotensin II inhibits $Na^+$,$K^+$-ATPase. In the case of extracellular high concentrations of potassium, $Na^+$,$K^+$-ATPase is activated. The mechanism by which the intra adrenal renin/angiotensin system is activated is unclear.

Vasopressin [also named antidiuretic hormone (ADH)], adrenomedullin, α-melanocyte-stimulating hormone (α-MSH), and endothelin-1 (ET-1) also stimulate the secretion of aldosterone, but to a much lower degree compared to angiotensin II and potassium. However, according to recent reports, endothelin potentiates angiotensin II- and ACTH-mediated stimulation of aldosterone production.

Adrenocorticotropic hormone, a product of a proopiomelanocortin protein such as MSH, is a potent acute stimulator of aldosterone secretion. ACTH is the main physiological regulator of glucocorticoid biosynthesis, and its secretion is inhibited by high glucocorticoid plasma concentration by means of a feedback mechanism at the level of the pituitary gland. Although from the physiological point of view there is not a clear relationship between ACTH and aldosterone, glomerulosa cells express ACTH receptors at a higher concentration (receptors/cell), compared to the corresponding fasciculata cells. At high levels, ACTH and angiotensin II stimulate aldosterone secretion to a similar degree. However, in long-lasting (chronic) exposures, ACTH reduces aldosterone levels, after the initial augmentation, to control levels, or even below control levels. Histological observations have determined that after chronic treatment with ACTH, the zona glomerulosa is reduced and the zona fasciculata is expanded. This result is not surprising because, for example, ACTH induces 17α-hydroxylase in the adrenal glands of cortisol-producing animal species. In addition, the pathophysiological importance of ACTH in the regulation of mineralocorticoids is underlined by the existence of ACTH-dependent, aldosterone-secreting, adrenal adenomas. The mechanism of action of ACTH involves production of cyclic AMP, and activation of cyclic AMP-dependent protein kinase A (PKA). Other mechanisms involving protein kinase C and extracellular calcium influx are well known and accepted.

## Glossary

**cytochrome P450** Heme protein with hydroxylase activity. The number 450 refers to the wavelength of maximal absorbance of the CO-cytochrome complex.

**$\Delta^4$ pathway** Biosynthetic pathway with majority of 3-keto-4-ene structures as intermediates.

**$\Delta^5$ pathway** Biosynthetic pathway with majority of 3β-ol-5-ene structures as intermediates.

**mineralocorticoid** One of the main steroid hormones synthesized by the adrenal cortex. Its function is focused on regulation of electrolyte balance through stimulation of renal sodium retention.

**steroid** Member of a family of organic compounds derived from cholesterol. The basic steroid structure consists of cyclopentane perhydrophenanthrene.

## See Also the Following Articles

**Glucocorticoid Biosynthesis • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Disorders, Genetic Basis of • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Receptor, Natural Mutations of • Mineralocorticoids and Hypertension • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Mineralocorticoid Disorders, Genetic Basis of

Ian Marshall and Maria I. New

*New York Presbyterian Hospital—Weill Medical College of Cornell University*

I. INTRODUCTION
II. SYNTHESIS AND ACTION OF MINERALOCORTICOID HORMONES
III. MINERALOCORTICOID RECEPTOR
IV. GENETIC DISORDERS
V. SUMMARY

**Mineralocorticoid biosynthesis and metabolism are affected by certain genetic disorders. In 11β-hydroxylase, 17α-hydroxylase, and aldosterone synthase enzyme deficiencies, the production of aldosterone is decreased, with elevation of mineralocorticoid precursors prior to the enzymatic block. Alternatively, aldosterone production can be increased, as occurs in glucocorticoid-remediable aldosteronism. The binding of aldosterone to the mineralocorticoid receptor (MR) may be altered as in pseudohypoaldosteronism, with resistance to aldosterone action at the MR. Finally, there can be binding of an agonist to the MR, with increased mineralocorticoid action as occurs in apparent mineralocorticoid excess.**

## I. INTRODUCTION

Mineralocorticoids are hormones whose function is to regulate the transport of electrolytes across epithelial surfaces. The major circulating mineralocorticoid, aldosterone, is a steroid hormone that is synthesized in the adrenal cortex and functions primarily in the maintenance of electrolyte equilibrium, which contributes to the stabilization of blood volume and blood pressure. Aldosterone binds to the mineralocorticoid receptor (MR) in its main target tissues, thereby promoting active sodium transport and excretion of potassium.

## II. SYNTHESIS AND ACTION OF MINERALOCORTICOID HORMONES

Aldosterone is synthesized exclusively in the zona glomerulosa of the adrenal cortex in a series of enzymatic steps from cholesterol (Fig. 1). Its intermediate precursors, deoxycorticosterone (DOC), corticosterone (B), and 18-hydroxycorticosterone (18-OHB), have some mineralocorticoid activity and are synthesized in all three adrenocortical zones; 18-OHB is synthesized predominantly in the zona glomerulosa and its secretion correlates with that of aldosterone. In addition to being 11β-hydroxylated to B, DOC can be 18-hydroxylated to 18-OHDOC, which is then 11β-hydroxylated to 18-OHB (Fig. 2). These three final steps in aldosterone synthesis are catalyzed by the P450 enzyme aldosterone synthase.

Because aldosterone is not stored in the adrenal glands to a significant degree, its production is regulated by changes in activity of one or more biosynthetic enzymes. The primary stimuli for these changes are the renin–angiotensin system and serum potassium ion concentration (Fig. 3). There are a number of minor modulators including adrenocorticotropic hormone (ACTH), sodium ion, dopamine, atrial natriuretic peptide, β-adrenergic agents, serotonin, somatostatin, and vasopressin.

## III. MINERALOCORTICOID RECEPTOR

Aldosterone binds to two different receptors, the high-affinity type I receptor and the more abundant low-affinity type II receptor. The type I receptor that has been identified as the mineralocorticoid receptor was first cloned in 1987 and consists of a 984-amino-acid protein that is encoded by a gene on chromosome 4. It is expressed predominantly in

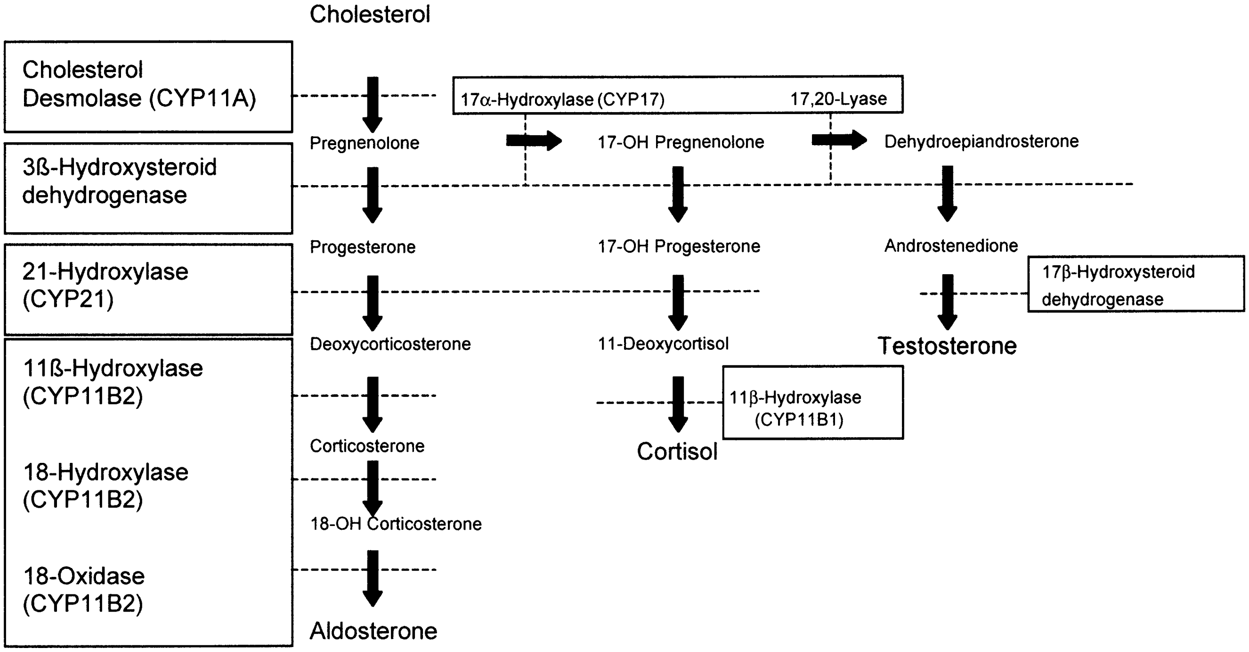
Cholesterol
Cholesterol Desmolase (CYP11A)
3ß-Hydroxysteroid dehydrogenase
21-Hydroxylase (CYP21)
11ß-Hydroxylase (CYP11B2)
18-Hydroxylase (CYP11B2)
18-Oxidase (CYP11B2)
17α-Hydroxylase (CYP17)
17,20-Lyase
Pregnenolone
17-OH Pregnenolone
Dehydroepiandrosterone
Progesterone
17-OH Progesterone
Androstenedione
17β-Hydroxysteroid dehydrogenase
Deoxycorticosterone
11-Deoxycortisol
Testosterone
11β-Hydroxylase (CYP11B1)
Corticosterone
Cortisol
18-OH Corticosterone
Aldosterone

FIGURE 1 Pathway of steroid biosynthesis. In addition to being 11β-hydroxylated to B, DOC can be 18-hydroxylated to 18-OHDOC. Reprinted with permission from New, M. I., and White, P. C. (1995). Genetic disorders of steroid hormone synthesis and metabolism. *In* "Genetic and Molecular Biological Aspects of Endocrine Disease," (R. Thakker, ed.), pp. 525–554. Ballière Tindall, London.

sodium-transporting epithelial cells of the kidney, the colon, and the exocrine glands (salivary and sweat glands), but also in nonepithelial tissues such as mononuclear leukocytes, the central nervous system, large blood vessels, and the heart. Aldosterone and cortisol have equal affinities for the MR and since normal circulating cortisol levels are 100- to 1000-fold higher than that of aldosterone, it would be expected that the ability of aldosterone to bind to the receptor would be limited. The intracellular enzyme 11β-hydroxysteroid dehydrogenase type 2 is co-expressed with the MR in renal tubular cells and catalyzes the conversion of cortisol to cortisone. This reaction protects the MR from saturation by cortisol and other glucocorticoids and therefore maintains the specificity of the MR.

Aldosterone promotes sodium conservation in its target tissues by interacting with the MR through activation of the amiloride-sensitive sodium channels (ENaC) and the $Na^+$,$K^+$-ATPase pump. It acts by binding to an intracellular receptor, and as a hormone–receptor complex, it interacts with hormone-responsive elements of the DNA. The interaction leads to the expression of specific proteins, whose candidate targets are the ENaC channels and the $Na^+$,$K^+$-ATPase pump.

## IV. GENETIC DISORDERS

### A. 11β-Hydroxylase Deficiency

Defects in 11β-hydroxylation result in virilizing congenital adrenal hyperplasia (CAH), often accompanied by hypertension. The abnormal adrenal steroid serum profile exerts a net mineralocorticoid effect, altering renal function and causing sodium retention and volume expansion.

A deficiency of enzyme 11β-hydroxylase is inherited as an autosomal recessive disorder, caused by inactivating mutations in the CYP11B1 gene located on chromosome 8. The gene consists of 9 exons, with the majority of mutations appearing to be random point mutations identified in exons 2, 6, 7, and 8. Although the enzyme defect is found in approximately 1 in 100,000 live births, the disease frequency is much higher in a consanguineous group of Moroccan Jews, where it occurs in 1 in 5000–7000 live births.

11β-hydroxylase catalyzes the conversion of DOC to corticosterone in the mineralocorticoid pathway and 11-deoxycortisol to cortisol in the glucocorticoid pathway. A deficiency of the enzyme with resultant low plasma cortisol leads to chronic elevation of ACTH, with subsequent increased

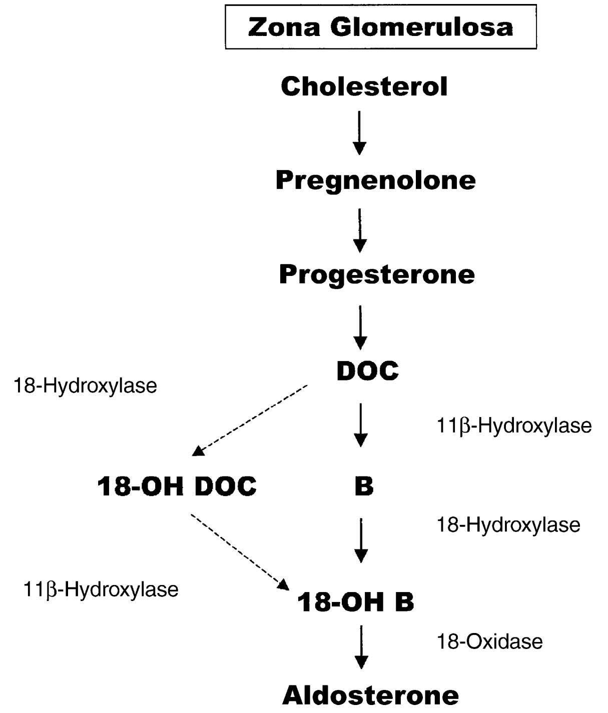

FIGURE 2 Synthesis of aldosterone in the zona glomerulosa.

synthesis and secretion of steroid intermediates proximal to the 11β-hydroxylase block and their non-11β-hydroxylated products. Accumulation of these precursors produces the typical phenotype that characterizes this disorder. Excessive production of DOC and its metabolites exerts a net mineralocorticoid effect. Sodium retention and volume expansion occur, with suppression of renin production. Hypertension occurs in approximately two-thirds of untreated patients, appears infrequently in infancy, and is variable in childhood. Hypokalemia is variable, developing concomitantly with sodium retention. Aldosterone production is low, secondary to low plasma renin activity and low serum potassium concentrations.

The precursors that accumulate in the glucocorticoid pathway are channeled into the androgen pathway and result in prenatal virilization, with varying degrees of genital ambiguity in the affected newborn female. Males and females may manifest signs of androgen excess at any phase of postnatal development, with progressive virilization, including precocious pubic hair, advanced somatic and epiphyseal development, and induced central precocious puberty later in childhood.

Treatment of 11β-hydroxylase deficiency consists of glucocorticoid replacement; this approach is effective because (1) it fulfills systemic steroid (glucocorticoid) requirements and (2) by providing feedback inhibition of ACTH release, it reduces the drive on the adrenal for the synthesis and secretion of precursor steroids. Therapeutic control is achieved by careful clinical monitoring, particularly in children when the dosage is continually adjusted, with particular avoidance of suppression of linear growth from overdosing.

Identification of the mutations affecting CYP11B1 has permitted prenatal diagnosis and subsequent treatment of the affected female fetus. This is achieved by the administration of dexamethasone to the pregnant mother by 8 weeks gestation to suppress excess adrenal androgen secretion and prevent virilization should the fetus be an affected female. Karyotyping and diagnosis by genetic analysis require chorionic villus sampling at $10\frac{1}{2}$ to 12 weeks or later by amniocentesis at 15 to 18 weeks gestation. If the fetus is determined to be a male or an unaffected female, treatment is discontinued. Otherwise, treatment continues to term.

## B. Aldosterone Synthase Deficiency

Deficiency of the aldosterone synthase enzyme, which catalyzes the three steps in the conversion of DOC to aldosterone (Fig. 1), results from mutations in CYP11B2, the gene that encodes this mitochondrial cytochrome P450 enzyme. Two types have been described. Type 1 results from deficiency of the 18-hydroxylase enzyme and is usually characterized biochemically by reduced levels of 18OHB and very low levels of urinary aldosterone metabolites. Type 2, which is characterized by increased levels of 18OHB and reduced urinary levels of aldosterone metabolites, results from deficiency of 18-oxidase enzyme.

Aldosterone synthase deficiency presents in infancy with renal salt wasting, which can lead to dehydration, shock and even death if not adequately treated. Infants can also present with failure to thrive. Plasma renin activity is increased secondary to low aldosterone levels. Cortisol synthesis is unaffected.

Patients are treated with mineralocorticoid replacement therapy in addition to sodium supplementation. Often, replacement therapy is not required after childhood when compensatory extrarenal salt-conserving mechanisms mature with age.

## C. Glucocorticoid-Remediable Aldosteronism

Glucocorticoid-remediable aldosteronism (GRA) is a familial form of low renin hypertension, which is inherited as an autosomal dominant disorder.

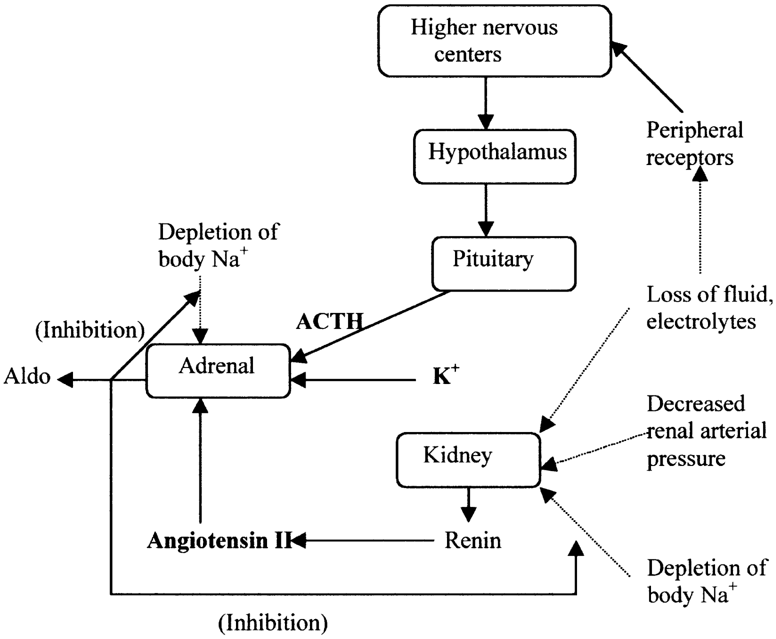

FIGURE 3 Control of the renin–angiotensin system. Reprinted with permission from Speiser, P.W., and New, M.I. (2002). Hormonal hypertension in childhood. *In* "Manual of Endocrinology and Metabolism, Third Edition" (N. Lavin, ed.), pp. 163–179. Lippincott Williams & Wilkins.

The disease is characterized by hyperaldosteronism and excessive secretion of two normally rare steroids, 18-oxocortisol and 18-hydroxycortisol. The presence of these two steroids suggests the simultaneous presence of 17α-hydroxylase and aldosterone synthase activities, which are normally limited to the zona fasciculata and the zona glomerulosa, respectively. Although there are clinical features similar to those found in other forms of primary hyperaldosteronism, the unique distinguishing feature of GRA is the complete and rapid suppression of aldosterone oversecretion by dexamethasone (glucocorticoid) administration.

GRA produces a volume-expanding, salt-sensitive form of low renin hypertension. Although hypertension is invariably present, hypokalemia and metabolic acidosis may be absent. Additionally, children demonstrate normal growth and development, which distinguishes this disorder from the aforementioned 11β-hydroxylase deficiency and apparent mineralocorticoid excess, discussed below.

Circadian measurement of plasma steroids in GRA patients has revealed that not only is aldosterone produced in excessive amounts, but is also high following ACTH stimulation, suggesting a zona fasciculata origin.

Other factors in addition to elevated aldosterone levels may be involved in the etiology of hypertension in this disorder. Whereas ACTH given in the dexamethasone-suppressed state reestablished the hypertension of the untreated state in GRA patients, aldosterone replacement alone in large doses over 5 days did not reestablish hypertension. Replacement of aldosterone, DOC, and 18-hydroxy-DOC in amounts restoring pretreatment levels of these steroids also failed to restore hypertension. Thus, a significant proportion of the hormonal effect in GRA hypertension may be derived from other ACTH stimulable steroids.

Qualitative steroid abnormalities in GRA have been identified, but the summed mineralocorticoid effects of these steroids at their physiological levels fall far short of the total mineralocorticoid effects observed. The steroids specific for GRA and identified as the 17α-hydroxylated analogues of 18-hydroxycorticostrone (18-OHB) and aldosterone, namely, 18-hydroxycortisol and 18-oxocortisol, respectively, suggest that the inner cortical zones, which express 17α-hydroxylase and are ACTH responsive, are responsible, thereby indicating a particular defect in adrenal zonation. An explanation for the elevated aldosterone and its unusual regulation by ACTH has been provided by genetic studies that have shown that a chimeric gene is created by misalignment of chromatids and unequal crossing over between genes during meiotic reduction in gametogenesis.

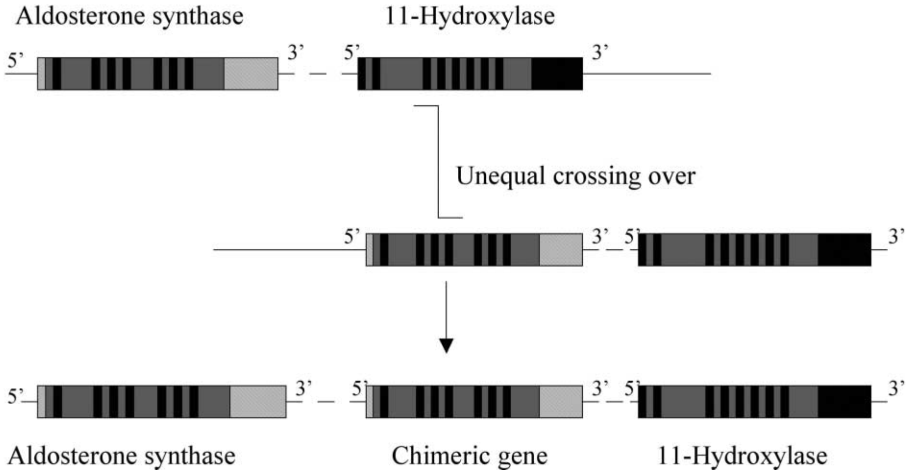

**FIGURE 4** Formation of chimeric gene product by misalignment of chromatids and unequal crossing over between genes during meiotic reduction in gametogenesis. Reprinted from Lifton, R. P., Gharavi, A. G., and Geller, D. S. (2001). Molecular mechanisms of human hypertension. *Cell* **104**, 545–556, with permission of Elsevier.

This occurs between the genes for CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase), which reside within a 30 kb stretch on chromosome 8. In affected individuals, a chimeric gene that has aldosterone synthase activity is produced but it is regulated by ACTH rather than by angiotensin II (Fig. 4).

Children with GRA are treated with glucocorticoids, and resolution of their hypertension usually occurs within 2 weeks after initiation of therapy. The response to glucocorticoids is variable in adults, often requiring the additional use of antihypertensive medications, such as spironolactone, which blocks the action of aldosterone at the mineralocorticoid receptor. Amiloride and triamterene have also been used in GRA patients.

## D. Pseudo Hypoaldosteronism Type I

Pseudo hypoaldosteronism type I is characterized by loss-of-function mutations that cause defective sodium transport due to the inability of aldosterone to exert its end-organ effect. Two genetically distinct forms have been identified, the autosomal dominant form and the recessive form.

The autosomal dominant form, also known as mineralocorticoid resistance, is caused by mutations in the MR of the kidney. As a result, aldosterone has no effect on the MR in the kidney. The disorder presents in infancy with salt wasting, dehydration, and failure to thrive, but does have a variable clinical presentation ranging from asymptomatic cases to severe salt wasting. Owing to the absence of responsiveness to aldosterone, there is markedly elevated plasma renin activity, with paradoxically elevated plasma and urinary aldosterone. Mineralocorticoid resistance is treated with sodium supplementation, which can often be discontinued after infancy as the salt loss may improve with age. Family members can be clinically asymptomatic but biochemically affected and can be identified by their plasma renin levels and aldosterone response to salt deprivation.

The recessive form of pseudo hypoaldosteronism results from loss-of-function mutations in one or more of the genes encoding the α-, β-, and γ-subunits of the amiloride-sensitive ENaC, which is present in the kidney as well as in the colon, sweat glands, salivary glands, and lungs. This disorder presents in early infancy with salt wasting, due to profound urinary sodium loss leading to hyponatremia, and is accompanied by hyperkalemia and metabolic acidosis. Because salt loss can occur from multiple organs, the presentation of the recessive form can be life-threatening if the disorder is unrecognized in the neonatal period. Biochemically it is also characterized by elevated plasma renin and aldosterone levels. Furthermore, children with this disorder can present with increased frequency of respiratory tract illnesses due to defective sodium-dependent liquid absorption in the airways. Treatment includes lifelong salt supplementation, often with the addition of potassium-binding resins to maintain normokalemia.

Gain-of-function mutations that affect the ENaC result in sodium retention, hypokalemia secondary to potassium wasting, and metabolic alkalosis, a disorder known as Liddle's syndrome (pseudo hyperaldosteronism). Liddle's syndrome is an autosomal dominant form of hypertension, characterized by suppressed plasma renin and aldosterone levels.

### E. Apparent Mineralocorticoid Excess

Apparent mineralocorticoid excess (AME) is a form of hypertension caused by mutations in the HSD11B2 gene, which result in a deficiency of the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 enzyme. The 11β-HSD type 2 enzyme maintains the specificity of the MR for aldosterone by catalyzing the conversion of cortisol to cortisone in the same cells in which the MR is expressed.

AME is a rare autosomal recessive disorder, having been identified in only approximately 60 patients over the past 20 years. To date, most patients with AME who have undergone molecular genetic analysis are homozygous for one of the different mutations, with only 3 compound heterozygote patients.

AME usually presents in early life with low birth weight and postnatal failure to thrive, hypertension, and persistent polyuria and polydipsia. The disorder is characterized by hypokalemic alkalosis, hyporeninemia, and undetectable serum concentrations of aldosterone. The diagnosis of AME can be made biochemically by measuring the ratio of urinary cortisol metabolites to cortisone, often measured as an increase in the sum of the urinary tetrahydrocortisol (THF) and allotetrahydrocortisol, divided by the concentration of tetrahydrocortisone (THE) [(THF + 5αTHF)/THE]. This ratio, which is normally 1.0, is elevated in patients with AME, with a predominance of THF.

The treatment of AME is primarily directed at the correction of hypokalemia and hypertension. Spironolactone, an MR receptor antagonist, is the medication of choice. Addition of potassium-sparing diuretics may be beneficial, but patients can become refractory to therapy. A reduction in dietary sodium and supplemental potassium can be beneficial.

### F. 17α-Hydroxylase Deficiency

17α-Hydroxylase deficiency is an extremely rare disease that has been identified in approximately 120 patients worldwide. The deficiency of 17α-hydroxylase results from mutations in the enzyme cytochrome P450C17, which functions both as steroid 17α-hydroxylase and as 17,20-lyase. The structural gene for cytochrome P450C17 has been mapped to chromosome 10q24.3. Deleterious mutations have been identified in numerous cases of 17α-hydroxylase deficiency.

The enzyme deficiency causes diminished production of cortisol and sex steroids, whose production requires the 17,20-lyase function of the same 17α-hydroxylase enzyme (Fig. 1). Both the adrenals and the gonads shared the enzyme defect, resulting in decreased biosynthesis of all androgens and estrogens. Untreated 17α-hydroxylase deficiency in females at pubertal age results in primary amenorrhea and lack of development of secondary sex characteristics. Male (46,XY) patients present with an undervirilized (i.e., infantile female) sexual phenotype at birth and, later, failure of pubertal development.

Reciprocal elevation of ACTH secondary to low cortisol increases synthesis via the 17-deoxy pathway, notably of the steroids DOC and corticosterone. As in 11β-hydroxylase deficiency, the formation of aldosterone is reduced secondary to suppressed renin as a result of excess DOC.

Because of massive overproduction of corticosterone, which is a weak glucocorticoid with serum concentrations 30 times normal, patients with this disorder rarely present with symptoms of adrenal insufficiency in early infancy. Diagnosis is often made in a young female or apparent female presenting at pubertal age with primary amenorrhea or lack of development of secondary sexual characteristics. There can be associated hypertension and hypokalemia at diagnosis. Alternatively, these latter findings may constitute the primary presentation at any age.

Treatment consists of glucocorticoid replacement in prepuberty to normalize elevated circulating mineralocorticoids and sex steroid replacement as appropriate for the phenotypic sex starting at pubertal age. In 46,XY patients, the testes may be abdominal, inguinal, or labial; if sex assignment is male, the gonads may be preserved. If the patient is reared as a female, estrogen replacement induces development of female secondary sexual characteristics and stimulates the increase in bone mass that normally occurs during puberty.

## V. SUMMARY

Adrenal steroid hormones are among the many factors regulating blood pressure. Mineralocorticoid abnormalities can cause clinical conditions such as hypertensive congenital adrenal hyperplasia due to 11β-hydroxylase deficiency, 17α-hydroxylase/17,20-lyase deficiency, and glucocorticoid-remediable aldosteronism. An inborn deficiency of the enzyme 11β-hydroxysteroid dehydrogenase can also explain the hypertension found in apparent mineralocorticoid excess. Because many of these disorders can be life-threatening, making a specific diagnosis can aid in designing an effective therapeutic regimen. Furthermore, understanding the etiology of the mineralocorticoid disorder can help in identifying other affected family members and in providing anticipatory management and genetic counseling in inherited disorders.

## Acknowledgments

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## Glossary

**aldosterone** Principal mineralocorticoid hormone produced in the adrenal cortex. Primary function is sodium reabsorption in the distal renal tubule.

**amiloride-sensitive epithelial sodium channel** This channel consists of three homologous subunits, $\alpha$, $\beta$, and $\gamma$, and mediates the reabsorption of sodium in the distal renal tubule.

**11β-hydroxysteroid dehydrogenase type II** An enzyme that is expressed predominantly in the kidney and catalyzes the conversion of cortisol to cortisone.

**mineralocorticoid** A steroid synthesized in the adrenal cortex that regulates electrolyte balance. Aldosterone is the principal circulating mineralocorticoid.

**mineralocorticoid receptor** The specific receptor for steroid hormones with mineralocorticoid activity.

**renin–angiotensin system** One of the principal regulators of the aldosterone biosynthetic pathway.

## See Also the Following Articles

**Glucocorticoid Biosynthesis • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Biosynthesis • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Receptor, Natural Mutations of • Mineralocorticoids and Hypertension • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Mineralocorticoid Effects on Physiology and Gene Expression

Anikó Náray-Fejes-Tóth and Géza Fejes-Tóth
*Dartmouth Medical School*

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**Mineralocorticoids, named for their ability to maintain the homeostasis of two important minerals, sodium and potassium, are a class of adrenocortical hormones. The major physiological effect of mineralocorticoids is to promote sodium retention and potassium excretion.**

## I. INTRODUCTION

Although the daily secretion rate of the adrenal gland mineralocorticoids is very low, i.e., only about 1/100th of that of glucocorticoids, this small amount is essential for life. In the absence of mineralocorticoids, such as occurs in Addison's disease, a life-threatening electrolyte and water imbalance develops.

The two main mineralocorticoid hormones are aldosterone and 11-deoxycorticosterone (DOC). The most potent mineralocorticoid, aldosterone, is produced by the zona glomerulosa of the adrenal cortex. Aldosterone was isolated in the early 1950s from a lipid extract of the adrenal cortex.

## II. PHYSIOLOGICAL EFFECTS OF MINERALOCORTICOIDS

Aldosterone has two major physiological functions: (1) it regulates the total amount of sodium in the body and, consequently, the extracellular fluid volume and (2) it regulates potassium homeostasis. Normal plasma sodium concentration is 136–145 m*M*; normal plasma potassium concentration is 3.5–5.0 m*M*. Changes in sodium levels lead to changes in plasma osmolality. Changes in plasma potassium levels alter membrane potential and, consequently, have major effects on excitatory tissues, such as those in the muscles, the heart, and the nervous system. Therefore, the levels of sodium and potassium in the plasma must be tightly regulated.

The main target site of aldosterone is the kidney, but aldosterone also affects sodium and potassium excretion by the colon and the salivary and sweat glands. In the kidney, the primary target segment of aldosterone is the cortical collecting duct. Mineralocorticoid effects on sodium and potassium transport take place in the major cell type of this nephron segment, the principal cells. A model of the cellular transport mechanisms involved in sodium reabsorption and potassium secretion by "tight" epithelia is shown in Fig. 1. Sodium enters the principal cells from the urine, through lumenal sodium channels (which are sensitive to the diuretic, amiloride), via the sodium electrochemical gradient; it is extruded from the cells by a $Na^+$- and $K^+$-transporting ATPase ("sodium pump") located in the basolateral membrane. The basolateral $Na^+$,$K^+$-ATPase also creates the driving force for potassium to exit from the cells into the urine via lumenal $K^+$-selective channels.

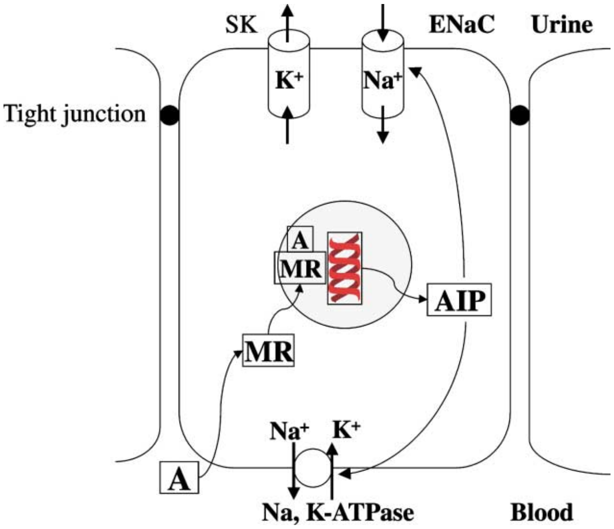

FIGURE 1 Model of aldosterone action in epithelial cells. Aldosterone (A) binds to mineralocorticoid receptors (MR), and the MR–aldosterone complex binds to specific hormone response elements on the DNA. Interaction of the activated MR with hormone response elements leads to changes in the transcription of specific, aldosterone-induced proteins (AIP). The AIPs modulate the activity and/or trafficking of the two most important effectors of aldosterone action, the epithelial $Na^+$ channel (ENaC) present in the apical membrane and the basolateral $Na^+$,$K^+$-ATPase. Activation of the $Na^+$,$K^+$-ATPase also creates the driving force for $K^+$ to exit from the cell to the tubule lumen though apical $K^+$-selective channels, which may explain the increased $K^+$ secretion following aldosterone. The tight junctions are impermeant for $Na^+$ and $K^+$, thereby preventing paracellular movement of the transcellularly transported ions.

Aldosterone stimulates active sodium reabsorption from the urine into the blood by a complex action, which results in increased activity of $Na^+$,$K^+$-ATPase as well as in increased conductive capacity of lumenal sodium channels. Although the effect of aldosterone on $Na^+$,$K^+$-ATPase is most likely due to direct stimulation of transcription of new mRNAs for this enzyme, the effect on sodium channels is more complex. The effect in this case includes an increase in the activity of pre-existing Na channels in the apical cell membrane, as well as an increase in the absolute number of channels in the apical cell membrane (see later).

Mineralocorticoid effects on Na transport in amphibian epithelia (which are frequently studied model systems) are usually divided into "early" and "late" phases. (It should be pointed out that the existence of these two phases in the mammalian collecting duct has not been clearly established.) The early response takes place about 0.5–3 h after hormone addition, and is probably mediated by activation of pre-existing apical membrane $Na^+$ channels. The late phase (several hours to several days after hormone addition) involves new $Na^+$ channel synthesis, $Na^+$,$K^+$-ATPase molecules, and secondary effects on $K^+$ and $H^+$ transport.

Because water movement follows passively that of sodium, an increase in sodium reabsorption necessarily results in an expansion of extracellular fluid volume and an increase in blood pressure. Therefore, chronic overproduction of aldosterone leads to pathological conditions accompanied by hypertension.

Although the main physiological effect of aldosterone is stimulation of $Na^+$ reabsorption, aldosterone also regulates the excretion of potassium by increasing $K^+$ secretion from the blood into the tubular lumen. This action takes place predominantly in the principal cells of the cortical collecting ducts. The exact cellular mechanism underlying the $K^+$-secreting response elicited by aldosterone is still not clear, but probably the bulk of the effect is mediated through an increase in the activity and expression of the basolateral $Na^+$,$K^+$-ATPase. Increased pumping of $Na^+$ out of the cell is coupled to increased pumping of $K^+$ into the cell. This event creates a favorable electrochemical driving force for $K^+$ to exit from the cell into the lumen through $K^+$-selective ion channels (SK channels). The hypothesis that aldosterone increases the number of $K^+$ channels in the apical membrane of cortical collecting duct cells by directly increasing their level of expression has not been confirmed. Thus, although it is widely acknowledged that aldosterone regulates renal $K^+$ handling, the question if this is only a secondary effect, coupled to its regulation of $Na^+$ reabsorption, is still open.

In addition to its effects on sodium and potassium transport, aldosterone also enhances the excretion of $H^+$ ions in the collecting ducts. $H^+$ transport takes place in the minor cell type of the collecting duct, the intercalated cells. The mechanisms by which aldosterone regulates acid secretion are not clear, and it is still debated whether aldosterone has a direct effect on the intercalated cells by increasing the expression of the $H^+$-ATPase, or whether the enhanced $H^+$ secretion is merely a consequence of the lumen negative voltage, created by increased sodium reabsorption in principal cells. Whatever the exact mechanism, the effect of aldosterone on acid secretion is probably not a major physiological regulatory function.

## III. RECEPTORS THAT MEDIATE MINERALOCORTICOID EFFECTS

Aldosterone, like other steroids, exerts its effects through binding to intracellular receptors, called mineralocorticoid receptors (MRs). Mineralocorticoids, like other steroids, enter the cell by passive diffusion. Ligand binding induces a structural change, i.e., activation, in the MR. The activated MR then interacts with specific regulatory segments, hormone response elements (HREs),on the chromatin. This interaction results in an increase (or decrease) of the transcription of specific genes. MRs are present in target tissues in the renal collecting duct system, the colon, and the sweat glands, for example; in addition, they can be found in the heart and in the muscular walls of vessels, as well as in the brain, where their function is still not clear.

The MR is a member of the nuclear receptor superfamily. Nuclear receptors are ligand-dependent transcriptional regulators that contain a highly variable N-terminal domain, a highly conserved DNA-binding domain, and a conserved C-terminal ligand-binding domain (LBD). Transcriptional activation function (AF) is localized to both the N-terminal region (AF-1) and the LBD (AF-2). The DNA-binding domain contains two type II zinc fingers, which anchor this domain to HREs.

Nuclear receptors bind to only one of two possible consensus sequence half-sites on DNA. These two half-sites can combine in various orientations and with different spacing, allowing binding of head-to-head, head-to-tail, or tail-to-tail receptor homo- or heterodimers. However, the diversity of HREs is insufficient to account for the observed spectrum of specificity in gene regulation by individual nuclear receptors. For instance, the MR, the glucocorticoid receptor (GR), and the androgen and progesterone receptors have identical DNA binding specificity but mediate very different biological actions. It should be noted that thus far no HRE has been identified that can distinguish between MR and GR. Thus, a central question in the biology of nuclear receptors is how distinct patterns of gene regulation are initiated by receptors with identical DNA sequence recognition profiles. It is logical to assume that receptor-specific actions are mediated through receptor-specific protein–protein interactions, but solid experimental evidence to support this hypothesis is still lacking.

In mineralocorticoid target tissues, in addition to MRs, GRs are also expressed. Although both types of receptors have high affinity for their respective ligands, they exhibit poor selectivity between endogenous glucocorticoids and aldosterone. In addition to glucocorticoids, the GR also binds aldosterone, although with lower affinity than cortisol. It was even more surprising to find that the MR *in vitro* has the same affinity for cortisol as for aldosterone ($K_d = 0.5–1.0$ n$M$). Because the plasma level of cortisol is from 100- to 1000-fold higher than that of aldosterone, the obvious question is how aldosterone can act as the physiological mineralocorticoid hormone in the presence of much higher concentrations of cortisol, which, in theory, should occupy all MRs. To explain this paradox, Funder and Edwards proposed that aldosterone selectivity of the MR is vested in the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). This enzyme catalyzes the dehydrogenation (oxidation) of the 11-OH group present in every biologically active glucocorticoid. The resulting oxidized glucocorticoid metabolites (such as cortisone or 11β-dehydrocorticosterone) are biologically inactive because they do not bind to the MR. Thus, 11β-HSD rapidly inactivates endogenous glucocorticoids (but not aldosterone; see later) in mineralocorticoid target cells. This hypothesis was proved right when a second isoform of 11β-HSD (referred to as 11β-HSD2) was characterized in aldosterone target cells. 11β-HSD2 has an unusually high affinity for endogenous glucocorticoids ($K_m$ in the nanomolar range) and catalyzes exclusively dehydrogenation, i.e., inactivation of glucocorticoids. Subsequently, the cDNA of 11β-HSD2 was cloned, and it turned out that 11β-HSD2 has very low degree of similarity to the type 1 isoform of 11β-HSD. 11β-HSD1 is expressed ubiquitously, and *in vivo* (mainly in the liver) it mediates activation of the inactive oxidized glucocorticoids to their active forms.

11β-HSD2, by lowering the intracellular levels of active glucocorticoids, protects the target cell MR from being occupied by glucocorticoids; thereby ensuring that aldosterone can act as the physiological mineralocorticoid hormone. Consequently, 11β-HSD2 has been called the "guardian of the MR." For such a protective mechanism, it's a prerequisite that the physiological ligand of the MR is not a substrate for this enzyme. Indeed, although aldosterone can exist in two forms (as an aldehyde and a hemiacetal), in the blood it occurs predominantly as a hemiacetal. In this molecule, the 11β-OH group forms a ring with the 18-aldehyde group and therefore 11β-HSD2 cannot oxidize the 11β-OH group in aldosterone.

The importance of 11β-HSD2 becomes clear if we consider the severity of the syndrome that develops if this enzyme is inactive: in the congenital disease of apparent mineralocorticoid excess (AME), aldosterone levels are low, but all the symptoms of hyperaldosteronism are present (high blood pressure, low plasma $K^+$ concentration, low plasma renin activity). This paradox occurs because deficient 11β-HSD2 activity in the kidney leads to high intracellular cortisol concentrations, which saturates the MRs and exerts mineralocorticoid activity. An acquired, milder form of 11β-HSD2 insufficiency occurs in patients who consume large amounts of licorice. The active ingredient of licorice, glycyrrhetinic acid, is an inhibitor of 11β-HSD2.

The critical importance of the MR in mediating the physiological effects of mineralocorticoids is clear from experiments with genetically engineered animals with targeted elimination of the MR (MR knockout mice) and from studies of patients with hereditary mutations of the MR. Homozygous MR knockout mice die a few days after birth due to pseudohypoaldosteronism; patients with loss-of-function mutations of the MR, survive, but also develop pseudohypoaldosteronism.

## IV. MINERALOCORTICOID-INDUCED GENES AND PROTEINS

There is a general agreement that mineralocorticoids stimulate $Na^+$ reabsorption by changing the transcriptional activity of specific genes. However, the exact mechanism(s) and the genes involved are just beginning to be uncovered. The two most important effector molecules in aldosterone-induced $Na^+$ reabsorption are (1) the epithelial Na channel (ENaC) present in the apical (lumenal) membrane of $Na^+$-absorbing cells and (2) the basolateral $Na^+$,$K^+$-ATPase (see Fig. 1).

### A. Epithelial Na Channel

The key event in transepithelial $Na^+$ transport in the collecting duct and other aldosterone target cells is $Na^+$ uptake, from lumen to cell, through ENaCs. ENaCs consist of three homologous subunits: α, β, and γ. ENaCs residing in the apical membrane of native epithelia are thought to consist of heterotrimeric complexes; however, the exact stoichiometry of the subunits in the apical membrane is still debated.

The critical role of ENaC in the regulation of Na homeostasis is demonstrated by at least two inherited diseases associated with mutations in ENaC subunits. Certain mutations in the β or γ subunits lead to Liddle's syndrome, characterized by constitutively elevated $Na^+$ reabsorption, and, as a consequence, hypertension, whereas loss-of-function mutations in ENaC lead to severe Na depletion and hypotension.

Although observations from several laboratories strongly indicate that aldosterone increases the *de novo* synthesis of ENaC subunits, there is no consensus about the subunit in which changes occur, the tissue-specificity of this effect, and, most importantly, the temporal correlation of the induction in

ENaC subunit mRNA with apical $Na^+$ permeability. It seems likely that the early increase in apical $Na^+$ permeability is mediated by postsynthetic modifications (such as phosphorylation or methylation) that enhance the translocation of ENaC subunits to the apical membrane or activate pre-existing channels in the apical membrane. Recent evidence strongly suggests that an important regulation of $Na^+$ reabsorption occurs via increased surface expression of ENaC subunits in the apical membrane. This effect is probably due to an enhanced rate of exocytosis of ENaC molecules.

## B. $Na^+$,$K^+$-ATPase

In $Na^+$-transporting epithelia, a basolateral $Na^+$,$K^+$-ATPase is responsible for pumping $Na^+$ out of the cell and $K^+$ into the cell, against their electrochemical gradients. By this action the $Na^+$,$K^+$-ATPase maintains the driving force for $Na^+$ to enter the cell via apical ENaCs. A regulatory role for aldosterone on the Na pump has been well established by studies in both amphibian and mammalian epithelia. Aldosterone was shown to stimulate the activity of $Na^+$,$K^+$-ATPase by rapidly increasing its mRNA and protein synthesis in amphibian cells. However, this rapid increase in mRNA and protein levels has not yet been unequivocally demonstrated in mammalian cells. Thus, the question as to whether the observed increase in $Na^+$,$K^+$-ATPase activity and expression is secondary to changes in intracellular $Na^+$ concentration (as a consequence of increased $Na^+$ permeability of the apical membrane) following aldosterone is still unanswered. In addition to increasing the synthesis of $Na^+$,$K^+$-ATPase, aldosterone might also increase translocation of the enzyme into the basolateral membrane, an action that would be analogous to increased insertion of ENaCs into the apical membrane.

## C. Early Aldosterone-Induced Genes

The observation that the Na-absorbing effect of aldosterone, at least in mammalian cells, seemed much faster than the increased rate of synthesis of ENaC or $Na^+$,$K^+$-ATPase suggested the existence of early, aldosterone-induced genes. According to a generally accepted hypothesis, the products of these genes would mediate the increased activity or trafficking of ENaC.

Although early studies on two-dimensional gels detected several aldosterone-induced proteins (AIPs), these proteins remained unidentified. However, a new and exciting development in the search for mediators of aldosterone action is the recent identification of two aldosterone-induced early gene products: serum- and glucocorticoid-induced kinase and K-ras2.

### *1. Serum- and Glucocorticoid-Induced Kinase*

Serum- and glucocorticoid-induced kinase (sgk), a serine/threonine kinase, was originally identified as the product of a gene that is rapidly induced by dexamethasone in mammary epithelial cells, but until recently, no function was ascribed to this kinase.

In cultured rabbit cortical collecting duct cells, synthesis of sgk mRNA and protein is rapidly induced by physiological doses of aldosterone; this occurs as well as in the rat collecting duct and colon following *in vivo* aldosterone treatment. In two cell lines (M1 and in A6 cells) that originate from collecting ducts but do not express MRs, glucocorticoids transcriptionally up-regulate sgk, with a concomitant increase in amiloride-sensitive $Na^+$ transport. Coexpression of sgk in *Xenopus* oocytes with ENaCs results in a three- to sevenfold increase in amiloride-sensitive current. This is not due to a nonspecific effect on vesicular traffic, because sgk does not increase surface expression of the $K^+$ channel, ROMK.

The sgk-induced increase in $Na^+$ current in oocytes is due to an increase in the number of ENaCs present in the plasma membrane and not to changes in the open probability or kinetics of ENaCs. This effect resembles that of aldosterone in epithelial cells. Mutational analysis of ENaCs and *in vitro* studies suggest that the effect of sgk on ENaC surface density is not mediated by direct phosphorylation of ENaC subunits. Thus, it seems that sgk regulates a protein involved in the vesicular traffic of these transporters, rather than the transporters themselves. Endogenous targets of sgk phosphorylation that mediate its biological effects have not yet been identified.

Although sgk is regulated at the transcriptional level by steroids, its activity is regulated by phosphorylation via a phosphatidylinositol-dependent pathway. Activation of phosphatidylinositol 3-kinase (PI3-kinase) leads to activation of two other kinases, phosphoinositide-dependent kinase 1 (PDK1) and PDK2, which, in turn, activate sgk. Because insulin and other hormones that stimulate the PI3-kinase pathway are able to stimulate $Na^+$ absorption, sgk might serve as a more general mediator of the hormonal regulation of $Na^+$ transport rather than as a mediator of aldosterone action alone.

### *2. K-ras2*

The small G-protein K-ras2 has been shown to be induced in amphibian A6 cells by high concentrations of aldosterone. This effect is most likely mediated by the glucocorticoid receptor, because A6 cells do not express functional MRs. Although the effects of K-ras2 on Na$^+$ currents in oocytes are inconclusive, antisense oligonucleotide and overexpression experiments in A6 cells suggest a possible role for this gene in corticosteroid-induced Na$^+$ transport. The role of K-ras2 in the mammalian collecting duct has not been established, and because the expression of K-ras2 mRNA is not induced by aldosterone *in vivo*, its physiological significance in mineralocorticoid action, at present, is uncertain.

### 3. *Other Early Effects of Aldosterone*

There is also evidence that the action of aldosterone is accompanied (and possibly mediated) by increased carboxymethylation of proteins, including that of βENaC. Synthesis of the key enzymes involved in this reaction is not increased by aldosterone, but their activity is. This raises the possibility that an aldosterone-induced early gene is responsible for the activation of these enzymes.

## V. NONCLASSICAL ALDOSTERONE TARGETS

In addition to its effects on ion transport in epithelial cells, aldosterone, under certain conditions, has been shown to have effects in other tissues, such as the brain, the heart, and vascular smooth muscle cells. These effects include elevation of blood pressure following intracerebral ventricular infusion of aldosterone, increase in salt appetite, interstitial fibrosis in the heart, and rapid effects on the activity of the Na/H exchanger. The identity of the receptors that mediate such effects of aldosterone are still unclear; in certain cases the effects might occur via MRs that are "unprotected" by 11β-HSD2, and in other cases "nonclassical" membrane receptors and "nongenomic" effects have been implied. No such membrane receptor for mineralocorticoids has been cloned, and the significance of these receptors and actions is not yet known.

## VI. SUMMARY

The primary function of aldosterone, a mineralocorticoid synthesized in the zona glomerulosa of the adrenal glands, is to stimulate the reabsorption of sodium from renal tubular fluid in the distal nephron of the kidney. A concomitant excretion of potassium and hydrogen occurs, in part due to the lumen (tubule) negative potential difference generated by active sodium transport.

Aldosterone exerts its effects through intracellular mineralocorticoid receptors. Aldosterone- activated receptors bind to specific elements on the chromatin, inducing the transcription of specific mRNAs. Although mineralocorticoid and glucocorticoid receptors serve different biological functions, aldosterone and cortisol have similar affinities for the mineralocorticoid receptors. Because the plasma concentration of aldosterone is about 1/1000th that of cortisol, in aldosterone target tissues (the renal collecting duct and salivary and sweat glands) the enzyme 11β-hydroxysteroid dehydrogenase type 2 inactivates glucocorticoids, leaving the aldosterone molecules without competition, and thus able to bind to their receptors. The congenital absence of this enzyme develops into a fatal condition, known as apparent mineralocorticoid excess.

The main effectors of aldosterone action on Na$^+$ reabsorption are the epithelial Na$^+$ channel, expressed in the apical membrane of Na$^+$ reabsorbing cells, and the basolateral Na$^+$,K$^+$-ATPase. Aldosterone increases the expression of both of these proteins. In addition, aldosterone rapidly increases the insertion of active ENaC subunits into the apical membrane and might activate ENaCs already present in the apical membrane.

Serum- and glucocorticoid-induced kinase, a serine/threonine kinase, has been recently identified as the product of an early-induced, aldosterone-regulated gene. This kinase is capable of increasing ENaC current and insertion of ENaC molecules into the plasma membrane and thus is probably an important mediator of the early effect of aldosterone on Na$^+$ reabsorption.

## Glossary

**aldosterone** The main mineralocorticoid hormone.

**epithelial sodium channel** Specific ion channel consisting of α, β, and γ subunits, expressed in the apical membrane of Na$^+$-reabsorbing epithelia.

**11β-hydroxysteroid dehydrogenase** An enzyme that catalyzes the oxidation of hydroxy groups in the 11β position of steroids. 11β-HSD2, the second isoform of this enzyme, has high affinity for endogenous glucocorticoids and is expressed specifically in aldosterone target cells.

**hormone response element** Regulatory DNA sequence in the promoter region of a gene; interacts with factors that bind hormone receptors, altering transcription of specific genes.

**mineralocorticoids** Hormones produced by the adrenal cortex; regulate sodium and potassium homeostasis.

**mineralocorticoid receptor** The specific target tissue receptor for mineralocorticoids. It has high affinity

($K_d$, $< 1$ n$M$) for aldosterone and for endogenous glucocorticoids such as cortisol.

**serum- and glucocorticoid-induced kinase** A serine/threonine kinase that is regulated transcriptionally and posttranscriptionally; its mRNA and protein expression is rapidly induced by aldosterone.

## See Also the Following Articles

**Glucocorticoid Biosynthesis • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Biosynthesis • Mineralocorticoid Disorders, Genetic Basis of • Mineralocorticoid Receptor, Natural Mutations of • Mineralocorticoids and Hypertension • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Mineralocorticoid Receptor, Natural Mutations of

Marie-Edith Rafestin-Oblin, Jérôme Fagart, and Maria-Christina Zennaro
*Xavier Bichat, INSERM U478, Paris, France*

I. INTRODUCTION
II. THE MINERALOCORTICOID RECEPTOR AS MEDIATOR OF ALDOSTERONE ACTION
III. ACTIVATING MUTATIONS OF THE MINERALOCORTICOID RECEPTOR
IV. LOSS-OF-FUNCTION MUTATIONS OF THE MINERALOCORTICOID RECEPTOR
V. SUMMARY

**The mineralocorticoid hormone aldosterone produces its effects through the mineralocorticoid receptor (MR), which, like other members of the nuclear receptor family, is a ligand-activated transcription factor that induces or represses specific target genes. Natural mutations in the gene coding for the MR can lead to either a loss-of-function or a gain-of-function.**

## I. INTRODUCTION

Aldosterone (Fig. 1), the mineralocorticoid hormone secreted by the adrenal gland, is a key regulator of sodium homeostasis and plays a central role in blood pressure regulation. Aldosterone produces its effects through the MR, which is expressed in the epithelial cells that line the distal tubule of the kidney, the distal colon, and the ducts of salivary and sweat glands. In these cells, MR-dependent sodium reabsorption is mediated by the apical epithelial amiloride-sensitive sodium channel (ENaC) and the basolateral $Na^+$, $K^+$-ATPase (Fig. 2). It is now clear that ENaC and $Na^+$, $K^+$-ATPase are not early aldosterone-induced proteins, and an active search for such proteins is currently in progress. Serum- and glucocorticoid-regulated kinase (sgk) has emerged as one such possible primary aldosterone-induced protein.

Aldosterone and another adrenal gland hormone, cortisol (the major glucocorticoid hormone in humans), bind to the MR with similar affinities; however, in epithelial cells, aldosterone is ensured access to the MR by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), whereas 11β-HSD2

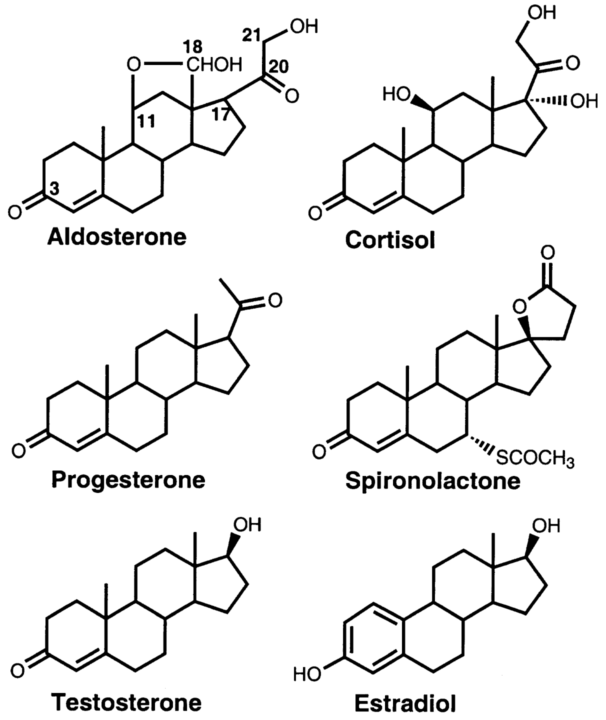

FIGURE 1 The key mineralocorticoid, aldosterone, and structurally similar compounds. Spironolactone is an aldosterone antagonist.

metabolizes cortisol to cortisone, which has no affinity for the MR. The MR is also expressed in some nonepithelial cells, such as the neurons of the hippocampus and the myocytes of the heart. However, the mechanism underlying the mineralocorticoid specificity of these cells is not clear.

Abnormalities in aldosterone secretion and in the production of steroids that activate the MR and mutations of 11β-HSD2, MR, or ENaC all affect the sodium balance in epithelial target cells. This article focuses on the mechanism of action of the MR and on the receptor mutations that have a functional impact.

## II. THE MINERALOCORTICOID RECEPTOR AS MEDIATOR OF ALDOSTERONE ACTION

The MR belongs to the nuclear receptor superfamily that includes the other steroid hormone receptors as well as receptors for thyroid hormone, vitamin D, and retinoic acid and numerous orphan receptors. Like the other nuclear receptors, the MR contains several functional domains (Fig. 3). The N-terminal part of the protein harbors an autonomous activation function. The cysteine-rich central region, containing approximately 90 amino acids, is the DNA-binding domain (DBD). This region is the one most completely conserved in the nuclear receptors; it contains two zinc finger structures, in which each zinc atom is coordinated by four cysteines. These structures are responsible for DNA–receptor interaction and dimerization. The sequence between the DBD and the ligand-binding domain (LBD), called the hinge region, is probably involved in the ligand-induced conformational changes of the receptor. The C-terminal part of the receptor, a large region containing approximately 250 amino acids, mediates numerous functions, including ligand binding, interactions with heat-shock proteins and transcriptional coactivators, dimerization, nuclear targeting, and hormone-dependent activation.

The human MR gene contains 10 exons. Two different 5′-untranslated exons (1α and 1β) are transcribed to form two distinct mRNA isoforms, MRα and MRβ, which encode the same protein. Exon 2, which contains the MR translation start site, encodes the N-terminal part of the receptor, exons 3 and 4 encode the DBD, and exons 5–9 encode the hinge region and the LBD (Fig. 3).

The crystal structure of the LBD has been solved for several receptors, either in their unliganded state (apo-receptor) or in their agonist-associated state (holo-receptor). A common fold organization has been described in which 11 to 12 α helices and one β turn are arranged as an antiparallel sandwich fold, in a three-layer structure surrounding the ligand-binding cavity. In the holo state the LBD is more compact and

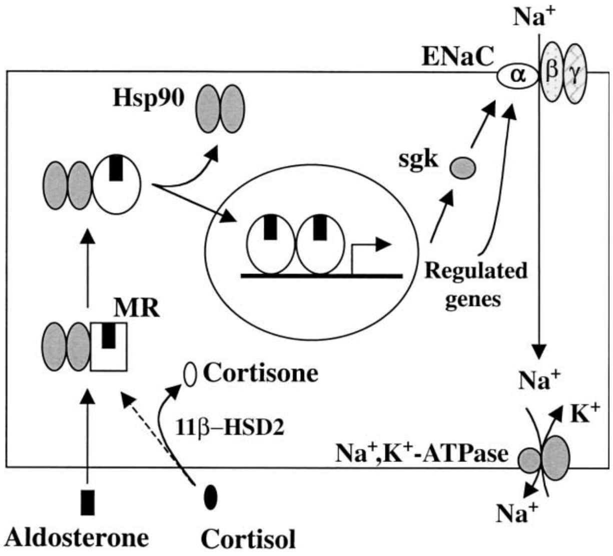

FIGURE 2 Mineralocorticoid receptor (MR)-dependent sodium reabsorption. ENaC, Amiloride-sensitive sodium channel; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; Hsp 90, 90 kDa heat-shock protein.

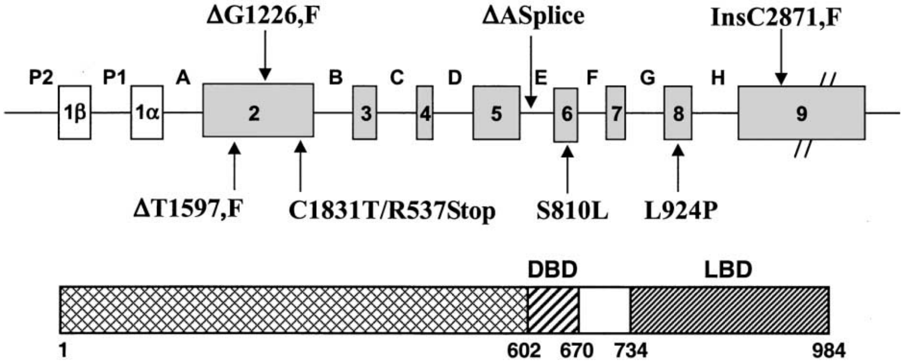

FIGURE 3 The human mineralocorticoid receptor gene (top) and the encoded protein functional domains (bottom). DBD, DNA-binding domain; LBD, ligand-binding domain. See text for discussion.

the helix 12 harboring the ligand-induced activation function is folded back toward the core of the LBD, creating an interface suitable for coactivator binding. It has not yet been possible to purify and crystallize the MR. Nevertheless, a three-dimensional model of the LBD of the human MR has been constructed using the crystallographic data for another member of the nuclear receptor superfamily as a template. Validation of the model by site-directed mutagenesis has made it possible to describe the organization of the MR LBD, to pick out the amino acids that delimit the ligand-binding cavity, and to identify those amino acids that interact with the polar functions of aldosterone. The 3-ketone moiety is anchored by Gln-776 and Arg-817, the 20-ketone moiety is anchored by Cys-942, and the 18- and 21-hydroxyls establish hydrogen bonds with Asn-770 (Fig. 4).

The aldosterone-dependent activation of gene transcription is thought to be a multistep process. In its unliganded state, the MR is found predominantly in the cell cytoplasm as a hetero-oligomeric complex in association with the heat-shock protein Hsp 90. The MR–Hsp 90 interaction appears to be required to maintain the receptor in a nonfunctional state and to fold the MR LBD in a ligand-binding-competent state. Aldosterone binding induces a change in the receptor conformation, thus allowing the associated proteins to dissociate and permitting the recruitment of transcriptional coactivators. Within the nucleus, the aldosterone-associated receptor binds as a dimer to hormone response elements in the promoter region of target genes and initiates hormone-mediated transcription through specific interactions with the transcriptional machinery.

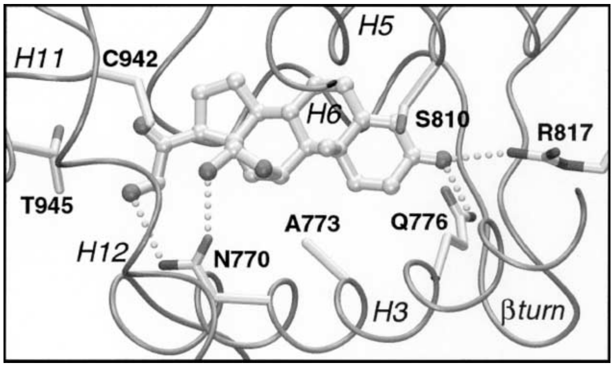

FIGURE 4 Aldosterone docking within the ligand-binding domain of the human mineralocorticoid receptor.

The contact between Asn-770 in helix 3 and the 21-hydroxyl group of aldosterone is crucial for the activation of the MR. In the absence of this contact, the MR remains in an inactive state—indeed, progesterone and the drug spironolactone, both of which lack the 21-hydroxyl group (Fig. 1), are potent mineralocorticoid antagonists.

## III. ACTIVATING MUTATIONS OF THE MINERALOCORTICOID RECEPTOR

A missense mutation in the MR LBD, resulting in the substitution of leucine for serine at codon 810 ($MR_{L810}$), has been detected in a 15-year-old boy with severe hypertension, suppressed plasma renin activity, and low serum aldosterone. Examination of the patient's family revealed that 11 $MR_{L810}$ carriers all developed hypertension before they were 20 years old, a rare trait in the general population, whereas the other members of the family had unremarkable blood pressure. All pregnancies among patients known to harbor this mutation have been complicated by a dramatic exacerbation of their hypertension, combined with the complete suppression of their renin–angiotensin system. Aldosterone levels, which are normally increased during pregnancy, were undetectable.

The $MR_{L810}$ function measured in cis–trans cotransfection assays, revealed a constitutive activity of $MR_{L810}$ that is not detected for the wild-type receptor ($MR_{WT}$). This activity, observed in the absence of any added steroid, may contribute to the hypertension in patients harboring this mutation. Several steroids have been tested for their ability to activate $MR_{L810}$. Estradiol and testosterone, steroids with a 17β-hydroxyl group (Fig. 1), activated neither $MR_{L810}$ nor $MR_{WT}$, whereas steroids with a 21-hydroxyl group, such as aldosterone and cortisol, activated both $MR_{WT}$ and $MR_{L810}$. Surprisingly, steroids without the 21-hydroxyl group did activate $MR_{L810}$, but they displayed antagonist properties when bound to the $MR_{WT}$. This is the case for the antihypertensive drug spironolactone (a synthetic steroid with a 17γ-lactone), and as a result, its use is contraindicated for $MR_{L810}$ carriers. Progesterone also activates $MR_{L810}$, and in this regard has the same potency as aldosterone. Progesterone levels normally increase 100-fold in pregnancy, and so it is likely that this hormone is responsible for the severe hypertension that is developed during pregnancy by $MR_{L810}$ carriers. The mechanism of early-onset hypertension in men is unclear. It has been suggested that 17α-hydroxyprogesterone may contribute to hypertension in men, because it is present at levels similar to those of aldosterone; furthermore, this derivative of progesterone is able to activate $MR_{L810}$.

The mechanism of $MR_{L810}$ activation is distinct from that of $MR_{WT}$. In a three-dimensional model of $MR_{L810}$, the side chain of Leu-810 in helix 5 projects into the ligand-binding cavity, thus making possible van der Waals contacts between Leu-810 and Ala-773 in helix 3. The implication of these contacts in the $MR_{L810}$ activation process was demonstrated in a site-directed mutagenesis study. Mutant receptors with amino acids bearing a short side chain at position 810 or 773 were not activated by a progesterone derivative, whereas $MR_{L810}$ was activated by the compound. The fact that the interaction between the steroid 21-hydroxyl group and Asn-770 becomes dispensable when a helix 3/helix 5 contact is created suggests that these contacts are interchangeable for receptor activation. Such a helix 3/helix 5 contact is present in the crystal structures of the progesterone and androgen receptors, with a methionine and a glycine at the position corresponding to Leu-810 and Ala-773 in the $MR_{L810}$. The conservation of this contact and its role in the $MR_{L810}$ activation by steroids lacking a 21-hydroxyl suggest that it is significant in activating nuclear receptors.

## IV. LOSS-OF-FUNCTION MUTATIONS OF THE MINERALOCORTICOID RECEPTOR

Pseudohypoaldosteronism type 1 (PHA1) is a rare inherited disease involving mineralocorticoid resistance characterized by severe neonatal salt loss accompanied by hypotension, despite high aldosterone levels. Affected patients also have marked hyperkalemia and metabolic acidosis and are resistant to the administration of exogenous mineralocorticoids. Treatment consists of salt supplementation, which can sometimes be discontinued after a variable period of time.

There are two forms of PHA1, autosomal recessive and autosomal dominant; in other cases, PHA can be sporadic. The recessive form, with severe and recurrent life-threatening episodes of salt loss, is caused by homozygous loss-of-function mutations in genes encoding the subunits of the amiloride-sensitive ENaC. The autosomal dominant form and the sporadic cases manifest with a milder clinical picture, and in these forms, salt loss gradually improves with age. Heterozygous mutations in the MR have been identified in four autosomal dominant cases and in one sporadic case (Fig. 3). These mutations include a nonsense mutation at position 1831 (Δ1831T), introducing a premature stop codon at position 537 (R537Stop). Two single base pair deletions (Δ G1226 and ΔT1597) introduce frameshift mutations, leading to a premature stop codon; the resulting proteins lack the entire DBD and LBD. A single base pair deletion in the intron E splice donor site changes the consensus sequence, suggesting that this mutation results in aberrant splicing. A missense mutation resulting in the substitution of proline for leucine at codon 924 ($MR_{P924}$) was found in a Japanese family with the dominant form of PHA1. *In-vitro* functional assays revealed that, unlike $MR_{WT}$, $MR_{P924}$ is not activated by aldosterone. Replacement of leucine by proline at position 924 has been proposed to affect receptor dimerization. Nevertheless, a change in receptor conformation cannot be ruled out, because the introduction of a proline within helix 10 could indeed alter the helical structure. Another frameshift mutation was detected in a sporadic case of PHA1. This mutation (InsC2871) affects the C-terminus of the LBD, introducing a new 54-amino acid sequence from codon 958 (the first stop being located at position 1012). Although the ligand-binding capacity of this mutant has not been investigated, it is likely that modification of the extremity of the MR LBD could impair the ligand-binding capacity of this mutant, as has already been reported for truncations or point mutations in this region.

## V. SUMMARY

Aldosterone is involved in the regulation of sodium homeostasis and plays a role in the regulation of blood pressure. It acts by binding to the mineralocorticoid receptor, a ligand-activated transcription factor that belongs to the nuclear receptor family. Loss-of-function mutations of the mineralocorticoid receptor have been identified in several patients with autosomal dominant or sporadic pseudohypoaldosteronism type 1. On the other hand, there has also been a description of a mineralocorticoid receptor activating mutation that causes early-onset hypertension exacerbated during pregnancy. This mutation modifies the selectivity of the receptor, so that progesterone and the antimineralocorticoid drug spironolactone become potent activators of the mutant receptor. This identification of naturally occurring activating and inactivating mutations of the mineralocorticoid receptor highlights its central role in the regulation of blood pressure.

### Glossary

**cis–trans cotransfection assays** Experiments designed to determine the transactivation activity of a receptor transiently expressed by a cell combined with a construct, including a receptor-regulated promoter driving a reporter gene.

**mineralocorticoid receptor** The specific receptor for aldosterone.

**steroid hormone receptor family** Subfamily of nuclear receptors consisting of the receptors for androgens, glucocorticoids, estrogens, mineralocorticoids, and progesterone.

### See Also the Following Articles

**Glucocorticoid Biosynthesis • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Biosynthesis • Mineralocorticoid Disorders, Genetic Basis of • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoids and Hypertension • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Mineralocorticoid Receptors

John W. Funder
*University of Melbourne and Prince Henry's Institute of Medical Research*

I. INTRODUCTION
II. BACKGROUND
III. EPITHELIAL MR AND THE PHYSIOLOGY OF ALDOSTERONE ACTION
IV. CO-EXPRESSION OF MR AND 11βHSD2 IN NONEPITHELIAL TISSUES
V. MR EXPRESSION IN THE ABSENCE OF 11βHSD2 IN NONEPITHELIAL TISSUES

**Mineralocorticoid receptors are members of the steroid/thyroid/retinoid/xenobiotic/orphan receptor family of transcription factors, most closely linked in terms of sequence and**

**subcellular mechanism of action with the glucocorticoid receptor, the androgen receptor, and the progesterone receptor.**

## I. INTRODUCTION

Although the time-honored definition of a mineralocorticoid hormone is that of promoting unidirectional transepithelial sodium transport, mineralocorticoid receptors (MRs) are found in varying abundance in both epithelial and nonepithelial tissues. In addition, MRs have equivalent affinity for aldosterone, the physiologic mineralocorticoid, and the physiologic glucocorticoids (cortisol and corticosterone), which circulate at ~1000-fold higher plasma levels. In classical mineralocorticoid target tissues, aldosterone access to MRs involves the expression of high levels of the enzyme 11βhydroxysteroid dehydrogenase type 2 (11βHSD2), which converts cortisol and corticosterone to their cognate, receptor-inactive 11-ketosteroids. In several nonepithelial tissues, 11βHSD2 is also co-expressed with MRs, consistent with such tissues (vascular smooth muscle, amygdala, and placenta) also being physiologic aldosterone target tissues. In other nonepithelial tissues, MRs are unprotected and thus presumably essentially always occupied by glucocorticoids: the function of such MRs remains obscure. Similarly unexplained to date is the differential effect of MR occupancy between 11βHSD protected and unprotected tissues. In protected tissues, cortisol and corticosterone mimic the effect of aldosterone, if and when they can access MRs, whereas in unprotected tissues, the glucocorticoids appear to function as MR antagonists and inappropriate occupancy by aldosterone may have very deleterious effects. Possible roles for glucocorticoid-occupied MRs as monitors and modulators of intracellular redox status are proposed.

## II. BACKGROUND

The hormone aldosterone was first isolated and characterized in 1953, and distinct MRs were identified 2 decades later. From the very first studies it was clear that MRs had a disconcertingly high affinity for the physiologic glucocorticoids, which circulate at orders of magnitude higher concentrations; it also became rapidly apparent that MR expression was not confined to classic aldosterone target tissues (kidney distal tubule, distal colon, sweat gland, and salivary gland) but was seen in a variety of nonepithelial tissues such as brain, heart, and vascular smooth muscle cells (VSMCs). In classical epithelial aldosterone target tissues, MRs are expressed at modest levels (~10,000 molecules/cell), as is the enzyme 11βhydroxysteroid dehydrogenase at much higher levels (~3.5 million molecules/cell). 11βHSD2 operates to convert receptor-active cortisol and corticosterone to their receptor-inactive 11-keto congeners, in the process generating NADH from its obligate co-substrate NAD; aldosterone is not similarly metabolized, as its 11βhydroxyl group cyclizes with the signature CHO (aldehyde) group at C-18, forming an 11,18-hemiacetal and thus protecting aldosterone from enzymatic attack and allowing it preferential access to MRs.

Immunocytochemical studies have shown that both unliganded MR and 11βHSD2 are extranuclear, but that on binding steroid the MR translocates to the nuclear compartment and binds to palindromic 15-mer response elements apparently shared with the glucocorticoid receptor (GR), progesterone receptor (PR), and androgen receptor (AR) and generally termed glucocorticoid-response elements. Many facets of the subcellular action of aldosterone have been assumed by inference from studies on GR/PR/AR, for several reasons. First, MRs are unstable in broken cell preparations, particularly in the absence of added molybdate, and thus are difficult to work with. Second, an effective aldosterone antagonist (spironolactone) was developed 40 years ago, and thus the impetus to develop antagonists by cellular and molecular screening was lacking. Finally, and most importantly, until relatively recently aldosterone appeared to be a relatively unidimensional hormone (sodium homeostasis), and the roles of the obviously more complex but still considered cognate "mineralocorticoid" receptor were tarred with the same brush. In the sections to follow, the clearly important epithelial roles of aldosterone and MR will be presented, focusing on areas that remain incompletely explored or enigmatic. Subsequently, the physiology (or currently, often the pathophysiology) of both protected (e.g., VSMCs) and unprotected (e.g., cardiomyocyte) MRs will be discussed. Finally, a possibly unifying hypothesis for MRs occupied by glucocorticoids, reconciling the apparently opposing actions in the two tissue types, will be put forward.

## III. EPITHELIAL MR AND THE PHYSIOLOGY OF ALDOSTERONE ACTION

As previously noted, MRs are expressed at modest levels in particular cells (e.g., principal cells in the renal collecting tubule) of established aldosterone target tissues. Aldosterone acts via MRs to regulate genes coding for pump subunits, channel subunits, or enzymes that activate them to increase sodium transport from the tubular lumen into the cell. As such, aldosterone forms a crucial endocrine link in a negative feedback loop, with its levels increased by sodium deficiency or loss, lowered plasma volume, or high potassium intake. Without aldosterone, animals require a high-salt diet to survive, with many species including human needing mineralocorticoid replacement therapy in addition; similarly, the MR knockout mouse dies ~10 days postpartum unless supplementary sodium is given.

In evolutionary terms, the drive was almost certainly that of retaining relatively scarce sodium in terrestrial environments. Accordingly, multiple mechanisms to elevate aldosterone secretion rate have evolved; in addition to the well-recognized effect of angiotensin II, potassium intake has been shown to be a powerful, independent elevator of aldosterone production. Redundancy of this sort is clearly an evolutionary advantage in the context of a commonly low sodium, the high potassium intake of a largely vegetarian diet, and the intercurrent fluid and electrolyte losses seen with a high incidence of diarrheal disease. In such a context, the evolution of a specific aldosterone synthase (CYP11B2) in the zona glomerulosa of the adrenal cortex—or, in some species, of conditions favoring aldosterone synthase activity of CYP11B1, the mechanisms of which are currently unknown—is of survival advantage, if aldosterone synthesis can be yoked to sodium status, as is obviously the case. In addition, the evolution of an aldosterone-specific MR (which does not appear to have been the case) or of mechanisms allowing aldosterone to selectively access and activate a less selective receptor is a necessary second part of such a control system.

In terms of evolution, MRs appear probably to have substantially antedated aldosterone synthase. If the MR is compared with the GR, its nearest homologue, the area of highest identity (90%) is the relatively short (66–68 aa) DNA-binding domain; in the ligand-binding domain, the extent of identity is 57%, and in the N-terminus <15%. In addition, the genes for the two receptors are located on different chromosomes. In contrast, aldosterone synthase and the glucocorticoid-specifying enzyme CYP11B1 lie adjacent to one another on chromosome 8 and are 94% identical over the whole coding region. The most common explanation offered for such a finding is one of relatively recent gene duplication, and the inference is that the more complex (CYP11B2, aldosterone synthase) derives from the less complex (CYP11B1). On the other hand, there is no formal proof of this, with its corollary that aldosterone followed corticosterone in evolutionary time and that MR preceded its "cognate" ligand.

Two other points need to be made regarding the classical epithelial MR. First, it is clear from studies in which 11βHSD2 is blocked by licorice ingestion or carbenoxolone administration, or is congenitally defective as in the syndrome of apparent mineralocorticoid excess, that the physiologic glucocorticoids mimic the effect of aldosterone when they occupy the MR in such circumstances. Similarly, when 11βHSD2 is absent or blocked, "pure" glucocorticoids selective for the GR and with no affinity for the MR will mimic the effect of aldosterone on urinary electrolytes, for example, providing further evidence for the pivotal role of 11βHSD2 in conferring steroid selectivity on aldosterone target tissues. An inference of the equivalent MR activity of mineralocorticoids or glucocorticoids in such circumstances is that on entering the nucleus the steroid–receptor complex is able to recruit a similar range of co-activators, although studies in this area for the MR are just beginning, lagging well behind comparable findings for other receptors.

In this context, however, another question arises. Unless 11βHSD2 acts as a physical constraint to the access of glucocorticoids to the MR, unlikely given its localization on the endoplasmic reticulum, it is inevitably a less than perfect protective mechanism, with its efficiency at reducing glucocorticoid access determined by the relative concentrations of enzyme and receptor and their (loosely speaking) relative affinity for the ligand. A rough calculation gives a figure of ~5% for corticosterone and ~15% for cortisol, as the amount of unmetabolized glucocorticoid that "leaks" past the protective enzyme to the receptor. In such circumstances, what the enzyme does is reduce active glucocorticoid receptor by approximately an order of magnitude—which would mean that cortisol/corticosterone concentrations postenzyme were still ≥10-fold those of aldosterone and that MR would be ≥90% occupied by glucocorticoid.

That this is not merely a rough estimate is shown by *in vivo* [$^3$H]aldosterone-binding studies in adrenalectomized rats. Rats were injected with tracer plus RU486 (to exclude [$^3$H]aldosterone from the GR), alone or with increasing half-log doses of nonradioactive aldosterone or corticosterone. Fifteen minutes later the animals were killed, and MR occupancy—by tracer or by added nonradioactive steroid—was determined in heart, hippocampus, kidney, and colon. In heart, corticosterone was ~30% as potent as aldosterone in binding the MR, consistent with the known differences in receptor affinity (corticosterone 3 × aldosterone) and plasma binding (corticosterone 10 × aldosterone). In the kidney, which unlike the heart expresses 11βHSD2, corticosterone was ~3% as potent as aldosterone, consistent with the enzyme adding another order of magnitude in specificity—but also with the bulk of the renal MRs being occupied under normal conditions by corticosterone, as predicted from the rough mathematical calculations.

One possible explanation for this apparent conundrum lies with the often forgotten co-substrate for 11βHSD2, i.e., NAD. If the enzyme is operant, in addition to converting hydroxyl to ketosteroids it is generating high levels of NADH. In a series of studies on the GR, it has been shown that thioredoxin binds to the DNA-binding domain and that a GR–dexamethasone complex is or is not transcriptionally active depending on the redox state of the cell. A similar scenario for glucocorticoid–MR complexes would thus explain why such complexes are inactive in the presence of ambient high levels of NADH, as is the case when the enzyme is operant, or become active when the enzyme is blocked. If such a redox dependence holds for glucocorticoid–MR complexes, as appears to be the case for GR–glucocorticoid complexes, then presumably an aldosterone–MR complex is not equivalently redox-sensitive, reflecting some otherwise minor differences between occupied MRs depending on the ligand. Whether or not this redox dependence holds awaits experimental investigation; in its absence, and in the meantime, there remains a continuing conundrum in terms of the selectivity of action, rather than binding, of epithelial MR.

## IV. CO-EXPRESSION OF MR AND 11βHSD2 IN NONEPITHELIAL TISSUES

Until recently, it appeared that 11βHSD2 was co-expressed with MRs in classical mineralocorticoid target tissues, but not in nonepithelial tissue, despite claims to the contrary; in the heart, for example, a report of their co-existence was predicated on cardiac levels of the enzyme <1% of those in the kidney, where it is expressed in only ~5% of the total number of cells. More recently, however, co-localization has been shown to occur in several areas, although the ascription of physiologic roles to such MRs is the objective of current studies. In the amygdala, for example, co-expression is thought to allow a degree of mineralocorticoid selectivity in terms of salt appetite, particularly necessary given the very high reflection coefficient of aldosterone vis-à-vis glucocorticoids at the blood–brain barrier. In the placenta, 11βHSD2 has long been ascribed the role of separating maternal and fetal glucocorticoids; a role in MR protection, however, seems possible given the demonstration that prostaglandin 15 dehydrogenase production in human placenta increases 100-fold in its sensitivity to cortisol (from an $IC_{50}$ of $10^{-8}$ $M$ to an $IC_{50}$ of $10^{-10}$ $M$) in the presence of carbenoxolone to block 11βHSD2. Finally, an elegant series of studies on the rapid nongenomic effects of aldosterone on the $Na^+/H^+$ exchanger in human VSMCs has shown that not only are such effects mediated via classical MRs, but also that such MRs are 11βHSD2 protected, in that in the presence of carbenoxolone cortisol at nanomolar doses mimics aldosterone but is without effect in its absence.

The vascular smooth muscle cell thus appears to be a candidate aldosterone target tissue by the same criteria and presumably to the same extent as the renal collecting duct or the distal colon. In addition to the acute studies described above, there are as yet unpublished reports of vasoconstrictor effects of near-physiologic doses of aldosterone on human forearm vasculature and a number of previous studies on isolated vessels or dispersed cells that were often consistent with alternative explanations given the high dosages of aldosterone commonly used. It would now seem incumbent upon the vascular biologists in the field to establish the physiologic actions of aldosterone, genomic and nongenomic, via the MR in VSMCs. Given the effects of elevated aldosterone in restoring plasma sodium and volume, a direct vasoconstrictor action of aldosterone appears to be not inconsistent with an overall action to compensate for fluid and electrolyte loss, at least in the short term pending restoration via altered intake and excretion.

That the VSMC MR may be of high pathophysiologic significance in cardiovascular disease is suggested by a number of recent findings on the vascular inflammatory responses following elevation of mineralocorticoid levels in salt-loaded rats. In such studies, the levels of inflammatory cells and markers (ED-1, osteopontin, monocyte chemoattractant protein-1, interleukin-6, etc.) are increased in and around coronary vessels within a week of administration and are very substantially blocked by co-administration of the novel MR selective antagonist eplerenone. What is important is that such lesions reflect inappropriate MR activation rather than aldosterone per se: animals receiving high salt, plus carbenoxolone to block 11βHSD2 and allow corticosterone access to and activation of coronary MRs, show inflammatory responses identical to those of mice treated with deoxycorticosterone and salt in parallel. This is of particular potential importance if the majority of VSMC MRs are physiologically occupied by glucocorticoids and are or are not transcriptionally active depending on the redox status of the cell.

## V. MR EXPRESSION IN THE ABSENCE OF 11βHSD2 IN NONEPITHELIAL TISSUES

The two best studied nonepithelial tissues in which MRs are expressed (often at high levels, as in the hippocampus) are a range of areas in the brain and in the heart. Such receptors appear normally to be occupied by glucocorticoids in what might be described as a tonic inhibitory fashion; if aldosterone is introduced to such receptors, in many circumstances its effects can be blocked (at comparable doses, reflecting the absence of 11βHSD2) by the physiological glucocorticoid. When rats are infused with aldosterone via the lateral ventricle of the brain at doses totally without effect when infused peripherally, it elevates blood pressure; co-infusion of corticosterone antagonizes the blood pressure rise, with infusion of corticosterone alone having no effect. That this neat study may not be the final word, however, is shown by subsequent experiments from the same laboratory, in which intracerebroventricular (icv) RU28318, a water-soluble MR antagonist, blocked the hypertensive response to 0.9% NaCl to drink normally shown by salt-sensitive rats. Similarly, icv RU28318 lowered blood pressure by 20–30 mm Hg in Wistar rats essentially independent of $Na^+$ status, again evidence against a simple tonic inhibitory role for MR occupancy by physiologic glucocorticoids in the circumventricular areas of the brain involved in blood pressure control.

In cardiomyocytes, MRs similarly "see" aldosterone and glucocorticoids differently, with corticosterone clearly not mimicking the anabolic effect of aldosterone on neonatal rat cardiomyocytes in culture. Though such studies show a functional difference between mineralocorticoid and glucocorticoid occupancy of MRs in such cells, they do not directly show a tonic inhibitory role for glucocorticoids in such circumstances. In a more recent study, however, such a role has been directly demonstrated, by observing the effects of cardiomyocyte-specific overexpression of 11βHSD2 (to levels of only ~10% of those in a renal principal cell, however) allowing aldosterone inappropriate access to cardiomyocyte MRs. Such mice demonstrate a syndrome of progressive cardiac dilation, cardiomegaly, and heart failure, with functional parameters improved significantly by administration of the selective MR antagonist eplerenone. It is important to emphasize that such mice are maintained on regular chow and drinking water and have no exogenous aldosterone; their cardiac pathology thus presumably represents grossly altered transcription under the influence of normal levels of aldosterone inappropriately occupying MRs. It is also important to note that in such animals the coronary vessels—as opposed to the heart muscle—appear normal, supporting the selectivity of the induced lesion.

Finally, a note of further speculation. If MRs have persisted throughout evolution, to be always essentially occupied by glucocorticoids in unprotected tissues such as the cardiomyocyte, it is possible that such glucocorticoid–MR complexes serve a physiologic function. The effects of redox state on GR–dexamethasone transcriptional activity and its proposed parallel role for MR–glucocorticoid complexes in the kidney were noted earlier. One possible physiologic role for an MR always occupied by glucocorticoid in the heart might thus be as a sensor of redox state, with transcriptional activation dependent not on ligand binding but on the metabolic state of the cell. In a recent study on the positional cloning of the combined hyperlipidemia gene HypLip1, a mutation in the cytoplasmic thioredoxin-binding protein TXNIP showed major alterations in the hepatic citric acid cycle, with sparing of ketone body and triglyceride production. Very high cardiac levels of thioredoxin/TXNIP were observed in both wild-type and mutant mice, consistent with a major role in metabolic regulation of redox state, and thus, a possible role for cardiac MRs in modulation of transcriptional activity in response to redox state.

## Glossary

**aldosterone** The physiological mineralocorticoid (salt-retaining) hormone, produced in the zona glomerulosa of the adrenal cortex and circulating at relatively low plasma levels.

**cortisol and corticosterone** Physiological glucocorticoid hormones, produced in the zona fasciculata of the adrenal cortex. In most species, cortisol is the predominant glucocorticoid, but in rats and mice, corticosterone is the sole glucocorticoid.

**11βhydroxysteroid dehydrogenase type 2** An enzyme that converts steroids bearing a hydroxyl group at carbon 11 (e.g., cortisol, corticosterone) to their 11-keto congeners (cortisone, 11-dehydrocorticosterone).

**epithelial and nonepithelial tissues** Epithelial tissues demarcate a boundary between the milieu interieur and the milieu exterieur (e.g., gut wall, kidney tubule), across which the transport of ions and water in either direction is possible. Nonepithelial tissues are entirely part of the milieu interieur (e.g., brain, vascular wall).

**NAD/NADH** Proton acceptor/donor pair; required cosubstrate for 11βhydroxysteroid dehydrogenase type 2.

**redox state** Ratio of total reducing equivalents (NADH, GSH, etc.) to their oxidized form (NAD, GSSG, etc.).

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin Peptides • Glucocorticoid Effects on Physiology and Gene Expression • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Biosynthesis • Mineralocorticoid Disorders, Genetic Basis of • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Receptor, Natural Mutations of • Mineralocorticoids and Hypertension • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Mineralocorticoids and Hypertension

David J. Morris[*] and Andrew S. Brem[†]

[*]*The Miriam Hospital* • [†]*Rhode Island Hospitals, Brown University Medical School*

I. INTRODUCTION
II. PROBABLE MECHANISMS OF ALDOSTERONE ACTION ON BLOOD PRESSURE
III. ALDOSTERONE-DEPENDENT CONDITIONS
IV. CONDITIONS MIMICKING ALDOSTERONE EXCESS

**Although a renal sodium-retaining factor isolated from extracts of animal adrenal glands had been known since the early 1940s, it was not until 1953 that elaborate sequential paper and column chromatography techniques allowed aldosterone to be isolated from beef adrenals. The mineralocorticoid activity of the hormone was established experimentally and through biological studies, and once the chemical structure of aldosterone was determined, it was shown to originate in the zona glomerulosa cells of the adrenal cortex. Patients with primary adrenal tumors containing glomerulosa cells demonstrated all the findings of excess aldosterone production: suppressed plasma renin levels, excessive sodium retention, potassium wasting, and hypertension.**

## I. INTRODUCTION

The mineralocorticoid activity of aldosterone has been established physiologically by administering the hormone to adrenalectomized rats and measuring in their urine the excretion ratio of $^{24}\text{Na}/^{42}\text{K}$. Aldosterone has been isolated from the urine of individuals known to be in positive sodium balance, including pregnant women and patients with congestive heart failure, cirrhosis of the liver, and nephrotic syndrome. The hormone is secreted by the zona glomerulosa cells of the adrenal cortex. Patients with primary adrenal tumors containing glomerulosa cells produce excess aldosterone, which leads to suppressed plasma renin levels, excessive sodium retention, potassium wasting, and hypertension (Conn's Syndrome).

Sodium intake and the rennin–angiotensin system principally regulate the synthesis of aldosterone, but hormone synthesis can also be stimulated by increases in serum potassium and, to a very limited degree, by adrenocorticotropic hormone (ACTH). Mineralocorticoids play a major role in sodium homeostasis and blood pressure regulation. Although the principal tissue targets for mineralocorticoids are sodium-transporting epithelial cells (e.g., in kidney, parotid gland, sweat ducts), mineralocorticoids also have significant potential actions in nonepithelial target tissues (e.g., vascular system, heart, and brain). Extra-adrenal *in situ* synthesis has been recently described in the vascular tissue. Studying patients with forms of low-renin hypertension has also led to the discovery of other relevant, albeit weaker, mineralocorticoids, including deoxycorticosterone and 18-OH-deoxycorticosterone.

## II. PROBABLE MECHANISMS OF ALDOSTERONE ACTION ON BLOOD PRESSURE

### A. Renal and Volume-Sensitive Processes

The renal sodium-retaining effects of aldosterone were first described over 50 years ago. In a series of elegant physiological experiments, it was demonstrated that after a latent period of 60 to 90 min, amphibian epithelia exposed to mineralocorticoids generated a progressive increase in mucosal to serosal sodium transport. That aldosterone-dependent rise in sodium flux was preceded by specific receptor binding of the steroid hormone; translocation of the receptor–steroid complex to the nucleus was dependent on *de novo* protein synthesis and consumed energy in the form of adenosine triphosphate (ATP). Further studies conducted on renal distal tubules and other aldosterone-responsive tissues expanded our understanding of mineralocorticoid-induced electrolyte transport. Now it has become clear that mineralocorticoids such as aldosterone can induce the synthesis of components of $Na^+,K^+$-ATPase and can directly increase the apical (mucosal) membrane conductance to sodium by enhancing the open probability for the sodium channel (ENaC) located within that membrane.

Mineralocorticoid receptors are found in the distal nephron, principally in the cortical collecting tubule. Under physiological conditions, the mineralocorticoid aldosterone affects the reabsorption of approximately 3% of the sodium filtered by the glomerulus. Continuous exposure to aldosterone results in a state of positive sodium balance and can expand the extracellular fluid space sufficiently to produce mild edema and hypertension. The progressive increase in extracellular volume induced by mineralocorticoid eventually reaches a limit, however, and renal tubular "mineralocorticoid escape" occurs. The escape, mediated largely through a decreased reabsorption of sodium in the proximal tubule, allows for sodium balance to be reestablished, but at a new steady state. Even with the activation of the escape process and normalization of the extracellular fluid space, the hypertension most often persists. Thus chronic mineralocorticoid exposure influences blood pressure in non-volume-dependent ways.

### B. Vascular Effects of Mineralocorticoids

Over the past decade, it has become increasingly clear that mineralocorticoids can and do exert specific effects on the arterial vascular network, independently of extracellular volume and systemic blood pressure. Perhaps the most intriguing observation has been that vascular smooth muscle cells have the ability to directly synthesize minute quantities of aldosterone and that angiotensin II induces CYP11B2, the enzyme responsible for aldosterone synthesis in vascular tissue. Aldosterone, in turn, augments the vascular effects of angiotensin II by directly up-regulating angiotensin II receptor (AT-1 type) number in vascular smooth muscle cells. Thus, these vascular angiotensin II–aldosterone interactions have significant implications for the entire systemic arterial network affecting vessel compliance (resistance), blood pressure, and cardiac function.

Chronic exposure to both angiotensin II and aldosterone promotes the generation of type I and type III collagen in the heart and aorta, resulting in an increase in interstitial fibrosis that is independent of blood pressure. Angiotensin II, unlike aldosterone, also inhibits collagenase activity, preventing or delaying the breakdown of preformed collagen. The net effect of the interstitial fibrosis induced by both aldosterone and angiotensin II is to decrease vessel compliance, leading to a “stiffer,” less resilient ventricle or artery. The diminished arterial compliance is a contributing factor in the evolution of systolic hypertension.

Aldosterone induces a series of specific ionic transmembrane transport processes in vascular smooth muscle cells, transiently changing the intracellular milieu prior to the sequence of structural remodeling. In experimental models, aldosterone exposure over 7–10 days increases the membrane conductance, allowing enhanced influx of sodium in vascular smooth muscle cells. This influx is mediated by mineralocorticoid receptors in vascular smooth muscle. The steroid- receptor binding can be blocked by spironolactone, and sodium channel activity can be directly inhibited by amiloride. Although the entry of calcium ions is not directly affected, there is a decrease in the expression of the membrane-bound sodium–calcium ion exchanger, which would result in a net increase in cytosolic calcium. Subtle changes in the cytosolic concentrations of both of these ions can “sensitize” vascular beds to contractile stimuli and boost vascular resistance.

Two compensatory mechanisms are activated in vascular smooth muscle in response to chronic mineralocorticoid exposure: the sodium pump activity and expression of ($Na^+$,$K^+$-ATPase) and efflux of potassium ions via the calcium-dependent potassium channel. The increase in sodium pump activity strives to restore normal intracellular sodium concentrations and the potassium efflux attempts to relax the smooth muscle cell, dropping vascular resistance. Chronic intracellular potassium depletion appears to be one of the factors linked to the stimulation of collagen formation.

### C. Central Nervous System Effects

Mineralocorticoids also exert their effects on blood pressure through actions in the brain. Although mineralocorticoid receptors have been observed in the hippocampus and hypothalamus, their role was difficult to determine until relatively recently. In a series of elegant experiments, Elise Gomez-Sanchez demonstrated that infusion of minute quantities of aldosterone into the ventricles of the brain induces hypertension in animals after 14 days. The hypertension could be blocked by the intraventricular infusion of either a mineralocorticoid receptor antagonist or by amiloride. The observed increase in blood pressure in this model could be enhanced with sodium loading but was not dependent on it. This central form of hypertension appears to be mediated by a generalized increase in sympathetic tone with an accompanying rise in vascular resistance. Thus concentrations of aldosterone, which would produce no visible systemic effect, can produce hypertension when placed directly into the brain.

## III. ALDOSTERONE-DEPENDENT CONDITIONS

### A. Adrenal Tumors

A full discussion of the clinical syndromes associated with aldosterone excess is beyond the scope of this article. Rather, the focus is to mention the key points on the more common conditions encountered in clinical practice. Primary hyperaldosteronism should be considered as a cause of hypertension in patients who present with hypokalemia and metabolic alkalosis or who develop those findings nearly simultaneously with the onset of diuretic treatment. Although plasma renin and aldosterone levels are dependent on dietary sodium and position (lying versus standing), the plasma renin is maximally suppressed in cases of primary hyperaldosteronism.

An adrenocortical adenoma is among the most common causes of primary hyperaldosteronism. These tumors are generally small, less than 2 cm in size, and hence are often difficult to identify radiographically. Aldosterone-producing adenomas often also generate 18-OH-corticosterone in concentrations exceeding 100 ng/dl. Patients with this type of tumor exhibit an unexpected drop in aldosterone secretion with changes in position. When plasma aldosterone levels are measured in the early morning, after patients remain supine overnight, and then are remeasured after the patient stands upright for 4 h, the values fall paradoxically on the standing sample. Adrenal carcinomas are quite rare but also can secrete aldosterone. These cancers are usually large and are easily seen on routine radiographic studies.

### B. Idiopathic Adrenal Hyperplasia

Bilateral nodular adrenal hyperplasia, or idiopathic hyperaldosteronism, is the next most common cause of excess aldosterone secretion. Both adrenal glands are involved, but the degree of involvement may not necessarily be symmetrical. In contrast to adrenal adenomas, plasma renin may be only partially suppressed and there is generally no paradoxical position effect on aldosterone secretion. There is some evidence that adrenal glomerulosa cells have increased sensitivity to the normal promoters of aldosterone secretion, including angiotensin II.

Direct bilateral adrenal vein sampling or adrenal imaging using $^{131}$I-labeled iodocholesterol may be required to make the diagnosis.

### C. Glucocorticoid-Remediable Hyperaldosteronism

Glucocorticoid-remediable (suppressible) hyperaldosteronism is a rare autosomal dominant inherited condition in which adrenal glomerulosa cells respond inappropriately to ACTH. Affected patients exhibit a chimeric gene crossover between the 11β-hydroxylase (responsible for cortisol synthesis) and the aldosterone synthase. Treatment with exogenous glucocorticoids such as dexamethasone suppresses the secretion of ACTH from the pituitary gland, thereby attenuating the release of aldosterone.

### D. Renal Renin-Dependent Hyperaldosteronism

Processes affecting renal arterial blood flow, including renal artery stenosis, are associated with enhanced release of renin from the juxtaglomerular apparatus. The enzyme renin cleaves angiotensin I from angiotensinogen, the precursor produced by the liver and released into the circulation. Converting enzyme found in plasma and in the tissues, including kidney, transforms the decapeptide angiotensin I to the octopeptide angiotensin II. Angiotensin II is a potent stimulus for the adrenal glomerulosa cells to secrete aldosterone. Thus, renal vascular disease is associated with both excessive release of renin, leading to elevated plasma angiotensin II, and increased aldosterone levels. Evaluation of the renal arteries is warranted with secondary hyperaldosteronism and worsening hypertension. A captopril renal scan and/or angiography may be required to establish the diagnosis.

## IV. CONDITIONS MIMICKING ALDOSTERONE EXCESS

### A. Syndromes of Mineralocorticoid Excess: Altered 11β-Hydroxysteroid Dehydrogenase Activity

The syndrome of apparent mineralocorticoid excess (AME) was first described in children who demonstrated sodium retention, potassium wasting, and hypertension without measurable elevations in either renin or aldosterone. Thus the severe hypertension in these children was initially categorized as pseudohyperaldosteronism. Later, it became clear that this syndrome was associated with mineralocorticoid receptor (MR) activation and that it could be successfully treated with spironolactone. Once the mineralocorticoid receptor was cloned, relative binding studies were performed. Quite unexpectedly, the MR demonstrated equal binding affinities for both aldosterone and cortisol. From these observations came the concept that an alternative regulator of steroid binding must exist over the MR to maintain its specificity. In the past decade, one regulatory modulator of steroid binding, the isoform of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD2), was isolated and cloned from mineralocorticoid-responsive tissues. 11β-HSD2 converts active endogenous glucocorticoids to their respective 11-dehydro derivatives and confers aldosterone specificity on the MR. 11β-HSD2 has a low $K_m$ for glucocorticoids, making it sensitive, and is dependent on the cofactor nicotinamide adenine dinucleotide ($NAD^+$) for its activity. As an enzyme, it appears to function physiologically only in the dehydrogenase mode. The 11-dehydro metabolites of cortisol and corticosterone are not able to directly activate mineralocorticoid receptors and induce a primary biologic effect.

Children with AME have homozygous mutations in the 11β-HSD2 gene, resulting in diminished 11β-HSD2 enzyme activity and failure of the "protective" mechanisms that selectively prevent glucocorticoids from accessing the MR in the distal nephron. This inappropriate stimulation of the MR in the kidney by glucocorticoids results in $Na^+$ retention, $K^+$ wasting, and hypertension associated with low plasma aldosterone and renin levels. Importantly, when challenged with exogenous cortisol, these children develop sodium retention and a marked increase in blood pressure, confirming that cortisol functions as a mineralocorticoid in the kidney, controlling both electrolyte transport and blood pressure. Licorice derivatives can mimic the hypertensive profile of AME. Licorice contains glycosides of glycyrrhetinic acid, which is a potent inhibitor of both isoforms of 11β-HSD.

Although increased renal sodium retention was thought to be the principal cause of hypertension in this disease, effects of nonmetabolized glucocorticoids in the vasculature due to diminished 11β-HSD local tissue activity may also be a factor in the development of hypertension in these children. Glucocorticoids and mineralocoticoids are known to potentiate the vasoconstrictive effects of both catecholamines and angiotensin II. It has been shown that glucocorticoids further amplify the contractile effects of catecholamines and angiotensin II when 11β-HSD enzyme activity is inhibited. In *in vitro* experiments using inhibitors of both vascular 11β-HSD1 and 11β-HSD2 dehydrogenase, including the use of 11β-HSD2 and 11β-HSD1 antisense oligonucleotide probes, prior glucocorticoid exposure enhanced catecholamine-induced vascular contraction. Thus, 11β-HSD activity in the control of sodium retention in the kidney and 11β-HSD activity in vascular tissue are important in the control of blood pressure.

### B. Liddle's Syndrome

Dysregulation of the sodium channel within the epithelial cells of the cortical collecting tubule can result in a syndrome that mimics hyperaldosteronism, but plasma renin and aldosterone values are maximally suppressed. Liddle's syndrome, a rare autosomal dominant disorder, results from a defective β-subunit of the epithelial sodium channel present in the cortical collecting duct cell apical membrane. This channel is normally activated by mineralocorticoids, but in this case is activated independently of hormone stimulation, resulting in sodium retention, potassium secretion, metabolic alkalosis, and hypertension.

## See Also the Following Articles

**Glucocorticoid Biosynthesis • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Mineralocorticoid Biosynthesis • Mineralocorticoid Disorders, Genetic Basis of • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Receptor, Natural Mutations of • Stress • Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids**

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# Monoaminergic and Cholinergic Control of the Anterior Pituitary

Edward J. Wagner
*Western University of Health Sciences, California*

I. INTRODUCTION
II. SYNTHESIS, REUPTAKE, AND METABOLISM OF BIOGENIC AMINES AND ACETYLCHOLINE
III. NEUROTRANSMITTER ANATOMICAL PATHWAYS
IV. OVERVIEW OF RELEASING AND ANTERIOR PITUITARY HORMONES
V. NEUROTRANSMITTER REGULATION OF ACTH SECRETION
VI. NEUROTRANSMITTER REGULATION OF GH SECRETION
VII. NEUROTRANSMITTER REGULATION OF PROLACTIN SECRETION
VIII. NEUROTRANSMITTER REGULATION OF TSH SECRETION
IX. NEUROTRANSMITTER REGULATION OF GONADOTROPIN SECRETION
X. SUMMARY

## I. INTRODUCTION

Anterior pituitary hormone secretion is regulated by aminergic and cholinergic transmitters. The degree of neurotransmitter regulation varies among the hormones. For some hormones, information concerning the regulatory mechanisms, the pharmacology, and/or the physiology for a particular neurotransmitter system is either scarce or replete with contradictions. Contradictory findings may arise from differences among species or experimental methodologies, or from a lack of specific pharmacological tools to study a transmitter system. Despite these problems, it is well established that aminergic and cholinergic transmitters alter adenohypophysial hormone secretion primarily by modulating the excitability of hypothalamic neurosecretory cells that contain releasing or inhibiting hormones that are secreted into the median eminence and carried to the anterior pituitary by the portal circulation. In some cases, these neurotransmitters can serve as the hypothalamic factor and have a direct adenohypophysial effect, or can act by another peripheral mechanism. This article provides a compact, concise review of the aminergic and cholinergic transmitter regulation of adenohypophysial hormone secretion. Five major hormones are discussed: adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin, thyroid-stimulating hormone (TSH), and the gonadotropins. The neurotransmitters and modulators covered include norepinephrine, dopamine, serotonin, acetylcholine, histamine, and, to a certain extent, epinephrine and melatonin.

## II. SYNTHESIS, REUPTAKE, AND METABOLISM OF BIOGENIC AMINES AND ACETYLCHOLINE

The first step in the biosynthesis of catecholamines such as dopamine, norepinephrine, and epinephrine involves hydroxylation of the aromatic amino acid substrate tyrosine by the rate-limiting enzyme tyrosine hydroxylase, which forms 3,4-dihydroxyphenylalanine (DOPA). DOPA is then rapidly decarboxylated by the ubiquitous enzyme L-aromatic amino acid decarboxylase (AADC) to form dopamine. In noradrenergic and adrenergic neurons, dopamine is further hydroxylated at the β-carbon of the phenylethylamine moiety by dopamine β-hydroxylase, thus rendering norepinephrine. Norepinephrine, in turn, is subject to an N-methylation reaction in adrenergic neurons by the enzyme phenylethanolamine-*N*-methyltransferase. Biosynthesis of indoleamines such serotonin and melatonin also involves the initial hydroxylation of the aromatic amino acid tryptophan by tryptophan hydroxylase, resulting in 5-hydroxytryptophan (5-HTP). As with the catecholamines, 5-HTP is then decarboxylated by AADC to form serotonin. In the pineal gland, serotonin is N-acetylated by the enzyme serotonin *N*-acetyltransferase to form *N*-acetylserotonin. This intermediate product is then methylated at the 5-hydroxy position by hydroxyindole-O-methyltransferase to yield melatonin. Histamine is formed from a single decarboxylation reaction in which the precursor amino acid histidine is acted on by the enzyme histidine decarboxylase. Likewise, acetylcholine is generated in a single enzymatic step involving two substrates: an acetyl group donated by one substrate (acetyl coenzyme A) is transferred to the other substrate (choline) by the enzyme choline acetyltransferase. All monoamine neurotransmitters and acetylcholine are packaged into synaptic vesicles by vesicular transporters that extrude protons from the vesicular lumen in exchange for the transmitters.

The primary means by which monoamine neurotransmitter action is terminated is by their removal from the synaptic cleft via reuptake into the nerve terminal. This is accomplished through membrane-bound transporters, with each one being responsible for the selective reuptake of a given amine. The reuptake process is dependent on the presence of sodium and chloride ions. Once inside their respective nerve terminals, dopamine, norepinephrine, and serotonin can then be oxidatively deaminated by monoamine oxidase (MAO) to form 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylethyleneglycol (DOPEG), and 5-hydroxyindoleacetic acid, respectively. DOPAC and DOPEG either can be transported out of the central nervous system, or can be acted on by extraneuronal catechol-O-methyltransferase (COMT) to yield homovanillic acid and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), respectively. Dopamine and norepinephrine can also be directly O-methylated by COMT to form 3-methoxytyramine and normetanephrine, and then deaminated by MAO, resulting in homovanillic acid and MHPG, respectively. The major metabolites of epinephrine are metanephrine formed by O-methylation by COMT, and vanillyl mandelic acid formed by subsequent oxidative deamination by MAO. Catecholamine metabolites such as DOPAC and MHPG can be subsequently conjugated with a sulfate group by the enzyme phenolsulfotransferase. The metabolism of histamine primarily involves an N-methylation reaction by the enzyme that produces *N-tele*-methylhistamine, histamine methyltransferase. Acetylcholine is rapidly cleaved and its action is thus terminated by acetylcholinesterases present in the synaptic cleft. Melatonin metabolites are formed primarily by hydroxylation at the C-6 position, followed by subsequent sulfation or glucuronidation conjugation reactions.

## III. NEUROTRANSMITTER ANATOMICAL PATHWAYS

Noradrenergic perikarya are organized in discrete clusters in various brain stem nuclei, e.g., the nucleus of the solitary tract and the locus coeruleus. Seven major noradrenergic cell groups are classified alphanumerically, $A_1$ through $A_7$. These cell groups send diffuse projections throughout the brain and spinal cord, including various regions within the hypothalamus. The $A_1$, $A_2$, and $A_6$ cell groups are the most relevant to the noradrenergic control of anterior pituitary hormone secretion. The cell bodies of dopaminergic neurons are also organized in discrete clusters, and are also classified alphanumerically ($A_8$–$A_{16}$). Dopaminergic cell groups, however, are more widely distributed than are noradrenergic cell groups, ranging from the ventral tegmentum of the midbrain to the olfactory bulb of the rostral forebrain. The dopaminergic cell groups that are principally involved in regulating anterior pituitary hormone secretion are located in the hypothalamus; these are the $A_{12}$, or tuberoinfundibular dopaminergic (TIDA), and the $A_{14}$ cell groups. Like the other catecholamines, the somas of adrenergic neurons are discretely localized into three distinct clusters within the medulla of the brain stem, and are alphanumerically classified as the $C_1$–$C_3$ cell groups. Each of these adrenergic cell groups has been reported to play a role in regulating the release of certain anterior pituitary hormones. Serotonergic neurons involved in regulating anterior pituitary hormone secretion have perikarya found primarily in the dorsal and median raphe nuclei located in the brain stem. Like the other monoamine neurotransmitter systems, serotonergic neurons are classified alphanumerically, designated as the $B_1$–$B_9$ cell groups. Serotonergic neurons send diffuse rostral projections to the hypothalamus and forebrain, and also project caudally to the spinal cord. Histaminergic cell bodies are discretely localized to the posterior hypothalamus, and these neurons send axonal projections to the forebrain, hypothalamus, and brain stem. The pertinent anatomical interrelationships between these aminergic neuronal groups and the hypothalamic neurosecretory cells containing the releasing and inhibiting hormones are depicted in Fig. 1.

The cholinergic neuronal systems involved in regulating anterior pituitary hormone release are less well defined. The highest density of cholinergic perikarya is found in basal forebrain nuclei, e.g., the nucleus basalis of Meynert, but these neurons project primarily to the hippocampus and cerebral cortex. Cholinergic cell bodies have been observed in the hypothalamic arcuate nucleus and lateral hypothalamus, but the projections of these neurons have yet to be thoroughly characterized. The neuromodulator and hormone melatonin is released from pinealocytes of the pineal gland into the general circulation. It is a lipophilic molecule, and as such it readily enters the brain and the cerebrospinal fluid.

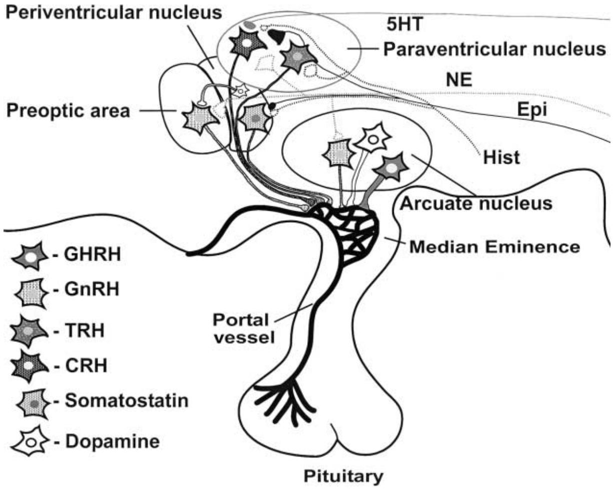

FIGURE 1 Schematic diagram that illustrates the neuroanatomical interrelationships between the ascending aminergic neurons and the hypothalamic neurosecretory cells that contain releasing or inhibiting hormones. 5HT, 5-Hydroxytryptamine (serotonin); Epi, epinephrine; Hist, histamine; NE, norepinephrine.

## IV. OVERVIEW OF RELEASING AND ANTERIOR PITUITARY HORMONES

The anterior pituitary is interconnected with the brain by way of the hypophysial portal vasculature. The superior hypophysial artery provides the blood supply to the median eminence, a circumventricular organ within the ventral hypothalamus that resides outside the blood–brain barrier. Portal vessels carry substances (e.g., neurotransmitters, peptides, releasing hormones) released from nerve terminals in the median eminence to the anterior pituitary, where they exert their influence over the secretion of specific hormones from specific cell populations.

Parvocellular neurosecretory cells projecting to and terminating in the median eminence originate from several hypothalamic nuclei. Most notable are the hypothalamic preoptic area nuclei, the periventricular nucleus, the paraventricular nucleus, and the arcuate nucleus. Table 1 lists the major hypothalamic

**TABLE 1 Releasing and Inhibiting Hormones Contained in Neuronal Cell Bodies in Hypothalamic Nuclei**

| Hypothalamic nuclei | Releasing/inhibiting hormone |
|---|---|
| Preoptic area | GnRH |
| Periventricular nucleus | Somatostatin |
| Paraventricular nucleus | CRH, TRH |
| Arcuate nucleus | Dopamine, GnRH, GHRH |

nuclei and the associated neurotransmitter or neuropeptide released from each of these regions.

The neurotransmitters and peptides released from parvocellular neurosecretory cells act on specific cell types in the anterior pituitary to effect changes in the secretion of specific hormones. Table 2 lists the pituitary cell types and the effects on pituitary hormone secretion produced by the various hypothalamic neurotransmitters and peptides.

ACTH is derived from posttranslational modification of proopiomelanocortin, and stimulates the secretion of glucocorticoid hormones (cortisol in humans) from the adrenal cortex in response to many different types of stressful stimuli. GH stimulates postnatal growth by promoting mitosis and protein synthesis. This promotion of growth is ultimately due to the GH-induced stimulation of insulin-like growth factor-I production in, and secretion by, tissues such as the liver and bone. Prolactin, a 199-amino acid polypeptide in its bioactive form, stimulates the synthesis of milk and its subsequent secretion from alveolar epithelial cells of the mammalian female breast. There is also considerable evidence for a regulatory role of prolactin in cellular and humoral immune responses in both physiological and pathophysiological (i.e., autoimmune disease) states. TSH exists as a dimer composed of glycosylated α- and β-subunits (96 and 110 amino acid residues, respectively), and elicits the release of thyroxine and triiodothyronine from the thyroid gland. These hormones play an important role in promoting maturation of the nervous system and in regulating metabolic rate and body temperature. The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are also both composed of α- and β-subunits. These hormones act on the gonads (testes in the male, ovaries in the female) to stimulate steroid hormone production (i.e., testosterone in the male, estrogen and progesterone in the female), follicle development and ovulation (in the female), and spermatogenesis (in the male). The aminergic and cholinergic regulation of the secretion of these anterior pituitary hormones, which is summarized in Table 3, is the subject of the next five sections.

## V. NEUROTRANSMITTER REGULATION OF ACTH SECRETION

Adrenocorticotropin hormone secretion is regulated by norepinephrine, epinephrine, dopamine, serotonin, acetylcholine, and histamine.

1. **Norepinephrine**. Neurons in the $A_1$ and $A_2$ noradrenergic cell groups increase corticotropin-releasing hormone (CRH), and thus ACTH release, following the activation of $\alpha_1$-adrenergic receptors in the parvocellular paraventricular nucleus (PVN) of the hypothalamus. Conversely, norepinephrine can decrease CRH release by activating $\alpha_{2}$- and β-adrenergic receptors in the PVN.
2. **Epinephrine**. Medullary adrenergic neurons emanating from the $C_1$, $C_2$, and $C_3$ cell groups increase CRH release by activating $\alpha_1$- and β-adrenergic receptors in the PVN. In the rat (but not in humans), $\beta_2$-adrenergic receptors may also account for some of the stress-induced increase in ACTH secretion caused by circulating epinephrine.
3. **Dopamine**. Dopamine causes a modest stimulation of hypothalamic CRH release via the activation of dopamine D1 receptors.

**TABLE 2 Hypothalamic Releasing and Inhibiting Hormones: Specific Effects on the Secretion of Anterior Pituitary Hormones**

| Releasing/inhibiting hormone | Pituitary cell type affected | Effect |
|---|---|---|
| CRH | Corticotroph | ↑ ACTH release |
| GHRH | Somatotroph | ↑ GH release |
| Somatostatin | Somatotroph, thyrotroph | ↓ GH, TSH release |
| Dopamine | Lactotroph, thyrotroph, somatotroph | ↓ Prolactin, TSH, GH release |
| TRH | Thyrotroph, lactotroph | ↑ TSH, prolactin release |
| GnRH | Gonadotroph | ↑ FSH, LH release |

TABLE 3 Summary of Aminergic and Cholinergic Modulation of Anterior Pituitary Hormone Secretion

| Transmitter/modulator (cell body location) | Anterior pituitary hormone[a] | | | | |
|---|---|---|---|---|---|
| | ACTH | GH | Prolactin | TSH | Gonadotropins |
| Norepinephrine (medulla) | ↑ ($\alpha_1$), ↓ ($\alpha_2$, $\beta$) | ↓ ($\alpha_1$) | ↓ ($\alpha_1$), ↑ (?) | ↑ ($\alpha_2$), ↓ ($\alpha_1$) | ↑ ($\alpha_1$), ↓ (?) |
| Epinephrine (medulla) | ↑ ($\alpha_1$, $\beta$) | ↑ ($\alpha_2$), ↓ ($\beta$) | — | — | — |
| Dopamine (hypothalamus) | ↑ (D1) | ↑ (?), ↓ (?) | ↓ (D2, D3), ↑ (D1) | ↓ (D2) | ↑ (?), ↓ (?) |
| Serotonin (raphe nuclei) | ↑ ($5HT_{1A,1C,\text{ and/or }2}$) | ↑ ($5HT_{1C/2}$) | ↑ ($5HT_{1A,2A\text{ and }2C}$) | ↑ (?), ↓ (?) | ↓ (?), ↑ (?) |
| Acetylcholine (basal forebrain) | ↑ (M,N) | ↑ (M) | ↓ (N) | ↓ (N) | ↑ (M), ↓ (?) |
| Histamine (hypothalamus) | ↑ ($H_{1\text{ and }2}$) | ↑ ($H_{1\text{ and }2}$), ↓ ($H_1$) | ↑ ($H_{1-3}$) | ↓ (?), ↑ ($H_2$) | ↑ ($H_{1\text{ and }2}$) |
| Melatonin (pineal gland) | — | — | ↓ ($MEL_{1A}$) | — | ↓ (?) |

[a] ACTH, Adrenocorticotropic hormone; GH, growth hormone; TSH, thyroid-stimulating hormone. The receptor subtype mediating a particular effect (when known) is indicated in parentheses. M, Muscarinic receptor; N, nicotinic receptor; D, dopamine; 5HT, 5-hydroxytryptamine; H, histamine; MEL, melatonin.

4. **Serotonin**. This amine increases ACTH secretion in rodents and humans by activating 5-hydroxytryptamine receptors ($5HT_{1A}$, $5HT_{1C}$, and/or $5HT_2$) located on CRH neurons in the PVN. Serotonergic neurons are involved in stress- and hypoglycemia-induced ACTH secretion.
5. **Acetylcholine**. This neurotransmitter increases ACTH secretion by increasing hypothalamic CRH release via the activation of muscarinic and nicotinic receptors.
6. **Histamine**. Histamine increases ACTH secretion in rodents and humans by activating $H_1$ and $H_2$ receptors located in the PVN. Histaminergic neurons appear to be involved in stress- and hypoglycemia-induced ACTH release.

## VI. NEUROTRANSMITTER REGULATION OF GH SECRETION

Growth hormone secretion is regulated by norepinephrine, epinephrine, dopamine, serotonin, acetylcholine, and histamine.

1. **Norepinephrine**. Norepinephrine decreases GH secretion by increasing the release of somatostatin. This central effect, due to the activation of $\alpha_1$-adrenergic receptors, has been demonstrated in dogs and rats, but not in humans.
2. **Epinephrine**. Epinephrine elicits an $\alpha_2$-adrenergic receptor-mediated increase in GH secretion in humans and rodents through a central mechanism involving an increase in growth hormone-releasing hormone (GHRH) release concomitant with a decrease in somatostatin release. On the other hand, central $\beta$-adrenergic receptor activation by epinephrine decreases GH release via an increase in somatostatin release. In addition, epinephrine can increase GH directly from the anterior pituitary, but this effect is minor compared to the central effect.
3. **Dopamine**. Dopaminergic agonists have been shown to increase GH release, as well as to decrease hypoglycemia-, levodopa-, and arginine-induced increases in GH release. Because dopamine can increase both GHRH and somatostatin release, it appears that this balance can change under different physiological conditions. Dopamine also decreases GH release via a direct action on the somatotroph. This may be important in regulating differentiation of adenohypophysial cells such as lactotrophs and somatotrophs.
4. **Serotonin**. The literature is replete with contradictory findings, especially with regard to human studies. In general, serotonin and 5HT receptor agonists increase GH release. Also, $5HT_{1C/2}$ receptor antagonists block the $\alpha_2$-adrenergic receptor agonist-induced increase in GH release. The stimulatory serotonergic effect is thought to be central—due presumably to a decrease in somatostatin release.
5. **Acetylcholine**. This neurotransmitter increases GH release under a variety of physiological conditions, such as the nocturnal increase in GH release and the exercise-induced increase in GH release. This is due primarily to an activation of muscarinic receptors. Cholinergic stimulation of GH release occurs via an inhibition of somatostatin release from the median eminence, and also via a direct effect on the somatotroph.
6. **Histamine**. The activation of $H_1$ receptors decreases pulsatile and opioid-stimulated GH secretion in rats, whereas in dogs the blockade of $H_1$ and $H_2$ receptors decreases the hypoglycemia- and opioid-stimulated increases in GH release, respectively. Similarly, in humans the blockade of

$H_1$ receptors decreases arginine- and opioid-stimulated GH secretion, and the blockade of $H_2$ receptors decreases nocturnal-, hypoglycemia-, and levodopa-induced GH secretion. Thus, in dogs and humans histamine facilitates GH secretion under a variety of physiological conditions, whereas in rats it is inhibitory.

## VII. NEUROTRANSMITTER REGULATION OF PROLACTIN SECRETION

Prolactin secretion is regulated by norepinephrine, dopamine, serotonin, acetylcholine, histamine, and melatonin.

1. **Norepinephrine**. Norepinephrine exerts a central, $\alpha_1$-adrenergic receptor-mediated tonic inhibition of prolactin secretion. On the other hand, central noradrenergic pathways may also be involved in the proestrous surge of prolactin, and in the stress-induced increase in prolactin secretion.
2. **Dopamine**. The predominant effect of dopamine released from tuberoinfundibular dopaminergic (TIDA) nerve terminals in the median eminence is a tonic inhibition of prolactin secretion caused by the activation of dopamine D2 receptors on lactotrophs. Centrally, activation of dopamine D2/D3 receptors increases neurochemical estimates of TIDA neuronal activity, resulting in a decrease in prolactin secretion. Conversely, central activation of dopamine D1 receptors decreases TIDA neuronal activity, and thus increases prolactin secretion.
3. **Serotonin**. Exogenous administration of $5HT_{1A}$, $5HT_{2A}$, and $5HT_{2C}$ receptor agonists increases prolactin secretion. In addition, serotonin and its precursor 5-HTP also increase prolactin secretion in both rats and humans. Moreover, central serotonergic pathways emanating from the dorsal raphe nucleus appear to facilitate the prolactin release observed during proestrus in rats and induced by suckling.
4. **Acetylcholine**. Cholinergic agonists decrease basal and suckling-, estrogen-, and opioid-induced prolactin secretion. This may be mediated via an activation of TIDA neurons, due presumably to a central mechanism, although a direct effect on the lactotroph has not been ruled out definitively. These inhibitory effects on prolactin secretion are due primarily to an activation of nicotinic receptors.
5. **Histamine**. Exogenous histamine increases prolactin secretion, and central histaminergic pathways emanating from the posterior hypothalamus appear to play an important facilitory role in the prolactin secretion induced by stress or by suckling. $H_1$, $H_2$, and $H_3$ receptors have all been implicated in the various roles of the amine in promoting prolactin secretion.
6. **Melatonin**. Increasing concentrations of melatonin, such as those that occur during the night or under conditions of decreased day length (i.e., short photoperiod), decrease prolactin secretion due to the interaction of the indoleamine with $MEL_{1A}$ receptors in the pars tuberalis. This interaction is thought to alter the secretion of a seasonal, peptidergic prolactin-releasing factor known as tuberalin.

## VIII. NEUROTRANSMITTER REGULATION OF TSH SECRETION

Thyroid-stimulating hormone is regulated by norepinephrine, dopamine, serotonin, acetylcholine, and histamine.

1. **Norepinephrine**. This amine plays a role in activating the hypothalamic–pituitary–thyroid (HPT) axis in response to cold. Norepinephrine can increase TSH release by activating central $\alpha_2$-adrenergic receptors and can decrease TSH release by activating $\alpha_1$-adrenergic receptors. There are conflicting reports on the effects of $\alpha_1$-adrenergic receptor activation in the thyrotroph.
2. **Dopamine**. Dopamine directly inhibits TSH release from the anterior pituitary in both rats and humans. There also appears to be a central component to the dopamine-induced decrease in TSH secretion. Both the central and adenohypophysial effects appear to be mediated by dopamine D2 receptors. Dopamine does not tonically inhibit TSH release under euthyroid conditions, but it may do so under hypothyroid conditions. Dopamine may also be involved in the diurnal variations in TSH secretion.
3. **Serotonin**. There appears to be a dual role of central serotonergic neuronal systems in regulating thyrotropin-releasing hormone (TRH) and thus TSH release. Serotonin may be involved in promoting the characteristic late-morning peak in TSH secretion. However, its involvement in the cold-induced activation of the HPT axis is equivocal. In addition, there is a serotonin-induced decrease in TSH that may be due to a peripheral regulatory mechanism, but a direct action of the amine on the thyrotroph seems

unlikely. The lack of truly specific pharmacological tools has made it difficult to ascribe serotonergic effects on TSH secretion to specific 5HT receptor subtypes.

4. **Acetylcholine**. There is some evidence from rat studies suggesting that central nicotinic receptor activation decreases basal TSH secretion, due possibly to the activation of TIDA or somatostatin neurons.
5. **Histamine**. Histamine increases hypothalamic TRH secretion in rats by activating $H_2$ receptors, but it also increases hypothalamic somatostatin release. This results in a net decrease in TSH secretion.

## IX. NEUROTRANSMITTER REGULATION OF GONADOTROPIN SECRETION

Gonadotropins (follicle-stimulating hormone and luteinizing hormone) are regulated by norepinephrine, dopamine, serotonin, acetylcholine, histamine, and melatonin.

1. **Norepinephrine**. Depending on the species, the gender, the season, and the point in the reproductive cycle, norepinephrine can exert both stimulatory and inhibitory influences on hypothalamic gonadotropin-releasing hormone (GnRH) and thus on FSH and LH secretion. As such, the amine can play an important role in both positive and negative feedback effects of gonadal steroids on the hypothalamic–pituitary gonadal axis. However, the predominant role of norepinephrine is to facilitate the preovulatory GnRH and thus LH surges, as well as the postcastration rise in GnRH and thus LH pulse amplitudes and frequencies. These latter effects arise from the activation of central $\alpha_1$-adrenergic receptors.
2. **Dopamine**. Dopamine appears to play a facilitory role in the postcastration rise in LH pulse amplitude. Conversely, dopamine released from TIDA nerve terminals in the median eminence appears to decrease LH secretion during the luteal phase of the reproductive cycle in ruminants.
3. **Serotonin**. What little work has been done concerning serotonergic regulation of gonadotropin secretion has yielded very inconclusive results. For example, in the rat, lesioning of serotonergic neurons is reported to reduce plasma levels of LH and to attenuate the preovulatory LH surge. On the other hand, 5HT receptor activation has also been shown to increase LH secretion, depending on the timing of agonist administration in relation to the preovulatory LH surge. Finally, other studies in male rats have shown that 5HT receptor activation is without effect on LH secretion.
4. **Acetylcholine**. This neurotransmitter increases FSH and LH release in rodents by activating muscarinic receptors. However, inhibition of acetylcholinesterase with the organophosphate diisopropylfluorophosphate (DFP) produces a biphasic effect on gonadotropin secretion. Low doses of DFP increase, and higher doses decrease, hormone secretion.
5. **Histamine**. There is some evidence to suggest a stimulatory role for histamine in regulating gonadotropin secretion, most notably in female rodents. $H_1$ and $H_2$ receptors appear to be involved. Histamine does not appear to exert a direct adenohypophysial effect, but may indirectly stimulate GnRH release.
6. **Melatonin**. Increases in melatonin concentrations like those observed under nocturnal conditions and those inextricably tied with short photoperiods reduce basal LH release from the rat pars tuberalis *in vitro*. In addition, melatonin decreases LH and FSH release by physiologically antagonizing the stimulatory effects of GnRH on gonadotrophs from neonatal rat pituitary gland. On the other hand, lesioning of the mediobasal hypothalamus of the Syrian hamster blocks the effect of short photoperiods and of melatonin on gonadotropin secretion. This latter finding suggests that, depending on the species, melatonin may also act centrally to effect a decrease in gonadotropin release.

## X. SUMMARY

Anterior pituitary hormones are regulated to varying degrees by aminergic and cholinergic neurotransmitters. With the exception of dopamine and perhaps melatonin, these neurotransmitters act upstream to regulate the excitability of hypothalamic neurosecretory cells that contain releasing and inhibiting hormones. Many of these neurotransmitters contribute to physiological and/or pathophysiological (i.e., hypoglycemia) regulatory mechanisms that exist for a particular adenohypophysial hormone. For example, norepinephrine produces a physiologically relevant stimulation of ACTH, TSH, and gonadotropin secretion. Norepinephrine may also inhibit gonadotropin secretion, depending on factors such as species, gender, and stage of development and of the reproductive cycle. Likewise, epinephrine also provides an important stimulatory signal for ACTH secretion, and increases GH secretion as well. Dopamine is unequivocally the principal prolactin-inhibiting factor, and it appears this amine is also capable of inhibiting both TSH and GH secretion. Serotonin plays an important stimulatory role in regulating ACTH and prolactin secretion, and may also promote GH release. Acetylcholine increases the release of ACTH, GH, and gonadotropins by activating primarily muscarinic receptors, and also inhibits prolactin and TSH secretion by activating nicotinic receptors. It is now becoming readily apparent that histamine provides a stimulatory signal in the control of prolactin and ACTH secretion, and may also stimulate GH secretion and inhibit TSH secretion. Finally, melatonin conveys environmental signals concerning short day length, in part by inhibiting both prolactin and gonadotropin secretion, which may thus provide behavioral cues critical for reproduction in seasonal breeders. Although the physiological relevance of certain transmitters (e.g., dopamine) regulating a given adenohypophysial hormone (e.g., prolactin) is well established, a broader and more cohesive consensus will undoubtedly emerge with further research in this fundamentally important field.

## Glossary

**adenohypophysis** Endocrine subdivision of the pituitary; composed of the pars distalis (i.e., the anterior lobe of the pituitary gland), the pars intermedia (i.e., the intermediate lobe of the pituitary gland), and the pars tuberalis.

**anterior pituitary** Major lobe of the adenohypophysis; composed of a heterogeneous mixture of cell types, such as corticotrophs, somatotrophs, lactotrophs, thyrotrophs, and gonadotrophs, each of which secretes specific hormones that act on particular end organs to exert biological effects.

**hypophysial portal vasculature** Circulatory system that connects the median eminence (perfused by the dense capillary network stemming from the superior hypophysial artery) to the anterior pituitary via portal vessels, through which releasing and inhibiting factors are transported.

**hypothalamus** Heterogeneous region of the brain comprising the ventral diencephalon; serves a diverse array of homeostatic biological functions, including the control of energy balance, reproductive and parental behavior, growth and development, emotional expression, and stress responses, due largely to its regulation of pituitary hormone secretion.

**median eminence** Highly vascularized region of the mediobasal hypothalamus that resides outside of the blood–brain barrier; contains a high density of neurosecretory terminals and serves as the primary site of release of releasing and inhibiting factors.

**neurotransmitter** Chemical messenger found in and released from particular nerve cells that possess the protein machinery necessary for its biosynthesis, reuptake and degradation; interacts with a distinct set of plasma membrane-bound proteins called receptors to produce an electrochemical signal associated with cell-to-cell communication.

**releasing and inhibiting hormones** Polypeptides or small molecule neurotransmitters that are released from the terminals of hypothalamic neurosecretory cells; these hormones are transported from the median eminence via the hypophysial portal vasculature to the anterior pituitary, where they act to stimulate or inhibit the release of specific adenohypophysial hormones.

## See Also the Following Articles

**Amino Acid and Nitric Oxide Control of the Anterior Pituitary • Cytokines and Anterior Pituitary Function • Neuropeptides and Control of the Anterior Pituitary**

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# Motilin

JOHN R. GRIDER, JOHN F. KUEMMERLE, AND KARNUM S. MURTHY

*Medical College of Virginia Campus of Virginia Commonwealth University*

I. INTRODUCTION
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**Motilin, a hormone produced by endocrine cells of the proximal small intestine, is released into the circulation in a cyclic manner. Motilin initiates the contractions of the migrating motor complex in the stomach and upper small intestine during the interdigestive period. Pharmaceutical agents that mimic motilin action are of interest in treating disorders of gastric emptying and colonic motility.**

## I. INTRODUCTION

The gastrointestinal tract is a rich source of hormones that are diverse in their chemistry, cellular localization, mechanism of action, and physiological function. These hormones, in conjunction with components of the enteric nervous system and the extrinsic autonomic nervous system, are responsible for controlling the processes by which ingested material is digested, absorbed, and distributed to other organs of the body, and for eliminating the unabsorbable waste material. Although a majority of the gut hormones are involved in regulating the postprandial actions initiated in response to ingestion of a meal, one hormone, motilin, has been shown to be particularly important in regulating the activity of the gut during the interdigestive period. This period is characterized by a repetitive increase in motor activity that originates in the proximal gut and moves distally; the moving front of intense contractile activity is termed the migrating motor (or myoelectric) complex (MMC). The MMC appears to be initiated by cyclic increases in the release of motilin that occur during the interdigestive period. It is generally thought that this migrating motor complex performs a housekeeping function, clearing the gut of residual materials and endogenous secretions between meals.

## II. STRUCTURE AND SYNTHESIS

Motilin is a 22-amino-acid peptide that was initially isolated from the canine gastrointestinal tract by J. C. Brown and colleagues; it was identified as the agent responsible for the increased gastric motility observed following duodenal alkalinization. The peptide was named motilin because of its ability to increase motor activity, that is, increase motility. Since the original identification and sequencing in the canine, motilin has been identified in several other species, including pigs, cats, rabbits, chickens, and humans. As can be seen from Fig. 1, the sequences of human and porcine motilin are identical and differ from that of canine motilin at positions 7, 8, 12, 13, and 14. Although the sequence in mammals is well conserved, the sequence of motilin in rodents such as rats and guinea pigs differs more widely.The gene encoding motilin has been mapped to the p21.3 region of chromosome 6 in humans and has been shown to contain five exons and four introns spanning about 9 kb. The motilin gene is somewhat unusual in that the active form of motilin is encoded in exons 2 and 3.

Motilin, like other peptide hormones, is synthesized as a larger precursor termed prepromotilin. The sequence of the cDNA encoding the precursor has been identified in a variety of species, including humans, pigs, rabbits, canines, and monkeys, and has been shown to be similar in most species. In each case, the prepromotilin contains a signal peptide of 25 amino acids; the signal peptide is cleaved to yield promotilin, which consists of the 22-amino-acid sequence of active motilin and two lysine residues that are the site of posttranslational processing, followed by a motilin-associated peptide of variable length and amino acid sequence.

| | |
|---|---|
| Human: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln |
| Monkey: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg- Ser-Lys-Gly-Gln |
| Porcine: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln |
| Horse: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Ser-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Arg-Gly-Gln |
| Sheep: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Val-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Tyr-Lys-Gly-Gln |
| Bovine: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Val-Arg-Arg-Met-Gln-Glu-Lys-Glu-Arg-Tyr-Lys-Gly-Gln |
| Canine: | Phe-Val-Pro-Ile-Phe-Thr-His-Ser-Glu-Leu-Gln-Lys-Ile-Arg-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln |
| Feline: | Phe-Val-Pro-Ile-Phe-Thr-His-Ser-Glu-Leu-Gln-Arg-Ile-Arg-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln |
| Rabbit: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Ser-Glu-Leu-Gln-Arg-Met-Gln-Glu-Arg-Glu-Arg-Asn-Arg-Gly-Gln |
| Guinea pig: | Phe-Val-Pro-Ile-Phe-Thr-Tyr-Ser-Glu-Leu-Arg-Arg-Thr-Gln-Glu-Arg-Glu-Gln-Asn-Arg-Leu-Arg |
| Chicken: | Phe-Val-Pro-Phe-Phe-Thr-Gln-Ser-Asp-Ile-Gln-Lys-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln |

FIGURE 1 Amino acid sequence of motilin in several species.

The motilin-associated peptide in human is known to be 65 amino acids long; however, its biological action and physiological role have not yet been identified.

## III. DISTRIBUTION

The presence of authentic motilin in the gastrointestinal tract has been demonstrated by a variety of techniques, including immunohistochemical methods and measurement of motilin immunoreactivity in tissue extracts. Motilin is located in specialized endocrine cells, termed the Mo, or M, cells, found among the epithelial cells comprising the mucosa, which is the innermost layer of the gastrointestinal tract. The M cell is characterized by cytoplasm containing small (180 nm) solid granules with a homogenous core and a closely applied membrane. These granules are round in human cells and round to irregular in cells from the dog. Motilin has also been reported to be costored with 5-hydroxytryptamine in a subpopulation of mucosal endocrine cells, although this remains a subject of debate. The highest concentrations of motilin are found in the most proximal section of the small intestine, the duodenum and the jejunum; lesser amounts are found in the ileum. Motilin-containing cells have been identified in the proximal intestine of most mammalian species, including humans, monkeys, dogs, cats, and rabbits. Motilin has also been detected in the gastrointestinal tract of a wide range of animals, including rodents (mouse, rat, guinea pig), amphibians (frog, turtle), and birds (chicken, quail). In general, in species in which the motilin prohormone has been isolated and characterized, the signal peptide and the motilin peptide are more closely similar in amino acid sequence (73–100% identity in motilin) than are the various species-specific motilin-associated peptides (23–59% identity). Measurement of mRNA levels leads to the same conclusion, i.e., that motilin is largely a hormone produced in mucosal endocrine cells of the duodenum and jejunum. A single mRNA species of 700 nucleotides has been isolated from the canine duodenum.

Authentic motilin is largely absent from the stomach. Recently, however, a novel peptide that shares a significant sequence homology (about 36% identity) to motilin has been identified in secretory granules of mouse, rat, and, more recently, human gastric antral endocrine cells. This peptide, named motilin-related peptide by Tomasetto *et al.* and ghrelin by Kojima *et al.*, may represent a new member of the motilin family of peptides. To date, preliminary reports indicate that this novel peptide may share similar biological properties with motilin but may produce effects distinct from those of motilin.

Although motilin is found primarily in the proximal gut, there has been a great deal of controversy as to the presence of motilin in nongut tissues. Much of the controversy arises from the use of different antisera with different specificities to search for the presence of the peptide, and the apparent overall much lower concentrations in brain as compared to gut. Nonetheless, motilin or its precursor has variously been reported to be present in the pituitary, in the Purkinje cells of the cerebellum, in the thalamus, and in the hypothalamus of numerous species. Recent studies by the group of T. L. Peeters have shown the presence of the cDNA encoding for the motilin precursor in hypothalamus, hippocampus, and cerebellum of the rhesus monkey. The same group has demonstrated mRNA for motilin in the guinea pig, rabbit, and human brain. Although it is likely that the presence of motilin in nongut tissues will continue to be debated, these studies seem to strongly support the presence of motilin in the central nervous system. The physiological role of motilin in the brain remains to be elucidated.

## IV. RELEASE

Perhaps one of the most interesting and distinctive characteristics of motilin is its pattern of release. Unlike levels of other gut hormones, motilin levels are not constant during the interdigestive period, but rather rise and fall in the blood in a regular, cyclic pattern. Peaks in motilin levels occur at intervals of 80 to 100 min. This cyclic pattern of motilin release has been demonstrated in numerous species, including humans and dogs. The peak of motilin release coincides with the end of a brief period of intense contractions in the stomach. This contractile period is phase III of the migrating motor complex, part of a cyclic pattern of contractions that move through the gut during the interdigestive period. As explained in the next section, it is generally accepted that the physiological role of motilin is to initiate this pattern of contractile activity in the stomach and proximal small intestine.

Although the cyclic pattern of motilin release has been well documented since its initial description in the late 1970s, the exact nature of the origin of this pattern remains to be fully elucidated. Many stimuli and inhibitors of motilin release have been identified, but none has adequately been shown to be responsible for controlling the physiologically relevant pattern of release. Studies of the role of the autonomic nervous system have shown that sympathetic inputs to the gut are not involved in the release of motilin. Vagal influences are also controversial because both cooling and electrical stimulation of the vagi can cause the release of motilin. A recent study by Sarr and colleagues demonstrated in the dog that the cyclic pattern of motilin release and the migrating myoelectrical complex were not affected by complete transection of all extrinsic nerves to stomach, small intestine, colon, pancreas, and liver, suggesting that neither afferent not efferent neural components of the central nervous system are responsible for the cyclic pattern of motilin release. Cholinergic mechanisms, however, are clearly important to the regulation of the release of motilin. The muscarinic antagonist, atropine, and the nicotinic antagonist, hexamethonium, can block the cyclical increase in plasma motilin in the dog. The increase in motilin that is elicited by cervical vagal stimulation is also blocked by atropine or hexamethonium. The release of motilin induced by the cholinergic agonist carbachol is, however, blocked by atropine but not by hexamethonium, suggesting a primary role of the muscarinic rather than the nicotinic receptor in mediating the release of motilin. It is likely that the cholinergic neurons involved in the physiological release of motilin are enteric neurons because, as previously indicated, vagotomy does not abolish motilin release.

The release of motilin is influenced by other neuropeptides in the gut. Bombesin has been shown to be a potent stimulator, and somatostatin a potent inhibitor, of motilin release in the dog. Agonists of the opiate receptors stimulate the release of motilin, although a physiological role of endogenous opioid peptides is unlikely because the opioid receptor antagonist, naloxone, has minimal effects on motilin release. 5-Hydroxytryptamine is also likely involved in the human gastrointestinal tract because an antagonist of the type 3 5-hydroxytryptamine receptor inhibits motilin release and the subsequent MMC contractions. Similarly, the same antagonists of the type 3 5-hydroxytryptamine receptor interrupt the MMC in the dog, although the effect may not be mediated through an inhibition of motilin release.

Intralumenal contents in the duodenum greatly influence the release of motilin. It is clear in dogs and humans that ingestion of a meal results in a cessation of the cyclic pattern of motilin release and disruption of the migrating motor complex. The causative agent and mechanism of this meal-induced change in pattern of release of motilin and of the MMC are not known. As stated previously, motilin was first isolated as the endogenous motility-stimulating agent released by the presence of alkaline solution in the canine duodenum. Subsequent studies in dogs and humans have shown that although pH changes can induce motilin release, their effect on physiological release of motilin is relatively small if the pH is adjusted to between 2 and 8. Biliary and pancreatic secretions have been postulated to be potential intralumenal agents that might influence the release of motilin, but their role in the physiological regulation of motilin release remains controversial. A recent study in humans showed that acute depletion of intraduodenal bile salts with the bile acid binding agent, cholestyramine, resulted in an increase in plasma motilin, suggesting that bile may have an inhibitory or negative feedback effect on motilin release.

Thus, although much is known about endogenous and exogenous agents that stimulate and inhibit the release of motilin from the duodenal mucosa, little is known of the physiological mechanisms responsible for the generation of the distinctive cyclic pattern of release or of the mechanisms responsible for the prompt cessation of this pattern on ingestion of a meal. These remain areas of controversy and intense investigation.

## V. PHYSIOLOGICAL ACTION

The physiological function of motilin is induction of gastric contractions that comprise phase III of the migrating motor complex. During the interdigestive period, a very distinct cyclic pattern of increased contractility occurs in the gastrointestinal tract at an interval of about 100 min. This pattern consists of distinct phases. Phase I is a period of quiescence lasting about 60–70 min, phase II is a period of increased but irregular activity lasting 20–30 min, and phase III is a period of intense and regular contractile activity lasting 5–10 min. Phase III is followed by a return to quiescence. This pattern of increased contractility begins in the gastric antrum and lower esophageal sphincter and moves distally through the entire length of the small intestine. The phase III contractile activity can be traced through the gastrointestinal tract and has been shown to take about 2 h to move completely through the gut. Thus, when the phase III activity reaches the distal small intestine, a new phase III is beginning in the stomach. The MMC has been postulated to fulfill a housekeeping role, continually clearing the stomach and intestines of secretions and intralumenal contents during the interdigestive period.

The cyclic pattern of motilin release is very similar to that of the MMC. The cyclic pattern of motilin release also has a period of about 100 min and has a peak that coincides with the end of phase III contractile activity in the stomach. Several lines of evidence support the generally accepted notion that motilin is responsible for induction of phase III contractions in the stomach. First, immunoneutralization of motilin with a specific antiserum in the dog disrupts the phase III contractile activity and prevents initiation of the MMC in the stomach. It should be noted, however, that phase III contractions can still be seen in the distal small intestine in the presence of neutralizing motilin antiserum, indicating a role for motilin in initiation of contraction in the proximal, but not distal, gut. Second, excision of the duodenum in the dog, to remove most of the motilin-secreting cells, impairs the normal MMC in the proximal, but not distal, gut. With time, however, MMC-like activity returns in these animals, suggesting that alternative pathways may fulfill the role of mediating the MMC after removal of the motilin cells. Third, infusion of motilin to reproduce physiological levels induces premature phase III contractile activity in the gastric antrum and duodenum, but not in the distal small intestine. This property of motilin has been demonstrated in humans and many other species. Two aspects of the response to motilin infusion are unusual. First, the intensity of the contractions during phase III is not dependent on the concentration of motilin, although increasing concentrations shorten the latency to initiation of phase III contractile activity. Second, the phase III contractions induced by infusion of motilin abate after a few minutes despite the continued presence of motilin. Thus, the response to exogenous motilin mimics very closely phase III contractile activity in terms of duration as well as contractile characteristics. It also suggests that motilin should be viewed more as the initiator of the physiological processes that produce the MMC, rather than as the mediator of the MMC.

Several other biological actions of motilin have been identified, although their physiological nature is controversial. Motilin has been shown to stimulate pepsinogen secretion in the stomach and generalized enzyme secretion from the pancreas, and contraction of the gallbladder and the lower esophageal sphincter. These effects are all normally associated with phase III of the MMC and have been postulated to be components of a general periodic activation of gut function induced by motilin. It should be noted, however, that the potency of motilin varies widely with respect to these biological effects and that the ability of motilin to induce these actions is also species dependent. Motilin has been shown to increase the emptying rate of liquids but not solids from the canine stomach, although this effect appears to be less consistent in normal human study participants. There is, however, evidence that motilin increases the rate of gastric emptying in patients with impaired gastric emptying due to diabetic gastroparesis. This effect has been most clearly shown for synthetic motilin-like agents known as motilides and is currently being investigated for potential therapeutic applications (see Section VII).

Finally, although motilin levels are very low or not detectable in the mucosa of the colon, motilin receptors have been identified in this region and colonic muscle had been shown to be very sensitive to exogenous motilin. Thus, motilin and motilides have been shown to be very potent in causing contractions of isolated colonic smooth muscle cells and in enhancing propulsive motility of the colon. Although it is not clear if this is a physiological action of motilin, the potential use of motilides as therapeutic agents to enhance colonic transit is currently an area of intense investigation.

## VI. MECHANISM OF ACTION

The action of motilin has been investigated in isolated muscle cells, in muscle strips, and *in vivo*. Depending on the species, motilin has been shown to act directly through an effect on smooth muscle cells and indirectly via activation of enteric neurons. These represent different phenomena. The physiological effect of motilin to cause phase III contractions of the MMC in most species, including humans and dogs, is mediated by an interaction with cholinergic neurons, as evidenced by the ability of muscarinic cholinergic antagonists to block initiation of the MMC. In the dog, 5-hydroxytryptamine neurons have also been implicated as a component of the neural pathway that mediates the physiological action of motilin. Depletion of 5-hydroxytryptamine by treatment with *para*-chlorophenylalanine or blockade of type 3 5-hydroxytryptamine receptors with selective antagonists eliminates the ability of motilin to initiate phase III contractions in the canine stomach. This effect is different in the human stomach, in which 5-hydroxytryptamine is involved in the release of motilin from endocrine cells; in the dog, there is no effect of 5-hydroxytryptamine drugs on motilin levels.

Studies of muscle strips and isolated muscle cells from several species have also identified a direct action of motilin on smooth muscle cells. In smooth muscle cells and muscle strips from human and rabbit stomach, small intestine, and colon, motilin has been shown to cause a direct contraction that is accompanied by an increase in intracellular calcium. The increase in calcium appears to be mediated via influx of extracellular calcium through L-type calcium channels.

Consistent with the pharmacological actions of motilin, specific receptors for motilin have been examined by the classical techniques of radioligand binding and autoradiography. Both techniques have been used in the past to demonstrate the presence of specific motilin receptors within the smooth muscle of the gastrointestinal tract of several species. Motilin receptors were shown to be highest in concentration in the antral muscle and to decrease caudally. Within the wall of the gut, the concentration of motilin receptors was highest in the circular muscle layer. Recent radioligand binding studies by the group of Poitras have confirmed the presence of motilin receptors in the smooth muscle of rabbit colon and human gastric antrum but have also provided evidence for the presence of a distinct motilin receptor type in enteric nerves. Using purified synaptosomal and smooth muscle membranes, it was shown that a variety of motilin agonists and antagonists demonstrate differential binding affinities for motilin binding sites in each membrane preparation, indicative of different receptor subtypes on enteric neurons and smooth muscle cells. These binding studies are supported by functional studies of the rabbit stomach, suggesting that neural receptors mediate the chronotropic effects of motilin whereas smooth muscle receptors mediate the ionotropic effects of motilin. It is interesting to note that, as with other gut neuropeptides, the neural receptors seem to be much more sensitive than the smooth muscle receptors.

The presence of specific motilin binding sites in the rabbit brain has been demonstrated using autoradiographic techniques. It is not clear if the motilin receptors on neurons of the central nervous system are the same as motilin receptors on enteric neurons. The physiological significance of these motilin receptors in the brain remains to be determined.

A human motilin receptor has been identified by screening known sequences of cloned orphan receptors. The original orphan clone, GPR38, has now been shown by Feighner *et al.* to bind both motilin and the motilide erythromycin; thus, the receptor has been renamed motilin-R1A (MTL-R1A). This receptor shares sequence homology (about 50%) with another group of receptors, known as the growth hormone secretagogue receptors, which mediate the action of the related peptide, ghrelin, or motilin-related peptide (see Section III). The motilin receptor is a member of the class of seven-transmembrane-domain receptors coupled to a heterotrimeric G-protein and appears to activate the phospholipase C signal transduction pathway. The presence of this receptor in the gastrointestinal tract was demonstrated by measurement of specific mRNA levels. Evidence for the presence of this motilin receptor was found in RNA isolated from human esophagus, duodenum, jejunum, ileum, and colon. In the colon, *in situ* hybridization revealed the presence of motilin receptor mRNA in enteric neurons co-expressing either nitric oxide synthase or choline acetyltransferase; in the duodenum, mRNA for the motilin receptor was evident also in smooth muscle cells. This study provides strong support for the presence of a specific motilin receptor in human smooth muscle cells and in enteric neurons. Whether this represents the only receptor or whether there are subtypes of the motilin receptor remains to be determined.

## VII. ERYTHROMYCIN AND MOTILIDES

Much of the current interest in motilin stems from a discovery made by Z. Itoh and colleagues in 1984. They observed that a 20-min infusion of the macrolide antibiotic erythromycin, administered to dogs intravenously (iv) at a dose of 30 μg/kg, induced powerful contractions in the stomach and duodenum. These contractions were very similar in duration to those of the phase III contractions of the MMC and moved caudally into the small intestine at the same velocity as the phase III contractions of the MMC. Almost simultaneously, a second study done by G. Zara and co-workers demonstrated that higher doses of erythromycin (7 mg/kg, iv) induced contractile activity throughout the small intestine whereas a lower dose (1 mg/kg, iv) induced a pattern of contractile activity similar to the phase III contractions of the MMC. Zara attributed the effects of erythromycin to an opiate-like effect whereas Itoh correctly recognized the effect as more motilin-like. These initial observations led to a thorough study of the effects of erythromycin and the related macrolide antibiotics. Subsequent studies have confirmed that erythromycin has similar effects to motilin in humans, inducing a phase III contractile pattern that moves through the gut in the same manner as the MMC. The macrolides have been shown to compete with radiolabeled motilin for binding sites in gut smooth muscle, enteric neurons, and brain tissue, and erythromycin has recently been shown to interact with the newly cloned motilin MTL-R1A receptor. The descriptive term "motilide" is thus derived from the words "motilin" and "macrolide," reflecting the fact that erythromycin and related macrolide antibiotics interact with the motilin receptor as potent agonists. These agents have been shown to act via the same mechanisms as motilin, causing direct smooth muscle contractions and activation of cholinergic muscarinic pathways. The motilides show the same species and tissue selectivity as does motilin. In general, their ability to mimic the actions of motilin at subantibiotic doses and their resistance to degradation have made them attractive models for the development of newer compounds with increased motilin-like activity and no antibiotic activity. Numerous new motilides have been synthesized based on the structural characteristics of erythromycin and have been evaluated for potential use as prokinetic agents in stomach and colon. Most interest has focused on their use in patients with decreased gastric emptying as a result of diabetic gastroparesis or functional dyspepsia. The potential for their clinical application is based on a report by Janssens *et al.* showing that gastric emptying in diabetic patients is greatly enhanced by erythromycin. Although the clinical trials of motilides such as ABT-229 and EM574 have been disappointing, the recent removal of cisapride and related prokinetic agents from clinical use may further stimulate development of even newer motilides with enhanced potency.

## VIII. SUMMARY

Although the mechanisms that govern the cyclic pattern of release and the exact stimulus for the release of motilin are not known, it is clear that the release of motilin is responsible for initiation of phase III contractions of the migrating motor complex in the stomach and upper small intestine during the interdigestive period in humans, dogs, and many other species. The physiological action of motilin appears to be mediated by specific motilin receptors located on enteric neurons, ultimately leading to activation of muscarinic cholinergic neurons in the gastric antrum. Specific motilin receptors have also been demonstrated on smooth muscle cells of stomach, small intestine, and colon, where their activation results in the influx of calcium and contraction of the smooth muscle cells. The actions of motilin are mimicked by the macrolide antibiotic erythromycin and a group of motilin agonists known as motilides. The motilides are currently being developed as pharmaceutical prokinetic agents to treat patients with impaired gastric emptying and colonic motility.

## Glossary

**enteric nervous system** Ganglionated nerve plexuses contained within the wall of the gastrointestinal tract. Neurons with cell bodies in these plexuses innervate other neurons, muscle cells, and secretory cells of the gastrointestinal tract and can stimulate or inhibit the activity of these other cell types.

**migrating motor complex** A repetitive pattern of contractile activity that begins in the stomach and moves caudally throughout the small intestine during the interdigestive period. This pattern of activity is also known as the migrating myoelectric complex and the interdigestive housekeeper.

**motilide** A macrolide antibiotic that acts as a motilin receptor agonist at subantibiotic doses and mimics the physiological and pharmacological effects of motilin.

**mucosa** Innermost layer of the gastrointestinal tract that is in intimate contact with lumenal contents. This layer is composed of epithelial cells with interspersed enteric endocrine cells.

## See Also the Following Articles

**Gastrin • Gastrointestinal Hormone-Releasing Peptides • Ghrelin • Vagal Regulation of Gastric Functions by Brain Neuropeptides**

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# Multiple G-Protein Coupling Systems

BRIAN HAWES

*Schering Plough Research Institute, New Jersey*

I. INTRODUCTION
II. G-PROTEIN-COUPLED RECEPTOR DIVERSITY LEADS TO ACTIVATION OF MULTIPLE G-PROTEIN SIGNALING PATHWAYS
III. MULTIPLE G-PROTEINS GENERATE DIVERSE SIGNALING PATTERNS
IV. DIVERSITY IN EFFECTORS
V. REGULATORS OF G-PROTEIN-COUPLED RECEPTOR SIGNALING
VI. SUMMARY

**G-Protein-coupled receptors (GPCRs) are the largest known family of cell surface receptors. GPCRs respond to a myriad of external stimuli. Cellular response to external stimuli is dependent on a wide array of variables. These variables provide the necessary tools to enable particular cells to respond in a specific manner to individual or multiple stimuli. Such stimuli can be physical or chemical in nature. The key**

**to an integrated response to the multitude of stimuli that are constantly bombarding a cellular system is specificity of response.**

## I. INTRODUCTION

The first layer of specificity in cellular response is the stimulus, and whether the stimulus is capable of provoking a response. The stimulus and the cell must be within a proximity that allows a response to occur. In addition, the stimulus must be of sufficient magnitude, frequency, or duration to elicit a response. Further, the cell must be capable of recognizing the stimulus and in a state in which a response is possible. Receptors are proteins that convey cellular responsiveness to specific stimuli and initiate the intracellular signaling within the stimulated cell that results in a specific response. Several types of receptors are present on the cell surface, in the intracellular milieu, or on intracellular organelles.

G-Protein-coupled receptors (GPCRs) respond to many stimuli, including light, monoamines, peptides, and chemicals of specific structure. These receptors are composed of seven transmembrane-spanning domains with an extracellular amino terminus and an intracellular carboxyl tail. GPCRs can be grouped into three broad categories: rhodopsin-like, calcitonin related, and metabotropic. The majority of GPCRs that have been identified are rhodopsin-like. These receptors have been the focus of a tremendous research effort over the past two decades.

As the nomenclature implies, GPCRs interact with G-proteins to mediate intracellular signaling. G-Proteins are composed of three subunits, α, β, and γ. In the absence of external stimuli, G-proteins are bound to GPCRs and are inactive. Agonist stimulation of GPCRs results in increased guanosine triphosphate (GTP) exchange for guanosine diphosphate (GDP) on the α-subunit of the associated G-proteins. Binding of GTP to the α-subunit reduces the affinity of the α-subunit to the βγ-heterodimeric subunit. This decrease in affinity allows the G-protein to dissociate into two active entities, the free α-GTP subunit and the free βγ-subunit. Both α-GTP and the βγ-subunit can potentially activate a number of intracellular signaling pathways. Intrinsic GTPase activity of the α-subunit deactivates α-GTP. The α-subunit returns to an inactive GDP-bound state, and reassociates with the βγ-subunit thereby rendering both signaling entities of the G-protein inactive. The multitude of GPCRs, the variety of potential α, β, and γ combinations, and the numerous intracellular signal transduction pathways provide the foundation for conferring specificity of cellular response to external stimuli.

## II. G-PROTEIN-COUPLED RECEPTOR DIVERSITY LEADS TO ACTIVATION OF MULTIPLE G-PROTEIN SIGNALING PATHWAYS

As already noted, the first layer of specificity in cellular response is the stimulus. The next layer providing specificity in GPCR-mediated intracellular signaling is the receptor. Hundreds of GPCRs have been identified and characterized. These receptors can be divided and subdivided based on nucleotide and amino acid sequences and ligand interactions. There are many illustrations of ligands that can activate multiple receptors or multiple receptor subtypes. For example, there are several receptors that are activated by adrenaline. These receptors fall into the categories of α- or β-adrenergic receptors. Each of these categories is subdivided into groups, including α1, α2, β1, β2, and β3 receptors. The α receptor types are further delineated to α1a, α1b, α1c, α2a, α2b, and α2c receptor subtypes. These receptor subtypes can be distinguished pharmacologically and functionally and bestow great diversity of cellular response. Similarly, multiple receptor subtypes have been identified for many other ligands, including those for cholinergic, purinergic, opioid, dopaminergic, serotonergic, and many peptide receptors.

Different receptor subtypes can mediate activation of the same G-proteins and regulate similar intracellular signaling pathways. For example M1 and M3 muscarinic receptors couple to $G_q$ to mediate phosphoinositide metabolism. Alternatively, different receptor subtypes may activate distinct G-proteins and regulate independent signaling pathways. The M2 and M4 muscarinic receptor subtypes activate $G_i$, which inhibits adenylyl cyclase rather than the $G_q$ pathway that is stimulated by M1 and M3. In addition, the pharmacology of a ligand at each receptor subtype may differ. The affinity of a ligand at one receptor subtype can be much higher compared to another receptor subtype. The degree of activation that a ligand can generate in one receptor subtype compared to another can also be variable. Thus, the potency and efficacy of a ligand at one receptor subtype may be quite distinct compared to another receptor subtype. The response of a cell to a particular stimulus is dependent on the receptor subtype that is expressed by the cell. Cells can express one receptor subtype or multiple receptor subtypes that respond to the same ligand. The cellular response to a particular stimulus is the sum of the responses contributed by each type of activated receptor. A ligand can have a focused response in a cell that expresses one receptor subtype (Fig. 1A), while generating a pleiotropic response in another cell that expresses multiple receptor subtypes (Fig. 1B). Furthermore, the cellular expression ratio of various receptor subtypes can distinguish the response of individual cells to specific stimuli. For example, if a cell expresses a preponderance of receptor subtype R1 (Fig. 2A), the cellular response will be similar to that of cells that express subtype R1 alone (Fig. 1A). In contrast, in cells that express an equal amount of receptor subtypes R1 and R2 (Fig. 2B), the response will be a mixture of R1- and R2-mediated signaling, assuming R1 and R2 have similar affinities and pharmacological properties with a specific ligand. By comparison, a cell expressing primarily receptor subtype R2 will exhibit a response reflecting R2 receptor activation (Fig. 2C). The response may be quite distinct from that elicited by receptor subtype R1 stimulation, even though R1 is expressed to some degree on the cell surface. This provides another layer of specificity in cellular response to external stimuli. The response of an individual cell to a specific ligand is dependent on the receptor subtypes that are expressed and the relative levels of expression of each receptor subtype.

## III. MULTIPLE G-PROTEINS GENERATE DIVERSE SIGNALING PATTERNS

### A. Classes of α and βγ Subunits

In addition to the multitude of external stimuli and the massive diversity in GPCRs, another layer of specificity in cellular response is provided by the numerous combinations of G-proteins potentially expressed within a cell. G-Proteins are categorized by the α-subunit of the heterotrimeric complex. There are four classes of G-protein α-subunits as shown in Table 1. Each class of G-protein α-subunit contains two or more members; 17 α-subunits have been identified. The functions of the subunit classes of α and βγ are shown in Table 1.

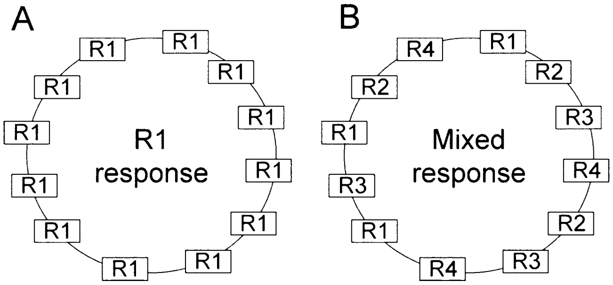

FIGURE 1 (A) Expression of a single receptor (R1), compared to (B) expression of multiple receptors (R1–R4), on a single cell.

#### 1. *αs*

The $G_{\alpha s}$ class consists of αs-type and αolf. Activation of αs-type G-proteins results in stimulation of adenylyl cyclase and subsequent increases in cAMP production. The αs class of G-proteins also can regulate $Ca^{2+}$ channel activity. Cholera toxin acts on αs G-proteins to inhibit intrinsic GTPase activity. The GTPase activity is the mechanism by which activated G-proteins are inactivated. Thus, cholera toxin enhances the activity of the αs class of G-proteins.

#### 2. *αi*

In contrast to αs G-proteins, activation of the αi class of G-proteins inhibits adenylyl cyclase, resulting in decreased cAMP production. This class of α-subunits also regulates $K^+$, $Ca^{2+}$ channels, and cGMP phosphodiesterase. There are several members of the αi class, including αi1, αi2, αi3, αo, αt-1, αt-2, αgust, and αz. With the exception of αz the activity of the αi class of G-proteins is inhibited by pertussis

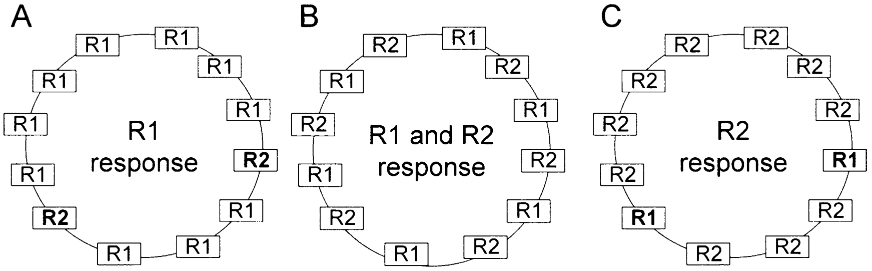

FIGURE 2 Cellular response is dependent on the ratio of receptor expression (see text for discussion).

TABLE 1 G-Protein Classification and Functional Activities

| Subunit class | Class members | Effector functionality |
|---|---|---|
| αs | αs, αolf | Adenylyl cyclase (stimulation), $Ca^{2+}$ channels |
| αi | αi1, αi2, αi3, αo, αt1, αt2, αgust, αz | Adenylyl cyclase (inhibition), $Ca^{2+}$ and $K^+$ channels, cGMP phosphodiesterase |
| αq | αq, α11, α14, α15, α16 | Phospholipase C |
| α12 | α12, α13 | $Na^+/K^+$ exchange |
| βγ | β1, β2, β,3 β4, β5, γ1, γ2, γ3, γ4,γ5, γ6 | Adenylyl cyclase, G-protein receptor kinases, $Ca^{2+}$ and $K^+$ channels, phospholipases A and C |

toxin. Pertussis toxin adenosine diphosphate (ADP)-ribosylates the α-subunit, rendering it unable to associate with receptor.

### 3. *αq*

A third class of G-proteins is αq. This class includes αq, α11, α14, α15, and α16. Activation of these G-proteins stimulates phosphoinositide metabolism. The activated α-subunit from this class of G-proteins directly activates phopholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to form diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). Both DAG and $InsP_3$ are intracellular effector molecules.

### 4. *α12*

The fourth class of G-proteins is α12. This class consists of α12 and α13. The α12 class of G-proteins regulates $Na^+/K^+$ exchange. This class of G-proteins is by far the least studied of the four classes and is thus the least well characterized.

### 5. *βγ*

The βγ-subunit of G-proteins also is an active signaling entity; βγ mediates numerous intracellular signaling events, including activation of certain subtypes of adenylyl cyclase, phospholipase C, and phospholipase A. The βγ-subunit also activates $K^+$ channels, and phosphatidylinositol-3-kinase. In addition, βγ binds to G-protein receptor kinases and transports the enzymes to membrane receptor substrates. Like the α-subunit, multiple subtypes of subunits β and γ have been identified. There are five β-subunits (numbered β1–β5) and at least six γ-subunits (numbered γ1–γ6). This theoretically creates 30 different combinations of βγ-subunits. Not all combinations are possible, however. For example, β2 and γ1 do not associate with each other, but β2 and γ2 do form a heterodimer. By comparison, β1 is structurally very similar to β2, but is able to form a dimer with either γ1 or γ2. In addition, some subtypes are expressed only in specific tissues. For example, β1γ1 is expressed only in the retina. Extensive studies to determine which β- and γ-subunits associate with certain α-subunits in particular cell types have not been completed. The multitude of heterotrimeric combinations that can be generated undoubtedly contributes to specificity of cellular response.

## B. Models of G-Protein Activation—α

Cellular response to external stimuli is dependent on which G-proteins are activated. In many cases, multiple G-proteins are activated simultaneously. Several models of multiple G-protein activation are possible, as depicted in Fig. 3. In the most simplistic model (Fig. 3A), two separate receptors, R1 and R2, are activated, and each receptor is coupled to a single type of G-protein (G1 and G2). The activated G-proteins then initiate independent intracellular effector pathways (E1 and E2). For instance, a single ligand could activate two receptor subtypes, with one receptor subtype coupled to $G_s$ and the other coupled to $G_q$. Activation of $G_s$ would lead to increased adenylyl cylcase activity, whereas $G_q$ activation would stimulate phospholipase C.

Alternatively, a single receptor can couple to multiple G-proteins. Indeed, many receptors are capable of coupling to several G-protein subtypes, depending on the cellular conditions. A single receptor coupling to two G-proteins can activate distinct intracellular signaling pathways (Fig. 3B). For example, one receptor could couple to both $G_i$ and $G_q$, resulting in a decrease in adenylyl cyclase activity and increased phospholipase C activity. It is also possible for the activation of distinct G-proteins to regulate the same effector system (Fig. 3C). For example, if a receptor couples to more than one subtype of $G_{\alpha i}$, both G-proteins mediate inhibition of adenylyl cyclase. G-Proteins from separate classes can also regulate the same effector. Multiple G-proteins may affect downstream signaling pathways in the same manner or may have opposing actions.

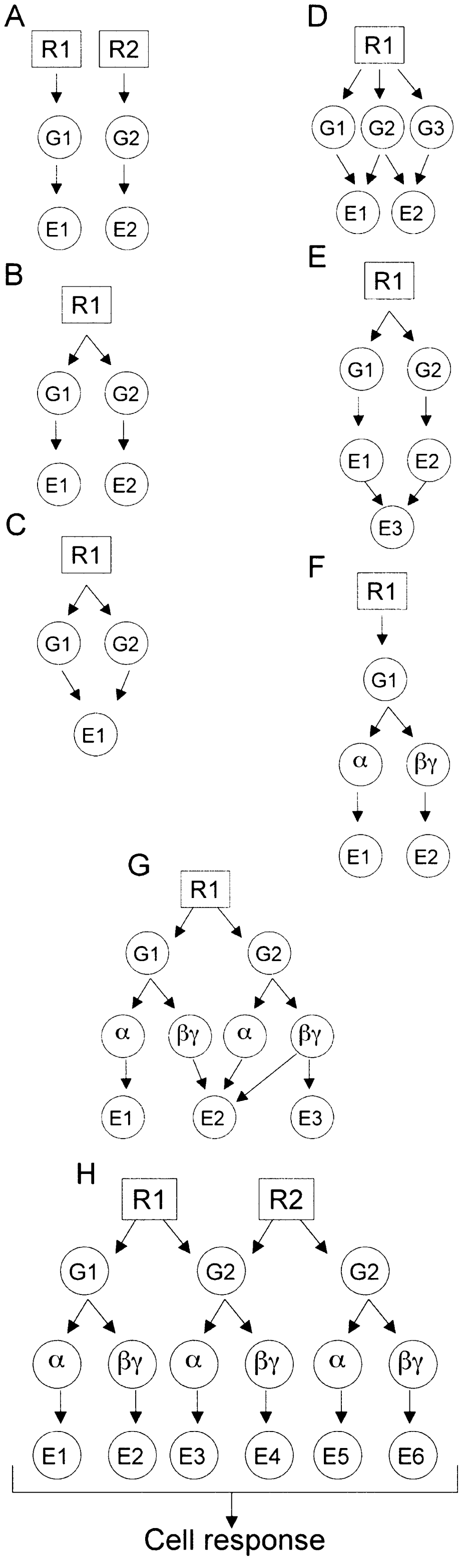

**FIGURE 3** Models of multiple G-protein and effector system activation. G1, G2. G-Protein types; R1, R2, receptor types; E1, E2, effector types.

For example, a receptor that couples to both $G_i$- and $G_s$-proteins would mediate both positive and negative regulation of adenylyl cyclase activity. In that case, effects on adenylyl cyclase would be dependent on the relative level of $G_s$ versus $G_i$ activation. Parameters that would contribute to this include the expression of $G_s$ and $G_i$, and the intrinsic ability of the receptor to couple to each G-protein.

Multiple G-protein coupling is not limited to just two G-proteins and two effectors. The model in Fig. 3D depicts a receptor coupling to three G-proteins. Two or more of these G-proteins may regulate the same effector while at the same time also modulating other effectors independently. An example would be a receptor coupling to two $G_i$ subtypes and a $G_q$-type receptor. The two $G_i$ subtypes decrease adenylyl cyclase activity whereas $G_q$ stimulates phospholipase C. The number of G-proteins to which a receptor is capable of coupling is dependent on which G-proteins are expressed and on receptor/G-protein coupling efficiency.

The effects of activation of distinct G-proteins may also intersect at a point in the intracellular signaling pathway downstream of the initial effector molecule (Fig. 3E). In this model, an individual receptor activates two distinct G-proteins, which, in turn, activate separate second-messenger effector pathways. Through distinct mechanisms, these pathways converge on a downstream effector. The convergent pathways may have synergistic or antagonistic effects. An example of a downstream effector that is regulated by distinct intracellular pathways initiated by activation of multiple G-proteins is mitogen-activated protein kinase (MAP kinase). Many diverse signaling pathways modulate MAP kinase activity, and it is common for a single stimulus to initiate numerous signaling mechanisms that eventually affect MAP kinase activation.

## C. Models of G-Protein Activation—α and βγ

As already noted, the α-subunit is not the only active signaling component of G-proteins. The βγ-subunit also interacts with effector systems. This adds a further level of complexity and another layer of specificity in cellular responsiveness. A simple model of α and βγ signaling is shown in Fig. 3F. A single receptor couples to a single G-protein. Following dissociation, two active subunits are formed, α and βγ, which each regulate an effector. For example, following activation of a $G_i$-coupled receptor, αi inhibits adenylyl cyclase and the βγ-subunit activates an isoform of phospholipase C. A more complicated model appears if multiple G-proteins are activated following receptor stimulation (Fig. 3G). In this model, a receptor activates multiple G-proteins, which each dissociate into active α and βγ components. Each α and βγ is capable of activating a number of effector systems. Some of these effectors are modulated by more than one α- or βγ-subunit, and the regulation may be synergistic or antagonistic. For example, the αq- and the βγ-subunits of $G_i$ both increase the activity of isoforms of phospholipase C. In contrast, the inhibition of one type of adenylyl cyclase activity may be counteracted by an increase in the activity of another class of adenylate cyclase by βγ-subunits. By comparison, the activity of some effectors may be dependent on only one specific α- or βγ-subunit. As shown in Fig. 3E, the effector systems may converge at another point downstream in the signaling pathway. With α- and βγ-subunits from multiple G-proteins activating a wide array of signaling intermediates, the potential for convergent signaling pathways increases. Likewise, some effector systems remain independent of other signaling pathways.

A model is shown in Fig. 3H demonstrating that the cellular response is a compilation of the activated signaling pathways. External stimuli activate one or more cell surface receptors, which are coupled to one or more G-proteins. The G-proteins each dissociate into α- and βγ-subunits, which activate one or more effector systems. These effector systems can be independent or convergent. The integration of these effector systems results in a dynamic system that can specifically respond to external stimuli.

## D. Example of Multiple G-Protein Signaling

An example of a complex intracellular signaling system mediated through multiple G-proteins is melanin-concentrating hormone (MCH)stimulation of MCH receptor-1 (MCH-R1) in Chinese hamster ovary (CHO) cells. Agonist stimulation of MCH-R1 results in activation of $G_i$, $G_o$, and $G_q$. MCH stimulates a decrease in adenylyl cyclase activity, an increase in phosphoinositide metabolism, an increase in intracellular free $Ca^{2+}$, and an increase in MAP kinase activation. Pertussis toxin completely abolishes the decrease in adenylyl cyclase activity and the increase in MAP kinase, but only partially blocks the increases in intracellular free $Ca^{2+}$ and phosphoinositide metabolism. Thus, the increase in MAP kinase is dependent on $G_i$ and/or $G_o$ activity, whereas changes in intracellular free $Ca^{2+}$ and phosphoinositide metabolism are dependent on $G_q$ and $G_i/G_o$ activity. Looking more closely at MAP kinase activity, $G_o$-dependent increases in MAP kinase in CHO cells utilize the α-subunit and are dependent on protein kinase C (PKC) activity. $G_i$-mediated MAP kinase activation in CHO cells utilizes the βγ-subunit and is independent of PKC. Depletion of cellular PKC inhibits MCH-stimulated MAP kinase activation by roughly 50%, indicating that both $G_i$- and $G_o$-dependent pathways are employed to mediate MAP kinase activity following MCH-R1 activation. Thus, the βγ-subunit of $G_i$ and αo activate distinct signaling pathways that converge to activate MAP kinase in this system. By comparison, αq, the βγ-subunit of $G_i$, and/or αo converge at a point higher in the signaling pathway to mediate increases in phosphoinositide metabolism and intracellular free $Ca^{2+}$.

## IV. DIVERSITY IN EFFECTORS

In addition to the subtypes of receptors and G-proteins, subtypes of several downstream signaling effectors have been identified. There are at least nine isoforms of adenylyl cyclase expressed in mammalian systems. These subtypes of adenylyl cyclase vary in tissue expression and interaction with specific G-proteins. Some of these isoforms of adenylyl cyclase are activated by the βγ-subunit, and some are inhibited. The isoforms of adenylyl cyclase that are expressed in a particular cell will have a profound influence on the intracellular signaling characteristics mediated by GPCR activation.

Several isoforms of phospholipase C have also been identified. There are at least 10 phospholipase C (PLC) genes grouped into three families: PLC-β, PLC-γ, and PLC-δ. The αq G-protein subunit activates all PLC-β isozymes, but is more effective at activating PLC-β1 and PLC-β3. By comparison, the βγ-subunit of $G_i$ activates PLC-β2 and PLC-β3. The combination of which PLCs are expressed and which are stimulated by activated G-proteins in a particular cell system contributes to linkage of the signaling pathways to a specific GPCR.

Another major intermediate common in G-protein-dependent signaling pathways is PKC. Similar to adenylyl cyclase and phospholipase C, at least nine isoforms of PKC have been identified. These isoforms are grouped into two broad categories based on structure. One class includes α, β-I, β-II, and γ. These isoforms of PKC all contain a conserved region, C-2. The other class of PKC isoforms includes δ, ϵ, ζ, μ, and θ, which lack the C-2 region. Most of these PKC isoforms are activated by DAG and by increased intracellular free $Ca^{2+}$. Because many activated G-protein subtypes stimulate DAG production and/or increase intracellular $Ca^{2+}$, PKC activity often is involved in GPCR-mediated intracellular signaling pathways. The various PKC isoforms phosphorylate specific proteins. GPCRs are therefore coupled to specific PKC-mediated protein phosphorylation, based on which G-proteins and PKC isoforms are activated. In addition, in some cases, PKC phosphorylates GPCRs and regulates receptor function.

Multiple subtypes of other intracellular signaling intermediates also exist. The wide array of potential signaling intermediates and pathways is too vast to describe in detail. The central theme, however, is that the diversity in intracellular signaling pathways provides the network required to achieve specific cellular response to external stimuli.

## V. REGULATORS OF G-PROTEIN-COUPLED RECEPTOR SIGNALING

In addition to the initiation of intracellular signaling pathways, cellular response also involves attenuation of GPCR activity. Several factors act in concert to decrease GPCR function through intracellular signaling pathways of their own. G-Protein-coupled receptor kinases (GRKs) are serine/threonine kinases that phosphorylate agonist-bound or activated GPCRs and initiate receptor internalization. There are seven mammalian GRKs grouped into three classes based on functional similarities. One class has the members GRK1 and GRK7. The second class includes GRK2 and GRK3. The third class consists of GRK4, GRK5, and GRK6. Activation of GRKs by various intracellular signaling mechanisms coupled with the diversity of GRK function provides another layer of specificity in cellular response.

Arrestins comprise another family of proteins that are involved in regulation of GPCR function. At least four arrestins have been identified. The arrestins are grouped into two classes based on sequence homology, function, and tissue distribution. Visual arrestin and cone arrestin (C-arrestin) comprise one class; β-arrestin1 and β-arrestin2 comprise the other class. These proteins are also involved in internalization of GPCRs, and thus regulate receptor function.

Proteins termed "regulators of G-protein signaling" (RGS proteins) are a third major modulator of G-protein-mediated signaling. These are GTPase-activating proteins that act on active $G_\alpha$ subunits to accelerate GTP hydrolysis, thereby decreasing G-protein activity. More than 20 RGS proteins are expressed in mammals. Some RGS proteins selectively modify specific $G_\alpha$ subunits, whereas other RGS proteins act more pervasively. The presence of RGS proteins within a cell can have profound effects on the cellular response to GPCR activation.

Regulation of G-protein-dependent signaling is not limited to the GRKs, arrestins, and RGS proteins. Other cellular agents, both known and unknown, can also regulate intracellular signaling. A cell must incorporate all of the signaling events and all of the regulatory actions to produce an integrated response to external stimuli.

## VI. SUMMARY

Many factors contribute to generation of a specific cellular response to external stimuli. Multiple receptors or receptor subtypes can be activated simultaneously by one or more stimuli. Activated receptors then positively couple to one or more G-proteins, creating two potential signaling entities, $G_\alpha$-GTP and $G_{\beta\gamma}$. There are several types of G-proteins, each varying in functional characteristics. Activation of multiple G-proteins engenders a cascade of signaling events that can be convergent with or independent of each other. Effector molecules that are regulated by G-proteins are also a source of diverse responsiveness. Effector subtypes can vary in responsiveness to specific G-proteins and provoke distinct downstream signaling events. Furthermore, multiple subtypes of proteins that regulate GPCR-mediated signaling (GRK, PKC, RGS proteins, arrestin, etc.) provide another layer of diversity in cell signaling. The receptors, G-proteins, effector molecules, and signaling regulators that are expressed in a particular cell will be determining factors in cellular responsiveness to external stimuli. The overall response will be dependent on the relative amount of expression of each of these factors within a given cell. A specific stimulus in one cell may produce a response that is completely different in another cell, depending on the relative expression levels of receptors, G-proteins, and other signaling intermediates. The integrated signaling systems bestow a cell with specific function.

## Glossary

**adenylyl cyclase** Cellular enzyme that generates increases in intracellular cAMP, leading to activation of protein kinase A, which phosphorylates and regulates a multitude of proteins.

**effector** Any of a number of enzymes or regulatory molecules within a cell that is stimulated or inhibited following receptor activation; responsible for transducing a cellular response to external stimuli.

**$G_\alpha$ and $G_{\beta\gamma}$** Guanyl nucleotide binding protein subunits $\alpha$ and $\beta\gamma$.

**intracellular signaling pathway** The series of chemical and physical reactions within a cell following external stimuli that lead to the overall response of the cell.

**ligand** Agent that activates a receptor, including neurotransmitters, hormones, small molecules, and photons.

**phospholipase C** Cellular enzyme that is activated by specific G-protein subunits; hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, which are second-messenger intracellular signaling molecules.

## See Also the Following Articles

**GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Receptor–Receptor Interactions**

## Further Reading

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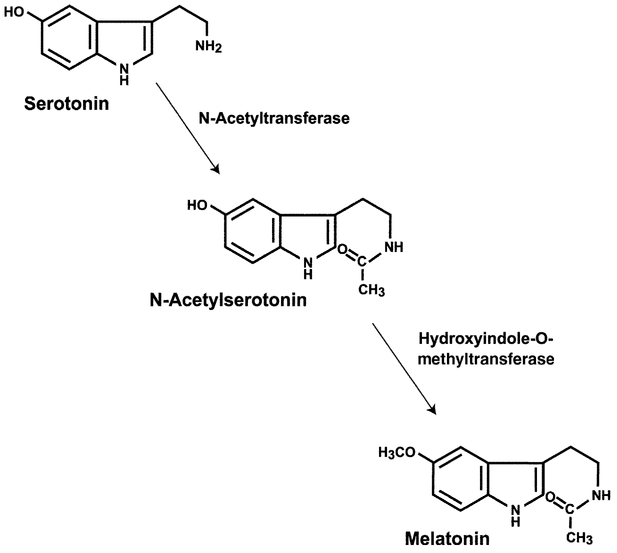

FIGURE 1 Synthesis of melatonin from serotonin.

photoreceptors are involved in entraining the endogenous circadian pacemaker and in regulating melatonin synthesis and secretion.

In mammals, the site of the ECP, or "biological clock," is the SCN of the hypothalamus. The SCN contains about 10,000 neurons and is a paired structure located in the anterobasal hypothalamus, just above the optic chiasm. The SCN receives external photic stimuli from the environment, maintaining entrainment to the 24-h day/night cycle. Photoreceptors in the retina send impulses to the SCN via a specific neural pathway, the retinohypothalamic tract, which is separate from the pathway that mediates vision. Bilateral enucleation probably abolishes all effects of light on circadian rhythms.

Communication between the pineal gland and the SCN is bidirectional. The pineal gland communicates with the SCN via melatonin, which is secreted into the CSF and into the bloodstream. Melatonin binds to specific SCN receptors, which mediate the phase-shifting effects of melatonin on circadian rhythms. The SCN restricts the synthesis and secretion of melatonin to an interval of approximately 12 h.

A polysynaptic neural pathway connects the SCN to the pineal gland (Fig. 2). From the SCN, impulses are transmitted to the paraventricular nucleus (PVN) of the hypothalamus and then to the intermediolateral column of the upper thoracic spinal cord. Preganglionic sympathetic neurons extend from the intermediolateral column to the superior cervical ganglion, adjacent to the internal carotid artery in the neck. Postganglionic sympathetic neurons then innervate the pineal gland. Norepinephrine is the neurotransmitter released from the postganglionic sympathetic neurons terminating on pinealocytes.

Pinealocytes possess both β1- and α1-adrenergic receptors. Norepinephrine stimulates the β1-receptors, leading to activation of *N*-acetyltransferase, the rate-limiting enzyme in melatonin synthesis. This action is potentiated by activation of α1-adrenergic receptors. The nerve endings of the sympathetic neurons terminating in the pineal gland have adrenergic receptors as well. These are α2-adrenergic autoreceptors, and their activation leads to a decrease in melatonin production. Activation of the sympathetic neurons terminating in the pineal gland is tightly controlled by the SCN. Nonspecific sympathetic stimuli, collectively known as the "fight or flight" response, do not activate the pineal gland. This may be partly due to active catecholamine reuptake present in the postganglionic sympathetic neurons innervating the pineal gland.

During the daily dark period, neural impulses from the PVN are transmitted to the pineal gland, where they stimulate melatonin synthesis and secretion. During the day, the SCN sends impulses that decrease the otherwise "on" signal of the PVN. When not inhibited by the SCN, the PVN stimulates

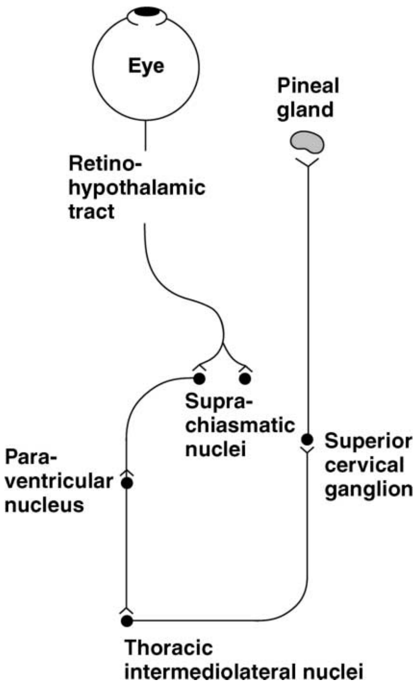
Eye
Pineal gland
Retino-hypothalamic tract
Supra-chiasmatic nuclei
Para-ventricular nucleus
Superior cervical ganglion
Thoracic intermediolateral nuclei

FIGURE 2 Neuroanatomy of the circadian system, depictjing neuroanatomic regulation of mammalian melatonin production.

melatonin production and secretion. As expected, drugs that affect sympathetic activation can alter melatonin secretion. Beta-blocking agents, which interfere with β1-adrenergic stimulation in the pineal gland, cause a reduction of melatonin production. Drugs that stimulate α2-receptors, such as clonidine, will decrease melatonin output, whereas drugs that block these receptors, such as yohimbine, increase melatonin production. Tricyclic antidepressants, which interfere with norepinephrine reuptake, increase melatonin production. Only extreme physical exercise, such as high-altitude marathon races, will lead to increased melatonin production. Drugs that affect melatonin production generally do so consistently throughout the night, so that they affect both the first and the second parts of the profile equally. This is important, because changes in the shape of the melatonin profile can, at least theoretically, lead to phase shifts of the ECP.

Even low levels of light exposure during the dark period can have an acute suppressant effect on melatonin production. This effect can occur only during the dark period, because melatonin secretion is already stopped by the SCN during the daytime (photoperiod). In almost all species studied, including humans, there is no corresponding stimulatory effect of darkness during the day; that is, dark exposure during the daytime does not lead to an acute increase in melatonin production. Thus, melatonin secretion is restricted to about 12 h during the night. The acute suppressant effect of light is critical for endogenous melatonin to augment entrainment of the ECP by the light/dark cycle (Fig. 3).

## IV. MELATONIN AS A PHASE MARKER

The phase of the ECP is best assessed by monitoring melatonin levels. Melatonin levels are typically low during the day and generally begin to rise about 14 h after a person's habitual waketime. In normal individuals, the time of the melatonin onset (MO) is very stable from day to day and can be used as a

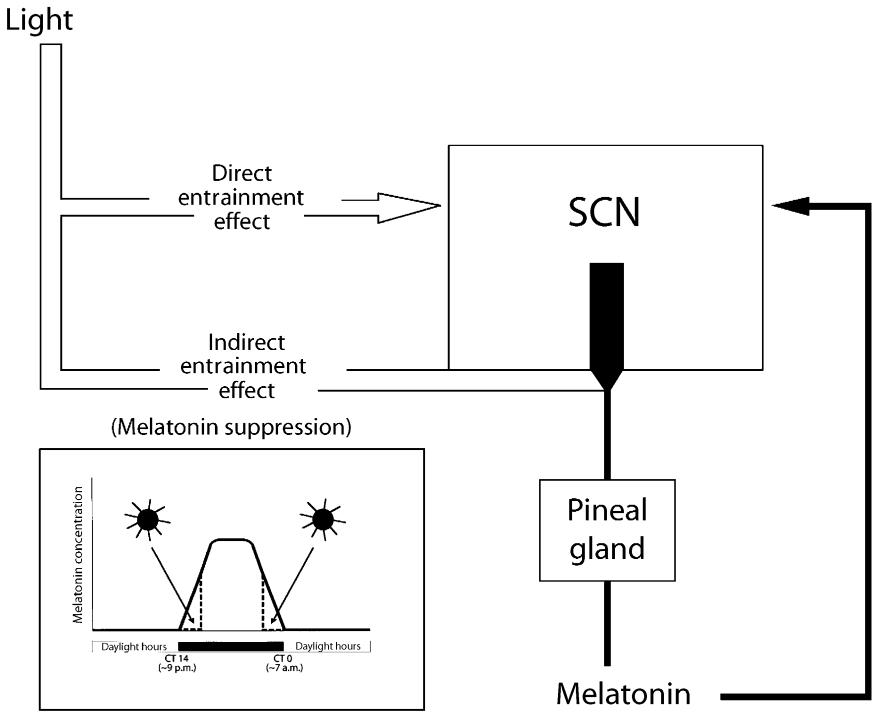

**FIGURE 3** Schematic diagram of some of the relationships between nighttime melatonin production by the pineal gland, the light/dark cycle, and an endogenous circadian pacemaker located in the suprachiasmatic nucleus (SCN). Acting on the SCN as described by the melatonin phase response curve (see Fig. 4) at any given time of the day or night, melatonin causes phase shifts opposite to those that light would cause (indicated by the opposing arrows). However, the suppressant effect of light pares the margins of the nighttime melatonin profile (vertical tapered arrow and inset) and reduces endogenous melatonin's stimulation of the melatonin phase response curve at the day/night transitions. This second (indirect) pathway for entrainment by light is particularly significant during shifts of the light/dark cycle. For example, morning light directly advances the endogenous circadian pacemaker and—by suppressing endogenous melatonin production in the morning—removes morning melatonin levels that would otherwise cause a phase delay.

marker to evaluate a person's circadian phase. Researchers often empirically use the time at which plasma melatonin levels reach 10 pg/ml as the MO, although other operational definitions can be used as well. Because sampling for melatonin is usually done in sighted people under conditions of dim light (less than 10–30 lux) to avoid the suppressant effects of light on melatonin secretion, the MO under these conditions is often called the dim light melatonin onset (DLMO). A person whose DLMO is occurring 14 h after waketime (2 h before bedtime) is said to be in normal phase. By frequently sampling overnight melatonin levels, it is also possible to map other points on the melatonin secretion curve. The time when melatonin levels start to fall (when melatonin synthesis stops) is called the synthesis offset (SynOff), and the time when melatonin levels decrease below 10 pg/ml is called the dim light melatonin offset (DLMOff). These phase markers could also be used to monitor a person's circadian rhythms, although the DLMOff may not be as reliable, because it is significantly influenced by the amplitude of melatonin secretion, which can vary markedly among individuals. Also, measuring the DLMOff, and particularly the SynOff, requires waking subjects in the middle of the night, whereas the DLMO can usually be measured prior to sleep onset. In addition to measuring melatonin in plasma, melatonin can also be measured in saliva. Levels in saliva are generally about one-third of those in plasma. Saliva collection is less invasive than blood collection and can easily be done at home.

## V. EFFECTS OF MELATONIN ON CIRCADIAN RHYTHMS

Animal studies show that melatonin is able to induce circadian rhythm phase shifts at both behavioral and cellular levels. More than a decade ago, it was demonstrated in human subjects that melatonin administered during the late afternoon or early evening advanced the DLMO, whereas melatonin given in the morning delayed it (Fig. 4). These results allowed for the description of a melatonin phase response curve (PRC), which makes possible accurate predictions of the effect of exogenously administered melatonin based on the time of administration.

The melatonin PRC reveals that there is an approximately 12-h interval during which exogenous melatonin will advance the endogenous pacemaker and a 12-h interval during which melatonin will delay it. Not surprisingly, the melatonin PRC is about 12 h out of phase with the light PRC. This would be expected because melatonin is thought to act as a chemical signal for darkness. The melatonin PRC is most helpful when it is expressed in circadian time (CT; DLMO = CT 14) rather than clock time. This allows the melatonin PRC to be used effectively in people whose circadian rhythms are out of phase with their environment (for example, immediately after a transmeridian flight). The advance zone of the melatonin PRC is generally from CT 6 to CT 18 and the delay zone is from CT 18 to CT 6. This information can be used therapeutically in a number of clinical situations. Figure 5 illustrates the fundamental ways in which bright light and melatonin should be scheduled according to clock times to correct circadian phase disorders.

## VI. ABNORMALITIES IN CIRCADIAN RHYTHMS

### A. Blindness

The circadian rhythms of blind people are often abnormal. Many blind people have circadian rhythms that are not entrained to the 24-h day/night cycle with a period longer (or rarely shorter) than 24 h; that is, they have "free-running" circadian rhythms. In these blind free-runners, the circadian rhythms governed by the SCN, such as melatonin secretion, core body temperature, and sleep propensity, shift later and later each day. Because of their constantly drifting circadian rhythms, BFRs spend much of their lives "out of phase." When they are out of phase, they often experience adverse effects, including nighttime insomnia and daytime somnolence. Only BFRs who constantly change their sleep and wake hours in accordance with their drifting ECP will have normal sleep. Another subgroup of blind people appears to be entrained but at an abnormal phase: the MO may stably occur at any time of the day or night, and thus they are always out of phase with their environment. These people may have chronic problems with insomnia and daytime somnolence. Still other blind people may have normally and stably phased circadian rhythms. Melatonin can be used successfully to treat circadian rhythm disorders in blind people. It is typically given in the evening or just before bedtime at doses of 0.5 mg (or possibly less). It is not yet known if lower doses of melatonin are needed to maintain entrained rhythms compared to doses needed for correction of abnormal rhythms.

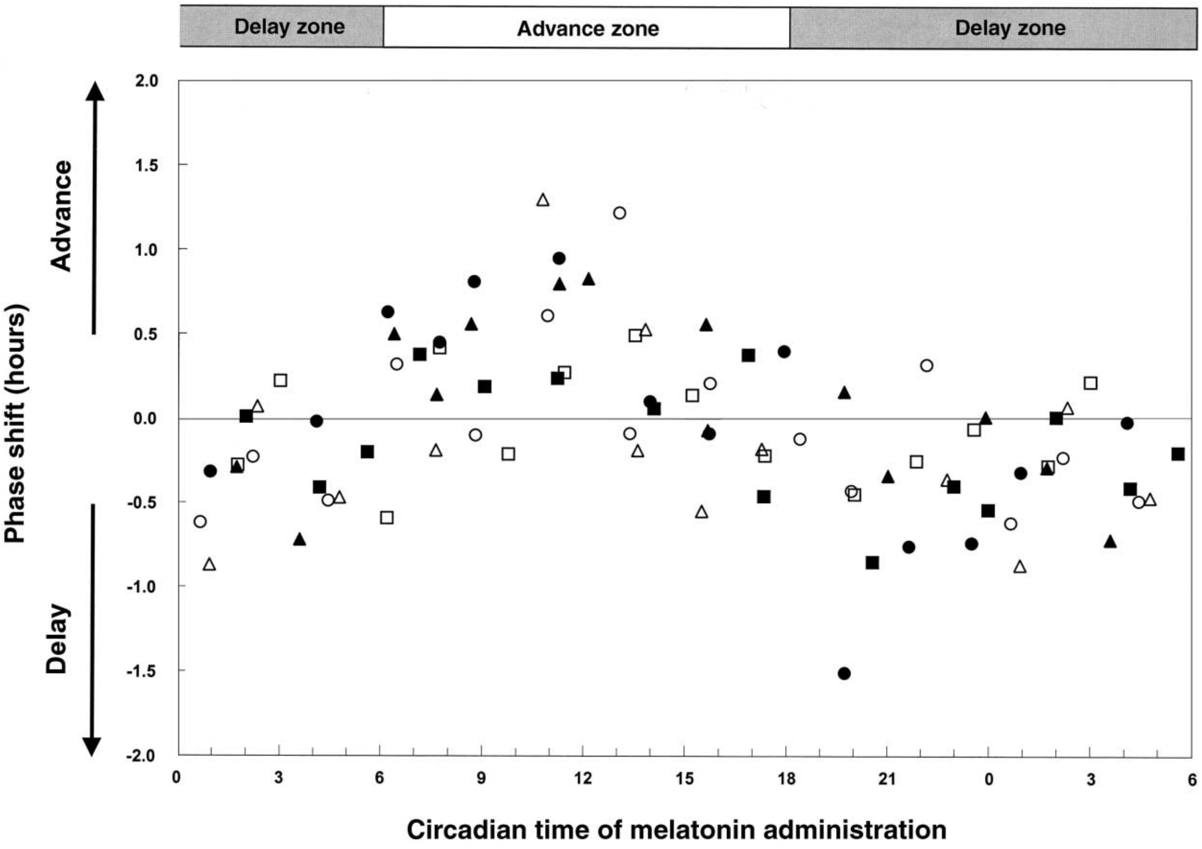

**FIGURE 4** Individual phase response curves from six subjects (five females and one male; the single male is represented by the filled circle) given melatonin (0.5 mg) for four consecutive days on 12 occasions. Phase advances and delays are plotted against circadian time (CT) of administration (CT 14 = baseline dim light melatonin onset). Exogenous melatonin causes phase advances when given between CT 6 and CT 18, and it causes phase delays when given between CT 18 and CT 6.

## B. Advanced and Delayed Sleep Phase Syndromes

Sighted people can also have endogenous circadian rhythms that are out of phase with their environment and with their desired sleep time. People who suffer from advanced sleep phase syndrome (ASPS) have sleep times that are advanced in relation to normal clock time and in relation to their desired sleep time. These people feel excessively sleepy in the evening, fall asleep early and suffer from early morning awakening. This condition develops more commonly in the elderly. Delayed sleep phase syndrome (DSPS) is just the opposite. People with this syndrome have sleep delayed in relation to clock time and in relation to their desired sleep time. They are unable to fall asleep until late in the night, typically after 1 AM, and they have difficulty awakening in the morning. This condition is more prevalent in the young. Both bright light and melatonin have been effectively used to treat circadian phase disorders in sighted people (see Fig. 5).

## C. Jet Lag

People who travel across multiple time zones are usually affected by jet lag, because their endogenous circadian rhythms are out of phase for the first few days in the new time zone. To hasten adaptation to the new time zone, sunlight exposure is scheduled to be obtained at specified times and, perhaps more importantly, to be avoided at specified times, for the first few days after arrival. For example, after traveling six or more time zones to the east, sunlight should be avoided in the morning and obtained in the afternoon for 1–2 days, after which it should be obtained in the morning for a day or two. Low doses of melatonin can be taken in the afternoon for a day

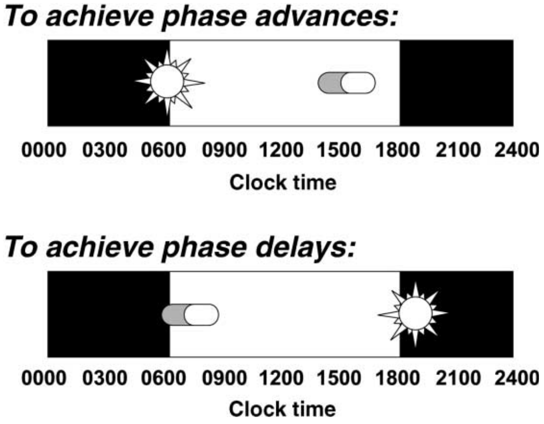

**FIGURE 5** Phase-shifting effects of light and exogenous melatonin. In order to cause a phase advance, light should be scheduled in the morning and melatonin should be administered in the afternoon. In order to cause a phase delay, light should be scheduled in the evening and melatonin should be administered in the morning.

or two before travel (see Fig. 5) and also for the first few days after arrival, with the timing of administration then adjusted according to local time.

## D. Shift Work

Shift workers can suffer extraordinary physical and psychological consequences from constantly being out of phase. The effects of shift work may be especially difficult as people age. People who permanently work day or evening shifts do not typically have chronobiological problems. The most significant problems are seen in those who do at least part of their work at night. Almost all night shift workers rotate shifts, if only between work days and days off. In a typical week they may work five nights and then try to normalize their schedule to daytime hours on days off in order to maximize time with family and friends. Other shift workers constantly rotate actual work schedules. They may, for example, do a few day shifts, a few evening shifts, and then a few night shifts within a week or so. With these schedule disturbances, they may never fully synchronize with their environment, and consequently they often suffer from insomnia during their desired sleep hours and somnolence while at work. Shift workers can also have impaired productivity and work performance. For all of these reasons and because shift work is so prevalent, this topic has been the subject of intense investigation. Both bright light and melatonin treatments can be used to help the circadian rhythm disturbances of shift workers (see Fig. 5). Avoiding bright light at certain times is also helpful. However, the practical use of these phase-resetting agents in shift workers is complicated by their constantly changing schedules and their individual preferences.

## E. Winter Depression

Seasonal affective disorder (SAD) is another condition that appears to stem from disturbed endogenous circadian rhythms. SAD is a condition characterized by recurrent bouts of depression that occur predictably every fall or winter as the days become shorter, with spontaneous improvement every spring or summer as the days lengthen. All patients with SAD have depression, but they also commonly have increased sleep time, hyperphagia (especially carbohydrate craving), weight gain, low energy, low motivation, and social withdrawal.

Bright light exposure is the treatment of choice for SAD. A typical treatment will start with 1–2 h of light treatment (10,000 lux) every morning; morning light is thought to be antidepressant because it causes a corrective phase advance. After patients respond, they can then decrease the duration of light exposure to less than 30 min per day. A subgroup of atypical subjects responds best to bright light exposure in the evening.

Studies into the effects of melatonin on the treatment of SAD have lagged behind studies of light therapy and, so far, very few have been published. However, according to the phase shift hypothesis of SAD, low doses of melatonin in the afternoon and evening should be therapeutic in the typical SAD patient and low doses in the morning should be therapeutic in the atypical SAD patient. In the phase-delayed type of SAD patient, the optimal amount of phase advance seems to be about 1.5 h. Combining light and melatonin may make the most sense for some people, because adding melatonin to light treatment will allow a shorter light treatment duration, which is often desirable. Using melatonin alone without light treatment is another option; however, this is not always feasible, because the doses of melatonin required to cause the necessary phase advance cause somnolence in some SAD patients, who seem to be very sensitive to melatonin's soporific effect. This effect can often be eliminated by using very low doses of melatonin (e.g., 0.075–0.125 mg) given every few hours in the afternoon and evening; however, even these doses will be sedating in some people. It should be emphasized that to achieve the full therapeutic effect of the phase advance caused by morning light or evening melatonin, it is important that a person not advance their sleep/wake cycle. That is, the ECP needs to be advanced in relation to the sleep/wake cycle.

## VII. SAFETY OF MELATONIN

In addition to phase-shifting effects, melatonin can have soporific effects, particularly at high doses. About 30% of people will experience sleepiness as a side effect of melatonin administration. If melatonin is taken incorrectly, it can potentially cause adverse consequences via its effects on sleep and circadian rhythms. For example, someone taking melatonin for jet lag could potentially retard the normalization of circadian rhythms by taking melatonin at the wrong time. Another example would be the development of somnolence when melatonin is taken during the day. Besides predictable consequences such as these, there have been no reports of serious irreversible adverse effects due unequivocally to melatonin. In the United States, where melatonin is easily available without a prescription, millions have used it over the past few years. However, people who choose to take melatonin should do so with the understanding that not much is known about the possible consequences, if any, of its long-term use.

## VIII. SUMMARY

Progress has been made during the past 20 years elucidating the physiology of the pineal gland and its primary secretory product, melatonin. Endogenous melatonin in humans probably augments entrainment of the ECP by the light/dark cycle. The production and secretion of melatonin are regulated by the ECP in the SCN of the hypothalamus. Both light exposure and exogenous melatonin can influence the ECP and, consequently, melatonin secretion.

Normally phased endogenous rhythms are thought to be important in maintaining good physical and mental health. Disturbances in the ECP can lead to sleep and mood disorders. Both melatonin and light can be used effectively to treat these. Ongoing work in this area will help refine the current therapeutic approaches and will find improved ways of manipulating body rhythms to help maintain optimum health.

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## Glossary

**advanced sleep phase syndrome** A disturbance in which sleep occurs intractably early.

**blind free-runners** Totally blind individuals whose circadian rhythms drift (usually later) each day.

**delayed sleep phase syndrome** A disturbance in which sleep occurs intractably late.

**dim light melatonin onset** The time when melatonin levels rise under dim light conditions; a useful marker for the endogenous circadian pacemaker phase.

**endogenous circadian pacemaker** Regulates most, if not all, circadian rhythms; located in the suprachiasmic nucleus.

**phase response curve** A mathematical plot that describes how light and melatonin affect endogenous circadian pacemaker phase.

**suprachiasmatic nucleus** The collection of neurons in the hypothalamus where the endogenous circadian pacemaker is located.

## See Also the Following Articles

**Endocrine Rhythms: Generation, Regulation, and Integration • Melatonin Receptor Signaling**

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# Melatonin Receptor Signaling

Margarita L. Dubocovich and
Monica I. Masana
*Northwestern University Feinberg School of Medicine*

I. MELATONIN PHYSIOLOGY
II. MELATONIN RECEPTORS
III. MELATONIN RECEPTORS AS THERAPEUTIC TARGETS

**Melatonin (5-methoxy-*N*-acetyltryptamine), one of the main hormonal outputs of the circadian system, relays photoperiodic information by providing dark timing cues to target tissues. Melatonin receptors receive and transduce the melatonin message to influence daily and seasonal rhythms of physiology and behavior in vertebrates.**

## I. MELATONIN PHYSIOLOGY

Synthesis and release of the hormone melatonin in the vertebrate retina and pineal gland follow a circadian rhythm that peaks during the dark period of the daily light/dark cycle (the photoperiod) (Fig. 1). In the absence of light cues, the endogenous circadian rhythm of pineal melatonin synthesis and release is driven by circadian pacemaker cells (the "clock") located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which sends the signal to the pineal gland via a multisynaptic pathway through the superior cervical ganglia. In the retina, a local clock mechanism governing melatonin synthesis and release drives the circadian melatonin rhythm. In both the retina and the SCN, the clock is entrained to a 24-h period by environmental changes in the light/dark cycle; the changes in light are sensed by the retina directly and conveyed to the SCN via the retinohypothalamic tract.

Melatonin is synthesized from serotonin through the sequential action of two enzymes: the rate-limiting enzyme arylalkylamine *N*-acetyltransferase (AANAT), which acetylates serotonin to yield *N*-acetylserotonin (NAS), and the enzyme hydroxyindole-*O*-methyltransferase (HIOMT), which transfers a methyl group from *S*-adenosylmethionine to the 5-hydroxy group of *N*-acetylserotonin. The daily fluctuations in AANAT and HIOMT activity are regulated directly by the activation of α- and β-adrenergic receptors in the pineal gland, followed by transcriptional and posttranscriptional mechanisms leading to an increase in activity of the rate-limiting enzyme AANAT, a decrease in serotonin content, and an increase in levels of NAS and melatonin (MLT). Melatonin synthesized in the retinal photoreceptors is secreted locally and is known to inhibit dopamine release. Melatonin and dopamine can act as signals for the dark and light, respectively, mutually inhibiting each other and functioning as major players in dark/light adaptation. Although AANAT and HIOMT have been localized primarily to the pineal and retinal tissues, these enzymes are also present at lower levels in other brain and peripheral tissues. At present, the functional relevance of extrapineal and retinal melatonin is unknown.

## II. MELATONIN RECEPTORS

### A. Molecular Structure and Pharmacology

The first described biological activity of melatonin can be traced back to 1917 when McCord and Allen discovered that extracts of bovine pineal glands caused blanching of *Rana pipiens* tadpole skin. Aaron Lerner used this bioassay to isolate melatonin from pineal extracts, which led to the elucidation of its chemical structure in 1958. The property of melatonin that allows it to aggregate pigment granules (melanosomes) of amphibian dermal melanophores was used to demonstrate the existence of melatonin receptors and to establish in cultured *Xenopus laevis* melanophores that activation of melatonin receptors inhibited cAMP formation through coupling to a pertussis toxin-sensitive G-protein.

Critical to the discovery of mammalian melatonin receptors was the characterization of the radioligands [$^{3}$H]melatonin and 2-[$^{125}$I]iodomelatonin, Thus, 7S NGF appears to be a storage form of NGF and is apparently restricted to this tissue (and perhaps some other rodent submandibular glands and snake venoms).

As was first determined with the 2.5S preparation, βNGF is composed of two identical polypeptide chains, associated noncovalently, and thus the 7S complex is a symmetrical heterohexamer. Sequence determination of 2.5S NGF, one of the earliest proteins to be so analyzed, indicated that the longest chain contains 118 amino acids ($M_w = 14259$) and each subunit has three intrachain disulfide bonds, which are paired 1–4, 2–5, and 3–6. Their unique conformational arrangement, subsequently determined from X-ray crystallographic studies, is a hallmark of the NGF super family (see later). These studies also showed that the N-terminal octapeptide is not present in approximately 50% of the chains and that somewhat lesser amounts of each subunit are missing the C-terminal arginine residue. βNGF isolated from purified 7S NGF has considerably less of both modifications. The importance of these regions to receptor binding, and hence activity, has been extensively studied. Basically, the N-terminal region including part of the octapeptide sequence is important for interaction with TrkA (one of the two NGF receptors; see later) but the C-terminal arginine does not contribute to interactions with either receptor. The 7S complex is maximally stable at neutral pH and dissociates above or below pH 7 (primarily due to the dissociation of one or the other of the kallikrein subunits). In contrast, the polypeptides making up the β-subunit are very tightly associated and require denaturing conditions to cause chain separation. Thus, the βNGF ligand always functions as a dimer.

As expected for proteins of this type, NGF is synthesized as a larger precursor molecule, and the sequence of this entity, termed prepro-βNGF, has been determined from the cDNA. Due to the use of an alternative initiation codon, two precursors are formed but both yield the same mature protein. The sequence of the human precursor is shown in Fig. 1, with an indication of the alternative initiation site. Similar sequence information is now available for NGF from a variety of other species, including the mouse, cow, chicken, guinea pig, and cobra. The prepro-βNGF gene is located on human chromosome 1 at p22 and on mouse chromosome 3.

Comparison of the βNGF sequence against the limited database available at the time of initial studies of NGF suggested a distant but significant relationship with proinsulin; this was strongly supported by similarities in functional responses of the respective target cells. This correctly presaged that NGF was a part of what has turned out to be a greatly expanded field of hormones and hormone-like substances that primarily function in autocrine and paracrine fashions. However, eventual structural data disproved the NGF-insulin relationship; rather, NGF is a member of another family of homologous factors collectively termed neurotrophins. In mammals there are three other known members: brain-derived neurotrophic factor (BNDF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4). NT4 is sometimes referred to NT4/5 due to some early confusion about the identity of these two neurotrophins. Two additional neurotrophins, NT6 and NT7, have been identified in lower species but do not have mammalian counterparts. Pairwise comparisons of these sequences (within a single species) show about 50% identity (Fig. 2). There is some variability in length that is manifested at both termini and in short insertions in the BNDF and NT4 sequences. However, the half-cystines are conserved, as is the overall structure of each factor (all four native neurotrophins and two artificial heterodimers have been solved by X-ray crystallographic analysis). Although there are distinct residues involved, the receptor-binding regions are also located in similar places (with the exception of the N-terminal region of NGF).

## III. STRUCTURE

Although crystals of βNGF were obtained in 1975, the three-dimensional structure was not obtained until 1991. When it was determined, it described a unique and somewhat unexpected fold (Fig. 3). Each subunit has an elongated structure as opposed to a strictly globular one and there are four extended polypeptide segments that form two pairs of twisted antiparallel β-sheets, giving rise to the extensive, highly hydrophobic interface ($\sim 3000\ \text{Å}^2$) between the two protomers making up the dimer. A “cystine knot” composed of all three intrachain links characterizes one end of this “bundle.” Two of these form a circular structure and the third passes through it, providing a quite distinct signature motif. At the opposite end, the β-sheets terminate in three hairpin loops, whereas a fourth, looser loop caps the cystine knot end. The two subunits are oriented in a parallel fashion and a not inaccurate description suggests the overall structure is like a bouquet of flowers.

In addition to the other neurotrophins, which have very similar conformations, there are three other families of hormones/growth factors that share

```
GAG AGC GCT GGG AGC CGG AGG GGA GCG CAG CGA GTT TTG GCC AGT GGT CGT
GCA GTC CAA GGG GCT GGA TGG CAT GCT GGA CCC AAG CTC AGC TCA GCG TCC
GGA CCC AAT AAC AGT TTT ACC AAG GGA GCA GCT TTC TAT CCT GGC CAC ACT

                   -121                                        -110
                     M   S   M   L   F   Y   T   L   I   T   A   F
GAG GTG CAT AGC GTA ATG TCC ATG TTG TTC TAC ACT CTG ATC ACA GCT TTT

                       -103        -100
 L   I   G   I   Q   A   E   P   H   S   E   S   N   V   P   A   G
CTG ATC GGC ATA CAG GCG GAA CCA CAC TCA GAG AGC AAT GTC CCT GCA GGA

        -90                                     -80
 H   T   I   P   Q   V   H   W   T   K   L   Q   H   S   L   D   T
CAC ACC ATC CCC CAA GTC CAC TGG ACT AAA CTT CAG CAT TCC CTT GAC ACT

                    -70                                     -60
 A   L   R   R   A   R   S   A   P   A   A   A   I   A   A   R   V
GCC CTT CGC AGA GCC CGC AGC GCC CCG GCA GCG GCG ATA GCT GCA CGC GTG

                                -50
 A   G   Q   T   R   N   I   T   V   D   P   R   L   F   K   K   R
GCG GGG CAG ACC CGC AAC ATT ACT GTG GAC CCC AGG CTG TTT AAA AAG CGG

    -40                                     -30
 R   L   R   S   P   R   V   L   F   S   T   Q   P   P   R   E   A
CGA CTC CGT TCA CCC CGT GTG CTG TTT AGC ACC CAG CCT CCC CGT GAA GCT

                -20                                     -10
 A   D   T   Q   D   L   D   F   E   V   G   G   A   A   P   F   N
GCA GAC ACT CAG GAT CTG GAC TTC GAG GTC GGT GGT GCT GCC CCC TTC AAC

                             1                                   10
 R   T   H   R   S   K   R   S   S   S   H   P   I   F   H   R   G
AGG ACT CAC AGG AGC AAG CGG TCA TCA TCC CAT CCC ATC TTC CAC AGG GGC

                                     20
 E   F   S   V   C   D   S   V   S   V   W   V   G   D   K   T   T
GAA TTC TCG GTG TGT GAC AGT GTC AGC GTG TGG GTT GGG GAT AAG ACC ACC

         30                                      40
 A   T   D   I   K   G   K   E   V   M   V   L   G   E   V   N   I
GCC ACA GAC ATC AAG GGC AAG GAG GTG ATG GTG TTG GGA GAG GTG AAC ATT

                     50                                      60
 N   N   S   V   F   K   Q   Y   F   F   E   T   K   C   R   D   P
AAC AAC AGT GTA TTC AAA CAG TAC TTT TTT GAG ACC AAG TGC CGG GAC CCA

                                 70
 N   P   V   D   S   G   C   R   G   I   D   S   K   H   W   N   S
AAT CCC GTT GAC AGC GGG TGC CGG GGC ATT GAC TCA AAG CAC TGG AAC TCA

     80                                      90
 Y   C   T   T   T   H   T   F   V   K   A   L   T   M   D   G   K
TAT TGT ACC ACG ACT CAC ACC TTT GTC AAG GCG CTG ACC ATG GAT GGC AAG

                100                                     110
 Q   A   A   W   R   F   I   R   I   D   T   A   C   V   C   V   L
CAG GCT GCC TGG CGG TTT ATC CGG ATA GAT ACG GCC TGT GTG TGT GTG CTC

                            120
 S   R   K   A   V   R   R   A  OPA
AGC AGG AAG GCT GTG AGA AGA GCC TGA
CCT GCC GAC ACG CTC CCT CCC CCT GCC CCT TCT ACA CTC TCC TGG GCC CCT CCC TAC CTC
AAC CTG TAA ATT ATT TTA AAT TAT AAG GAC TGC ATG GTA ATT TAT AGT TTA TAC AGT TTT
AAA GAA TCA TTA TTT ATT AAA TTT TTG GAA GC
```

**FIGURE 1** The nucleic and amino acid sequences of human prepro-βNGF. The amino acid positions are numbered beginning with the first amino-terminal residue of the mature βNGF molecule. The signal peptide consists of residues −121 to −104 whereas the propeptide is composed of residues −103 to −1. The initiation site is at position −121, with an alternative site at position −119, both of which are indicated by bold text.

the cystine knot/four-strand β-sheet core structure and thus form a superfamily with the neurotrophins. Although each family contains several members, they are represented by platelet-derived growth factor, transforming growth factor-β and chorionic gonadotropin. However, substantial differences, including protomer orientation and the presence of interchain disulfide bonds, distinguish the families. Beyond the conservation of the participating half-cystines, there is no recognizable sequence relatedness between members of the superfamily. This motif has also been found in some other nonhormonal molecules.

The crystal structure of the 7S complex defines the interactions between the kallikrein-like subunits

```
          1        10         20           30         40
NGF  -----SSSHPIFHRG EFSVCDSVSV WVG--DKTTATD IKGKEVMVLG
BDNF HSDPARRHSDPARRG ELSVCDSISE WVTAADKKTAVD MSGGTVTVLE
NT3  ------YAEHKSHRG EYSVCDSESL WVT--DKSSAID IRGHQVTVLG
NT4  ---GVSETAPASRRG ELAVCDAVSG WVT--DRRTAVD LRGREVEVLG
                   * * ***** *  **         *   *  * **

               50         60                70         80
NGF  EVNI-NNSVFK QYFFETKCRD PNPVDS-------GCRG IDSKHWNSYC
BDNF KVPV-SKGQLK QYFYETKCNP MGYTKE-------GCRG IDKRHWNSQC
NT3  EIKT-GNSPVK QYFYETRCKE ARPVKN-------GCRG IDDKHWNSQC
NT4  EVPAAGGSPLR QYFFETRCKA DNAEEGGPGAGGGGCRG VDRRHWVSEC
                 *** ** *                ****  *  ** * *

              90          100        110
NGF  TTTHTFVKAL TMDGKQ-AAWR FIRIDTACVC VLSRKAVRRG
BDNF RTTQSYVRAL TMDSKKRIGWR FIRIDTSCVC TLTIKRGR--
NT3  KTSQTYVRAL TSENNKLVGWR WIRIDTSCVC ALSRKIGRT-
NT4  KAKQSYVRAL TADAQGRVGWR WIRIDTACVC TLLSRTGRA-
           * **          **  ***** ***  *
```

**FIGURE 2** Alignment of the amino acid sequences (single-letter code) of human nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). Numbers correspond to positions in the NGF sequence. Residues important for p75 receptor binding are indicated by boldface type and residues of NGF known to interact with the TrkA receptor are shaded. Conserved residues are marked with an asterisk. Dashes represent gaps introduced for alignment purposes. Note that, because of differences in the lengths of the N-termini of the different neurotrophins, homologous positions in different molecules do not have equivalent numbering.

and those of βNGF. The αNGF subunits, which show a "locked zymogen" conformation consistent with their unprocessed state, bind to the core pleated sheets and the γNGF molecules interact mainly with the C-terminal region of the βNGF subunit. This latter interaction includes the C-terminal arginine residue of βNGF, which is bound in the active site of the γNGF. These interfaces are in the range of 2000–2500 Å$^2$. The two γ-subunits also have a major interaction (~2600 Å$^2$) but there is no contact between the α-subunits. The zinc ions occur at the interface (~1600 Å$^2$) of the α/γ-subunits and each contributes two metal ligands. Although α/β and β/γ dimers are stable in solution under certain conditions, α/γ dimers are not. Both kallikreins are monomeric in solution.

## IV. BIOSYNTHESIS/EXPRESSION

The signal peptide of human prepro-βNGF is 18 residues in length and the pro sequence contains 103 residues, yielding a full-length zymogen of ~30 kDa. *In vivo* and *in vitro* labeling experiments had initially detected a 22-kDa precursor that could be converted

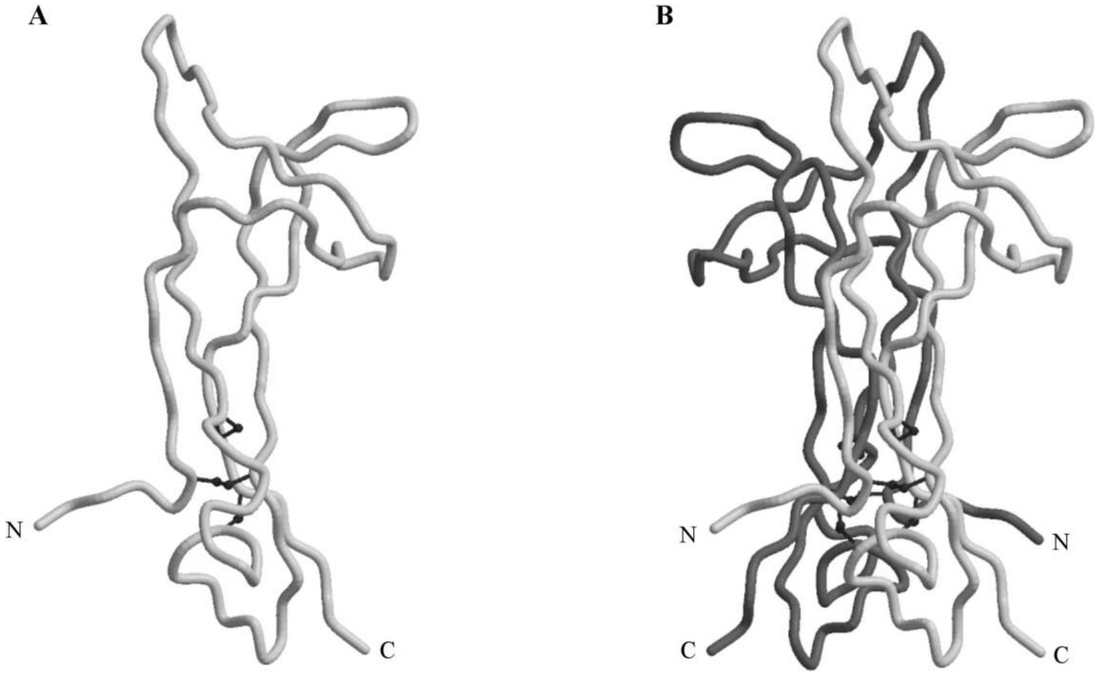

**FIGURE 3** Three-dimensional representation of nerve growth factor. (A) The unique tertiary fold for the individual NGF subunit, consisting of the "cystine knot" (indicated by the dark ball-and-stick representation). (B) The NGF dimer with one subunit represented in a darker color for clarity.

by the γ-subunit, among other proteases, to the mature form, and this species was consistent with an intermediate arising from cleavage at a pair of arginine residues. However, the high concentrations required and the lack of specificity argued against γNGF as the pro-βNGF-processing enzyme *in vivo*, and recent studies have identified furin, which is involved in other similar processing events, to be the causative entity. There is also a C-terminal extension, usually of two residues, that is removed in the mouse submandibular protein, probably through the agency of the γ NGF, but this is not apparently the case with the NGF of other species. A possible role for pro-NGF as a preferred ligand for the p75 receptor (see later) has been recently postulated.

NGF is expressed in both glia and neurons in the peripheral and central nervous systems (PNS and CNS), both during development and in the adult, as well as in several nonneuronal tissues. In keeping with the initial identification, NGF is expressed by non-neural target cells of afferent sympathetic and nociceptive sensory neurons and provides support for them via retrograde transport, the basis of the "neurotrophic hypothesis" (see later). The expression of NGF is also up-regulated in some of these tissues in response to injury and inflammation. This is particularly true of Schwann cells, in which, in the adult, it is synthesized only at very low levels, but following injury it is substantially induced by cytokines. In the CNS, the NGF gene is expressed in neurons, the highest levels of mRNA being found in the hippocampus and the cerebral cortex. NGF is also synthesized in astrocytes and microglia and a variety of factors and conditions also affect these levels.

Generally, NGF appears to provide target-derived support for CNS neurons and NGF synthesis by both neurons and support cells is enhanced following injury or other deleterious conditions. However, it should be noted that there have been many identifications of NGF synthesis and expression that are not fully explained. This includes the immune system, in which many types of leukocytes express βNGF, including mast cells, monocytes, T and B lymphocytes, and macrophages. This suggests a major role for NGF in the neural-immune axis, but descriptions of this important physiological system lack detail.

## V. RECEPTORS

βNGF interacts with two cell surface receptors: TrkA is a member of the receptor tyrosine kinase (RTK) family and elicits most of the known classical βNGF biological responses; the other receptor, p75NTR, is a pan neurotrophin receptor that binds all of the neurotrophins with similar affinity. The p75NTR induces a largely different set of responses but can also modulate the binding of βNGF to TrkA. TrkA was identified from a chimeric oncogene that contained a portion of tropomyosin fused to the endodomain of an RTK, thus giving rise to the name tropomyosin-related kinase (Trk). The identification of TrkA followed by several years the isolation and characterization of p75NTR and resolved several conflicting reports regarding the signaling entity responsible for the survival and differentiation activities of NGF.

TrkA is a typical single-pass transmembrane RTK. The extracellular part is glycosylated and contains five domains: (1) a cysteine-rich region, (2) three leucine-rich repeats, (3) a second cysteine-rich region, and (4 and 5) two immunoglobulin (Ig)-like domains. NGF binds to the most proximal Ig-domain (domain 5) and a structure of the NGF-domain 5 complex has been determined. Similar structures have also been determined for TrkB and TrkC, the specific receptors for BDNF and NT3. The importance/role of the other domains in TrkA function is less clear. The TrkA-binding site on βNGF has been deduced from several mutagenesis/chemical modification studies and confirmed by the three-dimensional structure. Basically, it is composed of three regions of NGF; the first two have been termed the "specificity patch" and the "conserved patch." The third is less well defined and involves residues in two of the hairpin loops at the end of the molecule opposite the cystine knot. The specificity patch is made up of residues in the N-terminus and the conserved patch is made from residues in the β-sheet core. The residues of TrkA that contact the specificity patch are in a quite hydrophobic pocket that is considerably more hydrophilic in TrkB and absent altogether in TrkC. This interaction is quite consistent with a number of derivatives with various N-terminal modifications that show decreased affinity for TrkA. The conserved patch is made up of several hydrophobic residues and is actually contributed by side chains from both βNGF protomers. There is great deal of similarity in this region in all the neurotrophins and this presumably reflects the commonality of this binding site in all the Trks. The last binding site occurs on the linker segments that connect domain 5 with the transmembrane segment. The βNGF residues involved are less well defined because this interaction has not been observed in the NGF–domain 5 complex.

The p75NTR is also a transmembrane glycoprotein with a single spanning segment. It has a relatively small endodomain containing a "death domain" motif. It also contains a less well-characterized segment of 29 residues, located near the membrane; this segment has been designated "chopper" and can induce cell death when incorporated into other proteins or when expressed in a membrane-bound form. The role of either of these domains in p75NTR activity is unclear. The ectodomain contains four cystine-rich sequences (each containing three disulfide bonds) that are similar to those found in the TNF receptor. As a result, a plausible model has been proposed (there are no crystallographic data). All four appear to be required for NGF binding, with the second being of most importance. The p75NTR binding site appears to be composed of two groups of residues—one contributed by loop 3 (near the cystine knot) and one by a group of basic residues in loops 1 and 4 at the opposite end of the molecule. The former seems to represent a common site found in the other neurotrophins as well. The latter is more specific to NGF.

There is substantial indirect evidence that p75NTR and TrkA can interact and it has been suggested that the high-affinity binding observed for NGF results from such a complex. However, physical evidence for the formation of a p75NTR-TrkA complex, with or without NGF, is lacking. Model building suggests that it would be possible for both receptors to bind NGF simultaneously, which would place the cystine knot end of NGF away from the membrane (given the orientation of NGF in complex with the TrkA domain 5). Association of p75NTR with this complex requires an orientation that is inconsistent with domain 2 of p75NTR providing a major binding site. For that to be achieved, the cystine knot must be located toward the membrane. Because both receptors can function in the absence of the other, it is possible that NGF binds to p75NTR in one orientation with the isolated receptor but in the opposite way in a ternary complex.

## VI. CELL SIGNALING

Signaling of all receptors is to some degree cell specific. Most of the information available for NGF signaling is derived from studies with PC12 cells, a rat pheochromocytoma line. These cells differentiate into a phenotype quite similar to that of sympathetic neurons on exposure to NGF (among other agents). Although not strictly neuronal cells, they have been extremely valuable in defining the signaling properties of the NGF receptors. As with other RTKs, the ligand-activated form of TrkA is a dimer. It is stabilized by the phosphorylation of two tyrosine residues in the activation loop and has two additional principal sites for downstream signaling. Tyrosine 490 (Y490), in the endodomain juxtamembrane region, is a docking site for Shc and FRS2, which are scaffolds for assembling signaling complexes, leading to the activation of Ras/ERK1/2 and phosphatidylinositol 3-kinase (PI3K) among other moieties. The former is associated with the differentiative responses whereas the latter is related to survival. Tyrosine 785 (Y785) similarly binds phospholipase C-γ leading to its activation as well. There are additional entities that have also been implicated, such as Src and Abl, but the mechanisms leading to their activation are not well understood.

The p75NTR is activated by NGF to produce ceramide by the activation of sphingomyelinase. The death domain may be responsible for this activity. This receptor also binds and activates tumor necrosis factor receptor-associated factors (TRAFs), leading to the production of nuclear factor κ (NF-κB). Interestingly, TrkA also activates this cytoplasmic transcription factor, but in an Shc-dependent fashion. It has also been suggested that p75NTR may function in a ligand-independent manner to produce cell death based on the overexpression of the intracellular domain. At least some of these effects may be mediated through RhoA and actin cytoskeleton rearrangement.

## VII. PHYSIOLOGY

βNGF has a multiplicity of roles in the development and maintenance of the nervous system. It also has less well-defined activities in other tissues. During development, it functions primarily with sympathetic and selected sensory neurons in the PNS and with striatal and cholinergic neurons in the CNS. The PNS activities were the basis for the initial identification by Levi-Montalcini and Hamburger and, indeed, it was the determination that NGF is specifically taken up and retrogradely transported to the perikarya of dependent peripheral neurons the led to the elucidation of the neurotrophic hypothesis. However, NGF, as well as other neurotrophic factors, certainly plays some role in maintaining these neurons before synapses are formed.

The availability of βNGF during development is important in determining the phenotypic rate and numbers of βNGF-responsive sensory neurons in the adult. A combination of βNGF antibody-blocking experiments and gene knockout experiments in mice has identified two populations of neurons that are reduced by as much as 80% in the absence of βNGF (the dorsal root ganglion and trigeminal ganglion). The antibody-blocking experiments also suggest that a subset of sensory neurons is βNGF dependent during development, including the nociceptive neurons. A role for βNGF in sensory neuron regeneration is also indicated by its neurite proliferation activity and up-regulation. In adult animals, sensory neurons do not require βNGF for their continued survival. Sympathetic neurons show even a greater dependency on βNGF. In gene knockout mice (both TrkA −/− and βNGF −/− mice), more than 90% of the sympathetic neurons disappear in the cervical ganglion. These data are supported by βNGF-blocking antibody experiments in which the same phenotype is observed. Unlike the sensory neurons, βNGF is required for the continued survival of the sympathetic neurons in the cervical ganglion of adult animals. In the CNS, there are similar observations, particularly in the gene knockout animals for the basal forebrain cholinergic neurons. However, it is also clear that the dependency of neurons on trophic stimulation is complex and even transient. Many neurons depend on more than one factor, and these allegiances can change with time.

There is clearly less neuronal dependency on NGF in adult animals, in which activities are more focused on synaptic function, synaptic plasticity, and repair. As a rule, target-derived support becomes less important although it does not completely disappear. However, other activities of NGF may become more important in the adult animal. One of these is a critical role for endogenous βNGF in inflammation and pain suggested by the increased levels of βNGF in damaged or inflamed tissue. Cytokines, such as interleukin-1β (IL-1β), typically involved in tissue damage and inflammatory processes, increase βNGF levels both *in vitro* and *in vivo*. Administration of antibodies that block βNGF prevents the heat and the mechanical hyperalgesia that normally follow tissue inflammation, without affecting the inflammation. The basis for this action includes an up-regulation of peptide neurotransmitters expressed by neurons concerned with the detection of potentially painful stimuli (nociceptors). TrkA −/− animals do not have nociceptive neurons, supporting these observations.

## VIII. PATHOLOGY

The neurotrophic hypothesis that envisioned neurons as dependent on target-derived sources of NGF (and other neurotrophic factors) suggested that any disruption in this flow would lead to neuronal death. Thus, a putative link to neural degenerative diseases was readily forged. However, as it became clear that this dependency was reduced, this hypothesis lost attractiveness, particularly in view of the fact that NGF levels were particularly reduced in patients with Alzheimer's disease. However, further studies have suggested that defects in retrograde transport may be more important. Thus, there maybe a connection with NGF and neurodegeneration that can be eventually exploited. NGF has also been documented to be associated with a variety of autoimmune diseases and inflammatory diseases. Increased levels of βNGF in circulating plasma are found in patients suffering from multiple sclerosis, systemic lupus, and arthritis. The nature of the NGF involvement is unknown.

Early attempts to utilize NGF and other neurotrophins therapeutically were not successful, mainly because of a variety of undesirable side effects. There may well be potential therapeutic targets for PNS and CNS disorders for NGF (or small-molecule antagonists or agonists), but these must first deal with the substantial range of activities of this hormone that underlies the negative responses.

## IX. SUMMARY

Nerve growth factor has proved to be a scientific "Rosetta Stone" in that it has provided insights into many groundbreaking areas of endocrinology; over many years, initial insights have been ultimately found to have broad applicability to roles of other hormones and growth factors. It is appropriate, therefore, that the humoral substance first discovered gave rise to the term "growth factor" that is so widely used today.

## Glossary

**cystine knot proteins** Superfamily of proteins, including four growth factor families, all of which contain the distinctive motif made up of a circle of two disulfides (and the intervening peptide chains) through which passes a third disulfide bond.

**glial cells** Nonneuronal support cells of the nervous system. Schwann cells are found in the peripheral nervous system and astrocytes and oligodendrocytes are found in the central nervous system.

**neurotrophic factor** Any one of several hormonal substances that provide tropic support for neurons by interacting with cell surface receptors and stimulating the metabolic and transcriptional responses required for maintenance of viability, differentiation, and other activities.

**paracrine substances** Hormones and growth factors that act in a paracrine fashion are not transported systemically, but rather diffuse from the cell of origin to the target cell. If these are the same type of cell, the mechanism is said to be autocrine. Substances that are transported through the circulation are considered to be endocrine.

**prepro structures (zymogens)** Precursor forms of proteins that are exported from the cell by removal of the pre sequence (to initiate transfer into the endoplasmic reticulum) and then the pro peptide, to yield the active mature protein. Such structures are common for hormones and hydrolases, for example.

**receptor tyrosine kinases** A superfamily of plasma-membrane-bound glycoproteins that specifically bind hormones and growth factors, leading to activation of their intracellular tyrosine kinase domains and thus inducing any of several signaling cascades, usually by sequential activation of other kinases.

## See Also the Following Articles

**Folliculogenesis, Early • Neurotrophins**

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# Neuroactive Steroids

Andre H. Lagrange[*] and Martin J. Kelly[†]

[*]*Vanderbilt Medical Center* • [†]*Oregon Health and Sciences University*

I. INTRODUCTION
II. STEROID SYNTHESIS
III. REGULATION OF SYNTHESIS
IV. CELLULAR EFFECTS OF NEUROSTEROIDS/NEUROACTIVE STEROIDS
V. CLINICAL/PHYSIOLOGICAL RELEVANCE
VI. CONCLUSIONS

**Many steroids are produced within the central nervous system, allowing for the possibility of local control of hormone synthesis. Other steroids are produced peripherally but affect neural tissues via the bloodstream. Both centrally and peripherally produced neurosteroids have nongenomic effects; they alter a variety of neuronal functions, including those involving ion channels and ionotropic and G-protein-coupled receptors.**

## I. INTRODUCTION

Steroid hormones are a broad class of lipophilic, polycyclic chemicals that control a wide variety of physiological functions. Despite the diversity of their actions, these hormones have long been thought to have a common mechanism of action. By regulating gene expression, steroid hormones control physiological processes ranging from immune function to metabolism to reproduction. However, there is mounting evidence that these compounds have important functions that are independent of any genomic effects. One of the earliest indications for a nongenomic mechanism of steroid action was the report by Hans Seyle that progesterone and ring A reduced metabolites have very rapid, robust anesthetic actions. In particular, the steroid derivative 3α-hydroxy-5α-pregnane-11,20-dione (also known as alphaxalone) is a potent anesthetic that has been used in human surgery. The anesthetic effect is stereospecific in that the closely related isoform betaxalone (3β-hydroxy-5α-pregnane-1,20-dione) has essentially no anesthetic action.

The powerful actions of synthetic steroid derivatives led researchers to explore the possibility that there are endogenously produced steroids that might influence central nervous system (CNS) function. Various steroids and their derivatives have been detected in the brain; due to their lipophilic nature, brain steroid hormone levels are often higher than serum levels. In some rat models, brain levels have been estimated to be as high as 10–100 nM. Levels of some steroid hormones persist in the CNS even several weeks after peripheral production of hormone is halted by gonadectomy and adrenalectomy. Subsequent work has shown that these hormones are synthesized *de novo* within the CNS, and thus the name ‘neurosteroids’ was adopted. This article reviews some of the neurosteroid effects in different cellular and whole animal physiologic models.

## II. STEROID SYNTHESIS

### A. Overview of Steroid Synthesis

All steroid hormones are synthesized from cholesterol precursors (Fig. 1). Cytochrome P450scc is a hydroxylase that is responsible for side chain cleavage of cholesterol, which results in the formation of pregnenolone. From pregnenolone, there are two interdependent pathways, one leading to mineralocorticoid/glucocorticoids and another that produces sex steroids. Although progesterone is a sex steroid, it stands at an important crossroad within this pathway and may be converted to other sex steroids, to glucocorticoids, or to 3α,5α-reduced neurosteroids.

The glucocorticoid pathway starts with 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), which converts pregnenolone to progesterone. Progesterone can be converted by P450c21 to 11-deoxycortisone, which is then transformed to corticosterone and aldosterone by P450c11. The sex steroid synthetic pathway starts with P450c17, which converts pregnenolone or progesterone to 17-OH complexes. These compounds are then further converted to sex steroids by the same enzyme. The first

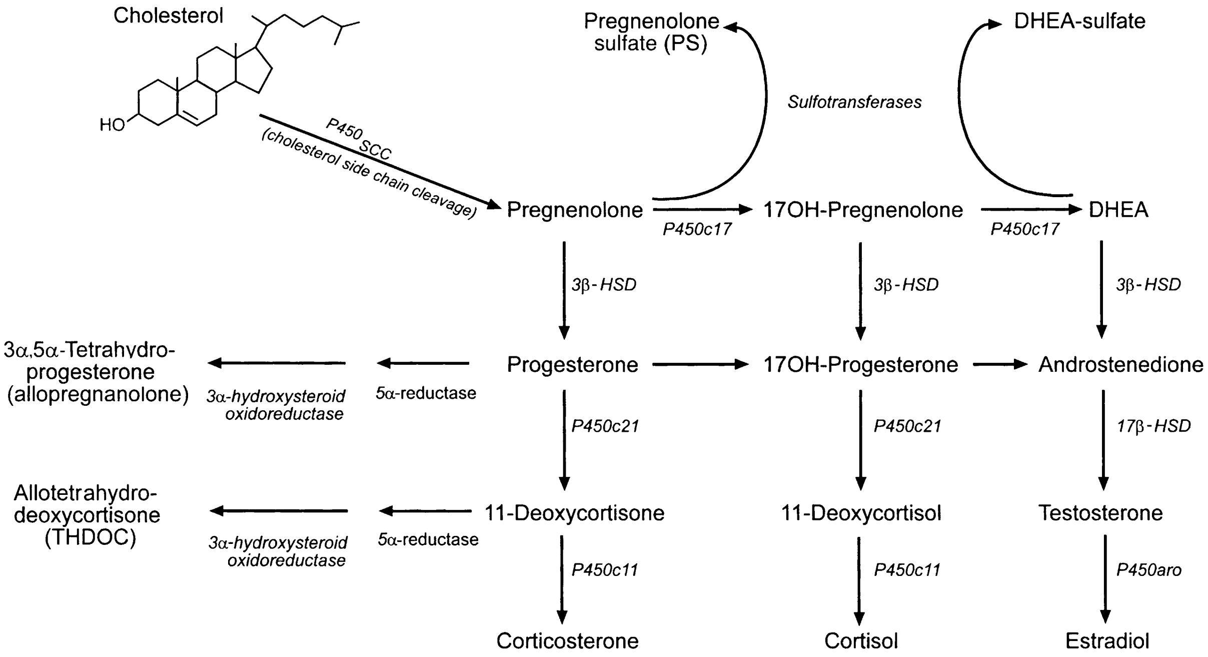
Cholesterol
HO
P450 SCC
(cholesterol side chain cleavage)
Pregnenolone sulfate (PS)
Sulfotransferases
DHEA-sulfate
Pregnenolone
P450c17
17OH-Pregnenolone
P450c17
DHEA
3β-HSD
3β-HSD
3β-HSD
3α,5α-Tetrahydro-progesterone (allopregnanolone)
3α-hydroxysteroid oxidoreductase
5α-reductase
Progesterone
17OH-Progesterone
Androstenedione
P450c21
P450c21
17β-HSD
Allotetrahydro-deoxycortisone (THDOC)
3α-hydroxysteroid oxidoreductase
5α-reductase
11-Deoxycortisone
11-Deoxycortisol
Testosterone
P450c11
P450c11
P450aro
Corticosterone
Cortisol
Estradiol

**FIGURE 1** Steroid synthetic pathway from the precursor cholesterol. The major enzymes are shown along with the intermediate steroid compounds in the synthetic pathway to the neuroactive steroids [e.g., allopregnanolone, tetrahydrocorticosterone, pregnenolone sulfate, dehydroepiandrosterone sulfate, and 17β-estradiol]. DHEA, Dehydroepiandrostenedione; 3β-HSD, 3β-hydroxysteroid dehydrogenase; see text for discussion of the different P450 enzymes.

sex steroid (other than progesterone) is dehydroepiandrostenedione (DHEA), which may be converted by 17β-hydroxysteroid oxidoreductase to androstenedione and then by 3β-HSD to testosterone, and is subsequently aromatized (P450aro) to 17β-estradiol (E2).

## B. Neurosteroids and Neuroactive Steroids

The gonads and adrenal glands produce steroid hormones (Fig. 2). They reach the brain, the spinal cord, and the peripheral nerves via the bloodstream. These hormones, which then have specific, nongenomic affects on neural tissue, are often referred to as "neuroactive steroids," to differentiate them from the neurosteroids, which are synthesized within the nervous system. For example, progesterone that is synthesized in the gonads or in glial cells can act as a neurosteroid or can be converted to other neurosteroids. Although the distinction between neuroactive steroid and neurosteroid may be heuristically useful, it is becoming clear that this is an artificial distinction. Steroids and steroid intermediates, including pregnenolone, progesterone, and DHEA, are made both in the CNS and in peripheral tissues. These lipophilic hormones easily cross the blood–brain barrier and so the physiologic importance of peripherally derived versus CNS-synthesized steroid hormones remains very ambiguous.

Cholesterol can be synthesized *de novo* within the CNS from low-molecular-weight precursors (e.g., mevalonate). The rate-limiting enzyme for steroid hormone synthesis in both the periphery and CNS is P450scc. This enzyme is found in the mitochondria of oligodendrocytes, some astrocytes, and possibly a small number of neurons. Isolated glia are able to produce pregnenolone, which may serve as an intermediate for further steroid synthesis. The next step is the conversion of pregnenolone to progesterone by 3β-HSD. This enzyme is found in both neurons and glia and is widely distributed within the brain, including the cortex, hippocampus, amygdala, and midbrain. As mentioned previously, pregnenolone and progesterone serve as important intermediaries from which glucocorticoids, sex steroids, and 3α,5α-reduced and sulfate derivatives may be formed.

Glucocorticoids can be synthesized from progesterone through the action of P450c21 and P450c11. Although P450c11 enzymatic activity has been demonstrated within the CNS, the same is not true for P450c21. Therefore, it is unclear whether

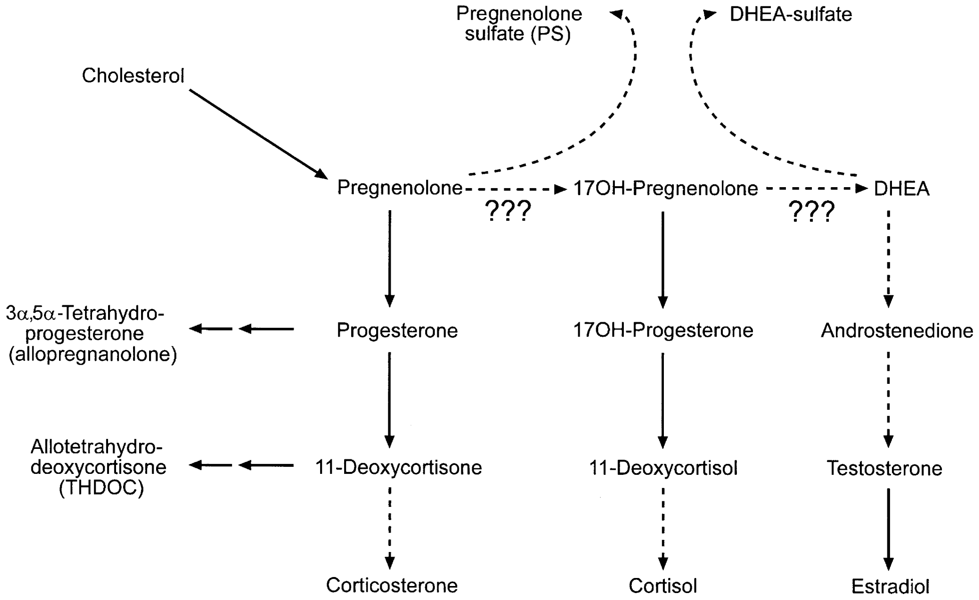

FIGURE 2 *De novo* synthesis of neurosteroids within the CNS. Steps that have been experimentally confirmed are represented by solid lines; steps for which the evidence is incomplete are shown as dashed lines. The enzymes mediating these steps are the same as those in the adrenals/gonads, with the exception of the conversion of progesterone to dehydroepiandrostenedione (DHEA). The P450c17 enzyme that mediates this conversion in the adrenal glands/gonads is not found in the adult CNS. Instead, if *de novo* synthesis of DHEA occurs within the CNS, it is probably mediated by an incompletely characterized $Fe^{2+}$-dependent enzyme.

there is *de novo* synthesis of glucocorticoids and the important reduced metabolite 3α,5α-tetrahydrodeoxycorticosterone (THDOC) in the CNS.

Within the sex steroid pathway, DHEA is both an early intermediary and a neurosteroid. Within the adult CNS, there is no P450c17 protein or mRNA. Thus, there should be no *de novo* synthesis of sex steroids through the usual pathway. However, DHEA is found to persist in the brain after gonadectomy/adrenalectomy or with inhibition of P450c17 by an antagonist. Recent work suggests there may be an alternative $Fe^{2+}$-dependent pathway in oligodendroglia and astrocytes for creating this steroid from progesterone. However, the physiological significance of this pathway remains uncertain, because high (10 mM), probably supraphysiologic, levels of $Fe^{2+}$ are necessary to produce significant quantities of DHEA. DHEA may be converted to androstenedione by 3β-HSD, and then to testosterone by 17β-hydroxysteroid oxidoreductase. However, although the enzymatic machinery for conversion of DHEA to testosterone is present, the physiological importance of this step in the CNS is uncertain. Unlike DHEA, the level of testosterone drops rapidly to undetectable levels after adrenalectomy/gonadectomy. Therefore, testosterone in the CNS is probably derived from peripheral sources. However, aromatase (P450aro) is found within the brain and can convert peripherally derived testosterone to 17β-estradiol.

The addition of sulfate moieties causes some very interesting and potentially important changes in the biological activities of neurosteroids. Pregnenolone sulfate (PS) and DHEA-sulfate (DHEA-S) are the most well-studied examples at this time. *De novo* synthesis of sulfated neurosteroids from isolated CNS cells has not yet been specifically demonstrated, and these compounds are known to be taken up from the blood. However, the brain contains the necessary sulfotransferases and sulfohydrolases to interconvert these compounds, and the levels of PS and DHEA-S in the CNS remain constant after adrenalectomy/ovariectomy, despite undetectable serum levels. It is therefore unclear whether sulfated steroids are synthesized *de novo* within the CNS or are derived from peripheral sources, or both.

Finally, the 3α,5α-reduced steroids are among the best characterized neurosteroids. Both 5α-reductase and 3α-reductase (3α-hydroxysteroid reductase) are found in neurons and glia in the pituitary, hypothalamus, medulla, thalamus, and cerebellum. These hormones can convert progesterone to 3α,5α-tetrahydroprogesterone (allopregnanolone) and corticosterone to 3α,5α-tetrahydrodeoxycorticosterone. These two neurosteroids have broad, powerful regulatory effects in several systems.

In summary, there is clear evidence for the *de novo* synthesis of neurosteroids in the CNS. The synthesis of progesterone from low-molecular-weight precursors has been established. The conversion of progesterone to allopregnanolone is well established, but synthesis of sex steroids and glucocorticoids is not as well documented. Progesterone is possibly converted to DHEA within the CNS, but testosterone, which is converted to estrogen, appears to be derived from peripheral sources. It remains completely unclear whether glucocorticoids or sulfated steroid derivatives are centrally or peripherally derived.

## III. REGULATION OF SYNTHESIS

The physiological regulation of neurosteroid synthesis remains largely unknown. Nonetheless, some early studies have shown that stress can change the levels of CNS steroid hormones. For example, significant elevations of pregnenolone and allopregnanolone have been observed in the frontal cortex following a swim stress in rats. Perhaps even more interesting is the fact that the stress of adrenalectomy/gonadectomy, although abolishing peripheral synthesis of steroid hormones, actually serves to increase brain tissue concentrations of allopregnanolone, PS, DHEA, and THDOC to physiologically active levels. Little else is known concerning the regulation of steroid synthesis in the nervous system. However, there is clear evidence that the levels of these hormones vary in response to diverse stimuli, and this remains a field wide open for further investigation.

## IV. CELLULAR EFFECTS OF NEUROSTEROIDS/NEUROACTIVE STEROIDS

### A. $GABA_A$

γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter that is distributed widely throughout the brain, activates two broad classes of GABA receptors, ionotropic $GABA_A$ and G-protein-coupled $GABA_B$ receptors. The G-protein-coupled $GABA_B$ receptors modulate a variety of effectors, including activation of $K^+$ channels and inhibition of $Ca^{2+}$ channels. $GABA_A$ receptors are composed of five subunits with an intrinsic chloride channel (Fig. 3). A vast majority of inhibitory postsynaptic currents in the brain are due to the activation of this conductance. Currently, there are six α, four β, three γ, one δ, one ε, one π,

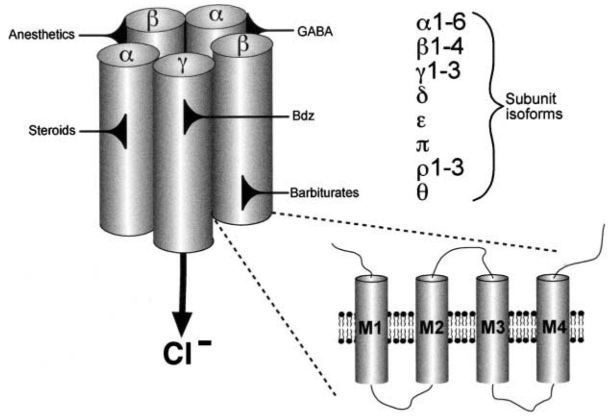

**FIGURE 3** γ-Aminobutyric acid type A (GABA$_A$) receptor heteropentameric structure with multiple allosteric modulatory sites. The different subunit isoforms are shown. The inset in the lower right shows the four transmembrane domains of each individual subunit.

three ρ, and one θ known subunit isoforms. Although there are an enormous number of possible subunit arrangements to produce receptors, only a relatively discrete number of actual arrangements have been demonstrated in brain. *In vivo*, these subunits combine to form heteropentamers, likely composed of two α, two β, and either a γ, δ, ϵ, π, or θ subunit. In addition to a GABA binding site, these proteins contain several other distinct allosteric modulatory sites for agents such as benzodiazepines (sedative-hypnotics), barbiturates (sedative-hypnotic/anesthetic), etomidate (general anesthetic), zinc, and furosemide (diuretic). Although the roles of these modulatory sites in normal physiology remain unknown, they are important targets for clinical manipulation. Examples include the use of phenobarbital in the treatment of epilepsy and propofol as a general anesthetic. As previously mentioned, the anesthetic action of the steroid derivative alphaxalone was one of the earliest indications for a neurosteroid effect. A variety of experimental paradigms have shown that, like several other general anesthetics, alphaxalone is an allosteric modulator of GABA$_A$ currents. Alphaxalone increases both the potency and efficacy for the GABA$_A$ receptor agonist, muscimol. Although alphaxalone does not directly activate GABA$_A$ receptors, it increases both the frequency of opening and the mean open time of the GABA$_A$ channels that have been activated by GABA. This modulation is mediated by a novel independent allosteric modulatory site on the GABA$_A$ receptors. Benzodiazepine antagonists do not inhibit the actions of alphaxalone. Instead of competing at benzodiazepine or barbiturate binding sites, the addition of alphaxalone actually increases the binding of these agents.

Further studies have shown that physiologically relevant levels of the endogenously synthesized neurosteroids allopregnanolone and THDOC enhance GABA$_A$ channel activity. A similar effect is seen with the compound 3α-OH progesterone (3α-OHP), thus the 3α-OH group appears to be necessary for biologic action. In contrast, betaxalone, corticosterone, hydroxycortisone, pregnenolone, and the biologically inactive 3β-OHP isomer have no effect in this system. Although this effect is mediated by a unique allosteric modulatory site, the exact binding site on the GABA$_A$ receptor remains unknown. However, there is some information that the subunit composition of GABA$_A$ receptors may be important in mediating the neurosteroid response(s). Oocytes injected with the $\alpha_2\beta_1\gamma_2$ combination have a greater neurosteroid modulation than do those expressing $\alpha_1\beta_1\gamma_2$ subunits. Those oocytes with no α-subunits ($\beta_1\gamma_2$ only) are much less sensitive to neurosteroids. Furthermore, the δ-subunit appears to convey resistance to neurosteroids. This effect of subunit composition is particularly interesting in light of the fact that the GABA$_A$ receptor subunit combinations throughout the brain vary with development and over the reproductive cycle. Furthermore, modulation of GABA$_A$ receptors by allopregnanolone also varies over the reproductive cycle, being most potent at times of high levels of serum estrogens.

In animal studies, allopregnanolone and progesterone enhance the ability of muscimol to impair the righting reflex (the ability to turn over during a fall). Furthermore, inhibition of endogenous allopregnanolone synthesis with a 30-min exposure to a 5α-reductase inhibitor reduces the muscimol sensitivity. This effect was reversed with administration of 5α-dihydroprogesterone, an intermediate compound between 5α-reductase and 3α-reductase. Patch-clamp recording from cortical neurons taken from these animals confirmed that this inhibition of endogenous allopregnanolone synthesis resulted in a smaller muscimol response compared to control. Thus, there is both cellular and behavioral data that neurosteroid modulation of GABA$_A$ responses may be physiologically important.

Interestingly, the sulfated neurosteroids PS and DHEA-S have exactly the opposite effect on GABA$_A$ receptor function. These steroids serve as noncompetitive antagonists that reduce the opening frequency of these channels and increase desensitization to GABA in neurons from multiple preparations, including primary chick spinal cord and rat hippocampal neurons, and in *Xenopus laevis* oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. The switch from a positive to negative allosteric $GABA_A$ receptor modulator seems to depend on the negative charge, because the inhibitory activity is retained when hemisuccinate is substituted for sulfate at the C-3 position. The interaction between steroid negative and positive modulators is not competitive, indicating that steroid negative and positive modulators act through distinct sites.

## B. Glycine Receptors

The glycine receptor is a ligand-gated chloride channel that is highly homologous to the $GABA_A$ receptors, so it is not a surprise that these receptors are modulated by neurosteroids as well. Although there is no effect of allopregnanolone or alphaxalone, the maximum response to glycine is enhanced by PS. The mechanism and physiological significance of this effect remains unexplored.

## C. Glutamate Receptors

Glutamate is the most widespread excitatory neurotransmitter within the brain. There are three broad classes of glutamate receptor: *N*-methyl-D-aspartate (NMDA), non-NMDA, and metabotropic receptors. As the name implies, the metabotropic receptor is a G-protein-coupled glutamate receptor, which, among other things, modulates the turnover of intracellular inositol trisphosphate. There are several variations of the non-NMDA receptors, which are activated by various ligands, including kainic acid and 3-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). All of these receptors have an intrinsic mixed sodium/potassium ion channel. Activation of this receptor/ion channel complex is the basis for many of the excitatory postsynaptic potentials within the brain. The NMDA receptors appear to be much more complicated. The receptors require multiple factors for activation. Opening of the intrinsic sodium/potassium/calcium channel requires binding of both NMDA/glutamate and glycine at two separate sites. There are additional sites for allosteric modulators, such as polyamine peptides. Even after binding of glutamate and glycine, the ion channel pore is blocked by $Mg^{2+}$. The pore can conduct current only if this magnesium blockade is removed by depolarization of the cell. If non-NMDA and NMDA receptors are simultaneously activated, the non-NMDA receptor-induced depolarization may be sufficient to remove the magnesium block of the NMDA receptors. Activation of NMDA receptors serves to depolarize the postsynaptic cells further. Moreover, some forms of this current are permeable to calcium, which may act as an intracellular messenger, resulting in long-term changes in postsynaptic cell function. The complex interactions among the glutamate receptors appear to play many important physiological functions, ranging from learning to excitotoxicity.

### 1. NMDA Receptors

Like the $GABA_A$ receptors, there are allosteric modulatory sites on the NMDA receptors. Pregnenolone sulfate acts at the neurosteroid modulatory site to increase the response to glutamate, but does not actually activate the receptor. In contrast, allopregnanolone sulfate (not allopregnanolone) is a negative modulator of NMDA currents. Finally, nanomolar concentrations of estrogen enhance NMDA-mediated excitatory postsynaptic potentials (EPSPs) in hippocampal CA1 pyramidal cells and high doses of this compound are associated with seizure activity in male rats. On the other hand, estrogens attenuate the neuronal damage seen after a variety of insults, including hypoglycemia, hypoxia, and glutamate agonists. It is currently unclear whether these various neurosteroids act by binding the same or different allosteric sites.

### 2. Non-NMDA Receptors

Estrogen increases the epileptogenicity of systemically administered kainate, more strongly in males than females. Based on *in vitro* intracellular recordings from CA1 pyramidal cells, administration of E2 increased synaptic excitability by enhancing the magnitude, but not the potency, of AMPA and kainate receptor-mediated responses. This effect is stereospecific, rapidly reversible, and does not appear to be due to a direct interaction between 17β-estradiol and the receptor channel. Rather, this effect appears to be mediated by activation of the cAMP/protein kinase A (PKA) pathway (Fig. 4). The actions of E2 are mimicked by PKA activators and blocked by PKA antagonists. The actual estrogen receptor remains unknown but possibly involves a novel binding site. Estrogenic modulation of non-NMDA receptors is seen robustly in a mouse line in which the α isoform of the classical estrogen receptor (ER-α) has been transgenically knocked out. Furthermore, this effect is not blocked by ICI 182,780, which is a potent antagonist at the ER-α and ER-β receptors. These effects may involve a G-protein-coupled receptor in that they are blocked

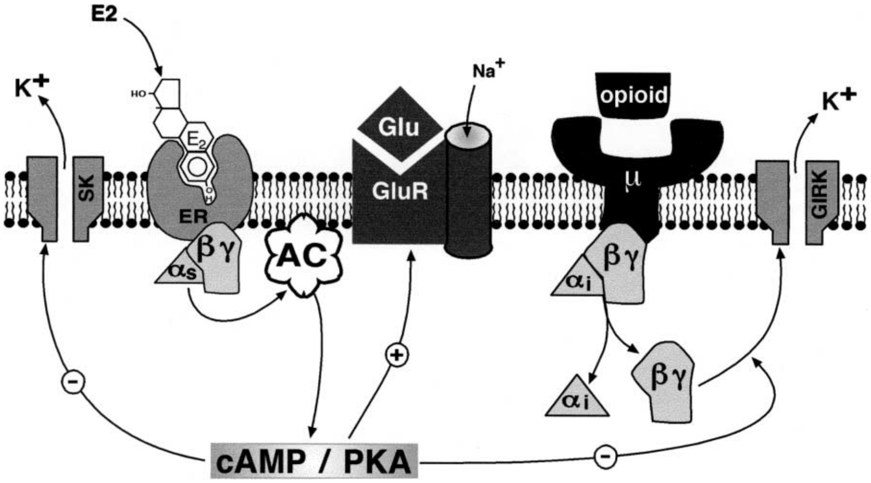

FIGURE 4 Schematic overview of the multiple rapid effects of 17β-estradiol (E2) in CNS neurons. In some cells (e.g., hypothalamic neurons), E2 binds to the estrogen receptor (ER), which stimulates adenylyl cyclase (AC) to produce cyclic adenosine monophosphate (cAMP), possibly via activation of $G_{\alpha s}$. Increased cellular levels of cAMP activate protein kinase A (PKA), which can uncouple μ-opioid (μ) receptors from their effector system (i.e., the inwardly rectifying $K^+$ channels, or GIRK) through phosphorylation of a protein [e.g., the $G_{\alpha i,o}$ (GIRK) channel]. In other cells (e.g., hippocampal CA1 pyramidal neurons), PKA can phosphorylate $Ca^{2+}$-dependent $K^+$ channels (SK), and inhibit their activity (outward $K^+$ current). Finally, PKA can phosphorylate a glutamate receptor/ionophore to increase its activity (inward cation current) in some neurons (e.g., hippocampal CA1 pyramidal neurons). Although this stylized figure shows the ER in the membrane, the actual subcellular localization of the receptor is not known.

by 5′-O-(2-thiodiphosphate) (GDPβS), which is a nonhydrolyzable GDP analogue that competitively inhibits G-protein activation by guanosine triphosphate (GTP), and they are mimicked by the toxin from *Cholerae vibrio* (cholera toxin), which is known to stimulate $G_{\alpha s}$.

## D. Calcium Channels

Neurosteroids also modulate N- and L-type calcium channels. Pregnenolone, THDOC, and PS, but not progesterone, all depress the maximal current up to 60%, as well as slow activation and deactivation of these calcium channels in hippocampal CA1 neurons. This effect may be mediated by an extracellular receptor because intracellular dialysis with PS has no effect. Furthermore, neurosteroids may work through action of G-protein-coupled receptors because pretreatment with toxin from *Bordetella pertussis* (pertussis toxin), an inhibitor of $G_{\alpha i,o}$, or intracellular dialysis with GDPβS blocks the effects of PS. The neurosteroid binding site mediating these effects is pharmacologically distinct from the $GABA_A$ modulatory site. Unlike PS actions at $GABA_A$ receptors, both the neurosteroids and their sulfated derivative have similar effects. The replacement of the sulfated group with acetate abolishes the physiological activity of PS, and progesterone has no effect.

In an independent series of experiments, picomolar concentrations of estrogen were shown to inhibit rapidly (within seconds) L-type calcium channels in acutely dissociated striatal neurons. Unlike estrogenic modulation of glutamate receptors, E2 coupled to bovine serum albumin (E2–BSA) readily mimicked the effect of free E2, suggesting an extracellular binding site. As with the other neurosteroids, the effects of estradiol may involve activation of G-proteins. Those cells that were dialyzed with 5′-O-(3-thiotriphosphate) (GTP-γ), which is a G-protein-activating GTP analogue that is more resistant to hydrolysis than GTP, had an irreversible suppression of $Ca^{2+}$ channels. Interestingly, a similar inhibition of L-type calcium current by estrogen, but not progesterone, has been demonstrated in vascular smooth muscle.

## E. Potassium Channels

Estrogen exerts complex control of neuronal potassium channels. Intracellular recordings in hypothalamic neurons have shown that perfusion of 17β-estradiol hyperpolarizes a subset of hypothalamic neurons in an *in vitro* preparation within seconds and that this effect reversed within minutes of washing out estrogen. Similar actions of E2 were demonstrated in hypothalamic ventromedial nucleus and amygdala neurons, even in the presence of protein synthesis inhibitors. The E2-mediated hyperpolarization of hypothalamic and amygdala neurons appears to be the result of the opening of an inwardly rectifying $K^+$ channel. In another set of hypothalamic neurons, the application of E2 causes a rapid depolarization that is due to the closure of a tonically active potassium conductance. It turns out that both the hyperpolarizing effects of E2 in one group of neurons and the depolarizing actions in another group are enhanced by coperfusion with a phosphodiesterase inhibitor. Interestingly, the hyperpolarizing effects of 17β-estradiol are mimicked by 8-bromoguanosine 3′,5′-cyclic monophosphate (8Br-cGMP), a cell-permeable analogue of cGMP, whereas this agent has no effect on that neuron subset that is depolarized by estrogen. Instead, application of 8-bromoadenosine 3′,5′-cyclic monophosphate (8Br-cAMP), a cell permeable analogue of cAMP, or stimulation of endogenous adenylyl cyclase with forskolin mimics the effects of estrogen to depolarize a subset of cells.

## F. G-Protein-Coupled Receptors

The discussion thus far has been limited to neurosteroid modulation of ionotropic receptors and ion channels. The effects of many neurotransmitters are also mediated by intracellular G-proteins, which subsequently serve to regulate a variety of intracellular processes. Estrogen is probably the most well-studied neurosteroid modulator of these receptors.

### 1. *μ-Opioid*

Activation of μ-opioid receptors hyperpolarizes hypothalamic neurons by opening a G-protein-coupled, inwardly rectifying potassium channel (Fig. 4). Perfusion of E2 *in vitro* results in a fourfold decrease in the potency of μ-opioid agonists to inhibit β-endorphin, but not gonadotropin-releasing hormone (GnRH) neurons. This effect is not mimicked by the inactive isomer 17α-E2, and selective estrogen receptor antagonists block this effect of E2. Estrogenic modulation of hypothalamic μ-opioid potency is mimicked either by stimulation of adenylyl cyclase with forskolin or by direct PKA activation with selective activators. Furthermore, selective PKA antagonists block the effects of E2. The apparent involvement of the cAMP/PKA pathway is highly reminiscent of estrogenic modulation of non-NMDA receptors. Moreover, the potency of the estrogenic effects is similar in both systems (effective concentration $EC_{50} \approx 10\,nM$) and is not mimicked by extracellular BSA–E2, implying that there may be a common receptor and/or intracellular mechanism mediating these estrogenic effects.

In hypothalamic neurons, $GABA_B$ and μ-opioid receptors are coupled to the same $K^+$ channels. Interestingly, E2 also produces a fourfold increase in the $EC_{50}$ of a $GABA_B$ agonist in the same β-endorphin neurons in which it reduces μ-opioid potency, thus implying that $E_2$ is modulating signaling pathways shared by both receptors.

### 2. *Catecholamines*

Voltage-independent, $Ca^{2+}$-activated $K^+$ currents underlie the long-lasting afterhyperpolarizations (AHPs) in a number of CNS neurons, including hippocampal, hypothalamic neurosecretory, midbrain dopamine, vagal motor, and cortical pyramidal neurons. These AHPs limit the firing frequency of neurons and are responsible for spike frequency adaptation in regular-firing cortical neurons and phasically bursting neurosecretory neurons. The slow AHP current is mediated by small-conductance, $Ca^{2+}$-activated $K^+$ (SK) channels, and three SK channel genes have recently been cloned. In hippocampal CA1 pyramidal neurons, these currents are regulated by monoamine neurotransmitters, which suppress the slow AHP. The effects of norepinephrine, serotonin, and histamine on the slow AHP are mediated via $\beta_1$, type 4 5-hydroxytryptamine (5-$HT_4$), and type 2 histamine ($H_2$) receptors, respectively. Interestingly, one of the long-term effects of corticosteroid is to increase the slow AHP in CA1 neurons, presumably via a genomic mechanism because protein synthesis inhibitors block these actions of the adrenal steroid. Recently, however, it was found that E2 inhibits the slow AHP current and potentiates the $\beta_1$-adrenergic receptor-mediated ($G_{\alpha s}$ coupled) inhibition of slow AHP current in CA1 hippocampal neurons (Fig. 4). Because the slow AHP is involved in spike frequency adaptation, these effects of E2 may be critical in increasing the firing frequency of hippocampal pyramidal neurons that are involved in arousal, attention, and memory.

## G. Summary

In summary, recent advances in our understanding of the mechanisms mediating neurosteroid action have helped support the idea that steroids may have a rapid, nongenomic effect to regulate neuronal function (Table 1). Although there are almost certainly multiple pathways, the current understanding allows us to differentiate these into two broad categories.

TABLE 1 **Summary of the Effects of Neuroactive Steroids**[a]

| Effect on | Prog | AlloPreg | THDOC | $E_2$ | PS | DHEAS |
|---|---|---|---|---|---|---|
| $GABA_A$-R | ± | $\uparrow I_{\max}$, $\uparrow$ potency | $\uparrow I_{\max}$, $\uparrow$ potency | — | $\downarrow I_{\max}$ | $\downarrow I_{\max}$ |
| Glycine-R | — | ∅ | — | — | $\uparrow I_{\max}$ | — |
| Non-NMDA-R | — | — | — | ± | $\downarrow I_{\max}$ | — |
| NMDA-R | ± | ∅ | — | ↑, ± | $\uparrow I_{\max}$ | — |
| $I_{Ca}$ | ∅ | ↓ | ↓ | ↓, ± | ↓ | — |
| $I_K$ | — | — | — | ↓ | — | — |
| μ-Opioid-R | — | — | — | ↓ Potency | — | — |
| $GABA_B$-R | — | — | — | ↓ Potency | — | — |

[a] Abbreviations: R, receptor; Prog, progesterone; AlloPreg, allopregnanolone (3α, 5α-tetrahydroprogesterone); THDOC, 3α, 5α-tetrahydrodeoxycorticosterone; E2, 17β-estradiol; PS, pregnenolone sulfate; DHEA-S, dihydroepiandrostenedione sulfate; $I_{\max}$, maximum response; potency, potency of agonist binding to receptor; ∅, no measured effect; ±, small or variable effect.

The oldest, most well-established mechanism of action is the modulation of $GABA_A$ and NMDA receptors by progesterone derivatives and sulfated neurosteroids. Although the actual binding site has not yet been identified, these steroids clearly alter neurotransmitter receptors by binding a specific allosteric modulatory site, resulting in well-defined changes in receptor kinetics.

Although the mechanism of estrogenic neuroactive steroid action is less clear, data from multiple systems may be converging on elucidating a single biochemical pathway. Biochemical studies in uterine tissue have shown that estrogen rapidly increases cAMP levels, thereby activating PKA. The ability of estrogen to rapidly uncouple G-protein-coupled receptors, inhibit non-NMDA glutamate receptors, and close potassium channels may all be mediated through cAMP/PKA. It is unknown whether this implies a common mechanism of estrogen action, or simply a single convergent point among multiple complicated pathways. The actual binding site for estrogen remains unknown as well. Several labs have shown a modulation of calcium channels. This may be mediated by a G-protein-coupled membrane estrogen receptor. Whether this is a completely different pathway or another example of the cAMP/PKA family remains undetermined at this point.

## V. CLINICAL/PHYSIOLOGICAL RELEVANCE

### A. Anesthetic

As previously mentioned, the first hint of neurosteroid existence was the discovery that progesterone and some of its derivatives have potent anesthetic actions. In fact, the drug althesin (mixture of alphaxalone and alphadolone acetate) has been used in human surgery.

### B. Regulation of Reproduction

The quintessential role of estrogen in the mammalian CNS is its negative and positive feedback actions on the hypothalamic–pituitary axis to regulate the reproductive cycle. In all mammalian species, the rapid negative feedback actions of estrogen on the hypothalamic GnRH neurons is a critical event for the activation and synchronization of GnRH neurons at the time of ovulation. Synchronous release of GnRH into the portal system produces maximal stimulation of pituitary gonadotrophs to secrete a 'surge' of luteinizing hormone (LH) to cause ovulation. An insight into the mechanism by which estrogen rapidly inhibits GnRH neurons was provided in the 1970s when it was found that E2 could rapidly inhibit hypothalamic neuronal activity. Therefore, the rapid effects of E2 on GnRH neurons may play a role in estrogen's negative feedback on the hypothalamus. In addition, estrogen regulates the reproductive axis through an effect on endogenous opioids. β-Endorphin neurons, originating in the hypothalamic arcuate nucleus, synapse directly on GnRH neurosecretory cells, and these neurons are an integral component of estrogenic negative feedback on the hypothalamic–pituitary axis. The opioid antagonist naloxone blocks the E2 negative feedback and advances the LH surge in women. Activation of μ-opioid receptors mimics E2 negative feedback on GnRH/LH release and has been shown to hyperpolarize directly, and thereby inhibit, GnRH neurons via opening G-protein-activated, inwardly rectifying potassium channels. As previously mentioned, the potency of μ-opioids to hyperpolarize β-endorphin neurons is greatly attenuated by brief application of estrogen (Fig. 4). Because β-endorphin is an endogenous ligand at μ-opioid receptors, these may serve as autoreceptors. By inhibiting β-endorphin autoinhibition, estrogen may cause increased β-endorphin release. Those cells that are impervious to this estrogenic effect (e.g., GnRH neurons) will remain sensitive to μ-opioids in the presence of increased concentrations of extracellular β-endorphin. Therefore, estrogen may serve to inhibit GnRH release through modulation of μ-opioid transynaptic regulation. The interaction between the direct inhibition and modulation of inhibitory input by estrogen has yet to be explored.

Neurosteroid modulation of reproduction may also be mediated by $GABA_A$ receptors. The activity of 3α-hydroxysteroid oxidoreductase is inversely correlated with E2 concentrations. Ovariectomy increases 3α-hydroxysteroid oxidoreductase activity, and this effect is abolished by subsequent administration of E2 (replacement therapy). Interestingly, allopregnanolone concentrations peak between proestrus and estrus in the rat, and the effects of allopregnanolone on the $GABA_A$ receptor vary over the reproductive cycle, being the most potent during the early follicular phase when the levels of serum estrogens are high. Furthermore, in ovariectomized rats, there is a nearly twofold reduction in the potency of allopregnanolone to modulate $GABA_A$ receptor.

### C. Striatal Function/Movement Disorders

Clinically, the basal ganglia (also referred to as the corpus striatum) are important target areas for estrogen's actions outside of the hypothalamus. The basal ganglia are actually composed of several nuclei, including the caudate putamen and globus pallidus. This area is important in the regulation of both movement and motivation. Dopaminergic input from the substantia nigra modulates the function of the basal ganglion and its dysregulation is involved in several diseases, including Parkinson's disease and tardive dyskinesia (TD). TD, a disorder that is characterized by involuntary movements, develops after prolonged exposure to dopamine-antagonist antipsychotic drugs (e.g., haloperidol). This disorder has a 2:1 higher incidence in women versus men. There are some other hyperkinetic movement disorders that are associated with estrogen. Women who are pregnant or receiving estrogen supplementation occasionally develop multifocal rapid movements (chorea gravidarum) that resolve when the pregnancy is over or when the estrogen supplementation is discontinued. In contrast, Parkinson's disease is a hypokinetic movement disorder associated with deficient dopamine. There are reports that there is a worsening of symptoms in women during the premenstrual period, when estrogen levels are falling. There is some preliminary evidence that postmenopausal estrogen replacement therapy is associated with a reduced risk of Parkinson's disease in women and a lower disease severity in women with early Parkinson's disease who are not yet taking 1,3,4-dihydroxyphenylalanine (L-DOPA, precursor for dopamine).

Estrogen has multiple short-term effects in the corpus striatum. It potentiates rotational behavior induced by amphetamine in ovariectomized, 6-hydroxydopamine-lesioned (unilateral) rats. This pronounced behavioral effect is recapitulated at the cellular level. Acute administration of E2 also increases dopamine turnover and amphetamine-induced striatal dopamine release within 30 min as measured by *in vivo* microdialysis. A similar effect has been shown in the nucleus accumbens, a target site of ventral tegmental dopamine neurons. Local injection of E2 (but not 17α-estradiol) rapidly ($<2$ min) potentiates $K^+$-stimulated dopamine release as measured by *in vivo* voltammetry.

In addition to modulating dopamine release, E2 affects the postsynaptic response to this neurotransmitter by uncoupling type 2 dopamine ($D_2$) receptors within the same period. This is consistent with an earlier report that acute E2 treatment reduces the number of caudate neurons that are inhibited by dopamine. Continued exposure to E2 (2 weeks) down-regulates $D_2$ receptor mRNA and attenuates $D_2$ inhibition of adenylyl cyclase in striatal neurons. Interestingly, $D_2$ receptors, like μ-opioid and $GABA_B$ receptors, are $G_{\alpha i,o}$-coupled receptors. In contrast, long-term exposure to E2 potentiates type 1 dopamine receptor ($D_1$)-stimulated adenylyl cyclase activity. Dopamine $D_1$ receptors are coupled to $G_{\alpha s}$, suggesting that the nature of estrogen's effects depends on the intracellular biochemical processes being activated. In fact, some of estrogen's actions may be mediated by alterations in G-proteins. Estrogen enhances the pertussis toxin-catalyzed adenosine diphosphate (ADP)-ribosylation of $G_{\alpha i,o}$, which indicates that E2 modifies the G-protein, possibly through protein kinase A phosphorylation. This is analogous to the rapid effects of E2 in the hypothalamus and hippocampus; therefore, this mechanism of 'uncoupling' $G_{\alpha i,o}$-coupled monoamine receptors may be via a common pathway in the CNS and peripheral tissues.

Although the striatum is a prime target for estrogen modulation of motor activity, there is virtually no ER-α receptor mRNA expression and very little ER-β mRNA expression in this dopamine-rich pathway, suggesting that E2 activates another receptor subtype in this region. Evidence for a membrane receptor for E2 is substantiated by studies showing that membrane-impermeable E2–BSA rapidly stimulates dopamine release from striatal slices. Other studies have shown that femtomolar concentrations of E2 and E2–BSA rapidly inhibit whole-cell L-type calcium currents in medium spiny (GABAergic) caudate putamen neurons.

### D. Epilepsy

Given the preponderance of data on neurosteroid modulation of $\text{GABA}_\text{A}$ and glutamate receptors, it is perhaps no surprise that there is strong evidence that both endogenous and exogenous neurosteroids can affect seizures. Studies in epileptic women have correlated high serum E2 levels with an increased risk of seizures and increased frequency of interictal epileptiform (IED) activity on electroencephalograms (EEGs). Exogenous estrogen administration reduces the latency and increases the severity of kainate-induced seizures in both male and female rats. Inversely, progesterone administration increases the electroshock- or kainate-induced seizure threshold in female rats. In women, high progesterone levels are associated with reduced IEDs. In fact, approximately one-third of epileptic women have a greater than twofold increase in their seizure frequency prior to their menses, when progesterone levels normally drop precipitously (catemenial epilepsy). This is particularly important in epileptic women with inadequate luteal phase syndrome, in which the progesterone levels do not stay sufficiently elevated for the entire luteal phase of the menstrual cycle. This results in irregular menstrual periods, infertility, and a significant increase in seizure frequency before and during menstrual periods. In some studies, it has been shown that administration of progesterone analog reduces seizure frequency by 39% in women with catemenial epilepsy.

At the cellular level, progesterone has a high affinity for intracellular progesterone receptors but has weak actions at $\text{GABA}_\text{A}$ receptors. However, allopregnanolone is devoid of activity at intracellular progesterone receptors but is a highly effective modulator of $\text{GABA}_\text{A}$ receptor complexes. There is increasing evidence that the antiseizure effects of progesterone may be mediated by conversion to allopregnanolone. Intravenous or intracerebroventricular administration of allopregnanolone has been shown to suppress seizures in animal models ranging from *in vivo* perforant path stimulation to injection of picrotoxin or bicuculline. Although both progesterone and allopregnanolone inhibit seizures, the effects of progesterone are greatly inhibited if coadministered with 5α-reductase inhibitors. Allopregnanolone may be an endogenous modulator of seizure threshold as well. Although there is no difference in serum allopregnanolone levels in epileptic and nonepileptic women, there is a significant increase in serum allopregnanolone within 15 min of partial-onset seizures.

## VI. CONCLUSIONS

The classic model of steroid hormone action is one in which (neuroactive) steroids are produced in the adrenals/gonads and travel through the bloodstream to the brain, where they alter protein synthesis by genomic actions at nuclear receptors. In contrast, neurosteroids, many of which are produced within the CNS, have nongenomic effects (alteration of a variety of neuronal functions, including those involving ion channels and ionotropic and G-protein-coupled receptors), allowing for the possibility of local control of hormone synthesis. Other steroids (e.g., testosterone) are taken up from the blood and may provide a mechanism for coordinating CNS and peripheral (outside the CNS) physiology. Additionally, there is a wide variety of steroid derivatives and they have an equally wide range of specific physiological actions. Given the broad range of neurosteroid action in various cellular physiological systems, it is not surprising that these compounds may be in involved in multiple arenas of CNS neurophysiology. There is good evidence at systemic and cellular levels for a role of neurosteroids in physiological/pathophysiological processes, ranging from reproduction to epilepsy. We have barely scratched the surface in understanding neurosteroid physiology and the prospect of expanding this field is exciting. In addition to increasing our understanding of normal physiology, steroid derivatives may offer important therapeutic options in the future. The broad range of action of neurosteroids may prove to be valuable ammunition in the arsenal of clinicians.

### Glossary

**dehydroepiandrostenedione** Neurosteroid that is synthesized in the central nervous system.

**γ-aminobutyric acid** The most prominent inhibitory neurotransmitter in the central nervous system.

**G-protein-coupled receptor** Membrane receptor that activates intracellular G-proteins.

**ionotropic receptor** Membrane receptor with an intrinsic ion channel.

***N*-methyl D-aspartate** Selective ligand for one class of glutamate receptors.

**P450aro** Cytochrome P450 (aromatase); enzyme that converts testosterone to estrogen.

**P450c11** Cytochrome P450 (11β-hydroxylase); enzyme that converts 11-deoxycorticosterone to corticosterone, 11-deoxycortisol to cortisol, and 18-hydroxycorticosterone to aldosterone.

**P450c17** Cytochrome P450 (17α-hydroxylase/C-17,C-20 lyase); enzyme that converts pregnenolone to 17α-hydroxypregnenolone, then to dehydroepiandrosterone.

**P450c21** Cytochrome P450 (21-hydroxylase); enzyme that converts progesterone to 11-deoxycorticosterone.

**P450scc** Cytochrome P450 (cholesterol side chain cleavage); enzyme that converts cholesterol to pregnenolone.

**pregnenolone sulfate** Neurosteroid that modulates glutamate channel activity.

**17β-estradiol** Female gonadal steroid that is a neuroactive steroid in the central nervous system.

**testosterone** Male gonadal steroid that is converted to 17β-estradiol in the central nervous system.

**3α,5α-tetrahydrocorticosterone** Neurosteroid that modulates $GABA_A$ channel activity.

**3α,5α-tetrahydroprogesterone (allopregnanolone)** Neurosteroid that modulates $GABA_A$ channel activity.

## See Also the Following Articles

**Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Membrane Steroid Receptors • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Hormone Receptor Family: Mechanisms of Action • Steroidogenic Acute Regulatory (StAR) Protein, Cholesterol, and Control of Steroidogenesis**

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# Neuropeptide Regulators of Juvenile Hormone Production

STEPHEN S. TOBE[*] AND BARBARA STAY[†]

[*]*University of Toronto* • [†]*University of Iowa*

I. INTRODUCTION
II. IS JUVENILE HORMONE PRODUCTION REGULATED?
III. ALLATOTROPINS
IV. ALLATOSTATINS

**The juvenile hormone of insects is a primary regulator of growth, metamorphosis, and reproduction in most insect species. As a consequence, it is essential that juvenile hormone production be precisely regulated so that it is present only during appropriate periods necessary for the control of these**

**processes. The presence of juvenile hormone at inappropriate times can result in disruption to metamorphosis and development and, in some cases, to disturbances in female reproduction. For this reason, complex mechanisms that regulate the production of juvenile hormone have evolved and, in many cases, neuropeptides are the primary regulators.**

## I. INTRODUCTION

In larval insects, the presence of juvenile hormone (JH) directs cells to continue development in a larval direction, whereas its presence in adult females directs specific cells to produce molecules associated with reproduction. In the absence of JH, metamorphosis of larval insects proceeds, either directly to the adult stage, in the hemimetabolous species (e.g., grasshoppers, bugs, cockroaches), or through an intervening pupal stage, in holometabolous species (e.g., beetles, moths and butterflies, flies, bees). The presence or absence of JH therefore determines the direction of development and reproduction in all insects at all stages of the life cycle. It is for this reason that the regulation of production of JH plays a central role in the life cycle of the insect. This article provides an overview of the neuropeptides that are known to regulate JH production in insects.

## II. IS JUVENILE HORMONE PRODUCTION REGULATED?

Is there evidence that JH production in insects is in fact regulated? As a central regulator of both development and physiology of the insect, the importance of JH and precisely controlled changes in its production cannot be overestimated. Figure 1 demonstrates that in both larval and adult stages, the production of JH undergoes dramatic and predictable changes. In the larval stages, these changes are associated with the ecdysis or molt, whereas in the adult, these changes are associated with the reproductive cycle and the production of eggs. In both cases, distinct peaks occur, and these are invariably associated with important physiological events,

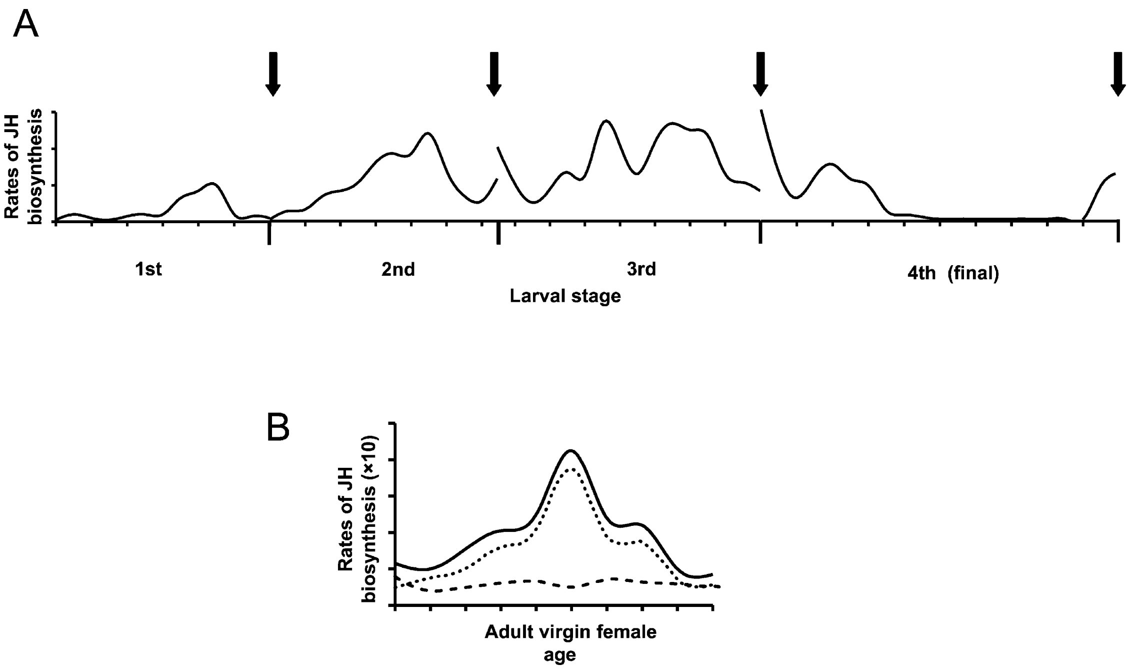

FIGURE 1 Juvenile hormone (JH) biosynthesis as a function of age throughout the larval stage (A) and in the adult virgin female (B) in the cockroach *Diploptera punctata*. Arrows denote the time of molting (A). (B) The results of unilateral cautery of the lateral neurosecretory cell region of the brain of virgin females that do not normally show a cycle of JH biosynthesis. The solid line represents JH biosynthesis by corpora allata on the unoperated side plus the corpora allata from the operated side; the line with long dashes represents JH biosynthesis by the single control unoperated corpus allatum; and the line with short dashes represents JH biosynthesis by the single operated corpus allatum.

including the time of deposition of the next cuticle in larvae and the synthesis of the yolk protein and its uptake by developing oocytes in adult females.

Profiles of JH production have been obtained for only a tiny number of existing insect species but all of these have revealed distinctive and predictable changes in production. In most cases, the periods of maximal production precede the larval molts or are associated with oocyte production in females. Rates of production of JH change dramatically over short periods of time, with changes of 10- to 20-fold being commonplace. In the very few studies that have examined the relationship between JH production and the circulating titer of hormone in the hemolymph, there is a high correlation. Accordingly, JH production mirrors the titer of the hormone.

Assuming that JH production is precisely regulated, how is this process in turn controlled? As with most other endocrine glands, the regulation of hormone production by the corpora allata is effected by neuropeptides and neurohormones. The principal neuropeptide regulators in insects are known as allatotropins (stimulators) and allatostatins (inhibitors) and a range of compounds that show such activity have been isolated. However, it should be noted that most assays for biological activity of these neuropeptides have employed short-term experiments *in vitro*, whereby the compounds in question are incubated with the corpora allata outside of the animal. In very few instances have the neuropeptides been shown to have high activity *in vivo*. Nonetheless, it is now widely believed that neuropeptides were some of the earliest neurotransmitters in the invertebrates and play a major role in the regulation of hormone production in all animals.

## III. ALLATOTROPINS

Nomenclature for insect neuropeptides is confusing. Peptides have been named on the basis of the function for which they were originally discovered, which may not be the true function or may be only one of several physiological functions. At present, there are two naming systems, a three-letter prefix, employing the first two letters of the genus and the first letter of the species of the insect in which the peptide was originally discovered, followed by the functional name of the peptide; and a five-letter prefix using the first three letters of the genus and the first two letters of the species (e.g., for *Manduca sexta* allatotropin: Mas-allatotropin or Manse-latotropin).

Neuropeptides known as allatotropins are responsible for the stimulation of JH production. Such stimulation is important to the insect, to raise the titer of JH in the hemolymph at appropriate times during development or reproduction. The allatotropin appears to exert its stimulatory effect directly on the corpus allatum, the site of biosynthesis and release of the JH. To date, a single type of allatotropin, a 13-amino-acid peptide, has been isolated and identified from the hornworm, *M. sexta*. The amino acid sequence of the Manse-allatotropin is Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe-$NH_2$.

This peptide has been demonstrated to stimulate JH production *in vitro* and structurally related peptides are known to occur in other members of the Lepidoptera, as well as in flies, mosquitoes, locusts, and cockroaches. However, these peptides do not show appreciable allatotropic activity (i.e., the ability to stimulate JH production) in these species, except for the Lepidopteran species. As with many peptides, the allatotropin peptide is not necessarily active at all times of the life cycle and the tissues of the insect show distinct periods of sensitivity. In fact, allatotropin was believed to be active only in adult hornworms, but more recent evidence indicates that it is active at specific times during larval life of some Lepidoptera. In addition, allatotropin exerts effects on tissues other than the corpora allata, including the stimulation of heart/dorsal vessel contraction and inhibition of ion transport across the larval midgut. Such actions are considered pleiotropic and emphasize the multifunctional nature of this peptide as well as of the allatostatins (see below).

Allatotropin is produced by cells in both the brain/central nervous system (CNS) of larvae and adults of *M. sexta* and is prominent in cells of the stomatogastric nervous system (e.g., ganglia and nerves along the foregut) and in the ganglia of the ventral nerve cord. In *Drosophila melanogaster* larvae, immunoreactivity to allatotropin is found in individual cells distributed in the lateral and tritocerebral regions of the brain and in the anterior portion of the ventral ganglion (Fig. 2).

The gene encoding the *Manduca* allatotropin has been cloned and characterized in this species as well as in other Lepidoptera and in *Drosophila*. It is expressed, as predicted from the above discussion, in several tissues, particularly the brain and ventral nerve cord, and at specific times during the life cycle (Fig. 2). The allatotropin peptide can be derived from each of three different precursor proteins and these proteins appear to arise as a result of alternative splicing of the allatotropin gene. The precursors also contain other allatotropin-like peptides that

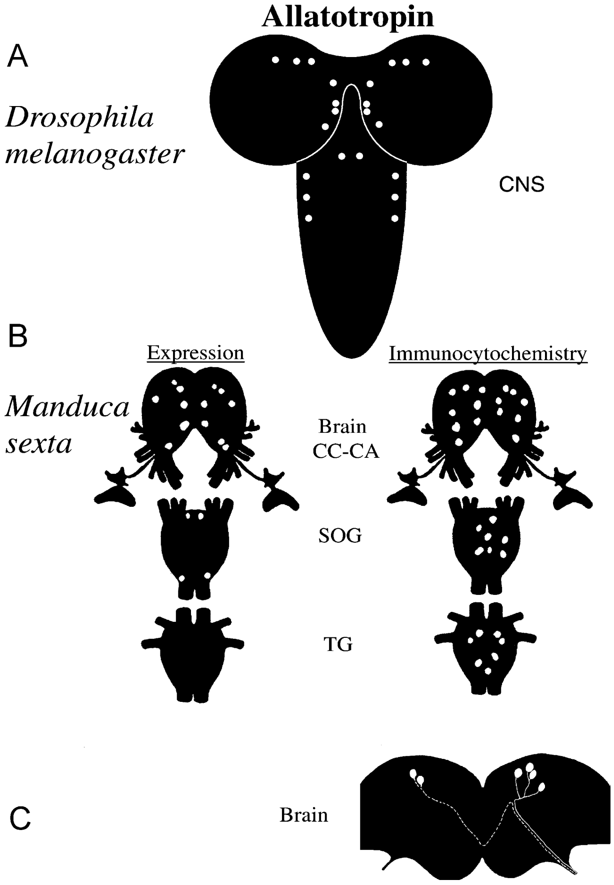

**FIGURE 2** Distribution of allatotropin immunoreactivity and expression in larval *Drosophila melanogaster* CNS (A) and in larval (fifth stage) *Manduca sexta* (B). (C) The pathway of cerebral allatotropin-immunoreactive neurons in the larval (fifth stage) *Manduca* brain. (A) *Drosophila* data from P. M. Koladich (2002), Ph.D. thesis, University of Toronto; (B) *Manduca* data from T. R. Bhatt (1998), Ph.D. thesis, University of Nevada, Reno; (C) data from Zitnan *et al.* (1995), modified and redrawn, with permission. CC, corpora cardiaca; CA, corpora allata; CNS, brain + ventral ganglion; SOG, subesophageal ganglion; TG, thoracic ganglion.

show biological activity. Allatotropin is expressed in the brain in larval stages (i.e., mRNA for allatotropin is present), indicating a potential role in the stimulation of JH production, in addition to the midgut function.

Alternative splicing is a common mechanism to increase the capacity and diversity of genes and their protein/peptide products and to regulate gene expression. In addition, the regulation of alternative splicing shows developmental and tissue specificity and may itself be hormonally controlled. It is significant that only a single allatotropin family has been identified to date, and the occurrence of alternative splicing may permit diversity in the function and developmental profile for the peptide.

## IV. ALLATOSTATINS

### A. Structure and Action

The two mechanisms that potentially regulate JH production involve either stimulation or inhibition of the biosynthetic process for the hormone within the corpora allata. The stimulation of biosynthesis would appear at first glance to represent the simplest means of regulating hormone production—in the presence of stimulatory factors, JH would be produced, presumably in a dose-dependent fashion, and in the absence of such factors, production would cease. An equally viable means of regulation is the inhibition of hormone production. In many species, this appears to be the primary mode of regulation of JH production; that is, corpora allata are normally inhibited or restrained in a dose-dependent fashion, and in the absence of the inhibitory signal, the glands are released and able to produce JH once again. However, in this instance, an increased dose of inhibitory factors would reduce JH production, and in most species studied to date, JH production cannot be completely inhibited by these factors, nor do corpora allata appear to be completely inhibited in the *in vivo* situation. It is likely that the corpora allata produce JH at a basal level, irrespective of the mode of regulation. That is, glands that operate by way of a stimulatory pathway do produce JH even when the allatotropin is not present, albeit at much lower levels than in the presence of the allatotropin (stimulation appears to be in the range of 4- to 10-fold). Conversely, glands that operate by way of an inhibitory pathway produce JH at low levels, even in the presence of the allatostatin. In both instances, such basal rates of JH biosynthesis are important, since they do indicate that the pathway for the production of JH is extant; the presence of the pathway permits a rapid response of the corpora allata to the neuropeptide signals. Glands that are completely inactive (and hence may lack the enzymes of the biosynthetic pathway for JH) may require a longer interval to respond to neuropeptide signals if biosynthesis of the enzymes is required.

The allatostatins or equivalent inhibitory factors have been hypothesized to exist for more than 50 years. Inhibition of the corpora allata and hence of JH production was inferred by studies on the effect of transection of nerves on the volume of the corpora allata, on production of eggs, or on the nature of the molt in larvae. Similar effects were subsequently obtained following cautery of selected regions of the brain, particularly following destruction of the lateral neurosecretory cells (see Fig. 3). Such operations resulted in the stimulation of JH production (see Fig. 1B) and a stimulation of the physiological processes regulated by JH. Such experiments led workers, including Berta Scharrer, to the conclusion that the corpora allata in cockroaches were normally inhibited by factors from specific neurosecretory cells in the brain.

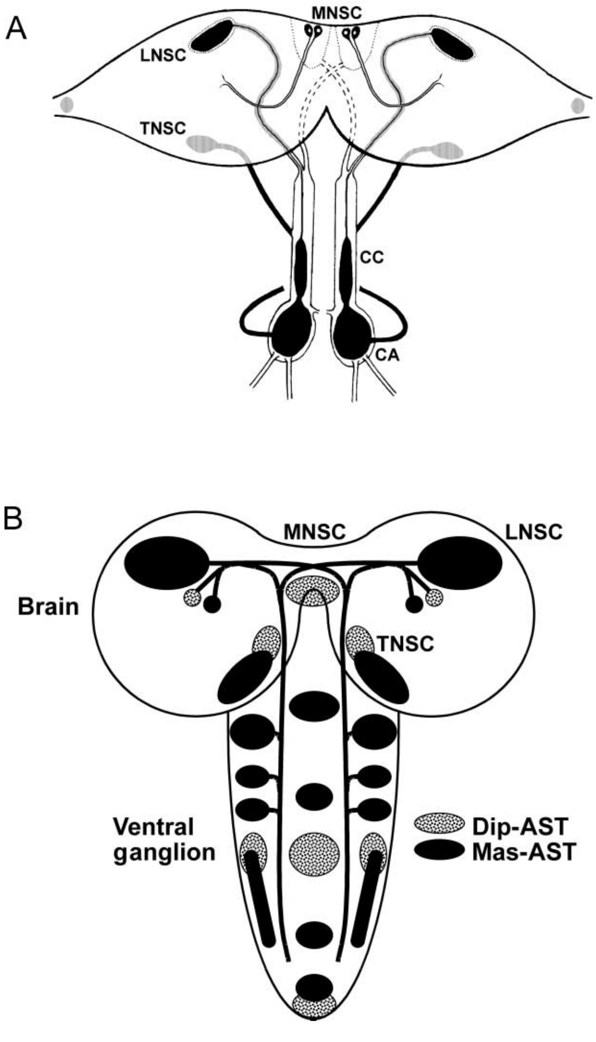

**FIGURE 3** Distribution of allatostatin immunoreactivity and expression in the brain and retrocerebral complex of adult female *Diploptera* (A) and CNS of larval *Drosophila* (B), dorsal view. In *Drosophila*, immunoreactivity to both cockroach and *Manduca* allatostatin is shown. In A, lightly shaded regions represent cell groups visible from the ventral perspective. MNSC, medial neurosecretory cells; LNSC, lateral neurosecretory cells; CC, corpora cardiaca; CA, corpora allata. Reprinted from Stephen S. Tobe (1999), Allostatins, *In* "Encyclopedia of Reproduction" (E. Knobil and J.D. Neill, eds). With permission from Elsevier.

The allatostatin neuropeptides can be categorized into three families, based on similarities in their amino acid sequences. The largest, most diverse, and most ubiquitous family is the cockroach allatostatins, or the Phe-Gly-Leu-amide family (FGLa) (see Fig. 4). All of these peptides share this common carboxyl-terminus. The five C-terminal amino acids can be generalized as the consensus sequence Tyr/Phe-Xaa-Phe-Gly-Leu-amide, although some minor substitutions do occur in some species (Fig. 4). To date, over 170 FGLa peptides have been either isolated or predicted on the basis of gene sequences and, although commonly called the cockroach allatostatins, actually occur throughout the insects as well as the crustaceans. Similar sequences or immunoreactivity to FGLa-like compounds has also been found in helminth worms, in mollusks, and in platyhelminths.

Figure 4 shows the 14 allatostatin peptides predicted from the coding region of the allatostatin gene of the American cockroach, *Periplaneta americana*, and the precursor polypeptide structure. Studies on other cockroach species have revealed that this family of peptides is remarkably conserved across the order Dictyoptera, suggesting important and ancient functions for the allatostatins. However, this function may not necessarily be the inhibition of JH production, since these peptides appear to be active only in this respect in cockroaches and closely related species such as crickets. In all other insect orders studies, the FGLa peptides are inactive in terms of JH biosynthesis *in vitro* but are potent myomodulators.

Cockroach allatostatins occur in all other orders that have been studied but at present, the identity of these compounds has been established definitively only in crickets and in selected members of the Lepidoptera and Diptera. It is clear that flies, including *Drosophila*, have different compounds capable of inhibiting JH production, but to date, these have not been sequenced. It will be particularly important to isolate and identify the *D. melanogaster* allatostatins in view of the genetic significance of this species and the availability of the genome sequence.

A second group of allatostatins has been isolated and identified from crickets. These peptides are characterized by tryptophan (W) residues at the N-terminal 2 and 9 positions. The generalized structure of these peptides is shown in Table 1 and this group is

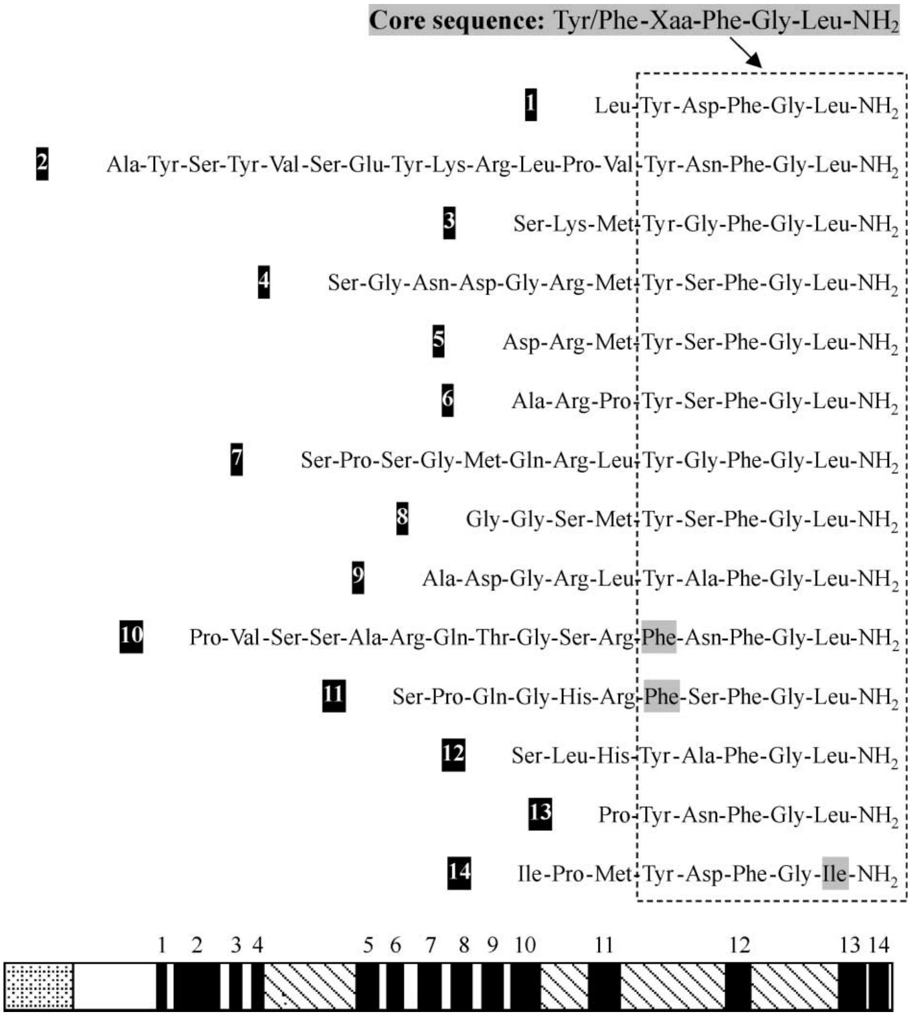
Core sequence: Tyr/Phe-Xaa-Phe-Gly-Leu-NH2
1 Leu-Tyr-Asp-Phe-Gly-Leu-NH2
2 Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val-Tyr-Asn-Phe-Gly-Leu-NH2
3 Ser-Lys-Met-Tyr-Gly-Phe-Gly-Leu-NH2
4 Ser-Gly-Asn-Asp-Gly-Arg-Met-Tyr-Ser-Phe-Gly-Leu-NH2
5 Asp-Arg-Met-Tyr-Ser-Phe-Gly-Leu-NH2
6 Ala-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH2
7 Ser-Pro-Ser-Gly-Met-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH2
8 Gly-Gly-Ser-Met-Tyr-Ser-Phe-Gly-Leu-NH2
9 Ala-Asp-Gly-Arg-Leu-Tyr-Ala-Phe-Gly-Leu-NH2
10 Pro-Val-Ser-Ser-Ala-Arg-Gln-Thr-Gly-Ser-Arg-Phe-Asn-Phe-Gly-Leu-NH2
11 Ser-Pro-Gln-Gly-His-Arg-Phe-Ser-Phe-Gly-Leu-NH2
12 Ser-Leu-His-Tyr-Ala-Phe-Gly-Leu-NH2
13 Pro-Tyr-Asn-Phe-Gly-Leu-NH2
14 Ile-Pro-Met-Tyr-Asp-Phe-Gly-Ile-NH2
1 2 3 4 5 6 7 8 9 10 11 12 13 14

**FIGURE 4** Schematic representation (bottom) of the amino acid sequence of the allatostatin precursor polypeptide in the cockroach *Periplaneta americana*. The hydrophobic leader sequence (signal sequence) precedes an untranslated region (clear), which is then followed by the allatostatin peptides. Black boxes represent individual allatostatins, which are numbered according to their position relative to the amino-terminus, and the amino acid sequences of each, corresponding to the numbers, are shown at the top. Acidic regions are indicated as diagonally striped areas. Shaded residues in the peptide sequences represent conservative substitutions.

commonly referred to as the $W_2W_9$ amide or $W_2W_9$a family of allatostatins. To date, these peptides are known to occur in crickets and in stick insects, but they are able to inhibit JH production only in crickets and at concentrations that are an order of magnitude greater than those of the FGLa family. The occurrence of these peptides in stick insects has necessitated a change in name of this family because the peptides are of variable length; hence, the designation $W(X)_6Wa$ is more appropriate. The structure of the gene encoding this family of peptides has recently been described in *Drosophila* although the peptides have yet to be isolated in this species.

A third group of peptides capable of inhibiting JH production in Lepidoptera is known as the PISCF family of allatostatins (see Table 1). Currently, only two members have been identified (isolated and sequenced). The *Manduca* peptide is a 15-amino-acid nonamidated, N-terminally blocked peptide originally isolated from *M. sexta* but subsequently found in other Lepidopteran species (Table 1). This peptide is unique, both from the structural perspective, in that it does not occur in any order other than Lepidoptera, and from the functional perspective, since the peptide is capable of inhibiting JH production completely (i.e., 100% inhibition), at least in

TABLE I **Allatostatin Amino Acid Sequences**

| |
|---|
| *Gryllus bimaculatus* W(X)$_6$Wa allatostatins |
| Gly-Trp-Gln-Asp-Leu-Asn-Gly-Gly-Trp-NH$_2$ |
| Gly-Trp-Arg-Asp-Leu-Asn-Gly-Gly-Trp-NH$_2$ |
| Ala-Trp-Arg-Asp-Leu-Ser-Gly-Gly-Trp-NH$_2$ |
| Ala-Trp-Glu-Arg-Phe-His-Gly-Ser-Trp-NH$_2$ |
| Ala-Trp-Asp-Gln-Leu-Arg-Pro-Gly-Trp-NH$_2$ |
| *Manduca sexta* PISCF allatostatin |
| gGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe-OH |
| *Drosophila melanogaster* PISCF allatostatin/flatline |
| gGlu-Val-Arg-Tyr-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe-OH |

*Manduca*. No other peptide is capable of reversibly inhibiting JH production to this degree. Furthermore, this peptide has no effect on JH production in non-Lepidopteran species and also appears to affect JH production in larvae and adults of selected Lepidoptera differentially.

A peptide very similar to the Manse-allatostatin (Manse-AST) has been identified in the genome of *D. melanogaster*, with a precursor organization similar to that of the *Manduca* precursor. This peptide is identical to Manse-AST, with the exception of a Tyr substituted for a Phe at N-position 4 of the peptide. This peptide does not inhibit JH production in flies but is a very effective modulator of heart muscle contraction, rapidly and reversibly suppressing contractions at specific developmental stages. For this reason, the peptide and associated gene have been named "*flatline*."

## B. Distribution of Allatostatins

Studies on the distribution of the allatostatins provide information not only on sites of release but also on potential target organs. Such information can provide insights into the functions of these peptides, which is particularly important for the allatostatins in light of their very large number, their extensive distribution, and the many different functions that they appear to perform (pleiotropism). Most of the information has been obtained from studies on the FGLa and the PISCF families and principally using immunocytochemistry and to a lesser extent *in situ* hybridization. In view of the role of the allatostatins in the inhibition of JH production, cells innervating the corpora allata can be expected to have immunoreactivity to these peptides. Figures 3 and 5 show diagrammatic representations and micrographs of the innervation of the corpora allata by cells in the brain. Innervation arises principally from the lateral neurosecretory cells although medial neurosecretory cells also appear to be important. The neurons arborize extensively within the corpora allata and there are apparent release sites within the glands. In the case of the *Drosophila* larval CNS, both FGLa immunoreactivity and PISCF immunoreactivity are apparent (Fig. 3B). In *Drosophila* as well, the transection of the nerves innervating the corpora allata, as in the cautery experiments with *Diploptera* noted in Fig. 1B, prevent the release of the peptides in the target tissue, thus releasing the corpora allata from the inhibition of the allatostatins. Not all allatostatins are released from the brain by way of axonal tracts to targets outside the brain. Within the brain, axons from the lateral, medial, and tritocerebral groups all arborize extensively and appear to innervate other neurons and thus can be regarded as interneurons.

Figure 5 also demonstrates extensive innervation of the hindgut of the termite by allatostatin-immunoreactive neurons and reveals the very wide distribution of allatostatin immunoreactivity in most insects. A similar distribution can be found in the midgut, foregut, and hindgut of many species, as well as in the reproductive system, in the optic lobes and antennal lobes, and throughout the CNS. Allatostatins are also found in high concentrations in the hemolymph (blood) serum and some hemocytes of cockroaches. Although it is not possible to define the serum source of the peptides, the extensive release sites observed in and on many tissues including the gut and CNS suggest that these tissues are important contributors.

## C. Receptors for Allatostatins

The clear action of the FGLa allatostatins as inhibitors of JH production in cockroaches and in crickets demands the presence of appropriate receptors in the target tissues, including the corpora allata. Since there are multiple allatostatins in these organisms, multiple receptors for the peptides may also exist. In addition, the corpora allata show major differences in sensitivity to the allatostatins at selected developmental times, suggesting that receptor occurrence or expression is regulated. In general, in adult females, the glands show a low level of sensitivity at times of high production of JH, whereas in larvae, although a similar relationship exists early in the stadium, at times of minimal JH production, the corpora allata also appear to be insensitive at later times in the stadium.

The other biological actions of the FGLa allatostatins, particularly the ability to modulate myotropic

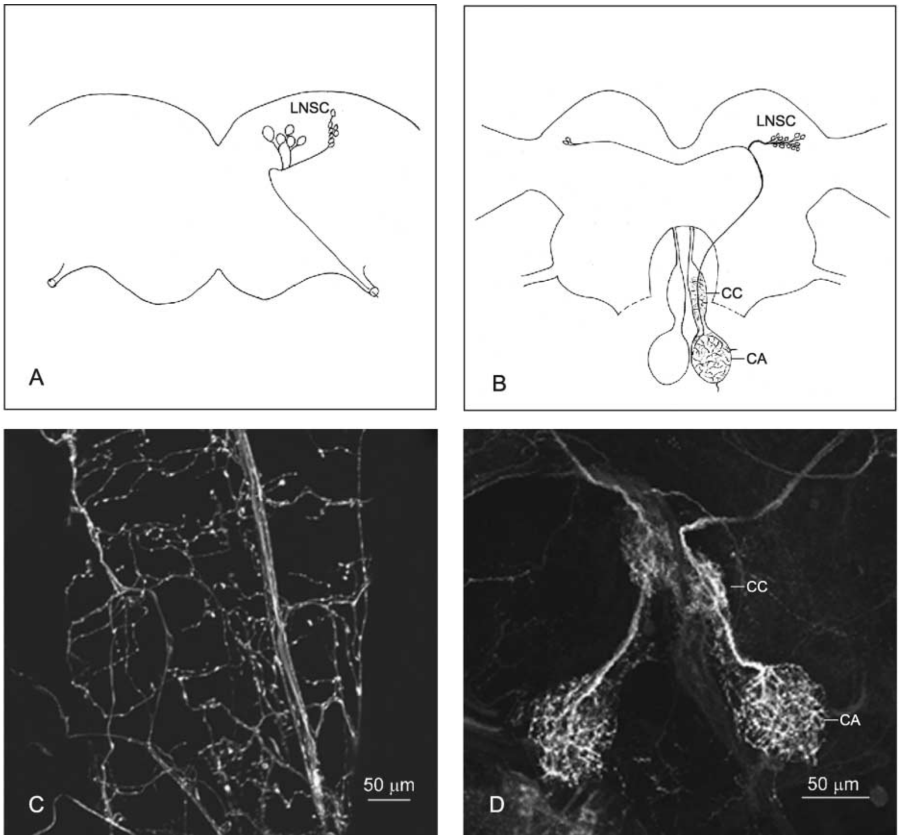

**FIGURE 5** Allatostatin immunoreactivity in *Diploptera punctata*, *Manduca sexta*, and *Reticulotermes flavipes*. (A) Diagram of cells in brain of last-stage larva of *Manduca* that innervate the corpora allata and are immunoreactive for Manse-AST (from Zitnan *et al*., 1995, *Journal of Comparative Neurology* **356**, pp. 83–100). (B) Diagram of cells in adult brain of *Diploptera* that innervate the corpora allata and are immunoreactive for Dippu-AST (from Chiang *et al*., 1999, *Journal of Comparative Neurology* **413**, pp. 593–602, Copyright 1999 Wiley. Reprinted by permission of John Wiley & Sons, Inc.). (C) Micrograph of nerves on the muscles of the colon of a termite, *R. flavipes*, that are immunoreactive for Dippu-AST. The varicosities are neuropeptide release sites. Stacked confocal microscope images of twenty-two 2 µm sections. (D) Micrograph of corpora allata and corpora cardiaca of a 45-day embryo of *Diploptera* that are immunoreactive for Dippu-AST. Stacked confocal microscope images of fifteen 2 µm sections. LNSC, lateral neurosecretory cells; CC, corpus cardiacum; CA, corpus allatum.

activity of the midgut and hindgut, also suggest the occurrence of multiple receptors or subtypes for these peptides. These actions are quite distinct from the inhibitory action on the corpora allata and may involve different signaling pathways (see Table 2). To date, two receptor subtypes have been cloned from *Drosophila* and at least one has been cloned from cockroaches. These receptors have been confirmed to be members of the G-protein-coupled superfamily and are structurally related to vertebrate galanin and somatostatin receptors, neither peptide of which is known to occur in insects. These receptors all exhibit seven membrane-spanning domains characteristic of the G-protein-coupled superfamily. The most conserved regions are the transmembrane domains facing the intracellular side of the membrane known to interact with G-proteins, which appear to be effectors both in the vertebrate system and in the insect system. The greatest difference in the receptors is on the extracellular side of the membrane in the ligand-binding region. This suggests that during evolution, the G-protein-binding domains were conserved, whereas the ligand-binding regions have undergone "rapid" change in response to the variety of ligands available.

As noted earlier, the different allatostatins of the FGLa family show different biological activities, with respect to both their ability to inhibit JH

TABLE 2 Rank Order of Effectiveness of Dippu FGLamide Allatostatins

| Peptide designation | Length (amino acid residues) | Muscle contraction | JH Production |
|---|---|---|---|
| Dippu-AST1 | 6 | 7 | 13 |
| Dippu-AST2 | 18 | 9 | 1 |
| Dippu-AST3 | 8 | 10 | 11 |
| Dippu-AST4 | 9 | 5 | 6 |
| Dippu-AST5 | 8 | 8 | 2 |
| Dippu-AST6 | 8 | 11 | 5 |
| Dippu-AST7 | 13 | 3 | 3 |
| Dippu-AST8 | 9 | 2 | 10 |
| Dippu-AST9 | 10 | 1 | 8 |
| Dippu-AST10 | 16 | 6 | 4 |
| Dippu-AST11 | 11 | 4 | 7 |
| Dippu-AST12 | 6 | 12 | 9 |
| Dippu-AST13 | 8 | 2 | 12 |

production and their ability to modulate myotropic activity (Table 2). With the elucidation of at least two receptors in insect systems, it is likely that these differences in biological activities are attributable to differences in the affinity of the receptors for the different allatostatins. The relative distribution of different receptor subtypes probably permits differences in the responses of target tissues.

The core region responsible for biological activity of the allatostatins is the C-terminal pentapeptide (Fig. 4). This sequence, which is conserved throughout the FGLa family of peptides, interacts with the extracellular surface-binding domain of the receptor(s) and shows full biological activity, albeit at high concentrations. This peptide appears to represent the minimal number of amino acid residues necessary for full potency. This suggestion has now been confirmed using expressed receptor in the frog oocyte expression system.

## D. Evolution of Allatostatins

The original function of the FGLa allatostatins is clearly unknown, but in view of the ubiquitous nature of the peptides in insects and other invertebrates, it is likely that they performed a myomodulatory function, as they do in modern species. Nonetheless, the remarkable sequence conservation in the cockroaches does suggest that these peptides are ancient and that their co-opting for the purposes of regulation of JH biosynthesis probably occurred before speciation. Thus, the various cockroach allatostatins can be regarded as orthologues.

The large number of extant peptides of the FGLa allatostatin family (at present, over 170 peptides have been isolated or predicted from gene sequences) might also indicate that specific allatostatins have specific functions, at least in cockroaches and related species. It is possible that at least some of the different allatostatins are not replaceable, effecting only narrowly defined functions. There is some suggestion of this in the different rank order of the allatostatins in terms of the ability to inhibit JH biosynthesis versus modulation of myotropic activity in cockroaches, whereby allatostatins that are the most active in terms of one function often show only very low biological activity in the other function (see Table 2). On the other hand, the great profusion of allatostatins suggests that the allatostatins may be replaceable for a particular function, reflecting some flexibility in the receptor-binding domain. The only region in which amino acid differences are not possible for retention of function is in the core region.

It is likely that the additional functions of allatostatins remain to be defined. At present, it is known that the FGLa family of peptides is able to:

(1) inhibit JH biosynthesis in cockroaches and crickets;
(2) modulate muscle contraction, both spontaneous and proctolin-induced;
(3) modulate neuronal activity in crab CNS;
(4) inhibit vitellogenin production in cockroaches; and
(5) modulate digestive enzyme activity in cockroaches.

The same allatostatin can exert several target-specific effects. The redundancy in the FGLa allatostatin function could allow for the proliferation of the peptides throughout evolution. Immunocytochemistry has putatively identified a very large number of different FGLa peptides in the invertebrates. In insects, in any given species in orders more recently evolved than cockroaches, there is a reduction in the number of members of the allatostatin family (based on gene sequences). For example, this number is reduced from 13 or14 in cockroaches to between 5 and 9 in flies and moths. It is significant, however, that there has been a concurrent loss in functionality of the FGLa peptides since they have no effect on JH production in these orders. At this point, it is impossible to determine whether the loss of inhibitory function (and hence a loss of receptor) preceded the reduction in number of the peptides. It is instructive to observe that in the locust, there has been a reduction in the FGLa family to 10 members and a loss in sensitivity to inhibition of JH production. Accordingly, a loss in biological activity may accompany the loss in the peptides expressed in a given species. In crickets, a family in which allatostatins are effective in the inhibition of JH production, there remain 14 putative peptides in the precursor deduced from cDNA sequences.

The neuropeptide regulators of corpus allatum function are important not only with respect to their modulation of JH production but also in their many other functions and in the insight they may provide into the evolution of neuropeptide families.

## Glossary

**corpora allata** Insect endocrine glands associated with and receiving innervation from the central nervous system; the site of production of juvenile hormone.

**juvenile hormone** Sesquiterpenoid compound, derived from farnesyl biphosphate, that regulates metamorphosis and reproduction in many insect species.

**orthologue** Peptides from a pair of genes in related species, derived from a single gene in the last common ancestor, which arose as a result of a speciation event; genes of different species that have a common origin. Orthologue assignments do not involve function.

**paralogue** When a peptide gene is duplicated giving rise to two copies in the genome, the two genes and their products are considered paralogous; involves a duplication and not a speciation event.

**pleiotropism** The phenomenon whereby one peptide or peptide family exerts multiple physiological effects on target tissues.

**preprohormone** The entire polypeptide encoded by an mRNA for a peptide hormone or hormones before processing by prohormone convertase enzymes to remove signal sequences and other nonfunctional regions.

**prohormone** The polypeptide encoded by an mRNA for a peptide hormone following removal of the signal sequence. Cleavage of the prohormone to individual peptides by prohormone convertases results in the release of the final peptide sequences with biological activity.

## See Also the Following Articles

**Insect Endocrine System • Juvenile Hormone Action in Insect Development • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormone Biosynthesis • Juvenile Hormones, Chemistry of**

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# Neuropeptides and Control of the Anterior Pituitary

Meghan M. Taylor and Willis K. Samson
*Saint Louis University School of Medicine*

I. INTRODUCTION
II. HYPOTHALAMIC COMMUNICATION WITH THE ANTERIOR PITUITARY: LONG AND SHORT PORTAL VESSELS
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V. SUMMARY

**Neuropeptides are short chains of amino acids that are produced in neurons throughout the body; when released, they bind to specific receptors on target cells, thereby communicating neural information to other neurons or to nonneural tissues (e.g., hormone-secreting cells of the anterior pituitary gland, vascular smooth muscle, and secretory glands). Neuropeptides access the anterior pituitary via a portal vessel system and set into action complex pathways of neuroendocrine control.**

## I. INTRODUCTION

Peptides are produced in neurons that have axons terminating at the base of the hypothalamus, in a specialized region named the median eminence; these neuropeptides gain access by diffusion to the unique vascular system that links the brain to the anterior pituitary gland. In this manner, neural signals are communicated to the hormone-secreting cells of the gland and, by controlling the release of those hormones, to the entire body. Each type of anterior pituitary hormone-secreting cell is regulated by a balance of stimulatory and inhibitory neuropeptides released from axon terminals in the median eminence, against a background of information (long loop feedback) received from factors secreted into the general circulation by the cells that are the targets of those anterior lobe hormones. Against that background of information from the periphery, it is the relative balance of releasing and release-inhibiting peptides reaching the gland, and the pattern of the exposure to those factors, that determine hormone production and secretion from the anterior pituitary. That balance defines the field of neuroendocrinology.

## II. HYPOTHALAMIC COMMUNICATION WITH THE ANTERIOR PITUITARY: LONG AND SHORT PORTAL VESSELS

The adult human pituitary gland weighs, under normal conditions, approximately 0.5 g, except in pregnancy, when the gland may double in size. Located in a protected cradle within the sphenoid bone, the pituitary measures approximately 1.0–1.5 cm in length and width and 0.5 cm in depth (Fig. 1). It is composed of the posterior lobe, which is neural in origin, and the anterior lobe, which is nonneural in origin. The anterior lobe, which contains the hormone-producing and -secreting cells of the gland, arises from the roof of the oral cavity

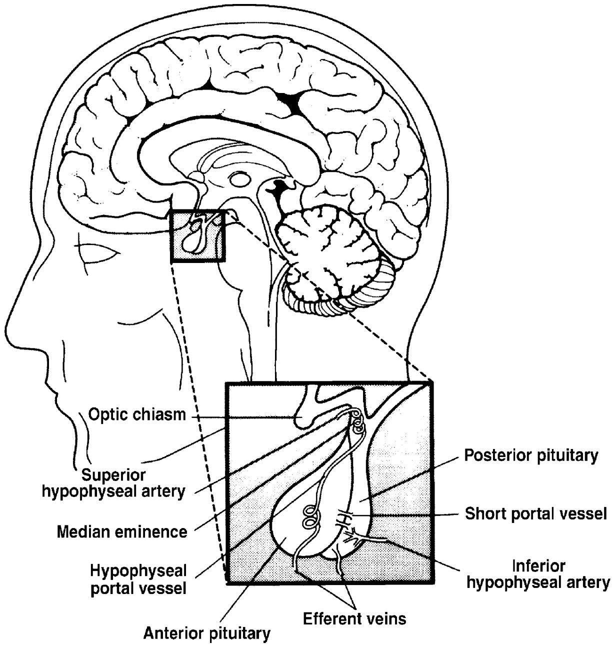

FIGURE 1 Location of the pituitary gland beneath the hypothalamus. The hypothalamo-pituitary portal vessels are idealized for simplicity. Reprinted from "Encyclopedia of Human Biology," Vol. 6 (1991), Academic Press, with permission.

**The appetitive drive for energy to sustain physiological well-being and to reproduce successfully at the opportune time are two instinctual urges essential for survival of the species against varied evolutionary pressures. Neuropeptide Y is an important neurochemical signal relaying information regarding these instincts.**

## I. INTRODUCTION

Since the energy cost of reproduction is high and reproductive function is sensitive to nutritional stress and metabolic demands, scarcity and abundance of energy resources must be perceived and relayed continuously to the neural processes governing reproduction. Recent insight into the diversity and interplay of signals in the hypothalamus, a brain structure involved in homeostatic integration, has elevated neuropeptide Y (NPY) to the top of the list of neurochemical signals that relay information bidirectionally between these instinctual drives (Fig. 1). This article collates our understanding of the neurobiology of NPY produced specifically in the arcuate nucleus (ARC) of the hypothalamus. Participation of NPY in neuroendocrine mechanisms that initiate and sustain the drive for energy intake and expenditure in an orderly manner on the one hand and those that regulate reproduction on the other are described. Subsequently, the ARC NPY links between appetitive and reproductive functions are addressed.

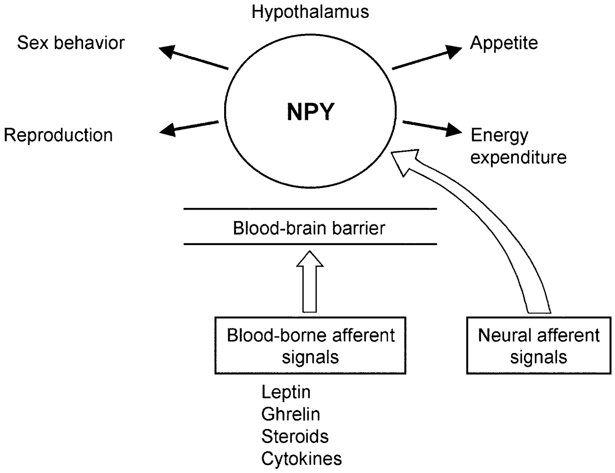

FIGURE 1 Neuropeptide Y (NPY) plays a pivotal role in the hypothalamus in regulating two neuroendocrine functions, reproduction and sex behavior (left) and appetite and energy expenditure to maintain body energy homeostasis (right). In addition to an interconnected circuitry in the hypothalamus (see Figs. 2 and 3), distinct afferent pathways play an important role in regulating NPY release at target sites. Afferent messages from the periphery are relayed by multisynaptic neural pathways through the spinal cord, brainstem, and lateral hypothalamus and then converge onto NPY neurons in the basal hypothalamus. NPY neurons also receive hormonal signals that cross the blood–brain barrier.

## II. NEUROANATOMY

NPY was isolated and chemically sequenced from the brain as a 36-amino-acid peptide closely related to members of the pancreatic polypeptide family. Since NPY is produced exclusively in neural tissue and has tyrosine residues at both the amino- and the carboxy-terminals, it was named neuropeptide tyrosine (Y). The NPY localized in various hypothalamic sites is derived from two main sources. The extrahypothalamic source is a cluster of neurons in the brainstem that co-express a few additional messenger molecules, the most prominent of which are the two catecholamines, norepinephrine (NE) and epinephrine. These NPY and catecholamine neurons are synaptically linked with neurons in the hypothalamus that express various neuroendocrine peptides, neuromodulators, and neurotransmitters. Under certain conditions, co-release of these messenger molecules is important in amplifying or restraining the postsynaptic response of hypothalamic target cells. A denser population of NPY-producing neurons distributed along the ARC and a subpopulation located in the dorsomedial hypothalamus (DMH) are the hypothalamic sources of NPY. NPY-producing neurons in the ARC transsynaptically regulate the synthesis and release of several neurohormones—gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone, somatostatin, corticotropin-releasing hormone, thyrotropin-releasing hormone, vasopressin, oxytocin, and dopamine. In addition, these ARC NPY neurons innervate other hypothalamic nuclei that regulate appetitive and sexual behaviors and thermogenic energy expenditure.

There is regional heterogeneity in the distribution of NPY neurons in the ARC to selectively regulate various neuroendocrine and behavioral functions. A subpopulation of neurons extensively innervate the median eminence (ME), a neurohumoral junction between the hypothalamus and the anterior pituitary gland. Within the ME, NPY neurons terminate in close proximity of nerve endings of neuroendocrine cells to regulate reproduction. NPY release in the ME evokes the efflux of neuroendocrine messengers into the hypophyseal portal veins for transport to target cells in the anterior pituitary gland. NPY itself is also released into the hypophyseal vessels to potentiate the effects of neuroendocrine hormones on pituitary target cells. ARC NPY neurons that project to the paraventricular nucleus (PVN) secrete NPY in discrete episodes to regulate appetitive behavior. Unlike NPY neurons in the brainstem, the ARC NPY neurons co-express agouti-related peptide (AgrP) and γ-amino butyric acid (GABA); each of these co-transmitters modulates the postsynaptic response of NPY on appetite and reproduction in distinctive ways. Within the ARC itself, NPY neurons are synaptically linked with proopiomelanocortin (POMC)-expressing neurons, an essential component of the neural circuitry involved in the regulation of appetite and reproduction. Since the ARC NPY neurons are strategically positioned inside the weak blood–brain barrier, they receive hormonal afferent messages from peripheral endocrine glands, leptin from adipocytes, ghrelin from stomach, steroids from gonads and adrenal cortex, and cytokines from immune cells (Fig. 1). The ARC NPY neurons also receive messages from ascending neural pathways via the catecholaminergic brainstem neurons and the orexin and melanin-concentrating hormone (MCH)-producing neurons in the lateral hypothalamus (LatH). Thus, the ARC NPY neurons perceive complex afferent messages and propagate command signals for the integration of a spectrum of neuroendocrine and behavioral functions.

Information obtained from investigations across many disciplines to gain insight into the physiology, cellular biology, and molecular biology of this discrete population of ARC NPY neurons in the integration of energy homeostasis and reproduction is summarized below.

## III. NEUROPEPTIDE Y AND APPETITE

### A. Physiological Orexigen

Among a wide variety of orexigenic neurotransmitters isolated from the hypothalamus, NPY has been found to be the most potent appetite-stimulating signal. Abnormal NPY secretion, as seen in diabetic and genetically altered rodents, produces relentless hyperphagia and increased rate of weight gain, culminating over time in morbid obesity. In laboratory rodents and other mammals, NPY synthesis in the ARC perikarya and storage in nerve terminals in the PVN precede mealtime (Fig. 2). Subsequently, NPY release is triggered by photoperiodically driven neurogenic stimuli from the timing mechanism in the brain. However, in subhuman primates and humans, all these antecedent neurosecretory events are independent of the photoperiodic clock and are entrained to mealtime.

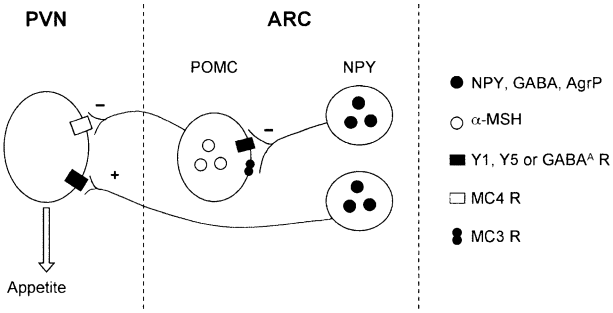

**FIGURE 2** A schematic representation of morphological and functional connectivities of neurons that co-express NPY, GABA, and AgrP in the ARC of the hypothalamus (referred to in this and subsequent figures as NPY neurons). These neurotransmitters/neuromodulators are released in the ARC and PVN to evoke appetite. A subpopulation of NPY neurons directly release NPY, AgrP, and GABA in the PVN to stimulate appetite via specific receptors. Another subpopulation of NPY neurons release NPY, AgrP, and GABA in the ARC to transsynaptically curtail the MC4 receptor-mediated restraint on appetite by attenuating the release of α-melanocyte hormone from POMC neurons. This curb on the tonic restraint on appetite is supplemented by AgrP-induced antagonism of MC4 receptors. For details, see text.

NPY release-generating signals evoke the synchronized discharge of high-amplitude episodes at a rapid pace in the PVN. This rhythmic NPY secretion initiates episodic feeding. NPY secretion subsides as animals consume a meal; however, in the absence of readily available food, the episodic NPY discharge persists unabated to sustain the food-seeking drive. This antecedent NPY release is indeed a physiological trigger for appetite as evidenced by findings that prior depletion of the readily releasable NPY stores in the PVN nerve terminals with antisense oligodeoxynucleotides, passive immunoneutralization of the released NPY with NPY antibodies, or blockade of NPY receptors with pharmacologic antagonists suppresses appetite. Although the daily feeding pattern persists in the complete absence of hypothalamic NPY in NPY null mutant mice, these mice do not display increased feeding in response to physiological challenges such as fasting and insulin deficiency, and the hyperphagia and obesity of leptin-deficient ob/ob mice are drastically reduced. Adaptive reorganization during early development within the hypothalamic appetite-regulating network and other hypothalamic orexigenic messengers produced elsewhere in the hypothalamus and co-expressed with NPY in the ARC compensate for NPY deficiency to reinstate the instinctive appetitive drive. Consequently, NPY is a physiologically relevant appetite transducer, and the altered frequency and amplitude of NPY discharge result in the disintegration of the hypothalamic control on energy homeostasis.

## B. Mechanism of Action

The orexigenic effects of NPY are mediated by the coordinated participation of three G-protein-coupled receptors, the $Y_1$, $Y_2$, and $Y_5$ receptors, in the ARC–PVN axis (Fig. 2). $Y_1$ and $Y_5$ receptor antagonists partially inhibit feeding, whereas germ-line deletions of any of these three receptors elicit varying degrees of altered feeding patterns and hyperphagia, modified response to varied diets, and obesity that manifests at different ages. Normally, a three-pronged interplay under the direction of NPY in the ARC–PVN axis initiates and extinguishes appetitive behavior. First, an increase in NPY and GABA release from the ARC NPY neurons in the PVN generates information to higher brain centers to evoke feeding. Concurrent release of AgrP from the ARC NPY neurons counteracts the tonic restraint exercised by melanocortin 4 (MC4) receptors and amplifies appetite (Fig. 2). Second, at the same time, increased NPY and GABA release within the ARC itself restrains POMC neurons, causing a decreased release of α-melanocyte-stimulating hormone (α-MSH) to diminish the tonic restraint on feeding. This timed and coordinated sequence of dual neurosecretory events, excitation by NPY and GABA and a curb on the tonic restraint on melanocortin signaling in the ARC–PVN axis, stimulates robust and sustained feeding (Fig. 2). Third, a gradual decrease in the release of NPY, GABA, and AgrP in the PVN, evoked by autofeedback through $Y_2$ receptors on NPY neurons in the ARC, inhibits feeding. Thus, the timely stimulation and termination of NPY release and co-expressed messengers in the hypothalamic ARC–PVN axis constitute an obligatory neurochemical signaling modality to stimulate and inhibit appetite in correlation with the meal pattern on a daily basis.

## C. Regulation of Release by Afferent Signals

A complex interplay among diverse neural and hormonal afferent signals under the direction of the central clock precisely times the release of orexigenic signals from NPY neurons in the ARC–PVN axis. The two major hormonal signals from the periphery, leptin from adipose tissues and ghrelin from stomach, restrain and augment, respectively, the ARC NPY efflux (Fig. 1). Neural afferents to the ARC NPY neurons from the ventromedial hypothalamus (VMH) relay inhibitory information generated by circulating leptin. A loss of this relay due to structural damage in the VMH results in hyperphagia and morbid obesity. Excitatory afferents from the LatH to ARC NPY neurons are relayed independently along the orexin and MCH neural pathways in response to reduced circulating leptin feedback. These neural pathways are strategically located to relay ascending visceral information via the spinal cord and brainstem to ARC NPY neurons for final processing and integration for energy homeostasis.

The most physiologically relevant afferent signals that regulate NPY secretion on a moment to moment basis are transmitted directly by the two functionally opposing afferent hormones, ghrelin and leptin (Fig. 1). Due to widespread action in the PVN, VMH, LatH, and other neighboring sites to produce tonic restraint, leptin is considered a major inhibitory signal. With this new understanding, it is possible to paint a clear picture of the sequential temporal interplay between hormonal afferent signals and hypothalamic effector pathways in governing the daily meal patterning. In anticipation of mealtime, the neural timing device, together with diminished leptin restraint from adipose tissue, stimulates high-frequency and high-amplitude ghrelin pulses from the stomach. This shift from inhibitory to excitatory afferents elicits NPY, GABA, and AgrP secretion from the ARC NPY effector pathway that, as described in the preceding section, propagates expression of hunger and the drive toward an energy source to replenish the depleted energy stores (Fig. 2).

Consumption of a meal reverses this chain of neural and hormonal events. First, there is a steady increase in leptin output, possibly stimulated by a gradual postprandial rise in pancreatic insulin secretion. As the tonic restraint by leptin on ARC NPY effector pathways is reinstated along with a steady decline in excitatory ghrelin generated by ingested food in stomach, appetite subsides. In the case of negative energy balance over long periods, such as that provoked by fasting, malnutrition, scarcity of food, or dieting, leptin secretion is drastically reduced but ghrelin output is markedly augmented. As a result of the concomitant suppression of tonic restraint and elevated stimulatory messages relayed by these functionally opposing hormonal afferents, NPY, GABA, and AgrP are hypersecreted to sustain the appetitive drive. Similarly, the high energy demands of lactation are met by hyperphagia induced by hypersecretion of ARC- and DMH-derived NPY in the PVN.

Afferent hormonal signals from other endocrine systems also employ NPY signaling in the hypothalamus to exert a modulatory effect on energy homeostasis (Fig. 1). Ovarian estrogens inhibit food intake in rodents by both decreasing NPY release in the PVN and increasing leptin secretion from adipocytes. A reduction of appetite and maintenance of reduced weight, consistently observed in response to estrogen therapy in women, are, therefore, likely a result of modified leptin–ARC NPY transmission. On the other hand, since adrenal glucocorticoids stimulate the production and release of hypothalamic NPY, it most likely contributes to hyperphagia and weight gain in patients on glucocorticoid therapy.

### D. Eating Disorders: Anorexia and Obesity

During infection, injury in the central nervous system, and other pathological afflictions, anorexia is the predominant condition that results in the loss of body weight and wasting. Under these pathophysiological conditions, the cytokines interleukin 1 and ciliary neurotropic factor are up-regulated. These cytokines efficiently down-regulate NPYergic signaling to produce anorexia and weight loss (Fig. 1). Indeed, NPY replacement therapy has proven beneficial in rodent models as it can counteract cytokine-induced anorexia and weight loss.

The incidence of obesity has increased to epidemic proportions worldwide. A large body of evidence endorses the view that genetic and environmental factors produce an imbalance in the tightly regulated feedback interplay between the peripheral leptin and ghrelin signals and the effector NPY neural pathways. This derangement promotes positive energy balance and enlarged fat tissue to store excess energy fuel. Basically, insufficient leptin restraint along with a rise in the excitatory ghrelin signal to the NPY network contributes to an environmentally induced increase in adiposity and morbid obesity. Recently, gene therapy to circumvent leptin insufficiency and to reinforce the restraint on NPYergic signaling has proven successful. A single injection into the hypothalamus of a nonpathogenic and nonimmunogenic adeno-associated virus vector encoding leptin reinstates the central tonic restraint and suppresses weight gain and adiposity for long periods in rodents consuming a diet rich in calories. This sustained efficacy of leptin gene therapy to suppress weight gain results from a voluntary reduction in food intake and increased energy expenditure. Both of these neural events, in turn, are elicited by a reduced efflux of orexigenic NPY and an increased efflux of anorexigenic α-MSH in the PVN.

## IV. NEUROPEPTIDE Y AND REPRODUCTION

### A. Anatomical and Functional Links with GnRH

Research spanning nearly two decades documents a crucial regulatory role of NPY in the hypothalamic control of reproduction. NPY is involved in the regulation of GnRH release, a primary brain peptide responsible for the maintenance of reproduction and sexual behavior in both sexes (Fig. 3). GnRH, produced by a network of neurons extending from the rostral septal–medial preoptic area (MPOA) to the ARC–ME caudally in the hypothalamus, stimulates the release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH is secreted into the hypophyseal portal veins in the ME in a pulsatile manner that represents an optimal mode of communication for pituitary gonadotropins to maintain reproduction in the two sexes (Fig. 3). Two oscillatory patterns of GnRH secretion have been observed in mammals. In general, GnRH is secreted in the form of low-amplitude pulses at more or less regular intervals throughout the 24 h period in males and through various stages of the reproductive cycle in females. These regularly spaced basal GnRH pulses in females are interrupted by an abrupt acceleration in the frequency and amplitude of GnRH discharge, culminating in the preovulatory LH surge release, an event that is essential for induction of ovulation (Fig. 3).

A distinct subpopulation of NPY neurons in the caudal ARC play a critical role in the excitation of pulsatile basal and cyclic GnRH discharge. NPY is also secreted in a pulsatile fashion in the ME with a frequency that coincides with that of GnRH. It acts synergistically with other hypothalamic excitatory signals, such as galanin and NE, to generate the basal GnRH pulses. In addition, NPY is a key excitatory signal for the initiation of the preovulatory secretion of GnRH. The sequential feedback actions of the ovarian steroids estrogen and progesterone on NPY neurons initially activate the synthesis of NPY in ARC perikarya and storage in terminal projections in the ME. This antecedent preparatory event culminates in the clock-driven hypersecretion of NPY, which, by a transsynaptic action on GnRH perikarya and dendrites in the MPOA and nerve terminals in the ME, generates a GnRH surge release into the hypophyseal portal veins (Fig. 3). Along with GnRH, NPY is also discharged into the hypophyseal portal vessels to potentiate the GnRH-induced preovulatory gonadotropin surge. Under certain

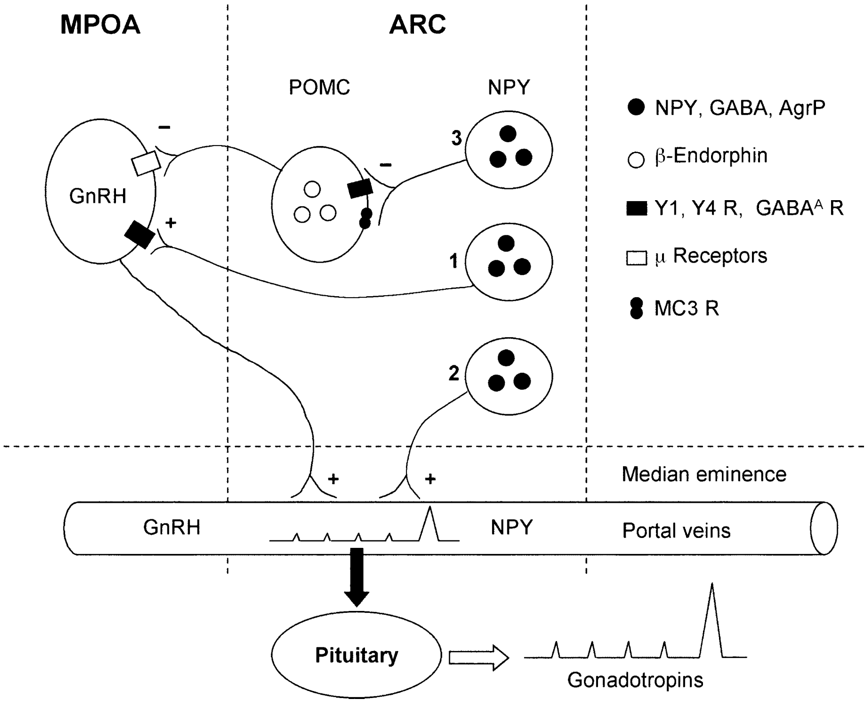

FIGURE 3 A schematic representation of morphological and functional connectivities of NPY-producing neurons in the ARC with the GnRH network in the MPOA and median eminence. NPY acts in three ways to modulate the basal and cyclic release of GnRH into the hypophyseal portal veins for transport to the pituitary gland to stimulate the release of gonadotropins: (1) Pulsatile discharge of NPY in the MPOA and median eminence stimulates GnRH release. (2) NPY is itself released into the hypophyseal portal veins to potentiate GnRH action in releasing pituitary gonadotropins. (3) NPY and GABA act transynaptically to inhibit opioid release from POMC neurons in the ARC. A decrease in tonic opioid restraint on GnRH secretion facilitates NPY-induced stimulation of GnRH and the preovulatory gonadotropin surge.

environmental insults and physiological challenges, disturbances in NPY pulse patterns inhibit both the basal and the cyclical discharge of GnRH, leading to depressed reproduction and infertility. For example, continuous NPY receptor activation produced by either NPY infusion or excessive endogenous NPY secretion, such as that manifested in genetically obese ob/ob mice and in response to fasting or diet restriction, suppresses reproduction. However, these adverse effects on reproduction correct themselves after normalization of NPY episodic signaling. In the complete absence of endogenous NPY, as in NPY null mutant mice, GnRH–LH surges are only partially attenuated because embryonic neuroendocrine reorganization compensates for the loss.

## B. Mechanism of Action

Morphological and experimental investigations have delineated distinct neural pathways mediating the excitatory and inhibitory effects of NPY on GnRH secretion (Fig. 3). NPY stimulates GnRH release on its own by acting through $Y_1$ receptors located on GnRH cell bodies in the MPOA and nerve terminals in the ME. This direct stimulatory action on GnRH is supplemented indirectly by modulation of the release of β-endorphin from POMC neurons by NPY. Normally, these endogenous opioids exert a tonic restraint on GnRH secretion. This restraint is curtailed by a coordinated interaction of NPY and GABA via $Y_1$ and $GABA^A$ receptors, respectively, on POMC neurons. Thus, an appropriately timed two-pronged action, the synergistic action of NPY with galanin (GAL), another excitatory neuropeptide produced in the hypothalamus, on GnRH neurons concurrent with a NPY–GABA-induced curb on opioid restraint, promotes GnRH secretion (Fig. 3).

On the other hand, experiments involving germline mutations of NPY receptors have identified NPY receptor subtypes that selectively participate in the inhibition of GnRH secretion by continuous NPY secretion. Under these conditions, overstimulation of $Y_1$ and $Y_4$ receptors located on POMC neurons in the ARC augments opioid restraint to turn off GnRH secretion. That these dual effects of NPY on GnRH secretion normally operate is endorsed by observations that a dampening of NPY signaling induced either by cytokine therapy or by germ-line deletion of NPY, $Y_1$, or $Y_4$ receptors in infertile ob/ob mice restores gonadotropin secretion and fertility.

## V. NEUROPEPTIDE Y AND NUTRITIONAL INFERTILITY

It has long been known that nutrition is one of the most important environmental factors to impact human reproduction. Rapid population growth and environmental degradation in the 20th century have severely diminished food resources worldwide. The chronic shortage of food in conjunction with droughts in underdeveloped nations has impacted fertility. Chronic undernourishment and short-term caloric imbalance retard the onset of puberty and diminish fertility in all mammalian species. Limited caloric intake alone or in concert with the energy demands of strenuous exercise adversely impacts reproductive function. Heavy energy demands during lactation are compensated for by hyperphagia and cessation of reproductive cycles. In several genetic models in rodents and humans, hyperphagia and attendant obesity are concomitant with disturbed reproductive cycles and infertility. Is there a commonality in neurochemical signaling that links the neuroendocrine control of energy homeostasis and reproduction and responds appropriately to challenges of varied nutritional environments?

The information in the preceding sections documents a pivotal role of the hypothalamic NPY pathway in governing the neuroendocrine control of energy homeostasis and reproduction. It is obvious that there is spatial and temporal specificity of NPY involvement in these two neuroendocrine functions. The neuroanatomical substrate engaged by the ARC NPY circuitry in regulating reproduction is the GnRH neuronal network resident in the MPOA–ARC–ME axis (Fig. 3). On the other hand, the NPY ARC–PVN pathway represents a final pathway for the synthesis, storage, and release of NPY and other appetite-regulating peptides (Fig. 2). The neural links with neighboring VMH and LatH regions are also important components of the hypothalamic orexigenic network in the daily management of energy homeostasis. Obviously, the neurochemical link between the circuitries that regulate reproduction and appetitive behavior lies in the commonality of the ARC as the source of NPY and other messenger molecules contacted by NPY neurons, all of which are vulnerable to internal and external environmental challenges of energy supply and hormonal imbalance (Fig. 4).

The intrinsic basal and cyclic patterns of NPY secretion in the MPOA–ARC–ME axis facilitate the secretion of the neurohormone GnRH to sustain reproduction. The rhythmic NPY secretion critical for imparting GnRH pulsatile secretion is modulated by the feedback action of gonadal steroids directly at the level of the ARC NPY subpopulation and the interconnected POMC–GAL pathways. An imbalance of gonadal steroid feedback produced by subnormal or inappropriate patterns of steroid production under nutritional stress disrupts the driving hypothalamic NPY GnRH signals, leading to depressed reproduction. Similarly, deficits in hypothalamic NPY synthesis and release during aging adversely affect GnRH secretion downstream, thereby leading to diminished reproductive and gonadal functions.

Aside from abnormalities in gonadal steroid feedback, deficits in energy fuels are sensed by the NPY network through an imbalance in leptin-ghrelin feedback (Fig. 4). A reduction in leptin feedback relieves the restraint on ARC NPY secretion, which, in concert with heightened stimulation by ghrelin,

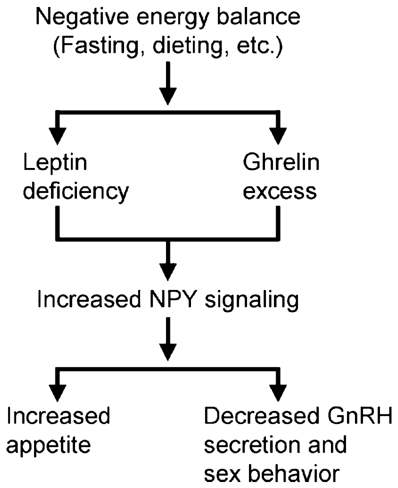

**FIGURE 4** Regulatory role of hypothalamic NPY in increasing appetite and concomitantly suppressing reproduction and sex behavior in response to negative energy balance. For details, see text.

up-regulates NPY secretion in both the orexigenic axis of the ARC–PVN and the reproductive MPOA–ARC–ME axis. The overall consequence is robust expression of appetitive behavior to replenish energy fuel through $Y_1$ and $Y_5$ receptors at targets both in the ARC and in the PVN. There is concurrent suppression of reproduction and sexual behavior as the inhibitory pathways involving $Y_1$ and $Y_4$ receptors on POMC and GnRH neurons and in other sites implicated in regulatory sexual behavior are mobilized. Thus, changes in information flow from the periphery to the NPY network due to severe depletion in energy fuels for short or long periods compromise the intricate communication in the neural network that evokes rhythmic GnRH–gonadotropin secretion. Fortunately, these deleterious effects on reproduction are transient because replenishment of energy fuels reverses these sequelae and reinstates the independent moment to moment control of NPY on energy homeostasis and reproduction. In sum, NPY is an essential messenger molecule in the hypothalamus that serves as a communication bridge between the neuroendocrine processes that regulate reproduction and those that maintain energy homeostasis.

## VI. SUMMARY

Information amassed during the past two decades affirms the concept that NPY produced by neurons in the ARC, a discrete subdivision of the hypothalamus, is an obligatory messenger molecule for commanding the two highly regulated innate drives in vertebrates: (1) appetite and the drive toward an energy source and (2) the urge to reproduce. A precise tracking of neural pathways originating in the ARC NPY perikarya has identified two distinct circuitries regulating each of these two hypothalamic regulatory functions. Within these circuitries, ARC NPY neurons, with the aid of intricate interconnections with other neurotransmitter/neuromodulator pathways within the hypothalamus and hormonal and neural afferent information from the periphery, transmit timed signals separately along these two circuitries. Subtle and progressive derangements provoked by environmental, genetic, and hormonal factors propagate molecular events governing the synthesis, release, and signal relay to reciprocally modify NPY transmission along the two circuitries. This commonality of neurochemical signaling has identified vulnerable loci for designing therapies to curb the epidemic of obesity and eating disorders without compromising fertility and, importantly, to alleviate nutritionally based infertility and reproductive disturbances.

## Glossary

**hypothalamus** Area at the base of the brain involved in maintaining body homeostasis and neuroendocrine control of pituitary hormone secretion and appetitive and sex behaviors. Various subdivisions of the hypothalamus, such as the median eminence, arcuate nucleus, ventromedial hypothalamus, lateral hypothalamus, paraventricular nucleus, and medial preoptic area, participate in the regulation of these varied functions.

**neurohormones** Hormones produced by neurons and released into the hypophyseal portal system in the median eminence of the hypothalamus for transport to the pituitary gland to stimulate or inhibit the release of hormones; for example, gonadotropin-releasing hormone stimulates gonadotropin secretion from pituitary gonadotrophs.

**neurotransmitters and neuromodulators** Chemicals produced by neurons to transmit messages to other neurons synaptically, for example, neuropeptide Y, γ-aminobutyric acid, agouti-related peptides, norepinephrine, α-melanocyte stimulating hormone, melanin concentrating hormone, and orexins.

**orexigen** Chemicals produced by cells to stimulate appetite.

**pituitary gland** An endocrine organ located ventral to the hypothalamus and connected to it neurally and by a specialized vasculature called the hypophyseal portal system.

**receptors** Proteins in target cells that avidly bind with specific hormones or neurotransmitters/neuromodulators to initiate intracellular signaling responsible for a biological response.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Eating Disorders • Growth Hormone-Releasing Hormone (GHRH) • Leptin Actions on the Reproductive Axis • Neuropeptides and Control of the Anterior Pituitary • Peptide YY**

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# Neurotensin

Michele Slogoff and B. Mark Evers

*University of Texas Medical Branch*

I. INTRODUCTION
II. THE NEUROTENSIN/NEUROMEDIN N GENE
III. THE NEUROTENSIN/NEUROMEDIN N PEPTIDE
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**Neurotensin, a peptide hormone that is produced in the hypothalamus, plays many important roles in regulatory and activation mechanisms in both the central nervous system and the gastrointestinal tract. Cloning of the neurotensin and closely allied genes and their receptors has provided insights into the physiological roles of these molecules, enhancing the potential for development of clinical/therapeutic applications.**

## I. INTRODUCTION

During the course of work to isolate the peptide substance P from bovine hypothalamus extracts, Carraway and Leeman discovered a new peptide that produced marked vasodilation of exposed cutaneous areas in rats. Sequencing of this novel peptide revealed a 13-amino-acid peptide that was named neurotensin (NT) because it was found in the brain and exhibited hypotensive activity. Subsequently, NT was isolated from the intestine, where it exhibits endocrine and possibly paracrine effects. In the central nervous system (CNS), NT has a neurotransmitter function and is involved in inhibition of dopaminergic pathways. In the gastrointestinal (GI) tract, NT affects GI motility, pancreatic and biliary secretion, and intestinal mucosal growth. Approximately a decade after the identification of NT, neuromedin N, a structurally related hexapeptide, was isolated from porcine hypothalamus. Neuromedin N has a peripheral distribution similar to that of NT and, in fact, has subsequently been found to be tandemly positioned with NT near the carboxy terminus of the precursor protein. A better understanding of the effects of NT in the CNS and the GI tract has been greatly facilitated by the cloning of the NT/neuromedin N (NT/N) gene, the cloning of the high- and low-affinity NT receptors, and the development of NT receptor antagonists. Moreover, the recent development of NT/N null mice by Dobner and colleagues will provide critical information regarding the precise physiologic role of NT in the CNS and GI tract.

The overview provided here primarily focuses on the most recent findings regarding the NT/N gene (structure, expression patterns, and molecular regulation), the NT/N peptide (structure, localization, and secretion), the NT receptors, and the central and peripheral effects of NT. Additional in-depth reviews on this subject are cited in the bibliography at the end of this article.

during embryologic differentiation. Between weeks 4 and 6 of gestation, cells destined to become the anterior lobe have budded off of what will become the roof of the mouth, organized into a spherical shape, and migrated upward toward the developing neural tube. This migration is halted by apposition to the base of the forebrain adjacent to the floor of the third cerebroventricle, which is the ventral aspect of the hypothalamus. At the same time, clusters of hypothalamic neurons are extending their axons downward toward the floor of the third ventricle, some stopping near the midline and others protruding out of the neural tube to lie alongside the developing anterior lobe (the. adenohypophysis), as a collection of axons terminating in what will be the posterior lobe (the neurohypophysis). Hypothalamic cells having axons that comprise the posterior lobe deliver two important neuropeptides to the general circulation, vasopressin and oxytocin. These neurons can be considered endocrine cells, because they release their peptides directly into the general circulation.

The cells that project to the floor of the third ventricle deliver neural products (i.e., peptides and biogenic amines) to a region that is invaded during the fifth and sixth weeks of gestation by mesenchymal elements (precursors of blood vessels), organizing into the vascular link between hypothalamus and the hormone-secreting cells of the anterior lobe. Those mesenchymal elements form the superior hypophyseal artery, a branch of the internal carotid artery, and this artery supplies blood to the hypothalamus and, via the portal vessels, the anterior lobe of the pituitary gland. The superior hypophyseal artery terminates in a series of fenestrated capillaries in the midline of the floor the hypothalamus, giving the tissue its characteristic tufted, vascular appearance. This protrusion on the ventral surface of the hypothalamus is called the median eminence and it is here that neuropeptides and biogenic amines access the portal vessel system for delivery to the anterior lobe. These neural factors are not released directly into the circulation, instead diffusing after release in the parenchyma of the median eminence through the fenestration of the capillary endothelial cells of the portal vessels and then out of those long portal vessels into the sinusoids of the adenohypophysis. Because these peptides are produced in neurons, but are delivered to their target tissue (the cells of the adenohypophysis) not via the general circulation but instead via a specialized circulation (the hypophyseal portal vessels), they are true neuroendocrine substances, as opposed to hormones per se. The superior hypophyseal arteries and thus the long portal vessels provide the majority of the blood flow to the adenohypophysis; the remaining supply comes from superficial, capsular arteries that arise from the inferior hypophyseal artery, which is a major source of flow to the posterior lobe.

Although only a minor component when compared to the contribution of the long portal vessels, a vascular connection between the posterior and the anterior lobes of the pituitary gland has been observed. These short portal vessels, formed from mesenchymal elements that give rise to the inferior hypophyseal artery, are an avenue by which the hormones (vasopressin and oxytocin) released into the general circulation draining the neurohypophysis might gain access to the cells of the adenohypophysis.

## III. HORMONE-SECRETING CELLS OF THE ANTERIOR PITUITARY GLAND

There are five major classes of hormone-secreting cells in the anterior pituitary gland. All are encapsulated in a dense collagenous matrix in the gland and are arranged in a sinsusoidal fashion adjacent to the thin-walled vascular elements. All five classes exhibit spontaneous (constitutive) secretory activity, but are controlled primarily by tropic factors delivered by the portal vessels (factors originating both in the periphery and in the hypothalamus). In addition, the secretory activity of these endocrine cells can be modulated by locally produced substances acting in a paracrine (after diffusion from neighboring cells) or autocrine (self) fashion. Tropic effects also are exerted by nonendocrine cells of the gland, particularly the folliculostellate cells found among the hormone-secreting cells.

Percentages of cell types in the gland vary with physiologic state, and cell size can similarly vary, dependent on physiologic state. For example, although lactotrophs (prolactin secreting cells) make up approximately 10–15% of the endocrine mass of the tissue under most conditions, during pregnancy and lactation they may contribute as much as 25% of the cell number. Hormone status in general also can affect the size of individual cell types. After menopause, when circulating gonadal steroid levels have fallen and their negative feedback effects are lost, gonadotrophs (cells producing luteinizing and follicle-stimulating hormones) increase in size (volume) by as much as two- to threefold.

In the past, cell types were classified by affinity for basic or acidic dyes; now, using more selective histologic techniques, cell types of the anterior pituitary gland are characterized by their hormone content (Table 1). The endocrine cells of the

**TABLE 1 Hormone-Secreting Cells of the Anterior Pituitary Gland**

| Cell type | Hormone content | Percentage of total cell number |
|---|---|---|
| Somatotroph | Growth hormone | 50% |
| Lactotroph | Prolactin | 10–25% |
| Corticotroph | Pro-opiomelanocortins (adrenocorticotropin, lipotropins, endorphins) | 15–20% |
| Thyrotroph | Thyroid-stimulating hormone | <10% |
| Gonadotroph | Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) | 10–15% |

adenohypophysis fall into two classes—those that produce and secrete unmodified protein hormones (prolactin, growth hormone, and adrenocorticotropin) and those that produce and secrete glycosylated proteins (thyroid-stimulating hormone, luteinizing hormone, and follicle-stimulating hormone).

## IV. REGULATION OF HORMONE SECRETION

Located beneath the thalamus at the ventral surface of the diencephalon forming the walls of the third cerebroventricle, the hypothalamus is small in size (about 4 g in adults) but is a major crossroad for emotional, autonomic, and endocrine circuitry within the brain. Afferents to the hypothalamus provide extero- and enteroceptive information that is then processed into the neural and hormonal signals responsible for maintenance of normal cardiovascular, renal, visceral, and endocrine function, as well as for the expression of appropriate and conditioned behavioral responses to the environment. Neurons in the hypothalamus are organized into dense clusters of cells called nuclei. These nuclei are integrative centers (microprocessors) that assess neuronal, and in some cases humoral, input and send afferent output (nerve fibers) to relay stations (other nuclei in brain) or to the region where the hypophyseal portal vessels form at the ventral surface of the hypothalamus, just at the point where the infundibular stalk penetrates the diaphragma sella. After diffusion into the portal vessels, these tropic substances control production and release of the anterior lobe hormones. Those tropic substances, which can be either small proteins (i.e., neuropeptides) or neurotransmitters such as dopamine or norepinephrine, can act to either inhibit or stimulate the release/production of hormones in the various anterior pituitary cell types.

### A. Growth Hormone

Growth hormone (GH), a protein hormone (191 amino acids) produced in somatotrophs of the adenohypophysis, circulates mainly free (i.e., not bound to carrier proteins) in plasma [plasma half-life ($t_{1/2}$) = 20 min; circulating levels are 2–4 ng/ml in adults (4–8 ng/ml in adolescents)]. Growth hormone acts to decrease blood amino acid levels, decrease blood urea nitrogen (positive nitrogen balance), increase DNA, RNA, and protein synthesis, decrease respiratory quotient due to increased fat oxidation, stimulate somatic growth, stimulate growth and calcification of cartilage, and, at high concentration, cause insulin resistance. Growth hormone secretion can be stimulated by exercise, arginine infusion, insulin-induced hypoglycemia, stress, dopamine, and α-adrenergics. Growth hormone is secreted in a pulsatile fashion, with a major daily secretory event occurring at the onset of sleep during Stages III and IV [non-rapid eye movement (REM) sleep]. Growth hormone secretion is inhibited by REM sleep, GH (autofeedback inhibition), insulin-like growth factors (long loop negative feedback), β-adrenergics, and hyperglycemia.

The hypothalamic component of feedback regulation of GH secretion has two major factors (Fig. 2). Growth hormone-releasing hormone (GHRH) is produced in neurons located in the arcuate nucleus and, above that, in the medial aspects of the ventromedial and dorsomedial hypothalamic nuclei. The predominant form of GHRH in humans is a 44-amino-acid peptide that is the final posttranslational product of a prohormone reported to be either 107 or 108 amino acids in length. GHRH is released into the median eminence in a pulsatile fashion, contributing thus to the pulsatile pattern of GH secretion. It acts by binding to a specific somatotroph receptor that is a member of the superfamily of G-protein-coupled, seven-transmembrane domain receptors. Binding results in activation of adenylyl cyclase, the formation of cyclic adenosine monophosphate (cAMP), an elevation in intracellular calcium levels, and both increased GH gene transcription and GH release. Non-cAMP-dependent signaling pathways may also mediate the effects of GHRH on the somatotroph.

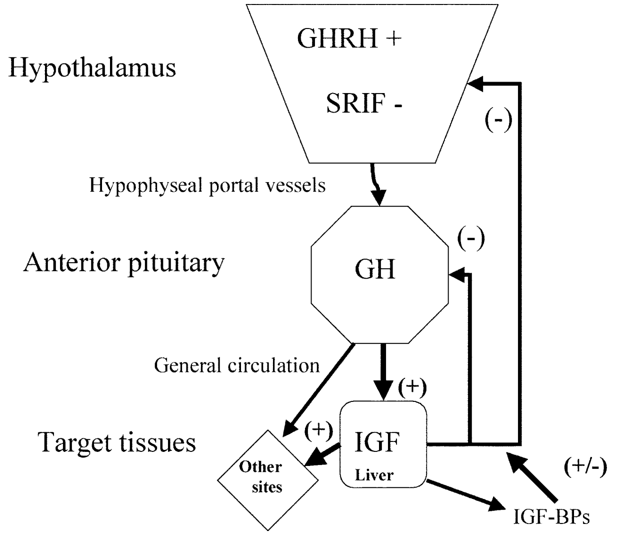

FIGURE 2 Hypothalamic regulation of growth hormone (GH) secretion. The 191-amino-acid hormone has a plasma half-life of 20 min; plasma levels are 2–4 ng/ml in adults and daily surges occur postprandially and at onset of sleep. GHRH, Growth hormone-releasing hormone; SRIF, somatotropin release-inhibiting factor; IGF, insulin-like growth factor; IGF-BPs, IGF-binding proteins.

Growth hormone release also is under inhibitory control, exerted by somatostatin produced in neurons of the anterior hypothalamus, adjacent to the ependymal lining of the third cerebroventricle (the periventricular zone). It, too, is produced by post-translational processing of a larger prohormone form (116 amino acids) and exists in both a 14- and a 28-amino-acid form. Somatostatin, or somatotropin release-inhibiting factor (SRIF), inhibits not only the release of GH, but thyroid-stimulating hormone and prolactin as well. The peptide exerts wide-ranging effects, most inhibitory, in a variety of other tissues. Multiple SRIF receptors have been identified, all being structurally related. In the pituitary gland, SRIF activates inhibitory G-proteins, resulting in decreased cAMP levels and lowered intracellular levels of free calcium. It acts to antagonize the actions of GHRH on adenylyl cyclase and blocks the effects of GHRH downstream of cAMP formation. The action to lower the cytosolic free-calcium levels is thought to be an important mechanism of action, mediated via an effect on voltage-gated calcium channels.

Studies utilizing opioid analogues have revealed the existence of a receptor mechanism, independent of GHRH and SRIF, that controls GH release. The endogenous ligand for that receptor was recently identified to be a novel 28-amino-acid peptide with a unique *n*-octanoyl modification of the serine in position 3 that is necessary for bioactivity. This peptide, called ghrelin, is produced in specialized endocrine cells of the gut, and some production in cells of the hypothalamic arcuate nucleus has also been reported. Thus ghrelin can access the adenohypophysis via the general circulation (from the stomach) or via the hypophyseal portal vessels following release into the median eminence. The ghrelin receptor is a G-protein-coupled, seven-transmembrane-spanning domain protein with homology to the motilin receptor. Binding of ghrelin results in activation of phospholipase C and the formation of inositol 1,4,5-trisphosphate ($InsP_3$). The physiologic relevance of the GHRH-like actions of ghrelin is still being established and the interactions between GHRH, SRIF, and ghrelin merit further scrutiny.

The nonhypothalamic control of GH release from the somatotroph includes, in addition to gut-derived ghrelin, the long loop negative feedback actions of the insulin-like growth factors (IGFs). Although GH can act directly to stimulate growth and metabolism in a variety of tissues, it is clear that its major actions are to stimulate the production and release of protein hormones that are structurally similar to insulin, hence the term IGFs. These peptide growth factors are produced primarily in liver, but also in bone, brain, prostate, and mammary tissues, where they can exert paracrine or true endocrine actions. In addition, the IGFs act in brain to inhibit GHRH release, and stimulate the release of SRIF, thus exerting long loop negative feedback on the hypothalamic regulation of GH secretion. The IGFs also can act directly in the adenohypophysis to antagonize the action of GHRH.

## B. Prolactin

Prolactin (PRL) is a protein hormone (198 amino acids.) produced in lactotrophs of the adenohypophysis; it circulates mainly free in plasma [plasma $t_{1/2} = 20–30$ min; plasma levels are 10 ng/ml in nonpregnant/nonlactating females (slightly lower in males and adolescents), slightly higher in lactating females, and 200–300 ng/ml during nursing]. PRL is cleared from the circulation in liver and kidney. The high catabolic clearance rate indicates very high synthetic capability of the lactotroph (45 ml/min/$m^2$). Pulsatile secretion occurs, with a minor circadian (daily) peak in early morning.

PRL was originally thought to be solely a hormone of reproduction. Indeed, PRL acts to stimulate mammary gland development (ductal and lobuloalveolar growth) and milk production in properly primed breast tissue. Prolactin also has been reported to organize the neural basis of maternal behaviors. In the adrenal cortex and ovary, PRL stimulates cytochrome P450 side chain cleavage enzyme (P450scc), resulting in the precursor for steroid synthesis. However, PRL has recently been recognized to have a broader spectrum of action, including immune modulation and potential actions on bone mineralization, vascular growth, and sodium homeostasis. Prolactin receptors belong both to a class of cell surface, tyrosine-kinase-linked receptors and to a class of intranuclear receptors (such as those for the steroid hormones). Promitogenic actions of PRL are related to both signaling pathways.

Prolactin secretion is stimulated by stress, hypothermia, tactile stimuli (vaginal or nipple stimulation), gonadal steroids (estrogens), and numerous neuropeptides (Fig. 3). The predominant hypothalamic regulation of PRL release is inhibitory in nature. Dopamine (DA), produced in the tubero-infundibular neurons of the arcuate nucleus, is released in a relatively constant fashion into the median eminence, and therefore PRL secretion is tonically inhibited. Dopamine exerts its inhibitory action on the lactotroph via binding to the $D_2$ subtype of the family of dopamine receptors. The receptor is another one of the G-protein-coupled, seven-transmembrane-spanning domain region proteins. Once activated by DA, the receptor links to a decrease in cellular levels of cAMP and inositol trisphosphates as well as to a decrease in intracellular calcium levels. The major cue for PRL release is the withdrawal of this inhibitory, dopaminergic tone, although stimulatory effects of some neuropeptides can be expressed even in the presence of physiologic levels of DA.

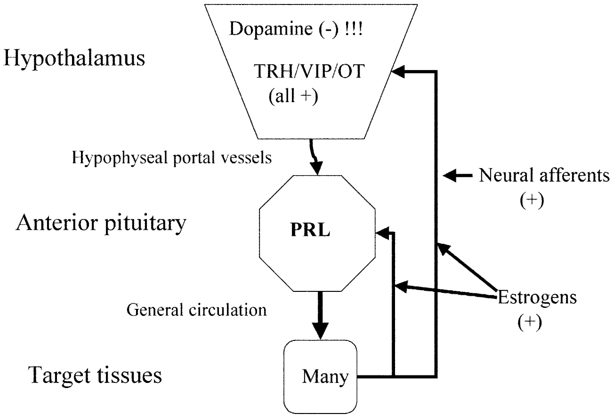

**FIGURE 3** Hypothalamic regulation of prolactin (PRL) secretion. The 198-amino-acid hormone has a half-life of 20–30 min; plasma levels are 10 ng/ml, except during pregnancy, laction, and stress. Minor daily surges occur in the morning. TRH, Thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; OT, oxytocin.

Stimulatory factors controlling PRL release originate in at least three locations. Estrogens of ovarian origin stimulate both PRL gene transcription and hormone release. Estrogens may also stimulate lactotroph proliferation by releasing a locally produced peptide, galanin, which then acts in a paracrine fashion within the gland. Neuropeptides of hypothalamic origin also can stimulate PRL release following delivery in the hypophyseal portal circulation. The most potent of these is the tripeptide, thyrotropin-releasing hormone (TRH), which is also the major regulator of TSH secretion. TRH stimulates PRL release even in the presence of physiologic, inhibitory levels of DA, a fact that separates it from the other peptidergic PRL-releasing factors (PRFs). TRH is the posttranslational product of a much larger prohormone that is produced in cells in the most medial aspects of the hypothalamic paraventricular nuclei. This is the major site of production that leads to peptide being delivered to the median eminence; however, TRH is produced in a variety of other sites. In the lactotroph, TRH binds to G-protein-coupled, seven-transmembrane-spanning domain receptors on the lactotroph, resulting in activation of phosphoinositide hydrolysis by phospholipase C and the subsequent activation of protein kinase C. The resulting elevation in intracellular free calcium levels certainly underlies the PRL secretory event, and perhaps even the elevation in cAMP levels that occurs concomitantly. Additional neuropeptides can stimulate PRL release from cultured pituitary cells and in some select situations *in vivo*. Both vasoactive intestinal polypeptide (VIP), a gut peptide also produced in neurons in the paraventricular nuclei, and oxytocin (OT), a neurophyseal hormone more traditionally considered part of the posterior pituitary system, can access the lactotroph following release into the median eminence and delivery by the portal circulation. Results from animal studies clearly demonstrate that both VIP and OT may be physiologically relevant PRFs, but this has not yet been established firmly in humans. OT may access the lactotroph via a second route, delivery by the short portal vessels connecting the posterior (neural) lobe with the adenohypophysis. In addition, there appears to be at least one additional factor (peptide?) of neural lobe origin that accesses the lactotroph via the short portal vessels, but the identity of this PRF has remained elusive.

## C. Adrenocorticotropic Hormone

Adrenocorticotropic hormone (ACTH; adrenocorticotropin) is derived from a large glycoprotein prohormone, pro-opiomelanocortin (POMC), which is produced in corticotrophs. Corticotrophs in the adenohypophysis produce mainly the 91-amino-acid protein β-lipotropic hormone (LPH; lipotropin) and the 39-amino-acid peptide ACTH. ACTH can be further processed to the 13-amino-acid long α-melanocyte-stimulating hormone (MSH), which stimulates pigment deposition in melanocytes. β-LPH can be further processed to β-endorphin (31 amino acids) and γ-LPH. Corticotrophs derived from the fetal intermediate lobe further process γ-LPH to β-MSH and produce γ-MSH from the N-terminal fragment of POMC.

ACTH circulates free in plasma with a half-life of 20 min and is cleared in kidney and liver. Levels under unstressed conditions are low (about 10 pg/ml) and secretion is pulsatile (20-min intervals); peak levels are present between 2:00 and 8:00 AM. ACTH stimulates activation of cytochrome P450 side chain cleavage enzyme, the rate-limiting step in cholesterol metabolism, and cholesterol esterase activities in the adrenal cortex, resulting in increased levels of glucocorticoids, mineralocorticoids, and adrenal androgens. At high levels, ACTH causes increased skin pigmentation because it also binds to the MSH receptor. ACTH acts via a membrane-bound receptor linked to adenylyl cyclase, increasing cAMP formation in hormone-producing cells of the adrenal cortex. The initial increase in cortisol secretion observed following ACTH infusion is due to increased adrenal blood flow and stimulation of conversion of cholesterol to pregnenolone (P450sccc activation). Prolonged actions of ACTH include stimulation of increased synthesis of the rate-limiting enzymes in adrenal steroidogenesis.

Secretion of ACTH is stimulated by stress, hypothermia, inflammatory substances (pyrogens), hypoglycemia, epinephrine (adrenaline), and several neuropeptides (Fig. 4). Cortisol from the adrenal cortex is the major inhibitory agent controlling ACTH production and release.

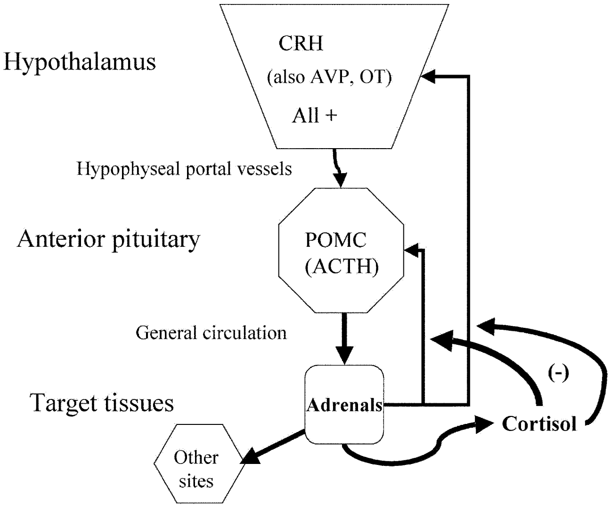

FIGURE 4 Hypothalamic regulation of adrenocorticotropic hormone (ACTH) secretion. The 39-amino-acid hormone has a half-life of 20 min; plasma levels are very low (picograms/milliliter) under nonstressed conditions. Secretion is pulsatile (20 min) and there is a daily surge between 2:00 and 8:00 AM. CRH, Corticotropin-releasing hormone; AVP, vasopressin; OT, oxytocin; POMC, pro-opiomelanocortin.

Neuroendocrine regulation of ACTH release is primarily stimulatory due to the neuropeptide, corticotropin-releasing hormone (CRH). CRH is a 41-amino-acid peptide produced in the parvocellular elements of the hypothalamic paraventricular nuclei. Actylcholine, serotonin, interleukins, and other cytokines all act to stimulate CRH release into the median eminence. At the corticotroph membrane, CRH binds a receptor similar to that for GHRH, resulting in activation, via G-protein coupling, of adenylyl cyclase. CRH also stimulates POMC gene transcription, probably via activation of protein kinase A. Within the hypothalamus, γ-butyric acid (GABA) and cortisol inhibit the activity of the CRH-producing neurons and therefore CRH release. Cortisol also acts directly in the anterior lobe to antagonize the action of CRH, resulting lower secretion and production of POMC-derived peptides. As was the case with PRL, there is credible evidence for the physiologic relevance of the ACTH-releasing actions of vasopressin (AVP) and oxytocin released into the median eminence or delivered via the short portal vessels; however, their role as ACTH secretagogues in humans has not been firmly established.

## D. Pituitary Glycoprotein Hormones

The three major pituitary glycoprotein hormones, thyroid-stimulating hormone, luteinizing hormone, and follicle-stimulating hormone, each having a molecular weight of approximately 30,000, are formed from two interconnecting amino acid chains. They all share a common α-chain but their unique β-chains give each hormone an individual, characteristic bioactivity.

### 1. *Thyroid-Stimulating Hormone*

Thyroid-stimulating hormone (TSH) circulates mainly free in plasma at levels of 1–4 ng/ml under normal conditions. TSH half-life is 50–60 min and it is cleared (degraded) primarily by the kidneys. Secretion is pulsatile, with a circadian rhythm characterized by highest levels between 9:00 PM and 5:00 AM and lowest levels between 4:00 and 7:00 PM. Physiologic surges occur in response to cold exposure (increases), heat (inhibits), starvation (inhibits), and stress (inhibits).

TSH stimulates thyroid hormone production and secretion by several mechanisms. Iodide transport into the thyroid gland is stimulated by TSH. TSH also stimulates thyroglobulin, iodotyrosine, and iodothyronine formation, and thus increases intracellular stores of thyroid hormones. Finally, TSH stimulates thyroglobulin proteolysis, as well as thyroxine (T4) and triiodothyronine (T3) release.

Hypothalamic control of TSH production and secretion (Fig. 5) is primarily exerted by the tripeptide thyrotropin-releasing hormone. Binding of TRH to its receptor (G-protein coupled) on the thyrotroph results not only in rapid secretion of TSH, but also in increased transcription of the β-subunit of TSH. These effects are thought to be signaled by TRH activation of adenylyl cyclase. There is also substantial evidence that TRH controls the final glycosylation (the addition of sugar moieties to the protein backbone of the hormone) of TSH, a step important for the bioactivity of the secreted product. Factors of hypothalamic origin (somatostatin and dopamine) can inhibit TSH secretion by a direct action in the adenohypophysis; however, by far the most significant negative regulation is exerted by T3 and T4 secreted by the thyroid gland (long loop negative feedback). The active agent in pituitary gland is T3, which either reaches the gland via the general circulation or is produced locally by conversion of T4. Triiodothyronine not only inhibits TSH gene transcription, it also down-regulates the TRH receptor.

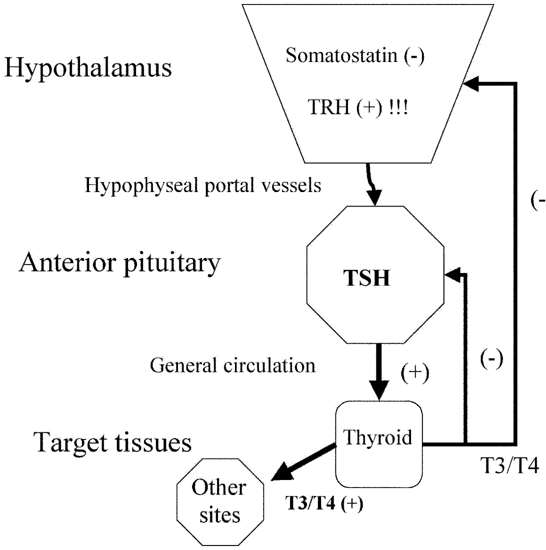

FIGURE 5 Hypothalamic regulation of thyroid-stimulating hormone (TSH) secretion. TSH is a glycoprotein ($M_w = 30{,}000$) with a half-life of 50–60 min; plasma levels are 1–4 ng/ml. Secretion is circadian, with peaks between 9:00 pm and 5:00 AM and a low between 4:00 and 7:00 PM. TRH, Thyrotropin-releasing hormone; T3, triiodothyronine; T4, thyroxine.

### 2. *Gonadotropins: Luteinizing Hormone and Follicle-Stimulating Hormone*

The 115-amino-acid-long β-subunits of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) confer unique biological activities to each hormone. The β-chain of LH is similar to that of human chorionic gonadotropin (hCG), thus common biologic activities are shared. Plasma levels of LH and FSH are expressed in terms of International Standard preparations (purified human hormones, as opposed to synthetic forms) and thus they are expressed as International Units (IU)/milliliter of plasma. LH and FSH both are secreted in a pulsatile fashion (circhorial in nature, about every 1–2 h) under the influence of gonadotropin-releasing hormone (GnRH), a 10-amino-acid peptide of hypothalamic origin. With the exception of puberty in males, when nocturnal pulses of GnRH entering the hypophyseal portal vessels cause increasing baseline and spike-like discharges of LH, gonadotropin levels remains fairly constant throughout adult life. Females also experience nocturnal gonadotropin pulses that increase in amplitude and frequency during puberty. During the reproductive years, the secretion of gonadotropins in women is circhorial (hourly) and low (basal LH, 0.8–26 mIU/ml; FSH, 1.4–9.6 mIU/ml). Monthly surges occur just prior to ovulation (LH, 25–57 mIU/ml; FSH, 2.3–21 mIU/ml). After menopause, gonadotropins are secreted in a circhorial pattern (LH, 1.3–13 mIU/ml; FSH, 0.9–15 mIU/ml).

In men, gonadotropin levels are low and fairly constant (LH, 1.3–13 mIU/ml; FSH, 0.9–15 mIU/ml). In women, gonadotropin levels fluctuate depending on stage of the menstrual cycle. After menopause, levels rise noticeably due to the absence of normal ovarian negative feed back (LH, 40–104 mIU/ml; FSH, 34–96 mIU/ml).

The plasma half-life of FSH is longer than that of LH (about 1 h for LH and 3 h for FSH) and the metabolic clearance rate for FSH (about 10 ml/min/m$^2$) is slower than that of LH (about 30 ml/min/m$^2$). These are at least two of the reasons why the midcycle surge of FSH is broader than that of LH. Both gonadotropins are degraded in the liver and kidneys, and some intact hormone is excreted in the urine. In females, LH acts in the ovary to stimulate follicular growth and rupture at ovulation. LH exerts luteotropic effects, stimulating estrogen and progesterone production. FSH also stimulates follicular growth. FSH further stimulates the conversion of androgens to estrogens by activating the enzyme aromatase in granulosa cells, and synergizes with estrogen to induce formation of LH receptors on those cells. In males, LH acts on Leydig cells to stimulate testosterone production. FSH induces LH receptors on those cells, and acts on Sertoli cells to stimulate spermatogenesis. Both LH and FSH signal through unique receptors that are coupled via G-proteins to adenylyl cyclase.

Neuroendocrine regulation of gonadotropin secretion (Figs. 6 and 7) is exerted primarily by the decapeptide hormone gonadotropin-releasing hormone (also called luteinizing hormone-releasing hormone, LHRH). GnRH binds to its cognate receptor (another G-protein-coupled, seven-transmembrane-spanning domain protein) and activates phosphoinositide hydrolysis (phospholipase C activity). The diacylglycerol formed activates protein kinase C and intracellular calcium levels rise, leading to stimulus–secretion coupling and release of LH and FSH. GnRH also stimulates transcription of the GnRH receptor gene and the LH and FSH genes.

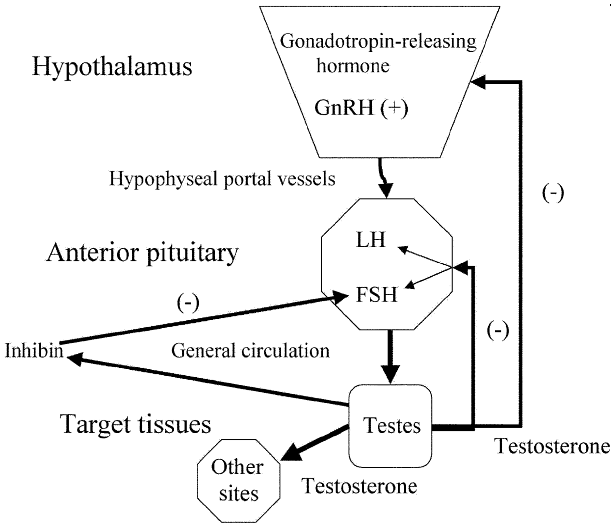

FIGURE 6 Hypothalamic regulation of gonadotropin [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] secretion in the male. LH half-life, 20–30 min; FSH half-life, 30–40 min; secretion of both hormones is low and constant. GnRH, Gonadotropin-releasing hormone.

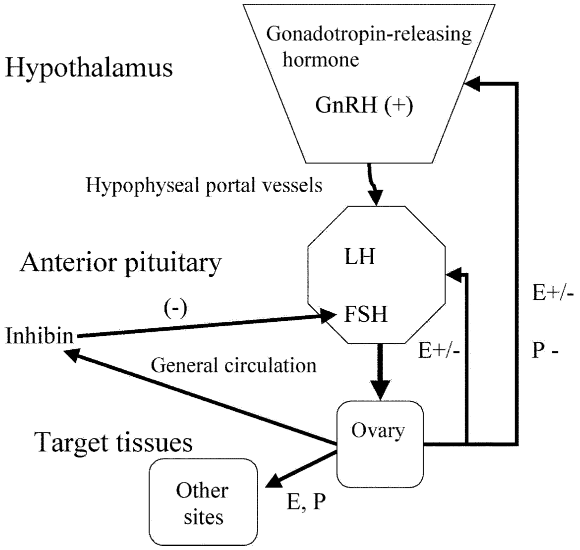

FIGURE 7 Hypothalamic regulation of gonadotropin [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] secretion in the female. LH half-life, 20–30 min; FSH half-life, 30–40 min; secretion is pulsatile, occurring hourly or monthly. GnRH, Gonadotropin-releasing hormone; E, estrogen; P, progesterone.

Sex steroids exert both positive and negative feedback effects on the hypothalamus and pituitary gland. In females, estrogen at low levels inhibits both LH and FSH secretion, whereas at higher levels the steroid actually stimulates LH β-chain gene transcription and sensitizes the gonadotroph to the actions of GnRH. Progesterone negative feedback is expressed primarily at the level of the GnRH neuron, decreasing pulse frequency of release of the decapeptide into the median eminence. In males, testosterone exerts negative feedback effects in the hypothalamus, similar to those exerted by progesterone in females, and direct inhibitory actions at the pituitary level, similar to those of estrogen in women.

Granulosa cells in the ovary, and Sertoli cells in the testes, also produce a heterodimeric hormone, composed of unique α-and β-subunits, that acts in the gonadotroph to inhibit FSH production and release. The production of this heterodimeric hormone, called inhibin, is in turn stimulated by FSH. It is in all likelihood the loss of inhibin production after menopause that explains the greater rise in plasma FSH levels, compared to LH levels, in the absence of ovarian feedback. Finally, it appears that a homodimer of the inhibin β-chain is produced in several tissues, including the pituitary gland. This homodimer, called activin, is thought to stimulate FSH production and secretion.

## V. SUMMARY

The unique vascular connection between the hypothalamus and pituitary gland established by the experiments of Jacobsen and Harris over 50 years ago is the anatomical basis for the science of neuroendocrinology. Peptidergic releasing and inhibiting factors produced in brain neurons, most localized to the hypothalamus, gain access to their target cells in the anterior lobe of the pituitary gland via this system of portal vessels. The control of peptide release into the median eminence is in turn controlled by neural afferents to the hypothalamus and by blood-borne substances that either cross the blood–brain barrier to act within the hypothalamus or act in the median eminence on the axon terminals containing those peptides, because there is no barrier at this vascularized site. Additional regulation of anterior lobe hormone secretion is determined by direct actions of those circulating hormones (long loop negative and positive feedback) and by intrinsic factors acting in paracrine or autocrine fashion within the adenohypophysis.

## Glossary

**adenohypophysis** The anterior pituitary gland.

**autocrine** Self-regulation; the release from a cell of a tropic factor that binds to and alters the activity of that same cell.

**median eminence** Floor of the third cerebroventricle in the hypothalamus; site of the fenestrated endothelium of the capillary loops of the portal vessels.

**neuroendocrine** Regulation by neural factors reaching the anterior pituitary gland via the portal vessels.

**neurohypophyseal** Neuronal projections from the supraoptic and paraventricular hypothalamic nuclei to the posterior pituitary gland.

**neurohypophysis** The posterior pituitary gland.

**paracrine** Regulation of neighboring cells by a tropic factor that, when released from one cell, diffuses to and acts on another cell.

**portal vessels** Venous system connecting the capillary loops formed by the superior hypohyseal artery in the median eminence of the hypothalamus with the sinusoids of the anterior pituitary gland.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Amino Acid and Nitric Oxide Control of the Anterior Pituitary • Cytokines and Anterior Pituitary Function • Follicle Stimulating Hormone (FSH) • Ghrelin • Growth Hormone (GH) • Growth Hormone-Releasing Hormone (GHRH) • Luteinizing Hormone (LH) • Neuropeptide Y (NPY) • Prolactin (PRL) • Thyroid Stimulating Hormone (TSH)**

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# Neuropeptide Y (NPY)

Satya P. Kalra and Pushpa S. Kalra
*University of Florida*

I. INTRODUCTION
II. NEUROANATOMY
III. NEUROPEPTIDE Y AND APPETITE
IV. NEUROPEPTIDE Y AND REPRODUCTION
V. NEUROPEPTIDE Y AND NUTRITIONAL INFERTILITY
VI. SUMMARY

## II. THE NEUROTENSIN/NEUROMEDIN N GENE

### A. Structure

The NT/N gene is highly conserved among species. The cDNA encoding the NT/N gene was first cloned from a canine enteric mucosal library by Dobner and colleagues. Using the canine cDNA probe, the rat NT/N gene was then isolated by screening a rat genomic library. The NT/N gene spans approximately 10.2 kb and is divided into four exons and three introns. The NT and neuromedin N domains are tandemly arrayed on exon 4. Subsequent cloning of the human NT/N gene demonstrated an open reading frame encoding a predicted precursor protein of 170 amino acid residues (Fig. 1). At the nucleotide level, the human NT/N precursor is 92, 91, and 81% identical to canine, bovine, and rat NT/N, respectively; the resulting precursor peptide is similarly conserved (Fig. 2).

### B. Expression

NT/N gene expression in the adult is predominantly localized to the small bowel, with increased expression noted in the ileum. In the adult, NT/N gene expression is not detected in the pancreas, stomach, or colon (Fig. 3). Interestingly, unlike the brush border enzymes (e.g., sucrase-isomaltase), the gradient of NT/N expression is not altered by changing the lumenal contents or the transposition of the intestine to other areas along the longitudinal gut axis. These findings suggest an intrinsic program of NT/N gene expression in the gut that is not affected by positional or nutrient alterations.

```
    CGGACTTGGCTTGTTAGAAGGCTGAAAG ATG ATG GCA GGA ATG  43
                                 Met Met Ala Gly Met

AAA ATC CAG CTT GTA TGC ATG CTA CTC CTG GCT TTC AGC  82
Lys Ile Gln Leu Val Cys Met Leu Leu Leu Ala Phe Ser

TCC TGG AGT CTG TGC TCA GAT TCA GAA GAG GAA ATG AAA 121
Ser Trp Ser Leu Cys Ser Asp Ser Glu Glu Glu Met Lys

GCA TTA GAA GCA GAT TTC TTG ACC AAT ATG CAT ACA TCA 160
Ala Leu Glu Ala Asp Phe Leu Thr Asn Met His Thr Ser

AAG ATT AGT AAA GCA CAT GTT CCC TCT TGG AAG ATG ACT 199
Lys Ile Ser Lys Ala His Val Pro Ser Trp Lys Met Thr

CTG CTA AAT GTT TGC AGT CTT GTA AAT AAT TTG AAC AGC 238
Leu Leu Asn Val Cys Ser Leu Val Asn Asn Leu Asn Ser

CCA GCT GAG GAA ACA GGA GAA GTT CAT GAA GAG GAG CTT 277
Pro Ala Glu Glu Thr Gly Glu Val His Glu Glu Glu Leu

GTT GCA AGA AGG AAA CTT CCT ACT GCT TTA GAT GGC TTT 316
Val Ala Arg Arg Lys Leu Pro Thr Ala Leu Asp Gly Phe

AGC TTG GAA GCA ATG TTG ACA ATA TAC CAG CTC CAC AAA 355
Ser Leu Glu Ala Met Leu Thr Ile Tyr Gln Leu His Lys

ATC TGT CAC AGC AGG GCT TTT CAA CAC TGG GAG TTA ATC 394
Ile Cys His Ser Arg Ala Phe Gln His Trp Glu Leu Ile

CAG GAA GAT ATT CTT GAT ACT GGA AAT GAC AAA AAT GGA 433
Gln Glu Asp Ile Leu Asp Thr Gly Asn Asp Lys Asn Gly

AAG GAA GAA GTC ATA AAG AGA AAA ATT CCT TAT ATT CTG 472
Lys Glu Glu Val Ile Lys Arg Lys Ile Pro Tyr Ile Leu

AAA CGG CAG CTG TAT GAG AAT AAA CCC AGA AGA CCC TAC 511
Lys Arg Gln Leu Tyr Glu Asn Lys Pro Arg Arg Pro Tyr

ATA CTC AAA AGA GAT TCT TAC TAT TAC TGAGAGAATAAATCA 553
Ile Leu Lys Arg Asp Ser Tyr Tyr Tyr

TTTATTTACATGTGATTGTGATTCATCATCCCTTAATTAAATAT        597
CAAATTATATTTGTGTGAAAATGTGACAAACACACTTATCTGTC        641
TCTTCTACAATTGTGGTTTATTGAATGTGTTTTTCTGCACTAAT        685
AGAAATTAGACTAAGTGTTTTCAAATAAATCTAAATCTTCAAAA        729
AAAAAAAAAAAAAAATGGGGCCGCAATT                        756
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**FIGURE 1** Human full-length cDNA clone of NT/N and predicted amino acid sequence of human prepro-NT/N. The neuromedin N and NT coding regions, located in tandem on exon 4, are highlighted in black. Modified from Dong *et al.* (1998), with permission from the *American Journal of Physiology.*

During development, expression of NT/N is noted in a well-defined spatial- and temporal-specific pattern. The earliest NT/N expression in fetal rats, identified by sensitive reverse transcriptase and polymerase chain reaction (RT-PCR) analysis, is

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Human:   MMAGMKIQLVCMLLLAFSSWSLCSDSEEEMKALEADFLTNMHTSKISKAHVPSWKMTLLNV  61
Bovine:   -----------+---------------------+-L------------S------+----  60
Dog:     ------------+-----------------------L------------S-+---------  61
Rat:      -I--++----+T--------------+++------L-----+--+--+SP----------  60

Human:    CSLVNNLNSPAEETGEVHEEELVARRKLPTALDGFSLEAMLTIYQLHKICHSRAFQHWEL 121
Bovine:   ---+-----Q------F-----++---F-+----------------+------------- 120
Dog:      --F------Q------F+----++---F------------------+---------+--- 121
Rat:      ---+-----+---+--+++++---+----LV--D-----+--++--+---+--------+ 120

Human:   IQEDILDTGNDKNGKEEVIKRKIPYILKRQLYENKPRRPYILKRDSYYY            170
Bovine:  -------+-----E------------------------------G----            169
Dog:     ----+--+-----E------------------------------G----            170
Rat:     -------H--+-TE------------------------------A----            169
```

**FIGURE 2** Analysis and comparison of the putative amino acid sequence of human NT/N to known sequences of the cow, dog, and rat. −, Identical sequences; +, similar sequences. Modified from Dong *et al.* (1998), with permission from the *American Journal of Physiology.*

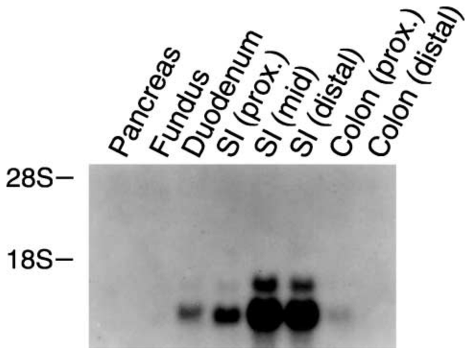

**FIGURE 3** Localization of NT/N mRNA in the rat. Rat NT/N cRNA was used to probe 10 μg of polyadenylated RNA. Northern blots are from the pancreas, fundus of the stomach, duodenum, three equal segments of small intestine (SI), and two equal segments of colon. Adapted from Evers *et al.* (1991), with permission from *Surgery*.

noted in the primitive foregut at 12 days of gestation. In early fetal development, expression of the NT/N gene is detected in multiple tissues, including colon, pancreas, liver, and stomach; this expression is transient and disappears by the end of the first week of life. In the intestine, NT/N expression is low in both the jejunum and the ileum of fetal rats; steady-state NT/N mRNA levels rise rapidly after postnatal day 1 to assume the adult topographical distribution of increasing NT/N expression along the jejunoileal axis of the small bowel. By postnatal day 28, the pattern of expression is that of an adult, with no expression detected in the pancreas, liver, and stomach, minimal expression in the jejunum, and the majority of NT/N gene expression in the ileum. Developmental expression of NT/N in humans follows a similar pattern; NT/N expression is found in the fetal colon and liver but is not apparent after 24 weeks of gestation. This widespread distribution of NT/N expression in the developing GI tract suggests the presence in the primitive gut of a shared ancestral stem cell that is capable of multidirectional differentiation.

In addition to the fetal colon expression of NT/N during a gestational stage in which the colon resembles the small bowel, expression of NT/N has been detected in human colon cancers. Approximately 25% of freshly resected colon cancers demonstrate NT/N gene expression, although NT/N expression is not apparent in the adjacent normal mucosa. Furthermore, NT/N gene expression has been detected in human colon cancer cell lines. Similar to the expression of sucrase-isomaltase or carcinoembryonic antigen (CEA), the re-expression of NT/N in certain colorectal cancers further suggests the reversion to a more fetal intestinal pattern. Similarly, NT/N expression has been noted in a variant of hepatocellular cancer (i.e., fibrolamellar cancer) as well as a nonfibrolamellar hepatocellular cancer (Hep3B). Taken together, these findings demonstrate a complex pattern of NT/N expression with well-localized and highly regimented expression in the adult GI tract, a more diffuse expression during fetal development, and re-expression in certain cancers.

## C. Molecular Regulation

We have analyzed the molecular factors regulating the constitutive expression of NT/N using the BON endocrine cell line, which was derived from a functioning human carcinoid tumor and established in our laboratory. BON cells, like the terminally differentiated N cells of the small bowel, express high levels of NT/N mRNA, synthesize and secrete NT peptide, and process the NT/N precursor protein in a fashion identical to that of the N cells. Transient transfection assays using the rat NT/N promoter identify the proximal 216 base pairs (bp) of 5′ flanking sequences essential for high-level constitutive NT/N expression. A critical element located in the proximal NT/N promoter binds both activator protein 1 (AP-1) and cAMP-responsive enhancer binding (CREB)/activating transcription factors (ATF) and is critical for NT/N expression in BON cells. Mutation of the cAMP response element (CRE)/AP-1 site almost completely abolishes NT/N expression. This functional "cross talk" between different transcriptional pathways converging on a single binding site within a promoter greatly enhances the combinatorial possibilities of these transcription factors to regulate gene expression. Dobner and colleagues have also shown that elements contained within the proximal 216 bp are important for inducible NT/N gene expression. In contrast to constitutive NT/N expression in BON cells, the elements responsible for NT/N gene induction in the rat pheochromocytoma cell line, PC12, involve the cooperation of multiple elements of the NT/N promoter, including a distal consensus AP-1 site, the proximal CRE/AP-1 site, a near-consensus CRE site, and a near-consensus glucocorticoid response element (GRE) (Fig. 4). Therefore, these results suggest that a different array of regulatory elements is required for the constitutive NT/N expression pattern in the gut, in which NT functions as an endocrine agent, compared with NT/N gene induction in the CNS,

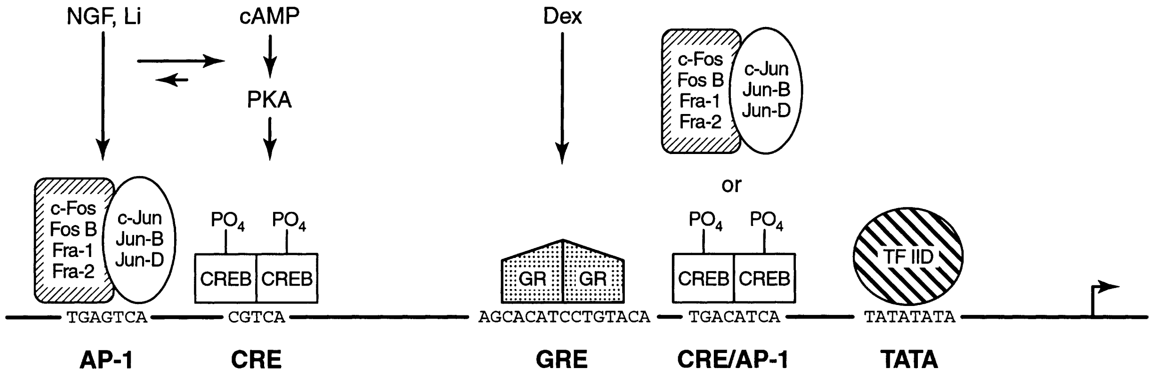

FIGURE 4 Schematic for cooperative regulation of NT/N gene transcription. NGF, Nerve growth factor; AP-1, activator protein 1; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; CREB, CRE binding protein; GRE, glucocorticoid response element; TF IID, transcription factor IID. Adapted from Dobner *et al.* (1992), with permission from *Ann. N.Y. Acad. Sci.*

in which NT serves as a neurotransmitter or neuromodulator.

The signal transduction pathways resulting in subsequent NT/N expression are also under investigation. In particular, the role of the Ras signaling pathway has been examined. Investigators in our group have shown that Ras (both wild type and activated) enhances expression of the NT/N gene in the Caco-2 human colon cancer cell line, most likely acting through the proximal CRE/AP-1 site. Furthermore, overexpression of Src kinase, another signaling protein in the Ras pathway, is associated with an increase in NT/N promoter activity. Mutation of the proximal promoter element inhibits Ras-mediated NT/N induction. Ras and Src can stimulate the binding activity of AP-1 proteins (e.g., c-Jun); therefore, we speculate that Ras, acting through AP-1 proteins, induces NT/N gene expression.

In addition to transcription factor binding, gene methylation regulates expression of various genes. Methylation appears to play a critical role in the expression of a number of genes during both normal development and malignant transformation. Investigators in our laboratory have shown, by complementary approaches, that DNA methylation plays a role in NT/N gene suppression in certain hepatocellular and colon cancer cell lines. Treatment with a demethylating agent, 5-azacytidine, partially activates the suppressed NT/N gene in the HepG2 hepatocellular cancer. Moreover, our group has shown that DNA methylation plays a role in NT/N gene silencing in the human colon cancer KM20 and that NT/N expression in the KM12C cell line is associated with demethylation of CpG sites in the NT/N promoter. These studies suggest that methylation status may be directly related to NT/N reexpression in certain cancers and may account for gene repression during development. In addition, we speculate that gene methylation may play a role, in combination with transcription factors, in providing the strict tissue-specific regulation of NT/N gene expression noted in various tissues.

## III. THE NEUROTENSIN/NEUROMEDIN N PEPTIDE

### A. Structure

NT is a peptide composed of 13 amino acids. The amino terminus is a pyrrolidone carboxylic acid and resists proteolytic degradation; the inactive degradation product is NT(1–8). The bioactive core, NT(9–13), resides in the carboxy terminus, which is highly conserved among species. The metabolism of NT(1–13) is rapid; therefore, the majority of circulating NT is in the form of the stable $NH_2$-terminal fragments, primarily NT(1–8) or, less commonly, NT(1–11). The neuromedin N peptide is structurally related to NT, with conservation of the carboxy terminus. Although distribution of neuromedin N is similar to that of NT, the effects of neuromedin N and NT may be different. NT is not lipophilic and thus does not penetrate the blood–brain barrier.

### B. Localization

In the GI tract, the NT peptide is localized to enteroendocrine cells (i.e., N cells), predominantly in the small bowel and proximal colon. Similar to the expression pattern of the NT/N gene, N cells are found in greatest abundance in the distal ileum mucosa. NT immunoreactivity has also been identified in the muscle layers of the gut, mostly in the myenteric plexus. Presumably, in this location, NT functions as a neurotransmitter as it does in the CNS. Within the brain, NT immunoreactivity is primarily located in the substantia nigra, periaqueductal gray matter, amygdala, nucleus accumbens, and some hypothalamic nuclei; lower concentrations are found in the caudate, hippocampus, and globus pallidus.

### C. Secretion

The precise signaling mechanisms regulating NT secretion are not entirely known. Fat in the proximal intestine appears to be the most potent stimulus. The initial NT release occurs within 10 min of eating, long before chyme reaches the ileum. Studies have shown that infusion of fat into the jejunum leads to an increase in NT release, whereas infusion of fat into the ileum does not. However, ileal resection abolishes release of NT. Atropine also abolishes this response but truncal vagotomy does not. These findings suggest that NT is released from the ileum via a humoral or neural stimulus from the proximal intestine. Furthermore, the process appears to be cholinergic, but not vagally mediated.

In addition to lipids, *in vitro* experiments using short-term ileal mucosal cell cultures demonstrate that the peptide bombesin directly stimulates NT release that is not abolished by atropine (in contrast to lipid-stimulated release). Catecholamines appear to stimulate NT release via a β-adrenergic receptor. Carbachol stimulates NT release in segments of perfused ileum but has no effect on basal NT release in short-term N cell cultures. In fact, carbachol inhibits β-adrenergic-stimulated NT release; the possible mechanism for these actions has yet to be elucidated. Similarly, substance P stimulates release of NT in neurons and in perfused ileum but not in short-term N cell cultures. Taken together, the data imply that there is more than one pathway affecting NT release (at least one pathway is cholinergically mediated and another is not) and that other mechanisms in the intact organism are required for the activation of release of NT by certain mediators.

## IV. NEUROTENSIN RECEPTORS

After secretion, the NT peptide must bind to a receptor in order to produce an effect. Three NT receptors have been identified. The first two receptors, the high-affinity NTS1 and low-affinity nts2, are G-protein-coupled receptors that differ slightly in structure. The recently identified third receptor, nts3, has an altogether different structure compared with NTS1 and nts2.

### A. NTS1 Receptor

The high-affinity NTS1 receptor is sensitive to $Na^+$ ions and guanosine triphosphate (GTP); both substances decrease the affinity of the receptor for NT. This high-affinity receptor is insensitive to levocabastine, an antihistamine-1 that blocks lower affinity binding sites. The NTS1 gene has been sequenced and encodes 424 amino acids (in rats) and 418 amino acids (in humans). The protein has seven transmembrane domains and is considered a G-protein-coupled receptor (Fig. 5). Expression of the NTS1 gene has been identified in the brain and intestine of rats and humans. *In situ* hybridization studies demonstrate high levels of expression in the diagonal band of Broca, medial septal nucleus, nucleus basalis magnocellularis, suprachiasmatic nucleus, supramammillary area, substantia nigra, and ventral segmental area in the brain. In the GI tract, the NTS1 receptor has been identified throughout the small intestine, large intestine, and liver. Following binding with NT, 60–70% of the NTS1 receptor internalizes in a temperature-dependent process; the receptor is not recycled to the cell membrane. After NT binds to this G-protein-coupled receptor, phospholipase C is activated, inducing phosphatidylinositol turnover and mobilization of intracellular calcium. Recent studies have shown that NT binding increases activation of extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK) (kinases in the Ras pathway). These findings suggest that, like other GI peptides (e.g., cholecystokinin and gastrin), NT activates the Ras pathway via its NTS1 receptor. These observations implicate the Ras pathway in the subsequent downstream effects of NT. Two nonpeptide receptor antagonists synthesized by Gully and colleagues effectively bind NTS1 and prevent many of the actions of NT. SR48692 has been shown to block a number of peripheral and central effects of NT but does not appear to inhibit the hypothermic and analgesic effects of NT. This antagonist binds to a binding site separate from but overlapping that of the NT peptide, thus blocking a portion of the receptor crucial for NT binding. Another antagonist, SR142948A, has been found to have a broader spectrum of activity and also inhibits the

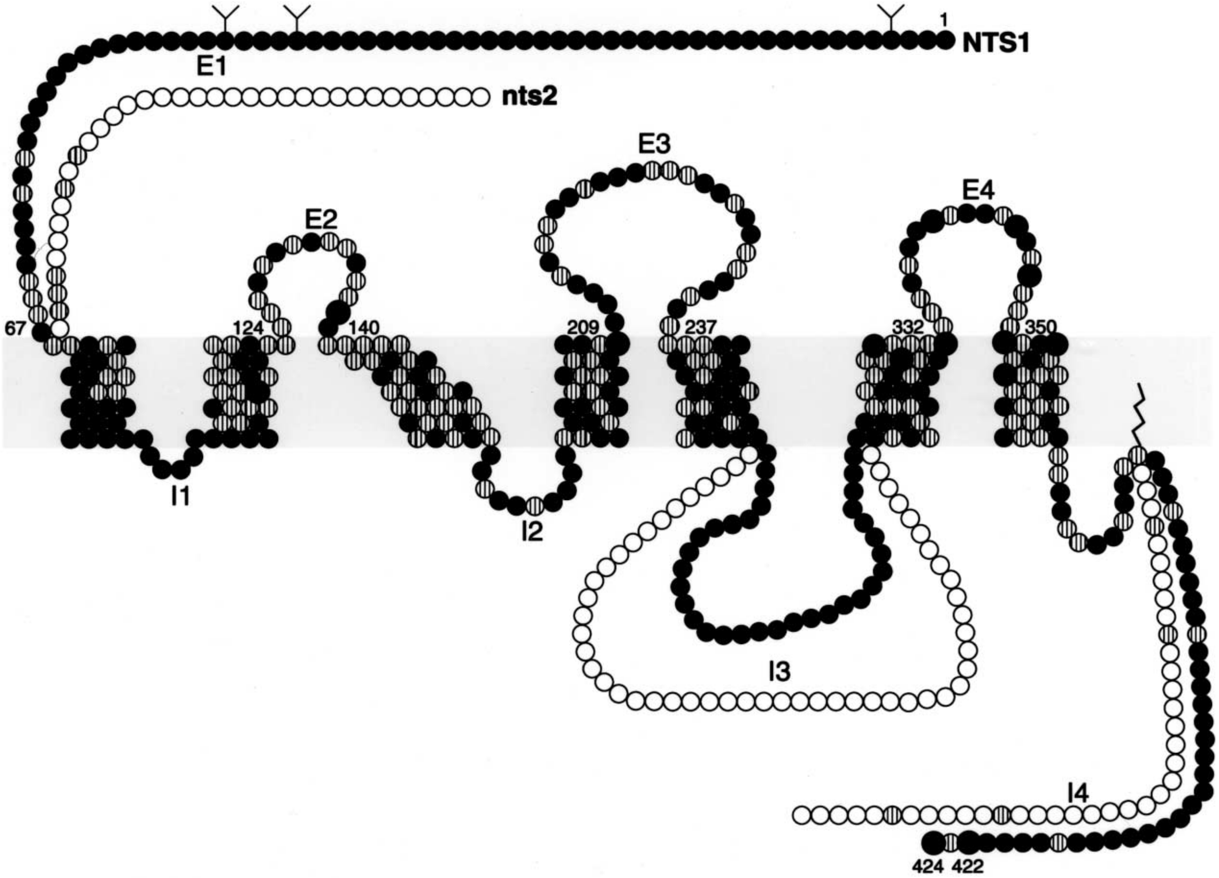

**FIGURE 5** Comparison of rat neurotensin receptors NTS1 (•) and nts2. The invariant residues between the NTS1 and nts2 receptors are noted by circles with vertical lines. Regions of the nts2 receptor that vary greatly from NTS1 and are noted by open circles. The N-terminus (1) and C-terminus (424) and the first residues of each extracellular segment of the NTS1 receptor are numbered. Glycosylation sites are highlighted (Y). Residues 422 and 424 are crucial for NTS1 internalization. The third intracellular loop (13) contains a region essential for coupling to phospholipase C. Adapted from Vincent *et al.* (1999), with permission from *Trends Pharmacol. Sci.*

hypothermia and analgesia induced by intracerebroventricular injection of NT.

## B. nts2 Receptor

The nts2 receptor is also a G-protein-coupled receptor with seven transmembrane domains. This receptor, in contrast to NTS1, is a low-affinity receptor that can be blocked by levocabastine and is relatively insensitive to $Na^+$ ions and GTP. The replacement of Asp by Ala or Gly in the second transmembrane domain is believed to be responsible for the decreased affinity of the receptor. This receptor has been localized mostly in the brain. It is specifically expressed in the olfactory system, cerebral and cerebellar cortices, hippocampal formation, and certain hypothalamic nuclei. The nts2 receptor is implicated in NT-induced analgesia. For example, intracerebroventricular injection of antisense oligonucleotides, which specifically block nts2 expression, inhibits NT-induced analgesia. These oligonucleotides have no effect on other receptors. Furthermore, SR48692, which does not block nts2, also does not inhibit NT analgesia.

## C. nts3 Receptor

In contrast to the first two receptors, the nts3 receptor is not a G-protein-coupled receptor but has a gp95/sortilin structure (Fig. 6). This receptor has an N-terminal signal peptide, a cleavage site for furin, a long lumenal domain, a single transmembrane domain, and a short cytoplasmic tail; it is stored in vesicles and inserted into the membrane in response to NT. No data are yet available regarding the specific physiologic functions of this newly cloned receptor.

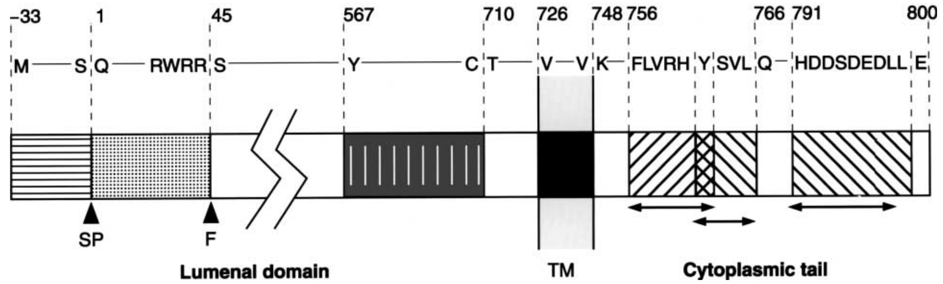

**FIGURE 6** The human nts3/gp/sortilin receptor. Numbers indicate the signal peptide (SP) (726–747), the propeptide released by furin (F) cleavage (1–44), the cysteine-rich domain homologous to Vps10p, a yeast receptor for carboxypeptidase Y sorting (567–709), the transmembrane (TM) domain (726–747), and the three internalization/sorting signals in the cytoplasmic tail homologous to the cation-independent mannose 5-phosphate/insulin-like growth factor-II receptor (456–765 and 791–799). Note the partial overlapping between two of these signal sequences. Adapted from Vincent *et al.* (1999), with permission from *Trends Pharmacol. Sci.*

## V. EFFECTS OF NEUROTENSIN

NT produces an array of effects in both the CNS and the GI tract. In the CNS, NT functions as a neurotransmitter or a neuromodulator, whereas in the periphery NT can exert its actions via endocrine, paracrine, or neuromodulatory effects.

### A. CNS Effects

NT produces significant CNS effects, including hypothermia and a naloxone-insensitive analgesia. NT blocks behaviors associated with activation of dopaminergic pathways, possibly by decreasing the binding efficiency of dopamine to its receptors. Centrally administered NT has been shown to mimic the actions of many antipsychotic drugs. NT, like antipsychotic medications, potentiates the sedation elicited by barbiturates and ethanol, decreases amphetamine-induced locomotor activity, and induces muscle relaxation and hypothermia. Administration of various antipsychotic drugs increases NT concentrations in the caudate nucleus and nucleus accumbens. This effect is preceded by an increase in c-*fos* mRNA levels in the dorsolateral stratum. Typical antipsychotic medications (e.g., haloperidol) induce NT/N expression in the striatum and nucleus accumbens. On the other hand, administration of atypical antipsychotic medications (e.g., clozapine), which are not associated with extrapyramidal side effects (EPSs), results in increased NT/N expression in the nucleus accumbens only. Recent studies using NT/N null mice demonstrate that NT is required for haloperidol-elicited activation of a specific population of striatal neurons; however, the incidence of catalepsy (the model for EPS in mice) was not different in NT/N null mice. These findings indicate that NT mediates the effects of a specific subset of antipsychotic agents but does not contribute to the generation of extrapyramidal side effects.

### B. Peripheral Effects

#### 1. *Vascular and Hemodynamic Effects*

The NT peptide was initially described as a "hypotensive peptide." In reality, the vascular and hemodynamic effects are more complicated than initially described. NT release can lead to either hypotension or hypertension by inducing either histamine release or adrenergic release, respectively. Other hemodynamic effects include decreasing blood flow to adipose tissue and increasing blood flow in the intestine without altering heart rate or blood pressure.

#### 2. *Immune Function*

Similar to other gut peptides, NT has an effect on immune function. Macrophages demonstrate enhanced phagocytosis in response to NT treatment. Mast cells respond to NT by releasing histamine; the response can be antagonized in rats with diphenhydramine (an antihistamine). In lymphocytes, NT can enhance proliferation, adherence, and chemotaxis. Because the small intestine has a high concentration of immunologically active cells, NT may affect immune function via paracrine effects. In rats, NT has also been shown to be a proinflammatory agent in the colonic inflammation associated with *Clostridium difficile* pseudomembranous colitis. This proinflammatory action is blocked by SR48692.

#### 3. *Effects on GI Nutrient Absorption, Motility, and Secretion*

Some of the physiologic effects of NT in the GI tract are related to motility. An infusion of NT mimics the consumption of a fat-rich meal (the most potent stimulator of NT release) in the following ways: NT decreases lower esophageal sphincter pressure, slows gastric emptying and intestinal transit while increasing colonic motility, and inhibits the migrating motor complex (MMC).

In addition to effects on GI motility, NT inhibits gastric acid secretion in a vagally dependent manner. Some debate exists regarding whether concentrations of NT that are present physiologically are sufficient to induce this effect. In contrast to gastric acid secretion, NT stimulates pancreatic bicarbonate and protein secretion in a dose-related fashion and has an additive effect in combination with secretin and/or cholecystokinin. NT potently stimulates biliary secretion of water, bicarbonate, and bile salts. Secretin is more effective than NT in stimulating secretion of bicarbonate and bile salts, but NT is more effective in stimulating secretion of bile water. A recent study demonstrates that NT also enhances jejunal absorption of conjugated bile acids and their return to the liver. Additional effects of NT include a net fluid secretion into the postduodenal small intestine and facilitation of lipid absorption from the proximal intestine.

#### 4. *Trophic Effects*

NT, given exogenously, has trophic effects on normal tissues as well as on certain cancers. Given in high doses, NT can induce growth of the gastric antrum, small intestine, and pancreas. In rats given an elemental diet, intestinal mucosal atrophy ensues; administration of NT can prevent this atrophy in the jejunum but not in other areas of the small bowel. NT can also augment the adaptive hyperplasia of intestinal mucosa associated with small bowel resection. Other studies demonstrate that NT stimulates mucosal growth in defunctionalized, self-emptying jejunoileal loops or isolated loops of small bowel (Thiry–Vella fistulas). Moreover, NT has been shown to restore gut mucosal integrity in rats and to prevent translocation of indigenous bacteria after radiation-induced mucosal injury.

In addition to its effects on normal tissues, NT has been shown to have a trophic effect on pancreatic and colon cancers that possess NT receptors. NT receptors have also been found in non-GI tumors, such as prostate cancer, in which NT has a similar trophic effect. Recent studies utilizing sensitive RT-PCR procedures have identified NT receptor expression in a majority of pancreatic adenocarcinomas. Although not universally expressed in human colon cancer cell lines, NT receptor expression has been identified in a more metastatic colon cancer cell line (i.e., KM20 cells). *In vitro* and *in vivo* experiments using the human pancreatic cancer line MIA PaCa-2, which possesses high-affinity NT receptors, have demonstrated growth in response to NT. NT treatment of athymic nude mice bearing MIA PaCa-2 xenografts results in increased tumor size, weight, and DNA and protein content. The NT receptor antagonist SR48692 inhibits these trophic effects. NT enhances colon carcinogenesis in rats and stimulates growth of MC-26 (mouse) and LoVo (human) colon cancer cells. These findings suggest a role for NT in the growth of GI cancers with high-affinity NT receptors. Therefore, analogous to current treatment strategies for endocrine-responsive breast and prostate cancers, NT receptor antagonists may play an adjuvant role in the treatment of certain GI and pancreatic cancers.

## VI. CONCLUSIONS/FUTURE PERSPECTIVES

Since its initial description almost 30 years ago, much has been learned regarding the regulation and actions of the NT peptide. The cloning of the NT/N gene in multiple species, the identification and cloning of the NT receptor genes, the synthesis of potent NT receptor antagonists, and the development of NT/N knockout mice are recent important developments that have greatly facilitated our understanding of these functions. Collectively, NT has been identified as an important contributory hormone for the maintenance of both gut structure and function. In the GI tract, NT affects intestinal motility, pancreaticobiliary secretion, fat absorption, and growth of normal and neoplastic tissues. In the CNS, an apparent important role for NT appears to be related to the blockade of dopaminergic pathways that have important ramifications in the effects of many psychotropic agents.

Future studies will further extend our current understanding of the effects of NT. Previous studies have identified the NT/N gene as a “model” intestinal gene to further delineate the complex differentiation pathways leading to gut development and maturation, as well as the process of fetal “dedifferentiation” noted in certain colon cancers. Understanding the factors regulating NT/N expression will yield important information on the regulation and attainment of the complete gut phenotype, thus not only providing a better understanding of normal gut development and function, but also providing a model to better understand the cellular events leading to gut neoplasia. The multitude of actions of NT, particularly its trophic and neuroleptic effects, suggest a potential future role for NT as a pharmacologic agent. The fact that NT is secreted in response to a fatty meal and induces delayed gastric emptying and intestinal motility suggests that NT may play a role in satiety and may be useful in the treatment of obesity. The trophic effects of NT on normal intestinal mucosa suggest that NT may be useful in stimulation of intestinal growth during periods of gut disuse, after small bowel resection, or with administration of chemotherapeutic agents. Furthermore, blocking NT receptors using potent receptor antagonists may be useful as adjuvant therapy in the treatment of certain NT receptor-positive colon and pancreatic cancers. Overall, the analysis of NT in the past has yielded important and clinically relevant information regarding its structure, expression pattern, role in development, secretion, and diversity of actions. It is anticipated that future studies, utilizing sophisticated molecular models and more specific receptor antagonists, will provide additional important information regarding the precise role of this novel peptide in GI and CNS functions, as well as exciting new therapies with diverse clinical implications.

## Glossary

| | |
|---|---|
| **Gp95/sortilin receptor** | Type of receptor that contains only one transmembrane domain. |
| **G-protein-coupled receptor** | Type of receptor that contains seven transmembrane domains. |
| **N cells** | Enteroendocrine cells localized to the intestine; produce and secrete neurotensin and neurotensin-related peptides; categorized as "open" cells, in which the apical microvilli are in contact with the intestinal lumen. |
| **neuromedin N** | Hexapeptide that is encoded on the same gene as neurotensin and has a similar distribution pattern. |
| **neurotensin** | Tridecapeptide that is found in the brain and gastrointestinal tract. |

## See Also the Following Articles

**Gastrointestinal Hormone-Releasing Peptides • GPCR (G-Protein-Coupled Receptor) Structure • Motilin**

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# Neurotrophins

Y.-A. Barde
*Friedrich-Miescher Institute for Biomedical Research, Switzerland*

I. NEUROTROPHIN: BIOCHEMISTRY AND MOLECULAR BIOLOGY
II. NEUROTROPHIN RECEPTORS
III. NEUROTROPHINS AND NEURONAL SURVIVAL
IV. NEUROTROPHINS AND NEURONAL PLASTICITY

**The neurotrophins constitute a small family of structurally related proteins. They are primarily known for their ability to affect key aspects of the biology of vertebrate neurons, including neuronal survival and death, dendrite and axonal elongation, and activity-dependent plasticity. The term "neurotrophin" was introduced subsequent to the finding that the sequence of the protein brain-derived neurotrophic factor was related to that of nerve growth factor. In mammals, two additional genes have been identified and they are designated neurotrophin-3 and neurotrophin-4. All four neurotrophins act through two structurally unrelated receptors designated Trk and p75. Neurotrophins can both prevent program cell death and cause it. In addition, they affect the shape of neurons as well as synaptic transmission.**

## I. NEUROTROPHIN: BIOCHEMISTRY AND MOLECULAR BIOLOGY

### A. Biochemistry

All neurotrophins are small (approximately 120 amino acids), basic (pI 9–10) proteins (Fig. 1). They are strongly similar in primary structure and are found in solution as noncovalently, tightly linked homodimers. They are secretory proteins and their cleavable leader sequence is followed by a pro-sequence of variable length [80 aa for the shortest one, human neurotrophin-4 (NT4)]. Cleavage of the pro-sequence occurs at a consensus sequence of the furin type found in all neurotrophins (R-X-K/R-R) to yield the mature and biologically active neurotrophins. However, recent evidence indicates that pro-neurotrophins may also be secreted and that in comparison with mature neurotrophins they have a higher affinity for the neurotrophin receptor p75 and a lower affinity for the Trk receptors. The functional consequence of this characteristic would be a shift toward the death-promoting activity of neurotrophins.

Each mature monomer comprises six cysteine residues involved in the formation of three disulfide bridges. The crystal structure of the neurotrophin dimers has revealed that the disulfide bridges are all grouped at one end of the molecule in the homodimers in an arrangement similar to that found in the transforming growth factor-β superfamily and in the platelet-derived growth factors.

Nucleotide sequences are available for several mammalian species, and approximately 50% of the amino acids are common to all neurotrophins, including the six cysteine residues. With the exception of NT4, neurotrophins are highly conserved between species. For example, there are no amino acid replacements in the mature sequence of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) in most mammals. Additional neurotrophin sequences have been reported in teleost fishes, but no neurotrophin sequence has been detected in the genome of *Caenorhabditis elegans* and *Drosophila melanogaster*. Similarly, these latter two genomes do not contain sequences corresponding to the neurotrophin receptors p75 or Trks.

### B. Molecular Biology

In human, *ngf* (nerve growth factor gene) has been localized to chromosome 1q21–q22.1, *bdnf* has been localized to 11q13, *nt3* has been localized to 12q13, and *nt4* has been localized to 19q13.3. The neurotrophin genes are large (at least 40 kb) and contain several small 5′ exons and a larger 3′ exon that contains most of the translated sequence. The neurotrophin genes encode several transcripts from at least four different transcription start sites. Also, they have short or long 3′-untranslated regions. These genes are transcribed as a major transcript of approximately 1.3 to 1.6 kb and often a larger transcript of approximately 4.3 kb. Their relative abundance depends on the tissue in question. In the brain, neurons are the major cellular site of neurotrophin gene expression and there are considerable differences in the degree of expression between different brain areas and developmental stage. Typically, gene expression is regulated by electrical activity. In particular, increased activity leads to rapid increased transcription of the *ngf* and *bdnf* genes, and decreased activity reduces the expression of *bdnf*. Outside of the central nervous system (CNS),

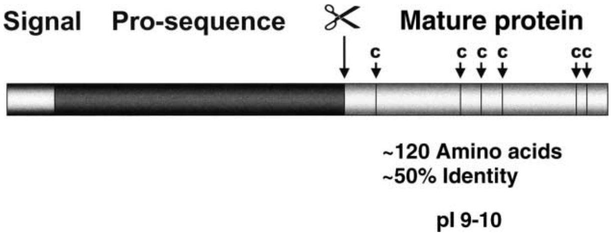

FIGURE 1 Neurotrophins are synthesized as pre-pro proteins. Cleavage occurs following a cluster of basic amino acids. The six cysteine residues indicated in the mature proteins are involved in the formation of three disulfide bridges. Neurotrophins occur in solution as tightly packed homodimers.

numerous cell types express the neurotrophin genes, including Schwann cells, skeletal muscle cells, and smooth muscle cells, as well as cells from heart and lung tissue. Expression has also been detected in cells of the immune system, including lymphocytes.

## II. NEUROTROPHIN RECEPTORS

Neurotrophin signaling is quite complex and diverse. There are two different receptor types that bind the neurotrophins, p75, and the Trks (Fig. 2). These receptors can mediate actions as diverse as promoting or preventing the death of neurons. Also, these two receptor types associate in the membrane, and these associations lead to increases in the specificity and affinity of the receptor complex.

### A. p75

The neurotrophin receptor p75 was the first member of a large family of receptors, which includes both tumor necrosis factor receptors and CD95, to be molecularly cloned. It was initially designated the nerve growth factor (NGF) receptor. This designation was revisited following the realization that all neurotrophins bind to this receptor and that the Trk receptors also bind neurotrophins (see below). At least two proteins are encoded by *p75*, one of them lacking three of the four cysteine-rich domains that characterize the extracellular domains of all members of the p75 family. Like all other members, p75 has no intrinsic catalytic activity and it transduces signals by associating with, or dissociating from, cytoplasmic partners. There are several protein association motifs in the cytoplasmic domain of p75, one being distantly related to the so-called "death domain" of other members of the family. Also of note is a short sequence of the intracellular domain of p75 that is related to the sequence of mastoparan, a 14-mer peptide derived from wasp venom and capable of directly activating G-proteins. The C-terminus forms a consensus sequence found in several other proteins associating with postsynaptic densities, such as postsynaptic density-95. A number of proteins have been shown to associate with the cytoplasmic domain of p75.

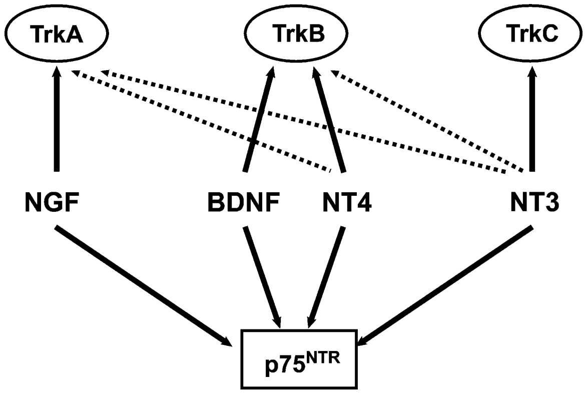

FIGURE 2 Neurotrophins bind two different kinds of receptors. All bind to the receptor p75 with an affinity of at least $10^{-9}$ M. They also bind to the Trk family of tyrosine kinase receptors. TrkC binds exclusively to NT3, TrkA shows a preference for binding to NGF, and TrkB binds preferentially to BDNF and NT4. NT3 also binds to TrkA and TrkB. All three Trk receptors associate with p75, and this association modulates both the binding specificity and the affinity of the Trk receptors.

All mature neurotrophins tested bind to p75 with an affinity of approximately $10^{-9}$ M. Recently, pro-NGF was demonstrated to bind to p75 with a 10-fold higher affinity than mature NGF and to bind to the TrkA receptor with a distinctly lower affinity than mature NGF.

### B. Trk Receptors

In mammals, three Trk receptors have been identified: TrkA, TrkB, and TrkC. TrkA binds NGF and to a lesser extent NT3, TrkB binds preferentially to BDNF and NT4, though NT3 can also bind to and activate TrkB, especially in the absence of p75 co-expression, and TrkC binds to NT3 exclusively (Fig. 2). As with the neurotrophins, additional Trk receptors have been identified in teleost fishes. Ligand binding leads to receptor-mediated tyrosine phosphorylation. This triggers the activation of pathways leading to some of the best known actions of the neurotrophins, including the prevention of programmed cell death and neuronal differentiation. Three main signaling cascades are activated by the Trk receptors and their substrates: the Ras/Raf/MEK/mitogen-activated protein kinase pathway, phosphatidylinositol 3-kinase, and phospholipase C-γ. At the level of the nerve terminals, the association of neurotrophins with Trk receptors triggers the process of retrograde transport, allowing signals to be transported from the terminals back to the cell body of neurons.

A number of splice variants have been described for the Trk receptors. The most abundant variants are forms of TrkB and TrkC that lack the kinase domain. These truncated TrkB receptors are often expressed in nonneuronal cells in the absence of TrkB.

### C. Expression of Neurotrophin Receptors

The expression of both types of neurotrophin receptors is tightly regulated. Typically, only specific subgroups of neurons express one particular Trk receptor. The specificity of the neurotrophins for subgroups of sensory neurons correlates strongly with the selectivity of Trk receptor expression on these neurons. In the peripheral nervous system (PNS), neurotrophins are often expressed in the peripheral targets of those neurons needing neurotrophins for survival in the embryo. In the adult, these target-derived neurotrophins regulate the functional properties of the neurons, including the levels of neurotransmitters and dendritic development. As in the central nervous system, some of the neurotrophin genes are also expressed by neuronal cell bodies, and BDNF in particular is expressed by some sensory neurons and transported in an anterograde manner. It is released by NGF-dependent neurons in the spinal cord, where it affects neurotransmission. p75 is co-expressed with the Trk receptors in many neuronal populations. Its expression profile is highly developmentally regulated. During postnatal development, p75 is down-regulated in most parts of the central nervous system, but it is rapidly induced after nerve lesion or seizure. This receptor is also expressed by many cells, such as neural crest cells, by the time they become postmitotic cells and/or migrate.

## III. NEUROTROPHINS AND NEURONAL SURVIVAL

### A. Neurotrophins Promote Neuronal Survival

Neurotrophins typically support the survival of embryonic neurons that die in their absence. Thus, antibodies to NGF, like the deletion of the *ngf* or *trkA* gene, lead to the virtually complete destruction of the peripheral sympathetic nervous system as well as to the loss of many neural crest-derived sensory neurons. BDNF, NT3, and NT4 also support the survival of subpopulations of peripheral sensory neurons, including those derived from epidermal placodes, which are not supported by NGF. With the exception of NGF, neurotrophins support the survival of embryonic rat motoneurons *in vitro*, and BDNF prevents their death *in vivo* after axotomy. NGF and to a lesser degree BDNF prevent the loss of cholinergic function seen after axotomy in adult animals. BDNF also supports the survival of dopaminergic neurons dissociated from the rodent mesencephalon as well as of retinal ganglion cells. In the mature nervous system, neurotrophins are involved in the maintenance of neuronal phenotypes; in particular, antibodies to NGF decrease the levels of enzymes synthesizing catecholamines as well as neurotransmitters such as substance P in the peripheral nervous system of adult animals. Neurotrophins also act on some nonneuronal cells, and NT3 in particular contributes to the division and survival of oligodendrocyte precursors. In aged, learning-deficient rats, the intraventricular injection of NGF can reverse the learning deficiencies in simple behavioral tests. Decreased levels of BDNF mRNA have been noted in the hippocampus of patients with Alzheimer's disease.

### B. Neurotrophins Cause Programmed Cell Death

Like other receptors belonging to the same family, such as CD95, p75 causes the death of neurons in the developing CNS. This occurs in the developing retina and spinal cord. Also, basal forebrain cholinergic neurons express p75 at high levels, and its complete elimination leads to a long-lasting increase in the number of basal forebrain cholinergic neurons. Cultured oligodendrocytes up-regulate p75 expression and they can be killed by the addition of NGF. NGF is not the only ligand able to activate p75 to cause cell death, and in developing sympathetic ganglia, BDNF also causes cell death through p75.

## IV. NEUROTROPHINS AND NEURONAL PLASTICITY

### A. Regulation of Process Outgrowth

Beyond their effects on the control of neuronal survival, neurotrophins also have pronounced effects on the size of neurons, on the rate of axonal elongation, and on the growth and branching of dendrites. In adult animals, the administration of NGF causes the length of dendrites of sympathetic neurons to increase. Conversely, antibodies to NGF decrease the length of these dendrites. When such experiments are performed in newborn animals, the increased number of dendrites caused by NGF administration is accompanied by a very large increase in the number of preganglionic axons. In mice carrying a deletion in the pro-apoptotic gene *bax,* the effects of neurotrophins on axonal elongation can be examined in the absence of effects on survival. In such animals, the lack of neurotrophins or of their corresponding Trk receptors causes a dramatic reduction in the number of peripheral axons. Neurotrophins are required for the elongation of peripheral nerves. Indeed, the acute application of a combination of function-blocking monoclonal antibodies causes a marked reduction in the elongation of both sensory and motor nerves. Also, the neurotrophins are able to attract sensory and sympathetic nerves. Similar mechanisms seem to operate in the CNS, as exemplified by work using live tadpoles and slices of the visual cortex. The effects of neurotrophins appear to be complex and different for apical or basal dendrites. The effects are also specific for each neurotrophin tested and the results indicate that each neurotrophin can act to modulate particular patterns of dendritic arborization. The regulation of cortical dendritic growth by neurotrophins requires endogenous electrical activity.

## B. Neurotrophins and Synaptic Transmission

Neurotrophins regulate the number of synapses and the efficacy of synaptic transmission. NGF levels modulate both the strength and the number of presynaptic inputs in sympathetic ganglia. In mice overexpressing BDNF in sympathetic neurons, increased numbers of synapses are observed, whereas in *bdnf* − /− animals, a decreased number of synapses are found. Presynaptic alterations and a decreased number of synapses are observed in the absence of TrkB and TrkC receptors.

Neurotrophins rapidly modulate neurotransmission, both in the PNS and in the CNS. Some of these effects occur very rapidly after the application of the neurotrophins. Within milliseconds, BDNF and NT4 cause depolarization and elicit action potentials in pyramidal cells of the hippocampus or cortex and in Purkinje cells of the cerebellum. This depolarization results from an increased conductance for sodium ions, and it is as rapid as that induced by the neurotransmitter glutamate.

BDNF modulates synaptic transmission in the hippocampus, as reflected by its role in long-term potentiation. This has been demonstrated in animals lacking *bdnf*, as well as by the use of specific monoclonal antibodies.

BDNF is not only stored in neurons, it is also released from internal stores by activity-dependent mechanisms. It is present in presynaptic nerve terminals and also in dendrites, where it co-localizes with the postsynaptic markers.

In the visual cortex, neurotrophins are involved in activity-dependent developmental plasticity. Neurotrophins influence the formation of ocular dominance columns in the visual cortex and promote the maturation of cortical inhibition.

## C. Neurotrophins and Behavior

Detailed explorations of the heterozygous *trkB* and of conditional mutants reveal that these animals become increasingly impaired over time with regard to their spatial learning behavior, similar to the phenotype in mice with hippocampal lesions. *Bdnf* + /− animals develop an enhanced aggressiveness and hyperphagia, accompanied by weight gain. BDNF is also necessary for canaries to learn new songs. Interestingly, testosterone treatment increases the levels of BDNF in the high vocal center.

## Glossary

**brain-derived neurotrophic factor** Protein purified from pig brain in the 1980s by monitoring its ability to prevent the death of peripheral sensory neurons.

**nerve growth factor (NGF)** The first neurotrophin to have been identified in the 1950s. It was purified on the basis of its ability to elicit neurite outgrowth from ganglionic explants. The extraordinary and unexplained abundance of NGF in the adult male mouse submandibular gland was a prerequisite for its early characterization.

**neurotrophin-3** Identified on the basis of sequence homologies to the previously sequenced proteins nerve growth factor and brain-derived neurotrophic factor.

**neurotrophin-4** First identified in *Xenopus laevis* cDNAs and subsequently detected in rodents and humans. Its sequence is more variable between species than the other three neurotrophins. It was initially designated neurotrophin-4 or neurotrophin-5 but it is likely to be the same protein in different species.

**p75** A glycoprotein of 75,000 Da that binds all neurotrophins. It was identified in the mid-1980s by expression cloning.

**tropomyosin receptor kinases (Trks)** A small group of closely related tyrosine kinase membrane receptors that are activated by neurotrophin binding. Trk was first recognized as an oncogene made up of the kinase domain of Trk fused with a portion of *tropomyosin*. The proto-oncogene was later found to be a nerve growth factor receptor.

## See Also the Following Articles

**Brain-Derived Neurotrophic Factor • Nerve Growth Factor (NGF)**

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# NGF

See *Nerve Growth Factor*

# Nitric Oxide

Derek P. G. Norman[*] and Anthony W. Norman[†]

[*]*Harvard University* • [†]*University of California, Riverside*

I. INTRODUCTION
II. CHEMISTRY, BIOSYNTHESIS, AND SECRETION
III. BIOLOGICAL ACTIONS OF NO
IV. SUMMARY

**Nitric oxide (NO) is one of the body's many hormones. It is unique in that it is the only animal hormone that is a gas. NO, however, is somewhat water soluble and is able to function as a chemical messenger, particularly in cells of the vascular endothelium, immune, and neural systems. Virtually all of the known biological actions of NO on the cardiovascular system are mediated by the activation of a guanylate cyclase, which produces the second messenger cyclic GMP that leads to vasorelaxation (lowered blood pressure).**

## I. INTRODUCTION

A relatively surprising addition to the family of chemical messengers is nitric oxide (NO). NO is a free radical gas of limited solubility in water. Because NO is noncharged, it can rapidly diffuse across cell membranes and into cells; it has been shown to act as both an intracellular and an intercellular (paracrine) messenger to elicit a wide spectrum of biological responses. A physiological function for NO was first established in the vascular system when the endothelin-derived relaxing factor (EDRF) could be quantitatively explained by the formation of NO. NO is now known to be an integral participant in the signal transduction processes associated with the vascular, immune, and neural systems.

## II. CHEMISTRY, BIOSYNTHESIS, AND SECRETION

The formation of NO is an enzyme-mediated reaction (see Fig. 1); the nitrogen donor is the amino acid L-arginine and the oxygen donor is molecular oxygen. The reaction is catalyzed by an NADPH-requiring nitric oxide synthase (NOS). NOS enzymes are structurally related to cytochrome P450 reductase and range in size from 13 to 160 kDa.

NOS exists both as a constitutive enzyme, which is regulated by Ca$^{2+}$ and the calcium-binding protein, calmodulin, and as an inducible enzyme, which is not

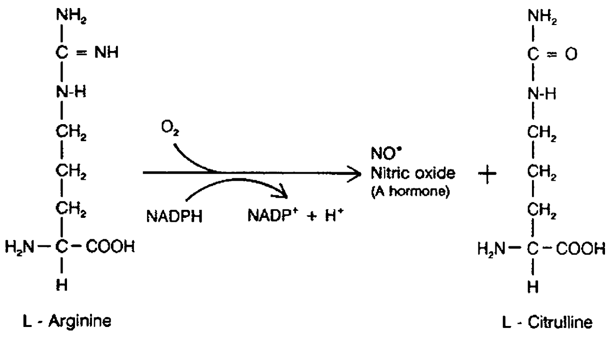

FIGURE 1 Enzymatic reaction catalyzed by nitric oxide synthase (NOS).

regulated by calmodulin. The regulatable forms of NOS are induced by interferon-α (INF-α), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and estradiol and are inhibited by glucocorticoids. The inducible forms of NOS are associated with components of the host defense immune system.

There is clear evidence that under some physiological circumstances the classical estrogen receptor, when occupied by its cognate hormone, estradiol, can initiate a signal transduction process that activates NOS. One novel aspect of this activation process is that the estrogen receptor for this biological response is localized not in the nucleus but in caveolae found in the cell's plasma membrane. Caveolae and caveolae-related membrane domains are enriched in molecules that play pivotal roles in intracellular signal transduction. In this setting, the steroid hormone estradiol is not working with its receptor to regulate the traditional response of regulation of gene transcription (over hours to days), but is instead generating a rapid response (over seconds to minutes) via a non-nuclear signal transduction process that results in NOS activation. This system of signal transduction has been particularly documented to occur in vascular endothelial cells where estradiol is known to modulate the local cell biology.

## III. BIOLOGICAL ACTIONS OF NO

The wide spectrum of biological actions of NO in the cardiovascular, nervous, and host defense systems is summarized in Table 1. Only the actions of NO in the cardiovascular system are discussed in this article.

**TABLE 1 Biological Actions of Nitric Oxide in the Cardiovascular, Nervous and Host Defense Systems**

| System | Response |
|---|---|
| Cardiovascular | |
| Smooth muscle | Initiate vasorelaxation; control of regional blood flow and blood pressure |
| Platelets | Limitation of aggregation and adhesion |
| Nervous | |
| Peripheral | Neurotransmission (penile erection, gastric emptying) |
| Host defense | |
| Macrophages | Defense against bacteria, fungi, protozoans, parasites, and viruses |
| Leukocytes | |
| Monocytes | |

After the generation of NO in the endothelial cell by the NOS enzyme, the NO diffuses to an adjacent smooth muscle where it acts as an agonist to initiate biological responses (see Fig. 2). Virtually all of the known biological actions of NO on the cardiovascular system are mediated by the activation of a soluble guanylate cyclase. The guanylate cyclase has a heme prosthetic group to which NO binds tightly to this moiety. The resulting activation of the guanylate cyclase results in the production of cGMP, which is then postulated to have actions on protein kinases, nucleotide-sensitive phosphodiesterases,

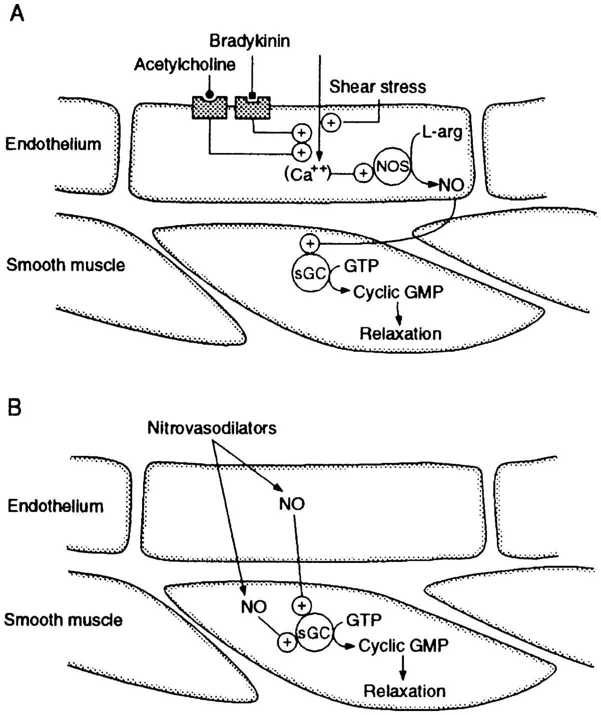

FIGURE 2 Model for the synthesis of NO in endothelial cells and its paracrine actions in smooth muscle cells. In (A), shear stress or receptor activation of vascular endothelium by bradykinin or acetylcholine results in an influx of calcium. The consequent increase in intracellular calcium stimulates the constitutive nitric oxide synthase (NOS). The nitric oxide (NO) formed from L-arginine (L-Arg) by this enzyme diffuses to nearby smooth muscle cells, in which it stimulates the soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP from guanosine triphosphate (GTP). This increase in cyclic GMP in the smooth muscle cells leads to their relaxation. In (B), nitrovasodilators such as sodium nitroprusside and nitroglycerin release NO spontaneously or through an enzymatic reaction. The liberated NO stimulates the soluble guanylate cyclase in the vascular smooth muscle cell, resulting in relaxation. [Adapted from Moncada, C., and Higgs, A. (1993). The L-arginine-nitric oxide pathway, *New Engl. J. Med.* **329**, 2002–2012.]

ion channels, or other unknown cellular proteins that are linked to the generation of the smooth muscle response of vasodilation and also to the inhibition of platelet adhesion and aggregation.

NO is a short-lived agonist in the cellular environment. NO is inactivated either by its chemical linkage to proteins (nitrosylation) or by oxidation to nitrite ($NO_2^-$) and then nitrate ($NO_3^-$).

## IV. SUMMARY

In the normal physiological state in humans, the distribution, composition, and volume of body fluids are held within relatively narrow limits, despite wide variations in the intake of water and $Na^+$. Such homeostasis or stability of the internal environment requires a multifactoral collaboration of the hormones and other physiological processes that affect electrolyte and water metabolism. A diverse array of hormones (aldosterone, angiotensin II, rennin, atrial natureatic protein or ANP, NO, endothelin, vasopressin, prostaglandins, and kinins) and signal transduction systems, each responding to different stimuli, is integrated to provide the kidney and cardiovascular system with coherent messages to effect the collective regulation of blood pressure, electrolyte concentration, and water volume.

## Glossary

**caveolae** Sub-domains of the cell's plasma membrane that have a typical flask-like membrane structure where signal transduction molecules may congregate.

**endothelium** The monolayer of cells lining the inner wall of the vascular system.

**estradiol** A steroid hormone that belongs to the family of estrogens.

**free radical** A molecule, usually short-lived and highly reactive, that contains an unpaired electron. All molecules containing an odd number of electrons are free radicals.

**nitric oxide** A molecule composed of one atom of oxygen and one atom of nitrogen, with the chemical formula NO, which chemically is a free radical. NO is one of the body's many hormones; it is unique in that it is the only animal hormone that is a gas.

## See Also the Following Articles

**Amino Acid and Nitric Oxide Control of the Anterior Pituitary • Calmodulin • Estrogen Receptor-β Structure and Function**

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# Non-Insulin-Dependent Diabetes Mellitus

**See *Diabetes Type 2***

basic fibroblast growth factor in human umbilical vein endothelial cells, and expression of granulocyte colony-stimulating factor and granulocyte/macrophage colony-stimulating factor in human endothelial cells. On the other hand, OSM has been reported to down-regulate the expression of cytochrome P450 in human hepatocytes.

## IV. BIOLOGICAL ACTIVITIES OF OSM (II)

OSM is secreted from activated T cells and monocytes stimulated by cytokines, T-cell activators, and phorbol 12-myristate 13-acetate (PMA) and plays roles in several inflammatory reactions. OSM increases the secretion of acute-phase proteins (APPs), such as haptoglobin, α1-antichymotrypsin, and fibrinogen, in liver cells at the early phase of inflammation. In the inflammatory process, the remodeling of the extracellular matrix is important for healing the damaged tissue induced by inflammatory responses. Matrix metalloproteinases (MMPs) are involved in extracellular matrix breakdown, and tissue inhibitors of metalloproteinases (TIMPs) inhibit the action of MMPs. Therefore, the balance between TIMPs and MMPs is important for the remodeling of the extracellular matrix. OSM induces TIMP-1 expression and inhibits IL-1β-induced TIMP-3 in cultured human synovial-lining cells. OSM induces the expression of MMP-1 and MMP-3 in astrocytes and of MMP-1 and MMP-9 in fibroblasts. Thus, OSM may be involved in wound healing by modulating the balance between TIMPs and MMPs. It is also known that another family member, IL-6, strongly affects inflammatory reactions. In fact, IL-6-deficient mice exhibit the reduced production of APPs and the delayed repair of the liver injury induced by carbon tetrachloride. Since OSM induces the IL-6 receptor in human hepatoma HepG2 cells and stimulates the production of IL-6 in cultured human endothelial cells, OSM affects inflammation not only directly, but also indirectly through the induction of other members of this family.

## V. STRUCTURE OF THE OSM RECEPTOR

It is known that different cytokines exhibit similar biological activities on the same cell type (functional redundancy). The functional redundancy among the cytokines of the IL-6 family is now well explained by their receptor structure. Functional receptors for this family of cytokines consist of multiple subunits including the common signal transducing subunit, gp130 (Fig. 1). The receptor complexes for IL-6 and IL-11 consist of a ligand-specific α receptor subunit and gp130. The binding of each cytokine to its specific α-subunit induces the dimerization of gp130. The LIF receptor consists of the low-affinity LIF-binding protein (LIFRβ) and gp130. LIF binding leads to heterodimerization of LIFRβ and gp130. The CNTF receptor is composed of the CNTF-specific α-subunit, LIFRβ, and gp130. Although OSM is a cytokine that binds with low affinity to gp130 directly, it is not enough to transduce its signals. In human, two types of functional OSM receptor are known: the type I OSM receptor is identical to the high-affinity LIF receptor that consists of gp130 and LIFRβ, and the type II OSM receptor consists of gp130 and the OSM-specific receptor β-subunit (OSMRβ). OSMRβ is expressed in a wide variety of cell types, including endothelial cells, hepatic cells, lung cells, skin cells, and many tumor cell lines. Although LIF and OSM share a number of biological functions in common, it is also known that OSM displays some specific biological properties that are not shared by LIF, e.g., growth inhibition of A375 melanoma cells and up-regulation of α1-proteinase inhibitor in lung-derived epithelial cells. Thus, many overlapping biological responses between hOSM and hLIF are mediated by the shared type I receptor, i.e., the LIF receptor, whereas OSM manifests its specific responses through the type II receptor. However, this is not the case for mOSM. Immediately after the isolation of the mOSM cDNA, it was recognized that there are some differences in biological activity between human and murine OSM. For example, it was shown that a more than 30-fold higher concentration of mOSM is required for the growth inhibition of M1 cells compared with hOSM. Likewise, mOSM is much less potent than hOSM in the inhibition of differentiation of mouse embryonic stem (ES) cells. Molecular cloning of mouse OSMRβ cDNA and reconstitution of the high-affinity functional OSM receptor revealed that mOSM transduces signals only through its specific receptor complex composed of gp130 and OSMRβ, but not through the LIF receptor (Fig. 2). Interestingly, hOSM binds to the mouse LIF receptor and transduces signals; however, it fails to transduce signals through the mouse OSM receptor. Thus, the biological functions of hOSM observed in mouse cells are likely to represent mouse LIF functions.

## VI. SIGNAL TRANSDUCTION PATHWAY

Cytokines bind to their receptors on the target cells and then initiate various downstream signaling cascades (Fig. 3). The IL-6 family cytokine receptors

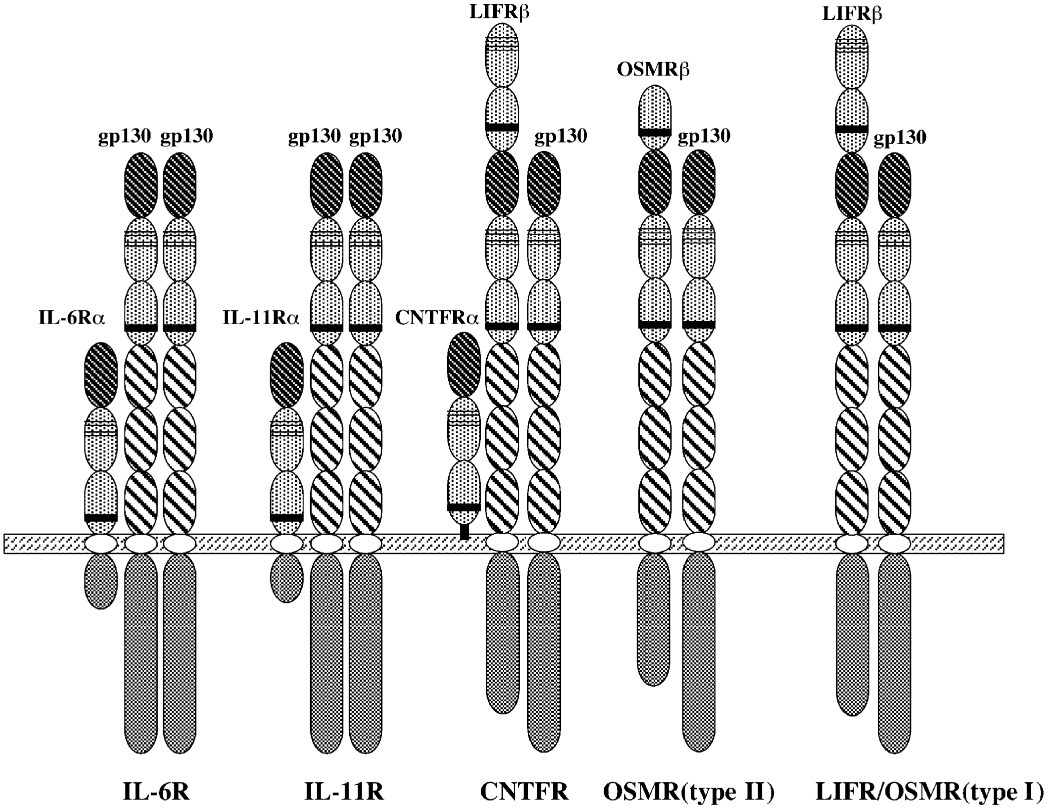

FIGURE 1 Composition of the IL-6 family cytokine receptor complex. IgG-like domains and fibronectin type III-like domains are shown as dark gray and diagonally striped symbols, respectively. The light gray area shows a cytokine-binding module containing the conserved cysteine residues (thin horizontal lines) and WS motifs (thick horizontal lines).

do not possess an intrinsic tyrosine kinase but utilize JAKs/STATs as major mediators of their signals. The first step in receptor activation is the ligand-induced homo- or heterodimerization of signal-transducing receptor subunits. As each signal-transducing receptor subunit binds one JAK (JAK1, JAK2, and Tyk2), dimerization of the subunits leads to the reciprocal phosphorylation and activation of JAKs. The activated JAKs phosphorylate tyrosine residues in the intracellular domain of the receptor, creating docking sites for STATs as well as various signaling molecules with an SRC homology 2 (SH2) domain. These molecules that are recruited to the receptors are then activated by JAKs. Phosphorylated STATs (STAT1, STAT3, and STAT5) then form homo- or heterodimers and translocate to the nucleus, where they are involved in gene regulation. A difference in signal transduction between type I and type II OSM receptors has also been reported. STAT5b is predominantly activated by the OSM-specific type II receptor in the A375 cell line. The IL-6-type cytokines stimulate not only the JAK/STAT signaling pathway but also the Ras/Raf/mitogen-activated protein kinase signaling pathway. It is known that several adapter molecules, such as SHP-2 (SH2 domain-containing protein tyrosine phosphatose 2), Grb2 (growth factor receptor-bound protein 2), and Gab1 (Grb2-associated binder-1), are involved in this pathway. Thus, OSM regulates complex biological responses, such as cell growth, differentiation, and apoptosis, through these signaling pathways.

## VII. SUMMARY

OSM is a member of the IL-6 cytokine family. Of all the members of this family, OSM is most closely related to LIF structurally and functionally. In addition, the genes for OSM and LIF are closely linked on the same chromosome. It is also known that the genes for their receptor β-subunits are present in close proximity on mouse chromosome 15. Co-localization of the OSMRβ and LIFRβ genes as well as OSM and LIF genes strongly suggests that they were created by duplication during evolution.

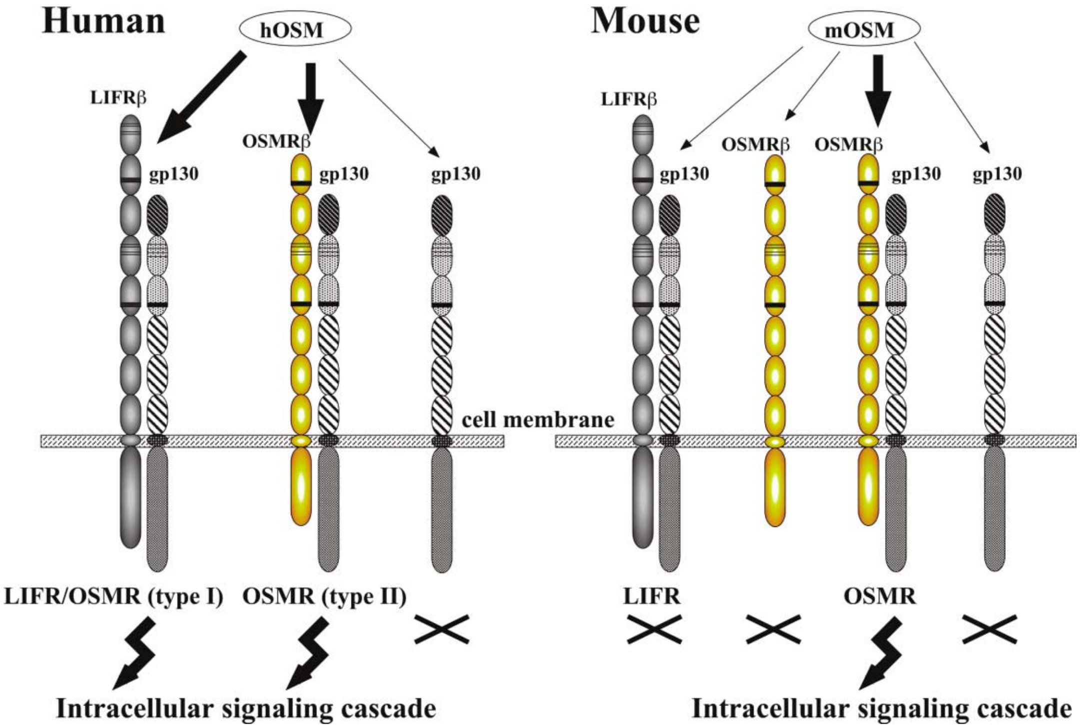

FIGURE 2 Formation of the functional receptor complexes for hOSM and mOSM. Thin arrows show low-affinity binding of OSM to each receptor component and thin arrows indicate high-affinity binding. Broken arrows indicate the relay of the intracellular signaling cascade. X, no signaling.

Since hOSM and hLIF showed various common biological activities, OSM has been thought to be the second LIF. However, hOSM also exhibits unique activities including growth inhibition of A375 melanoma cells, growth stimulation of Kaposi's sarcoma, and induction of TIMP-1 in fibroblasts. Molecular cloning of the hOSM receptor subunit and reconstitution of the functional hOSM receptor indicated that there are two types of functional receptor for hOSM. Although the type I OSM receptor is identical to the high-affinity LIF receptor and shares signaling pathways with LIF, the type II OSM receptor is utilized for OSM-specific signaling. The existence of two functional OSM receptors provides a molecular basis for the biological activities shared in common between LIF and OSM as well as for OSM-specific activities. It should be noted that mOSM uses only the OSM-specific receptor but not the LIF receptor.

## Glossary

**interleukin-6** A pleiotropic cytokine that acts on a wide variety of cells and exhibits many biological activities, e.g., the induction of B-cell differentiation to antibody-forming plasma cells, differentiation of myeloid leukemic cell lines into macrophages, megakaryocyte maturation, acute-phase protein synthesis in hepatocytes, and development of osteoclasts.

**Janus kinases (JAKs)** Intracellular tyrosine kinases with molecular masses of 120–140 kDa that bind to cytokine receptors. A typical kinase domain is located at the C-terminus, preceded by a kinase-like domain. Four JAKs (JAK1, JAK2, JAK3, and Tyk2) have been identified.

**leukemia inhibitory factor** A pleiotropic cytokine that was originally identified as a factor that inhibits leukemic cells and exhibits many biological activities, e.g., induction of monocytic differentiation of the murine leukemic cell line M1, suppression of differentiation of pluripotent embryonic stem cells, and inhibition of adipogenesis.

**signal transducers and activators of transcription (STATs)** A family of latent cytoplasmic transcription factors with an SH2 domain, members of which are activated by cytokines and translocate to the nucleus to participate in gene expression. Seven mammalian STAT genes (STAT1, 2, 3, 4, 5a, 5b, and 6) have been identified.

**tissue inhibitors of metalloproteinases** Specific inhibitors of matrix metalloproteinases, a family of enzymes that are responsible for the degradation of collagens, proteoglycans, and glycoproteins of the extracellular matrix.

## See Also the Following Articles

**Interleukin-6 • Leukemia Inhibitory Factor (LIF)**

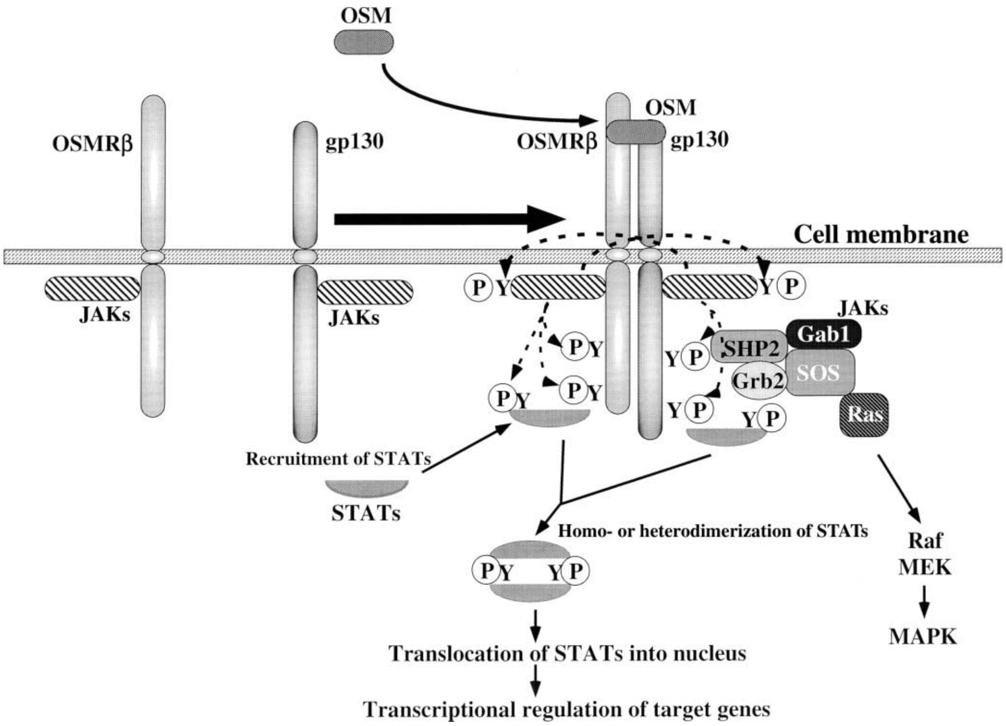

**FIGURE 3** Signal transduction pathways of OSM. Binding of OSM to the receptor components induces heterodimerization of the subunits, leading to the reciprocal phosphorylation and activation of JAKs. The activated JAKs phosphorylate the tyrosine residues of the receptor subunits, creating distinct binding sites for STATs and SHP2. The STATs recruited to the receptor are phosphorylated by JAKs, and homo- or heterodimerized STATs are translocated to the nucleus, where they regulate the transcription of target genes. The SHP2 recruitment is required for the mitogen-activated protein kinase (MAPK) pathway. Encircled P, phosphorylation; MEK, MAPK kinase; SOS, son of sevenless; Y, tyrosine residue.

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Mutations affecting the kit ligand (*Kitl*) and *Kit* loci in Steel (*sl*) and white spotting (*w*) mouse models, respectively, cause failures in the proliferation, survival, and migration of the PGCs. Similarly, signaling pathways elicited by members of the transforming growth factor-β (TGF-β) superfamily are necessary for PGC development and have been implicated in PGC migration and in the control of PGC proliferation. Knockout mouse models with mutations in bone morphogenetic protein 4 (*Bmp4*), *Bmp8b*, *Smad1*, or *Smad5* exhibit complete or nearly complete loss of the PGC population. More recently, double-mutant studies generating *Bmp2*$^{+/-}$, *Bmp4*$^{+/-}$ double heterozygotes have revealed co-operative functions of these related proteins in PGC development. Thus, multiple TGF-β superfamily ligands (i.e., BMP-2, BMP-4, and BMP-8B) signaling through the intracellular proteins SMAD1 and SMAD5 play key roles in PGC development.

In addition to soluble growth factors and the intracellular signaling proteins that mediate their response pathways, structural proteins that form contacts between cells and from cells to the extra-cellular matrix are important in PGC development. Knockout mice lacking the gap junction protein connexin 43 demonstrate decreased numbers of PGCs as early as E11.5. In contrast, integrin β1$^{-/-}$ chimeras demonstrate that integrin β1 is dispensable for early PGC differentiation but is essential for their migration to the putative gonad.

Germ cell-deficient (*gcd*) and atrichosis (*at*) mutant models exhibit early embryonic loss of PGCs, though the molecular causes underlying these defects are as yet unknown. Identification of the genes disrupted in *gcd* and *at* mutant mice will further elucidate the proteins required for PGC development and function.

## II. PRIMORDIAL FOLLICLE ORGANIZATION AND FOLLICULOGENESIS

Single layers of squamous granulosa cells surround oocytes during the perinatal period, and complex bidirectional communications established at this time between oocytes and these somatic cells function during all stages of follicle development and are crucial for female fertility. It is clear that signals from oocytes participate in the assembly of primordial follicles in the newborn ovary as knockout mice that lack factor in the germ line α (FIGα), an oocyte-specific helix loop helix transcription factor, fail to form primordial follicles and lose their oocytes within the first days of life. This suggests that FIGα regulates one or more factors that are critical for either the recruitment of granulosa cells to the oocyte or the adherence of these granulosa cells to the oocyte.

Recruitment of primordial follicles to initiate folliculogenesis is marked morphologically by oocyte and granulosa cell growth, and stages of subsequent follicular development are characterized by granulosa cell proliferation (see Fig. 2). Factors required for the initial recruitment of primordial follicles to form primary (one-layer) follicles are unknown. Oocyte–granulosa cell interaction is crucial to preantral

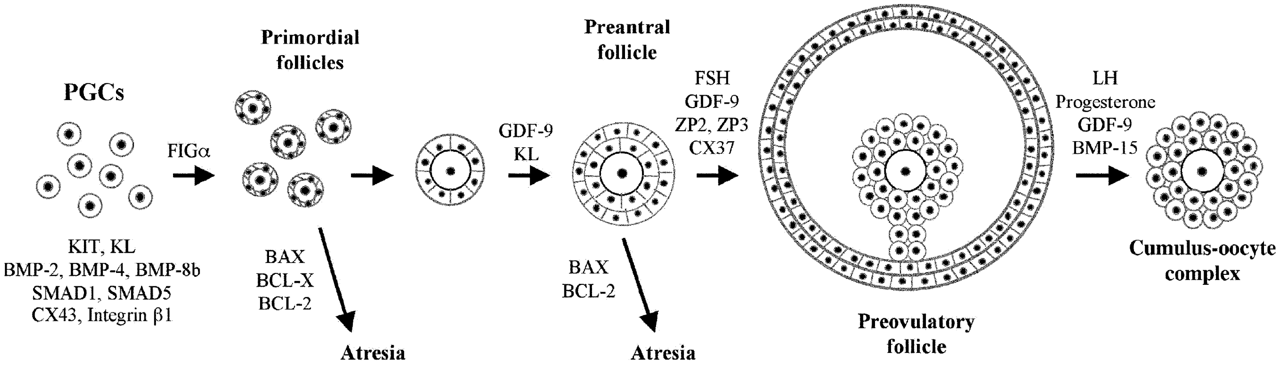

FIGURE 2 Stages of folliculogenesis and its key regulatory proteins. Primordial germ cells proliferate and migrate to the putative gonad during embryonic development and organize granulosa cells to form primordial follicles within a few days after birth in the mouse. Primordial follicles that are not lost to attrition are available to be recruited to the growing pool of follicles, first evidenced by a change in the shape of the granulosa cells from squamous to cuboidal. The mechanisms that regulate the measured recruitment of follicles over a female's reproductive lifespan are as yet unknown. Granulosa cells proliferate during the ensuing stages of folliculogenesis, and large antral preovulatory follicles develop in response to endocrine factors. At ovulation, cumulus–oocyte complexes are released from these dominant follicles, and the remnant cells undergo luteinization. Some of the factors that mediate steps in follicular development as demonstrated by phenotypes of mouse models are shown.

follicle development beyond the primary follicle stage. Growth differentiation factor-9 (GDF-9), a TGF-β superfamily member secreted by oocytes, kit ligand, which is derived from granulosa cells, and kit receptor, expressed on the oocyte surface, are key signaling proteins in these reciprocal interactions. Mice homozygous for a null allele at the *Gdf9* locus or for a hypomorphic *Kitl* allele exhibit female infertility due to blocks in follicular development before the formation of secondary follicles that contain two layers of granulosa cells.

Multilayered follicles become subject to regulation by extraovarian factors, namely, the gonadotropin hormones, and require follicle-stimulating hormone to achieve a crescendo of granulosa cell proliferation and become large antral preovulatory follicles. It is now appreciated that oocyte-derived factors influence granulosa cell proliferation and establish during these later stages of folliculogenesis characteristics unique to the cumulus granulosa cell population (i.e., the granulosa cells closest to the oocyte in the antral follicle as opposed to the mural granulosa cells closest to the follicle wall). For example, GDF-9 has been shown to: (1) stimulate the formation of the cumulus–oocyte complex (COC) by inducing hyaluronan synthase 2 and suppressing urokinase plasminogen activator; (2) promote both the elaboration and the response to prostaglandins in preovulatory granulosa cells, which in turn up-regulate progesterone production during ovulation; and (3) suppress the luteinization of cumulus granulosa cells by inhibiting the production of luteinizing hormone receptor. A related TGF-β superfamily protein, BMP-15, is also expressed in oocytes throughout most stages of follicular development and through ovulation and functions in a cooperative manner with GDF-9 to maintain the integrity of the COC and maximize female fertility in mice. Interestingly, the sheep orthologue of BMP-15, like mouse GDF-9, is crucial for early stages of follicular development and may also affect later events of follicle growth and ovulation. Point mutations occurring in the *BMP15* coding sequence in *Inverdale* (*FecX*$^{\mathrm{I}}$ mutation) and *Hanna* (*FecX*$^{\mathrm{H}}$ mutation) sheep cause defects beyond the primary follicle stage and infertility in homozygote ewes, but enhance ovulation in heterozygotes. Thus, the presence of the mutant BMP-15 in heterozygote sheep may interfere with or promote GDF-9 and/or BMP-15 homodimerizations or heterodimerizations or the interactions of these ligands with their receptors.

In addition to growth factors such as GDF-9 and BMP-15, oocytes produce and extrude structural proteins during follicular development that form their encasing zona pellucida matrix. Knockout mice lacking zona pellucida protein 3 (ZP3) or ZP2 demonstrate defects in early antral and preovulatory follicle development, respectively. Disrupted cumulus–oocyte complex formation and decreased ovulation in response to gonadotropins also contribute to both the infertility of the *Zp3* knockout and the subfertility of the *Zp2* knockout. Interestingly, blastocysts derived from *in vitro* maturation and fertilization of eggs from *Zp2*$^{-/-}$ and *Zp3*$^{-/-}$ females are not capable of completing development after transfer to pseudo-pregnant recipients. This suggests that zona matrix proteins are important in mediating granulosa cell signals to oocytes that optimize their later developmental potential. Interestingly, *Zp1* knockout females exhibit a more subtle subfertility phenotype, the first discernible compromise being a premature loss of zona integrity that affects early embryogenesis.

## III. CONTROL OF EARLY MEIOSIS AND APOPTOSIS IN OOCYTES

Mitotic divisions during PGC migration give rise to a complement of approximately 26,000 germ cells in the female mouse, and the size of this pool and the rate of its depletion are key determinants of the duration of reproductive potential. In addition to interactions with somatic cells during PGC migration and follicular organization, several factors intrinsic to the germ cells have proved critical to germ cell survival, including proteins involved in the control of early meiosis and apoptosis programs.

Several events of meiosis I precede the dictyate-stage block, which persists until ovulation. Replicated sister chromatids connected by axial elements condense and synapse with their homologous chromosomes by the pachytene stage; the maintenance of dictyate germ cells depends on cross-over exchanges that then occur between homologous chromosomes. Several proteins are known to play key roles between the zygotene and pachytene stages (Fig. 1). Chromosome condensation and the formation of synaptonemal complexes are blocked in knockout mice lacking ATM kinase or the DMC1 recombination protein, and in both of these models, female germ cells degenerate during embryogenesis. Translational control of synaptonemal complex proteins and perhaps other participants in early meiosis is exercised at this stage; knockout mice lacking CPEB or DAZL RNA-binding proteins have defects evident at the pachytene stage. Mismatch repair proteins encoded by the *Msh4* and *Msh5* loci are also critical for synapsis, and germ cells in these knockout mice have defects in the zygotene/pachytene stage and are lost within a few days postpartum. Similarly, oocytes in *Spo11* knockout mice, which lack an endonuclease that initiates recombination, die in the perinatal ovary. Thus, meiosis prior to the prophase I block requires the cooperative function of many factors and represents a period in oocyte development that is closely "monitored" to prevent the progression of abnormal gametes.

Functional antagonism between BAX and the anti-apoptotic factors BCL-X and BCL-2 regulates programmed cell death in oocytes. After the completion of PGC mitosis, apoptosis is key in controlling the size of the germ cell reserve during perinatal oocyte attrition and postnatally during follicular atresia in response to gonadotropin deprivation and environmental toxins. *Bax* knockout females have a reduction in postnatal loss of primordial and primary follicles, a threefold increase in the number of primordial follicles at sexual maturity, and an extended lifespan of ovarian function. Although late embryonic and perinatal oocyte loss is not measurably inhibited in the single knockouts, BAX is expressed in embryonic PGCs and has been implicated in the loss of PGCs in BCL-X hypomorphic mouse embryos. Like BCL-X, BCL-2 counters early oocyte apoptotic pathways; *Bcl2* knockout mice establish fewer primordial follicles, and many of these follicles are devoid of oocytes owing to germ cell degeneration. Conversely, oocyte expression of a *Bcl2* transgene increases the number of nonatretic maturing follicles, decreases follicular atresia and spontaneous oocyte loss, and suppresses oocyte apoptosis induced by doxorubicin. Thus, both loss-of-function and gain-of-function transgenic models have illustrated important *in vivo* roles for these apoptotic regulators in controlling the size of the finite oocyte pool after the completion of germ cell mitosis in embryogenesis and throughout the lifespan of ovarian function.

## IV. ACQUISITION OF MEIOTIC COMPETENCE AND COMPLETION OF OOCYTE MATURATION

In the periovulatory period, oocytes acquire competence to resume meiosis if separated from their surrounding granulosa cells, which normally control the prophase I block release. Coincident with the rise in serum luteinizing hormone (LH) and ovulation, oocytes from preovulatory follicles complete maturation; they undergo germinal vesicle breakdown, complete the reductional meiotic division, extrude the first polar body, and proceed to an arrest in metaphase of meiosis II.

Signals from the cumulus granulosa cells are critical to this progression. Defects in meiotic maturation are evident in oocytes from *Gja4* knockout mice lacking gap junction protein connexin 37, as well as in oocytes from connexin 43 knockout ovaries grafted into recipient mice to allow for follicular development. It is not clear from these models whether the meiotic phenotype reflects specific functions of these pores in discrete stages of oocyte maturation or whether the defects arise because normal intercellular communications within the follicle are never established. It has been speculated that gap junctions serve as a conduit for granulosa cell-to-oocyte movement of cyclic AMP (cAMP) prior to the LH surge, with oocyte cAMP being important in maintaining meiotic arrest. Consistent with this model, the LH surge induces connexin 43 phosphorylation changes, clearance of connexin 43 protein, and down-regulation of the connexin 43 mRNA. Paralleling these changes, decreases in oocyte cAMP levels, dependent on the activity of oocyte phosphodiesterase PDE3, allow for the resumption of meiosis. In addition to inhibiting oocyte maturation via cAMP, cumulus granulosa cells may provide stimulatory signals that promote meiotic progression. It has been hypothesized that granulosa cell-derived $Ca^{2+}$ and a lipophilic sterol (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol; meiosis-activating sterol) function in promoting meiotic resumption in response to the gonadotropin hormones. Investigating how these and other signals are transmitted to the oocyte and coordinate the regulation of meiosis in response to the hormonal environment *in vivo* represents an exciting field of ongoing research (see Fig. 3).

Oocyte factors are also important regulators in directing the completion of meiosis. Female knockouts lacking the endothelial isoform of nitric oxide synthase, expressed on the surface of oocytes, show compromised ovulation, delayed meiotic progression from metaphase I, and subfertility. The mechanism by which nitric oxide interfaces with the cell cycle machinery directing the metaphase I exit is not known, though mouse models have identified some factors relevant to metaphase transitions during mammalian oogenesis. For example, B-type cyclin proteins have been implicated in directing the commitment to complete both metaphase I and metaphase II as regulatory components of the maturation-promoting

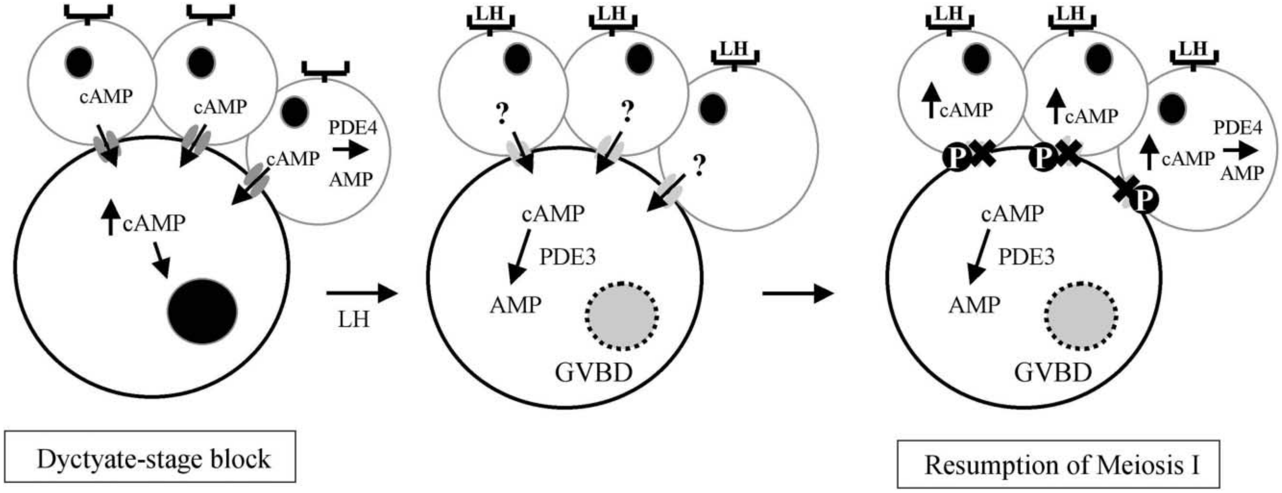

**FIGURE 3** A hypothetical model for the resumption of meiosis I. Prior to the preovulatory surge of LH, oocytes are maintained in a prophase I dictyate-stage block, in part by inhibitory signals from the surrounding granulosa cell compartment. It has been proposed that cAMP produced in the granulosa cells is communicated to the oocyte to maintain the dictyate nucleus and prevent germinal vesicle breakdown. The LH surge produces an increase in granulosa cell cAMP, which may affect the phosphorylation, closure, and clearance of gap junction channels by protein kinase cascades. This can be re-created by pharmacologically inhibiting granulosa cell phosphodiesterase (PDE4) activity, which promotes the resumption of meiosis in the absence of LH. It has been postulated that, prior to gap junction closure, a positive effector of meiosis ($Ca^{2+}$ or inositol 1,4,5-trisphosphate) is communicated to the oocyte, where it up-regulates phosphodiesterase enzyme (PDE3) and may mediate other intracellular events that promote oocyte maturation. The gap junction closure effectively isolates the oocyte as a compartment unto itself for the regulation of cAMP metabolism. The subsequent reduction in oocyte cAMP levels is a prerequisite for germinal vesicle breakdown and the release of the dictyate-stage block. Interfering with oocyte PDE3 blocks maturation even in the presence of LH and presumably an intact granulosa cell LHR response. *In vitro* studies have implicated PKA and PKC pathways as downstream mediators of cAMP effects in both the granulosa cells (releasing the meiotic block) and the oocyte (maintaining the meiotic block).

factor complex. B-type cyclin levels increase before each meiotic metaphase, and their ubiquitin-mediated degradation coincides with the metaphase–anaphase transition. Cyclin B1 is expressed in oocytes in this pattern and injection of cyclin B1 antisense mRNA causes defects during the completion of meiosis I and in the onset of metaphase II. Embryonic lethality in cyclin B1 knockout mice has thus far precluded *in vivo* studies of this protein's function in oocytes. Cyclin $B2^{-/-}$ females exhibit a subfertile phenotype, which has not yet been further characterized but may further establish the importance of this cyclin in mouse oogenesis.

The establishment of the block at metaphase II is important biologically to preclude parthenogenic activation of oocytes. This is compromised in *Mos* knockout mice lacking the oocyte-expressed MOS kinase, which is a component of the cytostatic factor complex implicated in maturation-promoting factor stabilization. Female *Mos* knockouts are subfertile and their oocytes have visible abnormalities in chromatin organization after the completion of meiosis I. A naturally occurring defect intrinsic to oocytes in the LTXBO mouse strain also causes a high incidence of parthenogenic activation, which is associated with delayed entry into anaphase I. Oocytes from these mice have elevated protein kinase C activity in late metaphase I, and inhibition of PKC in these oocytes promotes the timely onset of anaphase I and reduces spontaneous activation. These findings suggest that abnormal regulation of protein kinase C in the oocytes of this strain of mice is causal for their defects. Thus, LTXBO mice provide a valuable model for studying the molecular mechanisms underlying the late stages of oocyte meiosis and the onset of embryonic mitosis.

## V. FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT

Fertilization depends upon sperm–egg fusion molecules expressed by the gametes. Integrin α6β1 dimers and an associated tetraspanin protein, CD9, on the surface of oocytes are thought to be important in interacting with ADAM (a disintegrin and a metallprotease domain) proteins expressed by sperm, facilitating sperm binding and penetration. Corroborating this finding, knockout female mice lacking CD9 have severely compromised fertility owing to defects in the capacity for spermatozoa to bind to CD9 null oocytes.

Mammalian oocytes are normally released from their metaphase II block upon fertilization and this is marked morphologically by the separation of the second polar body. This represents the completion of oocyte maturation and the female gamete may be hereafter referred to as an egg. At least one mismatch repair protein, MLH1, though seemingly dispensable for meiosis I, is critical to the progression through meiosis II. *Mlh1* knockout females are completely sterile. Their oocytes develop, ovulate, and fertilize normally, but postfertilization development is blocked and extrusion of the second polar body is not accomplished. This unique block suggests the presence of a distinct checkpoint in meiosis II governing the completion of oocyte maturation, which involves the detection of aberrant DNA structures and arrests development in gametes with defective heteroduplex repair pathways.

Until sperm DNA decondensation and reorganization of a diploid nucleus are completed, many events of early embryogenesis cannot rely on *de novo* transcription from either parental genome, and factors that are required for embryonic development must be supplied by the oocyte. Disruptions of the maternal genome that cause phenotypes in embryonic development are termed maternal effect mutations and several have been recently characterized in mice using knockout technology. In each case, the gene product is normally accumulated in growing oocytes and persists in the early developing embryo, and the phenotype affects the offspring of homozygote females, regardless of their genotype or gender. Knockout females lacking a PHD-containing protein, ZAR1 (zygote arrest 1), are infertile due to a block in the development of their fertilized eggs to two-cell embryos. Similarly, a maternal effect gene encoding MATER (maternal antigen that embryos require) is necessary for development beyond the 2-cell stage and has been implicated in establishing embryonic genome transcription patterns. A third gene encodes DNMT1o, an oocyte-specific DNA methyltransferase critical for maintaining imprinting patterns established in the embryonic genome and the viability of the developing mouse during the last third of gestation. Transient nuclear localization of the DMTO1o protein during the 8-cell-stage embryo suggests that it normally functions during the fourth S phase and is required for the transmission of methylation patterns to each daughter cell produced in the 16-cell-stage embryo. Presumably, many other oocyte-derived factors mediate the complexities of early embryogenesis—both the dramatic morphological changes that herald early development and the compositional changes in mRNA and protein of reserves of eggs and zygotes around the time of fertilization.

## VI. CONCLUDING REMARKS

Our descriptive understanding of mouse oocyte and early embryo development has enabled us to invent transgenic technologies that hold the promise to reveal the molecular aspects of these biological processes. Future knockout models, multiple mutant studies, and the development of temporal and tissue-specific knockouts will allow us to more thoroughly examine the *in vivo* functions of gene products. The conservation of many genes and their functions across species indicates the potential of such studies in our pursuits to understand and affect the biology of female gameotogenesis in humans.

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## Glossary

**folliculogenesis** The process by which oocytes and ovarian somatic cells develop to ovulate a fertilizable egg. The first steps in follicle development are directed by intraovarian factors and result in proliferation of granulosa cells surrounding the oocyte and the recruitment of a layer of theca cells. The development of large antral follicles and ovulation depend on the actions of the pituitary gonadotropin hormones, follicle-stimulating hormone and luteinizing hormone. The stages of follicular development are summarized in Fig. 2.

**meiosis** A specialized cell division program completed during the formation of haploid gametes. In the first stage of meiosis (meiosis I), homologous pairs of replicated chromatids separate into daughter cells, and there is a reduction in both the DNA content and the ploidy. The second stage (meiosis II) involves the separation of paired chromatids. In mammalian female gametogenesis, the completion of each division results in the extrusion of a polar body so that one haploid egg is produced from the two divisions. There are arrests in meiosis at prophase I and metaphase II; these are released upon ovulation and fertilization, respectively. The stages of meiosis are summarized in Fig. 1.

**mitosis** Cell division that produces daughter cells with the same DNA content as the parental cell. The process begins after DNA replication and involves four phases as follows: (1) prophase, which is subdivided into stages describing chromosome morphology; (2) metaphase, when condensed chromosomes are aligned; (3) anaphase, when chromatids separate to opposite poles; and (4) telophase, which is followed by nuclear and cytoplasmic division.

**transgenic mice** Mice with heritable, engineered manipulations of the genome. These mice can have genes introduced for ectopic expression or can have endogenous genes or sequences altered. When the expression of an endogenous gene is completely abrogated, mice homozygous for the null allele are called knockout mice.

## See Also the Following Articles

**Corpus Luteum in Primates • Extracellular Matrix and Follicle Development • Fecundity Genes • Follicle Stimulating Hormone (FSH) • Folliculogenesis, Early • Implantation • *In Vitro* Fertilization • Ovulation**

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# Orphan Receptors, New Receptors, and New Hormones

David D. Moore

*Baylor College of Medicine*

I. INTRODUCTION
II. THE FIRST GENERATION OF THE NUCLEAR RECEPTOR SUPERFAMILY: IDENTIFICATION OF CLASSICAL RECEPTORS AND ORPHANS
III. THE NEXT GENERATION: LIGANDS AND FUNCTIONS FOR THE NEW RECEPTORS
IV. FUTURE GENERATIONS: FUNCTIONS OF THE CURRENT ORPHAN RECEPTORS
V. SUMMARY AND PROSPECTS

**There are 48 members of the nuclear hormone receptor superfamily in the human genome. Currently, these proteins can be divided into 23 conventional receptors with known ligands and 25 proteins, called orphan receptors, which do not have known ligands.**

## I. INTRODUCTION

The nuclear hormone receptor superfamily comprises two groups: the conventional receptors, those with known ligands, and the orphan receptors, those that do not have known ligands. The conventional receptors can be further subdivided into the classical receptors for a series of hormones and signaling molecules that were known to have nuclear receptors before any of the genes encoding these proteins were cloned, and the new receptors for more recently identified compounds. The ligands for the former group consist of estrogens, androgens, progestins, glucocorticoids, mineralocorticoids, vitamin D, thyroid hormone, and retinoic acid. Those for the new receptors include fatty acids, prostaglandins, oxysterols, bile acids, and xenobiotics, as well as an increasing number of synthetic ligands. Several of these synthetic ligands have already been found to be valuable therapeutic agents. This article focuses on the discovery of these new ligands, some of which were not expected to have such direct biologic regulatory functions, and their emerging roles in regulating lipid metabolism. The potential functions of several of the current orphans are also outlined.

## II. THE FIRST GENERATION OF THE NUCLEAR RECEPTOR SUPERFAMILY: IDENTIFICATION OF CLASSICAL RECEPTORS AND ORPHANS

The cloning and characterization of the cDNA encoding the glucocorticoid receptor (GR) by the laboratories of Keith Yamamoto and Ron Evans in 1984 and 1985 immediately revealed the existence of at least a small family of nuclear receptor proteins, based on the striking similarity of the GR cDNA sequence to that encoding the cellular proto-oncogene c-erbA. The subsequent isolation of clones encoding additional steroid receptors by the laboratories of Bert O'Malley, Pierre Chambon, and others resulted in the identification of the structurally conserved DNA-binding and ligand-binding domains shared by the family members and the initial characterization of the molecular mechanism of action of these receptors. In this mechanism, the DNA-binding domain functions to recognize specific sequences in appropriate target genes. The binding of the hormone to the structurally separate ligand-binding domain results in an allosteric effect that is now known to allow the binding of a series of different co-regulator proteins. Thus, hormone binding results in the recruitment of these co-regulator proteins to the target genes, where they exert their appropriate transcriptional regulatory effects.

However, the isolation of these additional receptors left open the question of what hormone might activate c-erbA. At the time, the main candidates were a small number of signaling molecules known to have nuclear receptors: thyroid hormone (triiodothyronine, T3), all-*trans*-retinoic acid (t-RA), 1,25-dihydroxyvitamin $D_3$, and dioxin. It did not take long for the laboratories of Ron Evans and Bjorn Vennstrom to identify T3 as the ligand for both c-erbA, which became the thyroid hormone receptor $\alpha$ isoform, TR$\alpha$, and the closely related TR$\beta$ protein. Thus, these proteins were the first to move from the orphan column to the conventional receptor column.

A similar strategy allowed the identification of a protein originally isolated as the target of hepatitis B virus insertions in hepatoma as the retinoic acid receptor $\alpha$ isoform (RAR$\alpha$), which was joined by the RAR$\beta$ and RAR$\gamma$ isoforms. The last receptors identified in this initial generation were the retinoid X receptors (RXR$\alpha$, $\beta$, and $\gamma$), which were initially identified as RAR relatives that were activated by high concentrations of t-RA, but were unable to bind it directly. The identification of the stereoisomer 9-*cis*-retinoic acid (9-*cis*-RA) as a specific agonist ligand for the RXRs was the first example of the use of what was then an orphan receptor to identify a new signaling molecule. Although the physiological significance of 9-*cis*-RA activation of the RXRs remains unclear more than 10 years after their identification, this exciting finding has led to the identification of specific synthetic RXR agonists that have recently received approval as cancer chemotherapeutic agents.

In 1996, estrogen receptor-$\beta$ (ER$\beta$) was the last of the classical receptors to be identified. This former orphan was found to be an alternative estrogen receptor on the basis of its sequence similarity to the previously identified ER$\alpha$. The existence of the $\beta$ isoform was not anticipated based on classical studies of estrogen function.

As these classical receptors were characterized, the list of the orphans that was initiated with estrogen receptor-related receptor-$\alpha$ (ERR$\alpha$) and ERR$\beta$ quickly began to lengthen. The current status of the family, as revealed in the human genome sequence and summarized in Table 1, is 48 total members, 14 of which are receptors for these classical ligands and 8 of which have more recently identified ligands as described below. The remaining 26 are still orphan receptors.

TABLE 1 Nuclear Hormone Receptor Subgroups

| Conventional receptors | |
|---|---|
| Classical | New |
| Steroid | Fatty acid |
| ERα, ERβ (3A1, 2), PR (3B3), AR (3B4) | PPARα, PPARδ, PPARγ (1C1–3) |
| GR (3B1), MR (3B2), VDR (1I1) | |
| Thyroid | Cholesterol/bile acid |
| TRα, TRβ (1A1, 2) | LXRα (1H3), LXRβ (1H2), FXR (1H4) |
| Retinoid | Xenobiotic |
| RARα, RARβ, RARγ (1B1–3) | PXR (1I2), CAR (1I3) |
| RXRα, RXRβ, RXRγ (2B1) | |
| **Orphan receptors** | |
| ERRα, ERRβ, ERRγ (3B1–3)[a] | |
| COUP-TFI, II (2F1, 2), ear2 (2F6) | |
| HNF-4α (2A1), HNF-4γ (2A2) | |
| SF-1, LRH-1, (NR5A1, 2) | |
| NGF-IB, Nurr1, Nor1 (4A1–3) | |
| RevErbAα, RevErbAβ (1D1, 2) | |
| RORα, RORβ, RORγ (1F1–3) | |
| TR-2, TR-4, (2C1-2) | |
| TLX (2E1), PNR (2E3) | |
| GCNF-1 (6A1) | |
| SHP, DAX-1 (0B1, 2) | |

*Note*. Conventional and orphan receptors are grouped based on ligand-binding properties. Many of the nuclear properties have a number of different names and/or different isoforms genereated by alternate splicing or promoter utilization, but only a single commonly used name is included here for each. See http://www.ens-lyon.fr/LBMC/laudet/NucRec/nomenclature_table.html for a more comprehensive list and GenBank accession numbers. In a standardized nomenclature for the nuclear receptors, each is referred to as "NR" followed by a three-character code based on evolutionary relatedness. This code is indicated in parantheses for each family member.

[a] Synthetic inverse agonist ligands have been identified.

In retrospect, it was misleading that the examples provided by these classical receptors clearly predicted that the "hormones" for the remaining orphans would share properties similar to those already described. Thus, it was anticipated that these new hormones would combine potent biologic regulatory effects with quite specific and high-affinity binding to their receptors. A major problem with this prediction was the significant discrepancy between the very limited number of remaining compounds with such properties and the much larger number of orphan receptors. The focus on high-affinity ligands stymied progress in this area for several years.

## III. THE NEXT GENERATION: LIGANDS AND FUNCTIONS FOR THE NEW RECEPTORS

### A. PPARα, PPARγ, and PPARδ

The next generation in the evolution of the understanding of the nuclear hormone receptors began in 1990 with the identification of a series of compounds previously known to increase numbers of peroxisomes in hepatocytes as ligands for a receptor termed the peroxisome proliferator-activated receptor (PPARα) (Table 2). This linkage was consistent with the prediction that a receptor ligand should have a potent biological effect. However, the fact that some of these ligands were active only at very high concentrations did not fit well with assumptions based on the classic hormones. This issue became even more significant when it was proposed that fatty acids were the endogenous ligands for the three PPAR isoforms. Although it is well known that consumption of different fatty acids can have significant effects on metabolism, it was not thought that such effects were due to a specific receptor for such fatty acids. Moreover, simple considerations of equilibrium binding dictate a basic trade-off between binding affinity and specificity that associates low-affinity interactions with decreased specificity. This argues strongly for quite limited specificity for binding of compounds that occupy a receptor at concentrations more than a million times higher than the picomolar

TABLE 2 Some New Ligands for Nuclear Hormone Receptors

| Receptor | Endogenous | Synthetic/exogenous |
|---|---|---|
| PPARα | Fatty acids, prostaglandins | Fibrates, other peroxisome proliferators |
| PPARδ | Fatty acids, prostaglandins | Carbaprostacyclin |
| PPARγ | Fatty acids, prostaglandins | Thiazolidinediones, new-generation anti-diabetic agents |
| LXR | Oxysterols | T09031 |
| FXR | Bile acids | GW4064, guggulsterone[a] |
| CAR | Androstanes[b] (bilirubin)[d] | TCPOBOP[c] (phenobarbital)[d] |
| PXR | Bile acids | Many drugs, catatoxic steroids and steroid antagonists |
| ERRs | ? | DES and 4-hydroxytamoxifen[b] |

[a] Antagonist.
[b] Inverse agonists.
[c] Rodent CAR only.
[d] Indirect activator.

levels associated with the effects of T3 and other high-affinity ligands. Thus, even though the concentrations of fatty acids required for PPAR activation may correspond to their endogenous levels, a signaling mechanism based on such poorly binding ligands must incorporate significant compromises in specificity relative to the conventional hormones. Such considerations predict that the PPARs would be unable to distinguish between various fatty acid species or even to exclude the effects of compounds not closely related to these endogenous ligands.

An elegant series of structural studies with the PPARs provided additional focus to these issues and addressed some of the associated concerns. These studies have revealed that the ligand-binding pocket of the PPARs is unusually capacious relative to more conventional receptors with high-affinity ligands. The ability of fatty acids to occupy this large pocket was directly confirmed by an X-ray crystal structure of eicosapentaenoic acid bound to PPARδ. This structure also confirmed the lack of specificity of such binding, since two quite different conformations of this flexible ligand were observed in the crystals.

Importantly, the crystal structures also explained the ability of additional synthetic ligands to bind these receptors with high affinity. A series of fibrate compounds associated with lipid-lowering effects were among the first compounds identified as activators of PPARα and were subsequently shown to be direct ligands. The initial identification of PPARγ as a factor promoting fat cell differentiation led to the identification of a class of compounds known as thiazolidinediones as relatively high-affinity PPARγ ligands, since such compounds were known to promote adipocyte differentiation. As expected, X-ray crystal structures demonstrate that the thiazolidinediones occupy a much larger fraction of the PPARγ ligand-binding pocket and make a good overall fit.

Based on both knockouts (genetically engineered null mice) and pharmacologic approaches, it is now clear that PPARα functions in the liver to stimulate fatty acid oxidation and PPARγ functions in fat cells to promote adipogenesis and expression of fat-specific genes. PPARγ also functions in macrophages to promote the return of cholesterol to the liver via the reverse transport pathway. The function of the third PPAR isoform, usually called PPARδ, is less clear. It is much more broadly expressed than the other two, and intriguing recent pharmacologic results suggest that PPARδ agonists may be therapeutically useful in treatment of the metabolic problems associated with obesity in syndrome X.

## B. Liver X Receptor-α, Liver X Receptor-β, and Farnesoid X Receptor

Three former orphans have been identified as receptors for primary metabolites of cholesterol with roles in cholesterol homeostasis. The first of these was liver X receptor (LXRα), which is activated by hydroxylated cholesterol derivatives called oxysterols. In agreement with the fact that these compounds had previously been identified as potential regulators of the expression of proteins involved in cholesterol homeostasis, and also its expression in the liver, LXRα knockout mice showed a profound defect in hepatic cholesterol metabolism. Normal mice are able to manage even relatively high levels of dietary cholesterol. However, the LXRα knockouts are unable to metabolize and eliminate the excess cholesterol, which accumulates in the liver. The closely related LXRβ isoform is also activated by oxysterols and is expressed in a number of tissues, including the liver, but the phenotype of the LXRα knockout animals demonstrates that LXRβ is unable to fully compensate for the loss of the former isoform. The LXRβ knockout also fails to show the same strong cholesterol accumulation phenotype as the LXRα knockout. However, the double-knockout animals have an even more severe phenotype, indicating some degree of functional overlap.

The phenotype of the LXRα knockout raised the possibility that LXR agonists could be useful in the treatment of hypercholesterolemia. Unfortunately, however, the synthetic agonist T09031 was found to significantly increase triglyceride levels in rodent models. This undesirable side effect is associated with the induction of the transcription factor sterol response element binding protein-1c (SREBP-1c), which promotes fatty acid synthesis.

In addition to their roles in lipid metabolism in the liver, the LXRs also function with PPARγ in the process of reverse cholesterol transport mentioned above. In this pathway, PPARγ activation in macrophages results in induction of LXRα expression, which, in turn, activates target genes responsible for cholesterol efflux, including the ABCA1 active transport pump and apolipoprotein E. Thus, LXR agonists may have beneficial effects in both the liver and the periphery if the triglyceride problem can be circumvented.

Another former orphan that functions in cholesterol homeostasis is farnesoid X receptor (FXR), which is directly activated by bile acids. Bile acids are downstream metabolites of cholesterol. They are produced in large amounts in the liver and are essential for the emulsification and absorption of dietary lipids. Although they are very efficiently reabsorbed in the gut after their release in bile, they represent the primary pathway for elimination of cholesterol from the liver. The potential function of FXR in bile acid and cholesterol homeostasis was supported by results with knockout animals, which show significant defects in these processes, including the inability to appropriately down-regulate bile acid biosynthesis in response to increased bile acid levels.

Like the cholesterol efflux from macrophages, this down-regulation is apparently also a consequence of a nuclear receptor cascade. In this case, activation of FXR by bile acids results in increased expression of an unusual orphan receptor named small heterodimer partner (SHP), which lacks a DNA-binding domain and functions to inhibit transactivation by other nuclear receptors. The orphan receptor liver receptor homologue-1 [LRH-1, also known as fetoprotein transcription factor (FTF)] is particularly sensitive to this repression and its activity is essential for the expression of the rate-limiting enzyme in bile acid biosynthesis, encoded by the *Cyp7A1* gene. Thus, the induction of FXR by bile acids results in decreased *Cyp7A1* expression via a pathway dependent on both SHP and LRH-1.

Like the LXRs, FXR is also a potential target for the modulation of cholesterol levels. Recently, guggulsterone, a natural product found to lower serum low-density lipoprotein levels in humans, was identified as an antagonist ligand for FXR. Treatment with this plant-derived steroid results in decreased accumulation of cholesterol in the liver of wild-type but not FXR knockout mice challenged with a high-cholesterol diet. Thus, it is possible that this or other, more specific FXR antagonists may be useful in the treatment of hypercholesterolemia.

## C. Constitutive Androstane Receptor and Pregnane X Receptor

The former orphans constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are each other's closest relatives within the receptor superfamily. Interestingly, they are in the same branch of the evolutionary tree as LXRα, LXRβ, and FXR. The final receptor in this cluster is the vitamin D receptor (VDR). All six of these receptors are expressed to at least some degree in the liver and all are RXR heterodimer partners. They are also all ligand dependent, with CAR and PXR sharing a rather complex overlapping network of xenobiotic agonists/activators and also target genes.

The term xenobiotic refers to all the compounds that organisms encounter that are not normal constituents of the body. This category includes an enormous range of agents present in foods, drugs, and environmental contaminants. It is well known that exposure to particular foreign compounds can increase the capacity of the liver to metabolize both the initial xenobiotic stimulus and other compounds. Both genetic and pharmacologic approaches have demonstrated that CAR and PXR mediate two particularly well-known xenobiotic responses.

In the first step of drug metabolism, called Phase I, a series of cytochrome P450 enzymes with a particularly wide range of substrates catalyzes the hydroxylation of xenobiotic compounds. In Phase II, a variety of other enzymes add a number of other functional groups, often to the site of initial hydroxylation. In general, this results in the inactivation and elimination of the xenobiotic substrate. Both CAR and PXR induce coordinate responses of Phase I and Phase II enzymes, as well as multidrug resistance transporters and other components of the drug metabolism pathway. Such responses are the basis for a class of clinically significant drug-drug interactions in which the presence of one drug affects the activity or half-life of another. In such interactions, the first drug is a direct activator of CAR or PXR, whereas the second is a substrate for the drug-metabolizing machinery.

The mouse PXR was first described as a receptor for a series of steroids and steroid antagonists that had previously been known to induce a characteristic xenobiotic response centered on the *Cyp3A* gene. A knockout of the mouse PXR gene confirmed its importance in this response, but also helped resolve a complication associated with the fact that the ligand-binding domain of the human PXR (also called SXR) diverges much more from the mouse PXR ligand-binding domain than is common within the superfamily. This divergent sequence had been shown to result in response to different agonist ligands. For example, the human receptor is potently activated by the antibiotic rifampicin, a well-known inducer of *Cyp3A* expression in human patients, but the mouse protein is completely unresponsive. As predicted from the activity of the isolated receptors, a mouse expressing the human PXR/SXR instead of its own PXR gains the ability to induce *Cyp3A* expression in response to rifampicin.

CAR shares a similar, if somewhat more complex, function. The barbiturate drug phenobarbital, used to treat seizures, also induces a characteristic xenobiotic response that centers on *Cyp2B* enzymes. Again, a knockout mouse model was used to demonstrate that this response is mediated by CAR. The pharmacology of this response is complicated somewhat by the fact that phenobarbital is not a ligand for either the mouse or the human CAR. Instead, it induces a specific translocation of the receptor from the cytoplasm of the hepatocyte to the nucleus. Little is known about this process, but it is thought to contribute to the activation of CAR by many other xenobiotics in addition to phenobarbital. Once in the nucleus, this receptor apparently relies on its ligand-independent or constitutive transcriptional activation function to stimulate the expression of appropriate target genes. Such constitutive activity is not a general characteristic of the ligand-dependent nuclear receptors described above. As described below, however, it is a common feature among the remaining orphan receptors.

The constitutive activity of mouse CAR can be further increased by a compound known as TCPOBOP (1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene), which is both a direct CAR agonist and a potent inducer of *Cyp2B* expression. It can also be blocked by androstanol and androstenol, which are inverse agonist ligands and can inhibit the activation of *Cyp2B* expression by TCPOBOP. As with PXR, the human and mouse CAR ligand-binding domains are divergent and neither of these murine CAR ligands binds the human receptor.

The functions of the two receptors in the xenobiotic responses overlap to a significant degree. For example, CAR is also able to activate *Cyp3A* expression and PXR can activate *Cyp2B*. They also share some activators, such as the antifungal agent clotrimazole, which can activate both human PXR and mouse CAR (but is a weak inverse agonist for human CAR). This overlap of targets and ligands is certainly not complete, however, and the specific and overlapping functions of these two receptors remain to be defined.

One area where they appear to show more specific activities is in their responses to endogenous compounds. Thus, PXR but not CAR has been associated with a protective response to toxic bile acids that is based on their function as both PXR ligands and substrates for *Cyp3A*. CAR, in contrast, functions to activate the clearance of bilirubin, a toxic breakdown product of heme that is specifically metabolized and eliminated by the liver.

## IV. FUTURE GENERATIONS: FUNCTIONS OF THE CURRENT ORPHAN RECEPTORS

In general, much less is known about those members of the nuclear receptor superfamily that remain orphans. In some cases, however, key insights into their roles have come from knockouts or other sources. The potential functions of some of the better characterized orphans will be briefly outlined here.

### A. ERRα, ERRβ, and ERRγ

The estrogen receptor-related receptors are closely related to the estrogen receptors and were among the first true orphans to be described. Like several of the other orphans, but not the classical receptors, they are evolutionarily ancient with an apparent homologue in *Drosophila*. The recent identification of the synthetic ER ligands diethylstilbestrol and 4-hydroxytamoxifen as inverse agonists strongly suggests that the identification of natural or endogenous ligands will soon relocate these proteins to the conventional receptor category. Although their overall functions remain uncertain, they also share DNA-binding sites, co-regulators, and target genes with the conventional estrogen receptors ERα and ERβ and may function to modulate estrogen signaling pathways.

## B. Steroidogenic Factor 1

In contrast to the isolation of many of the orphans using approaches such as low-stringency hybridization, steroidogenic factor 1 (SF-1) was first identified based on its ability to bind a series of related sites in the promoters of steroid hydroxylases. Also, unlike other orphans that bind DNA as homodimers or heterodimers, SF-1 binds as a monomer to an extended consensus element that includes a single copy of the nuclear receptor-binding hexamer. Like many other orphans, however, SF-1 functions as an apparently constitutive transcriptional activator.

The potential role of SF-1 in the regulation of adrenal steroidogenesis was strongly supported by the observation that the loss of SF-1 function in mice resulted in the absence of adrenals, gonads, and the ventromedial hypothalamus, as well as male-to-female sex reversal of internal and external genitalia. Thus, SF-1 is a key regulator of the development of important endocrine tissues. It is also expressed in these tissues in the adult, where its role remains an interesting question.

## C. Hepatic Nuclear Factor 4

Hepatic nuclear factor 4α (HNF-4α) is another orphan originally identified based on its ability to recognize specific sites, in this case in various promoters that are active in the liver. It binds these sites as a homodimer. Additional studies revealed that it is also expressed in the kidney, intestine, and pancreas, particularly the insulin-producing beta cells. A wide variety of target genes have been identified, including genes involved in fatty acid and cholesterol metabolism, glucose metabolism, urea biosynthesis, and liver differentiation. HNF-4α null mouse embryos die at a very early stage of development. The heterozygotes do not show an obvious phenotype. In humans, however, heterozygous loss of HNF-4α results in defective pancreatic beta-cell function and a characteristic syndrome called MODY (mature onset diabetes of the young). HNF-4α is MODY1; heterozygous loss of function of several other nonreceptor transcription factors that function in the beta cell results in a similar phenotype.

HNF-4 is another orphan that functions as an apparently constitutive transcriptional activator. Thus, it was a surprise when the recent X-ray crystal structure of HNF-4γ revealed a fatty acid occupying an unusually small putative ligand-binding pocket. This clearly suggests that the function of this important metabolic regulator could be modulated by appropriate synthetic ligands. However, its location in the structure and other lines of evidence suggest that this fatty acid may play a structural role more analogous to the zinc atoms in the DNA-binding domain than the much more dynamic functions of conventional nuclear receptor ligands. Thus, the potential ligand responsiveness of the HNF-4 isoforms remains to be established.

## D. Chicken Ovalbumin Upstream Transcription Factor I and II

These two closely related orphans are the mammalian homologues of an ancient orphan with unusually close relatives in *Drosophila* and a number of other invertebrates. The chicken ovalbumin upstream transcription factors can bind a wide variety of response elements as homodimers. They generally function as transcriptional repressors, but positive effects have been observed in some contexts. Although their activities appear very similar and their patterns of expression overlap significantly, the knockout of either chicken ovalbumin upstream transcription factor I (COUP-TFI) or COUP-TFII is lethal. In the former case, death is perinatal and is caused by multiple defects in development of the central and peripheral nervous system. In the cerebral cortex, for example, layer IV of the cortex is absent, a defect that is apparently secondary to a failure of the appropriate innervation of these neurons and subsequent cell death. In contrast, COUP-TFII null mice die at embryonic day 10 with a variety of heart and vascular defects. Interestingly, some of these defects resemble those observed in mice lacking angiopoeitin 1 or its receptor. The marked decrease in angiopoeitin expression in the knockouts suggests that COUP-TFII is an important upstream regulator of the expression of this important angiogenic factor. Thus, it is possible that modulation of COUP-TFII activity could control angiogenesis in pathologic processes including carcinogenesis.

## E. Nur-related Factor 1

Nur-related factor 1 (Nurr1) is one of three related receptors in a group that shows particularly flexible modes of DNA binding. Thus, nerve growth factor inducible B (NGFI-B) was the first orphan shown to bind with high affinity as a monomer, a property shared by the other two family members and also a subset of other orphans. At least in some cases, however, members of this family can also bind specific DNA sites as homodimers or as heterodimers with each other. In addition, Nurr1 and NGFI-B, but not neuron-derived orphan receptor 1 (Nor1), can bind distinct sites as heterodimers with RXR.

Of these three related orphans, the Nurr1 knockout showed the most dramatic phenotype, a complete loss of mesencephalic dopamine neurons. It is an interesting possibility that this orphan may be involved in disease processes that impact such dopaminergic neurons, such as Parkinson's disease and schizophrenia. Some support for this possibility is provided by the identification of Nurr1 gene mutations in a small number of schizophrenic patients.

### F. Reverse ErbAα

Reverse ErbAα (RevErbAα) is an orphan with an unusual genomic location: the coding region for its final exon overlaps with that of the final exon of the variant TRα2 isoform. It has a close relative, RevErbAβ, which is also linked to the TRβ locus but does not share a similar overlap. It is interesting that TRα and RevErbAα are also linked to RARα, whereas TRβ and RevErbAβ are linked to RARβ on a different chromosome, suggesting that a single, ancient duplication generated the two sets of isoforms for the three receptors.

A recent clue to the potential function of RevErbAα was provided by the unexpected demonstration that its expression is very strongly regulated by circadian rhythm. Somewhat less marked circadian variations were observed with RevErbAβ and also ROR isoforms, which can bind similar DNA-response elements. In RevErbAα knockout animals, circadian rhythms are significantly altered, but not absent. RevErbAα and RevErbAβ function as apparently constitutive repressors in co-transfection assays, and results with knockout models show that at least RevErbAα functions *in vivo* to represses the expression of Bmal1, a central component of the molecular circadian clock in mammals. Overall, these results confirm both the role of RevErbAα in the regulation of the circadian clock and the existence of redundant mechanisms in this important and complex pathway.

### G. SHP and DAX-1

SHP and dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome 1 (DAX-1) are unique orphans that lack a nuclear receptor DNA-binding domain. SHP directly interacts with a number of other nuclear receptors and inhibits their ability to activate transcription. As described above, results with SHP knockouts supported a specific role proposed for SHP in an FXR-dependent pathway for negative feedback regulation of bile acid biosynthesis. Interestingly, there are apparently also additional, redundant mechanisms for this process, since SHP null mice do show the expected loss of repression in response to a synthetic FXR agonist, but to a large degree maintain the repressive effect of high levels of dietary bile acids.

In contrast to SHP, which consists solely of a ligand-binding domain, DAX-1 includes an additional N-terminal domain. This domain has been associated with various DNA-binding activities, but the significance of this potential function remains uncertain. Loss of function of the human DAX-1 gene causes an X-linked form of adrenal hypoplasia congenita that is associated with hypogonadotropic hypogonadism. Like SHP, DAX-1 functions as a transcriptional repressor and it is thought that the loss of this repression function accounts for this phenotype. The transcriptional targets of DAX-1 remain unknown but several lines of evidence, including direct interaction and similar patterns of expression, suggest that it modulates SF-1 function.

## V. SUMMARY AND PROSPECTS

Two broad themes have emerged from studies of orphan receptors over the past 15 years. Starting with the TRs and RARs and continuing through the recent identification of the ERRs as potential targets of selective estrogen receptor modulators, the first is the identification of new ligands for orphan receptors. This has led to important and in some cases quite unexpected advances in several areas. Perhaps the most notable is the identification of the PPARs, LXRs, and FXR as key regulators of lipid metabolism. These and other receptors will continue to be important targets for the identification of new therapeutic approaches to regulate important metabolic pathways.

The potential therapeutic importance of these receptors highlights the significance of an intriguing question: Will ligands eventually be defined for all of the remaining orphans? This question can be further divided into whether such ligands will be endogenous ligands with important regulatory functions or synthetic compounds identified by high-throughput functional tests. In the former case, one might predict that many of the current orphans will remain orphans, since there is not a pool of remaining candidate compounds with the expected regulatory functions. Of course, this may be more reflective of a lack of imagination than a lack of such ligands, and it seems very likely that endogenous ligands will be identified for the ERRs and potentially other orphans. Nonetheless, several evolutionary arguments have suggested that the function of the progenitor of the superfamily may not have been ligand regulated and it is quite possible that other processes, such as phosphorylation, control the function of the remaining orphans.

Even if a significant number of the orphans do not have endogenous ligands, it remains an interesting possibility that synthetic ligands could be identified, for example, by efficient high-throughput screening technologies. The isolation of inhibitory peptides capable of binding to estrogen receptor isoforms with high affinity and specificity suggests a different approach to modulate the activity of orphans. With the continuing definition of the functions of the orphans from knockout studies and other studies, the motivation to identify appropriate ligands will be strong.

A second theme is the emergence of a number of important developmental functions of the orphans. Although the conventional receptor knockouts often show relatively limited phenotypes unless they are appropriately challenged, a number of the orphan receptor knockouts result in very early embryonic lethality or other dramatic developmental phenotypes. It is striking that comparisons of the complete human and *Drosophila* genome sequences reveal that the human nuclear receptors with *Drosophila* relatives are not the classical steroid thyroid and retinoid receptors, but the orphans. Though the existence of multiple isoforms of individual receptor types complicates matters, it is also appears that the knockouts of the mammalian receptors with the closest *Drosophila* relatives, such as the COUP-TFs and HNF-4α, show particularly strong embryonic phenotypes.

Whereas a number of such developmental functions have been identified for the conserved orphans, the apparent role of RevErbAα in circadian rhythm clearly suggests that there will be exceptions. It is a simple prediction that the orphans that remain less well characterized have some interesting surprises in store for us.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Crosstalk of Nuclear Recepors with STAT Factors • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Ligand Modification to Produce Pharmacologic Agents • Lipoprotein Receptor Signaling • Peroxisome Proliferator-Activated Receptors (PPARs) • Steroid Hormone Receptor Family: Mechanism of Action**

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# Osteogenic Proteins of the TGF-β Superfamily

U. Ripamonti

*Medical Research Council/University of the Witwatersrand, Johannesburg, South Africa*

I. INTRODUCTION
II. TRANSFORMING GROWTH FACTOR-β SUPERFAMILY
III. BONE MORPHOGENETIC PROTEINS/OSTEOGENIC PROTEINS
IV. ENDOCHONDRAL BONE INDUCTION BY TGF-β ISOFORMS
V. GEOMETRIC INDUCTION OF BONE FORMATION

**The normal repair and regeneration of bone constitute a complex process that is temporally and spatially regulated by soluble and insoluble signals. The bone morphogenetic proteins or osteogenic proteins, members of the transforming growth factor-β supergene family, are morphogens endowed with the striking prerogative to initiate *de novo* bone formation by induction in heterotopic extraskeletal sites of animal models.**

## I. INTRODUCTION

The three most important requirements for successful tissue engineering of bone are a suitable extracellular matrix substratum, capable responding cells, and soluble osteoinductive signals, which are members of the transforming growth factor-β (TGF-β) supergene family. The reconstitution of bone morphogenetic proteins/osteogenic proteins (BMPs/OPs) (the soluble signals) with biomimetic matrices (the insoluble substratum) provides a bioassay for *bona fide* initiators of bone differentiation as well as delivery systems for therapeutic local osteogenesis. Contrary to all the results obtained in the rodent bioassay, heterotopic implantation of naturally derived or recombinant human (h) TGF-β isoforms induces vigorous endochondral bone induction in the *rectus abdominis* muscle of the adult primate *Papio ursinus*. The binary applications of doses of recombinant hBMPs/OPs with relatively low doses of hTGF-β1 interact synergistically to rapidly induce massive heterotopic and orthotopic ossicles in the rectus abdominis muscle and calvarial defects, respectively.

The discovery that specific surface and geometric characteristics of sintered porous hydroxypatites can induce bone in heterotopic sites of primates in the absence of exogenously applied BMPs/OPs paves the way for the formulation and therapeutic application of smart porous substrata that lead to the formation of predictable tissue types via intrinsic osteoinductivity. The incorporation of specific biological activities into sintered hydroxypatites defined as geometric induction of bone formation elicits therapeutic osteogenesis in clinical contexts. The intrinsic osteoinductivity of porous substrata in primates indicates that the bone induction cascade is initiated by endogenously produced BMPs/OPs bound to the surface of the smart concavities of the substratum, with induction of bone as a secondary response. The concavities of the substratum are geometric regulators of growth endowed with shape memory, recapitulating events that occur in the normal course of embryonic development and appearing to act as gates that give or withhold permission to grow and differentiate. Extensive studies in animal models, particularly nonhuman primates, have made possible the use of both hOP-1 and hBMP-2 for craniofacial and orthopedic applications in clinical contexts.

## II. TRANSFORMING GROWTH FACTOR-β SUPERFAMILY

The initiation of bone formation during embryonic development and postnatal morphogenesis and osteogenesis involves a complex cascade of molecular and morphogenetic processes that ultimately lead to the architectural sculpture of precisely organized multicellular structures. Elucidating the nature and interaction of the signaling molecules that direct the generation of tissue-specific patterns during the initiation of endochondral bone formation is a major challenge of contemporary molecular, cellular, developmental, and tissue engineering biology.

Quantum leaps in these rapidly evolving fields have dramatically advanced our understanding of tissue induction and morphogenesis. First, the putative signaling molecules or morphogens, defined as form-generating substances capable of imparting specific different pathways to responding cells initiating the cascade of pattern formation and the attainment of tissue form and function, have been purified and cloned and their *in vivo* functions have been identified.

Nature relies on common yet limited molecular mechanisms tailored to provide the emergence of specialized tissues and organs. The distilled summary of this research effort is surprisingly simple: first, that tissue regeneration in postnatal life recapitulates events that occur in the normal course of embryonic development and morphogenesis and second, that both embryonic development and tissue regeneration are equally regulated by a select few and highly conserved families of morphogens. In addition, these gene products are members of the TGF-β superfamily, and purification and expression cloning yielded an entirely new family of protein initiators, the BMPs/OPs.

## III. BONE MORPHOGENETIC PROTEINS/OSTEOGENIC PROTEINS

The BMP/OP family is indeed an elegant example of nature parsimony in programming multiple specialized functions deploying molecular isoforms with minor variations in amino acid motifs within highly conserved carboxy-terminal regions.

Members of the BMP/OP and TGF-β families are pleiotropic morphogens that have potent and diverse effects on cell proliferation, differentiation, motility, and matrix synthesis. They are powerful regulators of cartilage and bone differentiation in embryonic development and in postnatal life and are soluble mediators of tissue morphogenesis and regeneration. They exert their biological activities through heteromeric serine/threonine kinase complexes of type I and II receptors and are predominantly synthesized as glycosylated homodimers with a carboxy-terminal region containing characteristic cysteine motifs. In addition to bone induction in postnatal life, BMPs/OPs are involved in inductive events that control pattern formation during embryonic morphogenesis and organogenesis in such disparate tissues as the kidney, eye, nervous system, lung, teeth, skin, and heart. These strikingly pleiotropic effects of BMPs/OPs spring from minor amino acid sequence variations in the carboxy-terminal region of the proteins, as well as in the transduction of distinct signaling pathways by individual Smad proteins after transmembrane serine/threonine kinase receptor activation.

The three mammalian TGF-β isoforms share limited homology with members of the BMP/OP family. A striking and discriminatory feature of the BMP/OP proteins is their ability to induce *de novo* cartilage and bone formation by induction when implanted in extraskeletal heterotopic sites of mammals as recapitulation of embryonic development. This ability, originally solely assigned to the BMP/OP family, has been extended to other TGF-β supergene family members including decapentaplegic and 60A gene products expressed early in *Drosophila melanogaster* development, demonstrating evolutionary conservation of related proteins from phylogenetically distant species.

## IV. ENDOCHONDRAL BONE INDUCTION BY TGF-β ISOFORMS

In the *bona fide* heterotopic assay for bone induction in rodents, the TGF-β isoforms, either purified from natural sources or expressed by recombinant techniques, do not initiate endochondral bone formation. More strikingly and recently, TGF-β isoforms themselves have shown a marked site- and tissue-specific endochondral osteoinductivity yet remarkably this occurs in primates only. Induction of osteogenic differentiation has also been shown, at least in rodents, by growth and differentiation factor-5 and Hedgehog proteins with an activity synergistically regulated and enhanced by recombinant hBMP-2.

The presence of several related but different molecular forms with osteogenic activity poses important questions about the biological significance of this apparent redundancy, additionally indicating multiple interactions during both embryonic development and bone regeneration in postnatal life. The fact that a single hBMP/OP initiates bone formation by induction does not preclude the requirement and interactions of other morphogens deployed synchronously and synergistically during the cascade of bone formation by induction, which may proceed via the combined action of several BMPs/OPs, resident within the natural milieu of the extracellular matrix of bone. Thus, it is likely that the endogenous mechanisms of bone repair and regeneration in postnatal life necessitate the deployment and concerted action of several of the BMPs/OPs resident within the natural milieu of the extracellular matrix. The presence of multiple molecular forms with bone inductive activity also points to synergistic interactions during endochondral bone formation. Indeed, a potent and accelerated synergistic induction of endochondral bone formation was shown with the binary application of recombinant or native TGF-β1 with hOP-1, in both heterotopic and orthotopic sites of primates (Fig. 1). Whether the biological activity of partially purified BMPs as shown in long-term experiments in the adult primate *P. ursinus* is the result of the sum of a plurality of BMP activities or is a truly synergistic interaction among BMP/OP family members deserves appropriate investigation. It is noteworthy, however, that in the identical orthotopic model, the long-term efficacy of single applications of gamma-irradiated hOP-1 delivered by a xenogeneic bovine collagenous matrix in regenerating large defects of membranous bone of the adult primate was demonstrated (Fig. 2). Ultimately, it will be necessary to gain insights into the potential distinct spatial and temporal patterns of expression of other BMPs/OPs during morphogenesis and regeneration elicited by a single application of hOP-1. *In vivo* studies should now design therapeutic approaches based on information about gene regulation by hOP-1.

The TGF-β isoforms are powerful inducers of endochondral bone when implanted in the *rectus abdominis* muscle of the primate *P. ursinus* at doses of 1, 5, and 25 μg per 100 mg of collagenous matrix as carrier, yielding large corticalized ossicles by day 90 (Fig. 3). Endochondral bone induction initiated by TGF-β isoforms expresses mRNA of bone induction markers including BMP-3 and OP-1. An additional and significant striking result is that the bone inductivity of TGF-β isoforms in the primate is site- and tissue-specific, with rather substantial

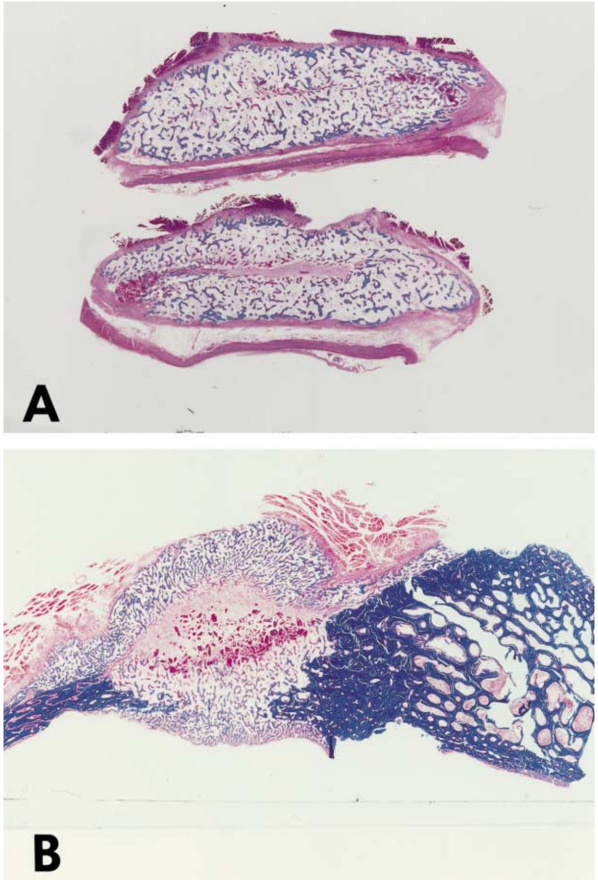

**FIGURE 1** Tissue morphogenesis by binary applications of recombinant hOP-1 and TGF-β1. (A) Generation of large ossicles that formed 30 days after implantation of 25 μg hOP-1 and 1.5 μg hTGF-β1 in the *rectus abdominis* of the adult primate *Papio ursinus*. (B) Tissue induction and morphogenesis of bone on day 30 on implantation of 100 μg hOP-1 in combination with 5 μg platelet-derived porcine TGF-β1, culminating in gross displacement of the pericranial tissues. Original magnification: (A) × 3.2 (B) × 2.8.

endochondral bone induction in the *rectus abdominis* muscle but absent osteoinductivity in orthotopic sites on day 30 and limited osteogenesis in orthotopic sites on day 90 (Fig. 4).

## V. GEOMETRIC INDUCTION OF BONE FORMATION

Importantly, to induce the cascade of endochondral bone differentiation, the soluble signals must be reconstituted with an insoluble signal or substratum that triggers the bone differentiation cascade. Bone regeneration in clinical contexts requires three key components: an osteoinductive signal, an insoluble substratum that delivers the signal and acts as a scaffold for the induction of new bone formation, and host recipient cells that are capable of differentiation into bone cells in response to the osteoinductive signals. All of the components are amenable to manipulation: the signals to be delivered and the nature of the carrier matrix, which additionally can be loaded with the responding cells and tissues. Although molecular biology has made quantum leaps in the mechanistic understanding of cellular and subcellular activities of soluble signals, less knowledge and fewer mechanistic insights have characterized the quest for optimal delivery systems. Such systems would include insoluble substrata that are inorganic and nonimmunogenic, carvable, and amenable to contouring for optimal adaptation to the various shapes of bone defects and initiating optimal osteogenic activity with relatively low doses of recombinant hBMPs/OPs. They would promote rapid vascular invasion, angiogenesis, and mesenchymal invasion when brought into contact with BMPs/OPs previously absorbed onto the carrier and would be capable of remodeling and resorbing once the regenerative processes are well under way. Finally, they would have optimal surface characteristics and geometric configurations, which are of critical importance in the induction of bone

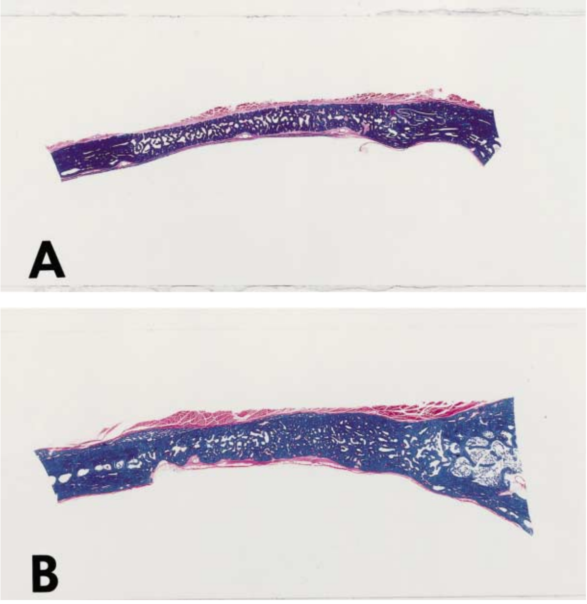

**FIGURE 2** Low-power microphotographs of calvarial specimens harvested on days 90 (A) and 365 (B) from the primate *Papio ursinus*. Complete reconstruction and regeneration of the defects after implantation of doses of 0.1 (A) and 0.5 mg (B) of gamma-irradiated hOP-1 delivered by 1 g of gamma-irradiated xenogeneic bovine collagenous matrix. Original magnification: × 2.5.

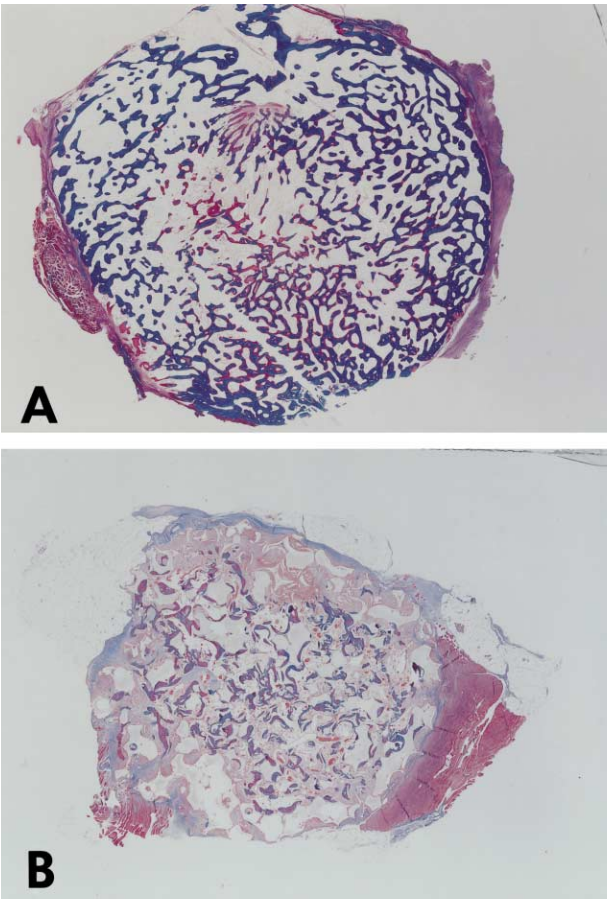

**FIGURE 3** Induction of bone formation by hTGF-β2 in the *rectus abdominis* muscle of the primate *Papio ursinus* on implantation of 5 μg hTGF-β2 delivered by the insoluble collagenous matrix as carrier (A) and 1 μg hTGF-β2 delivered by sintered highly crystalline porous hydroxyapatite (B) harvested on day 90. Original magnification: × 3.5.

with and without the exogenous applications of BMPs/OPs. The critical role of the geometry of the carrier substratum in the regulation of bone differentiation has been amply documented using different geometric configurations of collagenous matrix and porous hydroxyapatites, providing evidence that tissue induction and morphogenesis can be greatly altered by the geometry of the carrier.

Since regenerative phenomena recapitulate events that occur in the normal course of embryonic development, the observed multiple patterns of expression of BMPs/OPs in developing tissues and organs may help to devise therapeutic approaches based on recapitulation of embryonic development and ample evidence is accruing regarding the efficacy and safety of two members of the BMP/OP family now available in recombinant form, hOP-1 and hBMP-2, currently the subject of extensive preclinical and clinical research for orthopedic and craniofacial applications.

The finding that heterotopic bone induction in primates is initiated by naturally derived BMPs/OPs and TGF-βs, recombinant hBMPs/OPs and hTGF-βs, and sintered and highly crystalline hydroxyapatites with a specific geometric configuration indicates that heterotopic ossicles develop as a mosaic structure in which members of the TGF-β superfamily singly, synergistically, and synchronously initiate and maintain the developing morphological structures and play different roles at different time points of the morphogenetic cascade.

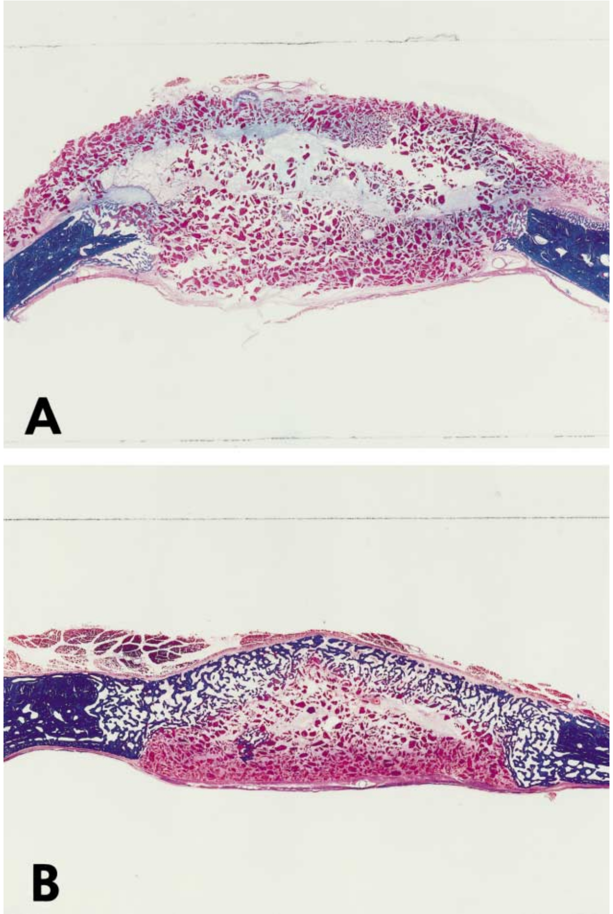

**FIGURE 4** Morphology of calvarial regeneration by hTGF-β2 in conjunction with collagenous matrix as carrier. (A) Lack of bone formation on implantation of 100 μg of hTGF-β2 on day 30 with prominent mesenchymal tissue influx and displacement of the collagenous matrix. (B) Limited osteogenesis only pericranially on implantation of 100 μg hTGF-β2. Note the delicate trabeculae of newly formed bone facing scattered remnants of collagenous matrix particles, embedded in a loose but highly vascular connective tissue matrix. Original magnification: × 2.8.

Biomimetic matrices endowed with intrinsic osteoinductive activity, i.e., capable of initiating *de novo* bone formation in heterotopic sites of primates even in the absence of exogenously applied BMPs/OPs, have been developed (Fig. 5). Our findings in nonhuman primates also demonstrate extensive bone formation by hOP-1 adsorbed onto sintered porous hydroxyapatites, indicating that predictable osteogenesis in clinical contexts for the treatment of craniofacial bone defects may be engineered using inorganic, nonimmunogenic, and carvable delivery systems that initiate osteogenesis with relatively low doses of recombinant morphogens, thus mimicking the macro- and microstructures of living bone.

The use of biomimetic matrices capable of initiating bone formation via intrinsic osteoinduction is quickly altering the horizons of therapeutic osteogenesis, leading to a quantum leap in bone tissue engineering. The geometry of the substratum profoundly regulates the expression of the osteogenic phenotype and is defined as geometric induction of bone formation. The intrinsic osteoinductivity regulated by the geometry of the substratum is helping to engineer morphogenetic responses for therapeutic osteogenesis in clinical contexts.

Members of the TGF-β supergene family, BMPs/OPs and TGF-β isoforms endowed with endochondral osteoinductivity in adult primates, are helping to engineer skeletal regeneration and tissue morphogenesis in molecular terms. This prediction is based on a surprisingly simple and fascinating concept: morphogens exploited in embryonic development can be reexploited for the initiation of postnatal morphogenesis and regeneration.

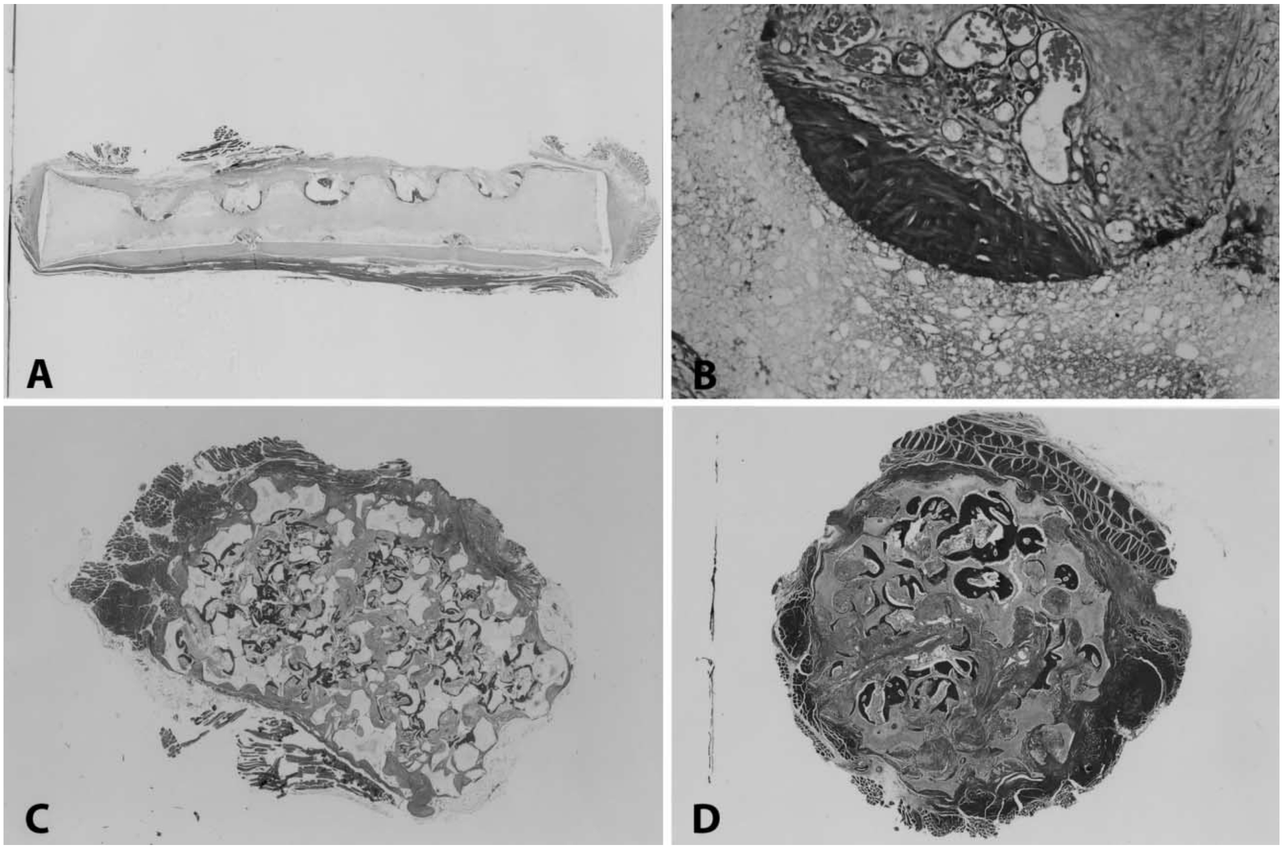

**FIGURE 5** Effect of geometry of the substratum on tissue induction and morphogenesis. (A) Monolithic discs of sintered highly crystalline hydroxyapatite with concavities on both planar surfaces were implanted in the *rectus abdominis* muscle of the primate *Papio ursinus* and harvested on day 90 after surgery. Bone forms only within the concavities of the substratum. (B) Note the vascular invasion and angiogenesis close to newly formed and induced bone. (C and D) Low-power photomicrographs of sintered porous hydroxyapatite specimens harvested from the *rectus abdominis* on day 90. Note the intrinsic and spontaneous induction of bone formation within the porous spaces of the hydroxyapatite, essentially initiating in concavities of the substratum. Original magnification: × 8.

## Acknowledgments

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The Bone Research Unit and the Medical Research Council acknowledge the CSIR Manufacturing and Materials Technology Unit for the preparation of the sintered hydroxy-apatite implants.

## Glossary

**bone morphogenetic proteins/osteogenic proteins** Pleiotropic glycosylated protein members of the transforming growth factor-β superfamily initiators of bone formation by induction.

**intrinsic osteoinductive activity** Induction of bone formation by specific geometric configurations of biomimetic matrices in the absence of exogenously applied bone morphogenetic proteins/osteogenic proteins.

**transforming growth factor-βs** Proteins secreted by transformed cells that can stimulate the growth of normal cells.

## See Also the Following Articles

**Bone Morphogenetic Proteins • Colony-Stimulating Factor-1 (CSF-1) • Thyroid Hormone Action on the Skeleton and Growth • Vitamin D and Cartilage • Vitamin D: Biological Effects of $1,25(\text{OH})_2\text{D}_3$ in Bone**

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# Osteoporosis: Hormonal Treatment

NORMAN H. BELL
*Medical University of South Carolina*

I. ESTROGEN
II. RALOXIFENE
III. SALMON CALCITONIN
IV. PARATHYROID HORMONE (1–34)
V. SUMMARY

**Estrogen, raloxifene, nasal salmon calcitonin, and parathyroid hormone (1–34) are the only hormones or hormonal analogues that have been approved by the Food and Drug Administration (FDA) for the treatment of osteoporosis in the United States.**

## I. ESTROGEN

It is well established that estrogen deficiency at the time of menopause leads to increases in skeletal remodeling and the rate of bone loss and, in some patients, to osteoporosis and fractures. It is also well established that restoration of estrogen levels via hormone replacement therapy (HRT) reduces skeletal remodeling and not only prevents bone loss but increases bone mineral density (BMD). Whether estrogen prevents fractures, however, is controversial. Analysis of the Study of Osteoporosis Fracture indicated that current estrogen use was associated with a 61% decrease in the incidence of fractures of the wrist and a 34% decrease in nonvertebral fractures. The incidence of hip fractures was significantly reduced in current users by 61% but only when HRT was begun within 5 years of menopause. In an open, randomized, placebo-controlled study from Finland of women treated with HRT beginning shortly after menopause, the incidence of nonvertebral fractures was significantly reduced by 61%. On the other hand, the incidence of fractures was not reduced by HRT in a prospective, double-blind, placebo-controlled study in postmenopausal women less than 90 years of age.

Earlier epidemiological studies indicated that HRT prevents myocardial infarctions and fatal heart attacks in postmenopausal women. However, the recent prospective, randomized, placebo-controlled Heart and Estrogen/Progestin Replacement Study in postmenopausal women with established heart disease found that the incidence of cardiovascular events was not different in HRT- and placebo-treated groups even though there was an 11% reduction in low-density lipoprotein cholesterol and a 10% increase in high-density lipoprotein cholesterol in the HRT-treated group. Previous to these findings, estrogen was considered to be effective with regard to treatment of coronary artery disease. In view of these findings, the use of HRT for prevention of coronary artery disease is no longer recommended. This recommendation has been confirmed. Recently, the Women's Health Initiative, a prospective, randomized clinical trial with conjugated equine estrogen and medroxyprogesterone acetate in 16,608 postmenopausal women, had to be stopped after 5.2 years because it was found that per 10,000 person years there were 7 more events of cardiovascular disease, 8 more strokes, 8 more pulmonary emboli, 8 more invasive breast cancers, 6 fewer colorectal cancers, and 5 fewer hip fractures in the patients than in the controls. Thus, the risks exceed the benefits from use of combined estrogen and progestin treatment in postmenopausal women.

## II. RALOXIFENE

Raloxifene is a member of a group of drugs known as selective estrogen receptor modulators (SERMs) that have a more favorable therapeutic profile than estrogen. Raloxifene is an agonist for bone but, unlike estrogen, is an antagonist for breast and uterine tissue. It is therefore safer than HRT. Raloxifene modestly reduces bone turnover and increases BMD and significantly decreased the incidence of fractures of the spine by 30% (at 30 mg/day) and by 50% (at 60 mg/day) in postmenopausal women. In preliminary studies, it reduced the incidence of breast cancer in postmenopausal women. Raloxifene is undergoing a large clinical trial, Study of Raloxifene and Tamoxifen, for comparison with tamoxifen, the current drug of choice, to determine its efficacy in the prevention of breast cancer. Whether raloxifene prevents coronary heart disease is not known. This question is being addressed in another large clinical trial.

## III. SALMON CALCITONIN

In the Prevent Recurrence of Osteoporotic Fractures study, nasal salmon calcitonin at 200 IU daily modestly reduced skeletal remodeling and increased BMD of the spine and significantly reduced the incidence of vertebral fractures by 33% in postmenopausal women. Nasal salmon calcitonin was ineffective in this regard at daily doses of 100 and 400 IU. Nevertheless, the drug is approved for treatment of postmenopausal osteoporosis.

## IV. PARATHYROID HORMONE (1–34)

Daily injections of parathyroid hormone (1–34) for less than 2 years significantly increased BMD and reduced the incidence of vertebral fractures by more than 60% and of nonvertebral fractures by more than 50% in postmenopausal women with one or more prevalent fractures. Parathyroid hormone (1–34) given by daily injection is anabolic for the skeleton and does not cause bone resorption.

## V. SUMMARY

Estrogen, raloxifene (a SERM), salmon calcitonin, and parathyroid hormone (1–34) are four hormones or hormonal analogues that have been approved for the treatment of osteoporosis. Each was shown to decrease skeletal remodeling and increase BMD by inhibiting bone resorption. Whether estrogen prevents fractures as well as coronary artery disease, however, has not been established. These questions may be answered by results of the Women's Health Initiative. Raloxifene was shown to diminish the incidence of vertebral fractures and has the advantage of inhibiting the growth of breast and uterine cancer and uterine tissue. It may prove useful for the treatment of breast cancer. Nasal salmon calcitonin at a dose of 200 IU daily was shown to reduce the incidence of vertebral fractures but doses of 100 and 400 IU daily were ineffective. Parathyroid hormone (1–34), an anabolic peptide, diminished the rate of both vertebral and nonvertebral fractures when given by daily injection.

## Glossary

**osteoporosis** A bone disease in which decreased bone mass and alteration in microarchitecture result in increased skeletal fragility and risk of fracture. It occurs in postmenopausal women as a consequence of estrogen deficiency.

**parathyroid hormone (1–34)** The N-terminal biologically active portion of parathyroid hormone, which is produced by the parathyroid glands and increases bone resorption and tubular reabsorption of calcium by the kidney.

**raloxifene** A selective estrogen receptor modulator that is an agonist for bone and, unlike estrogen, is an antagonist for uterine and breast tissue.

**salmon calcitonin** A hormone produced by the parafollicular C cells of the thyroid gland; it inhibits osteoclastic bone resorption and tubular reabsorption of calcium by the kidney. Salmon calcitonin has a different structure than human calcitonin and is more potent.

**selective estrogen receptor modulator** A drug that has a profile that is similar to or different than that of estrogen with regard to being an agonist or antagonist for skeletal, uterine, breast and other tissues.

## See Also the Following Articles

**Aromatase and Estrogen Insufficiency • Calcitonin • Estrogen and Progesterone Receptors in Breast Cancer • Estrogen Receptor Biology and Lessons from Knockout Mice • Osteoporosis: Pathophysiology • Parathyroid Hormone • SERMs (Selective Estrogen Receptor Modulators)**

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## III. CLASSIFICATION OF OSTEOPOROSIS

Osteoporosis can be categorized into primary and secondary forms based on the absence or presence of associated medical diseases that predispose to bone loss. Primary osteoporosis is the most common form and is due to age-related loss of bone from the skeleton. Secondary osteoporosis is associated with specific defined clinical disorders that lead to osteoporosis; some of the well-known disorders are given in Table 2.

In 1947, Albright divided osteoporotic patients into postmenopausal and senile groups. He clearly implicated the role of estrogen deficiency in postmenopausal osteoporosis. More recently, based on pathogenic mechanisms, Riggs and Melton used a new terminology: Type I, for postmenopausal osteoporosis, and Type II, for senile osteoporosis. This classification of Riggs and Melton does not include patients with osteoporosis resulting from other diseases and conditions. Appropriately, this latter group of osteoporotics has been classified as Type III. Type I osteoporosis characteristically affects women within 15 to 20 years of menopause, presents principally with vertebral crush fractures and Colle's fractures of distal forearm, and is due mainly to trabecular bone loss as a consequence of estrogen deficiency. Type II (age-related osteoporosis) occurs in both men and women 75 years of age and older and is usually manifested by hip and vertebral fractures. Type II osteoporosis is believed to be mainly due to age-related trabecular and cortical bone loss. Type III osteoporosis, or secondary osteoporosis, occurs in both men and women and is caused by a specific defined clinical disorder or medical treatment. A brief summary of the main features of these three osteoporotic types is shown in Table 3.

**TABLE 2 Major Causes of Secondary (Type III) Osteoporosis**

| | |
|---|---|
| Drug use | Corticosteriods<br>Anticonvulsants<br>Radiotherapy<br>Heparin |
| Gastrointestinal | Malabsorption syndromes<br>Primary biliary cirrhosis |
| Blood diseases | Myeloma<br>Thalassemia<br>Skeletal metastases |
| Endocrine | Thyrotoxicosis<br>Cushing's syndrome<br>Turner's/Klinefelter's syndrome<br>Primary hyperparathyroidism |
| Others | Immobilization<br>Transplantation<br>Alcoholism<br>Chronic renal failure<br>Osteogenesis imperfecta |

## IV. PEAK BONE MASS

Peak bone mass can be defined as the maximum level of bone mass attained in an individual as a result of normal growth. It is usually achieved in the first few years of the second decade of life, the early 20s. It is one of the two principal factors that determine lifelong skeletal health, the other being the bone loss. High peak bone mass provides a larger reserve later in life and offers a protective advantage when bone density declines as a result of aging or other causes. Achievement of peak bone mass is mainly determined by genetics, but nutritional, hormonal, and environmental factors and mechanical loading also influence bone mass.

Data from twin studies suggest that BMD at a number of skeletal sites has a strong genetic component and it is estimated that about 60–80% of peak bone mass is determined genetically. Family studies also provide evidence for a genetic impact on BMD; daughters of women with osteoporosis have a lower BMD than do daughters of women with a normal BMD. Another approach to identify specific genes that influence peak bone mass is to use the candidate gene approach. Using this approach, allelic variation in the vitamin D receptor was first shown to be associated with BMD. The association studies have expanded to other candidate genes known to play a role in normal bone physiology. Common allelic variations in the genes of collagen type I$\alpha$1, transforming growth factor-β (TGF-β), interleukin-6 (IL-6), and estrogen receptor have also been found to be associated with BMD in diverse populations. However, results of the association studies have been highly inconsistent. Recently, in a single extended pedigree, Johnson *et al.* reported the linkage of a genetic locus (named HBM, for high bone mass) in the human genome to a phenotype of very high spinal bone density. This HBM phenotype was further demonstrated to be due to a mutation in the low-density lipoprotein receptor-related protein 5 gene (LRP5). However, this mutation was not found in osteoporotic patients. There are probably several other major genes involved in the determination of bone mass.

Calcium is an important nutrient for attaining the peak bone mass. Higher calcium intake during skeletal growth period helps in achieving optimal

TABLE 3 **Classification of Osteoporotic Types**[a]

| Factor | Type I (postmenopausal) | Type II (senile) | Type III (secondary) |
|---|---|---|---|
| Age | 55–70 | 75–90 | Any age |
| Years past menopause | 5–15 | 25–40 | Any age |
| Sex ratio (F:M) | 20:1 | 2:1 | 1:1 |
| Fracture site | Spine | Hip, spine, pelvis, humerus | Spine, hip, peripheral |
| Bone loss | | | |
| Trabecular | +++ | ++ | +++ |
| Cortical | + | ++ | +++ |
| Contributing factor | | | |
| Menopause | +++ | ++ | ++ |
| Age | + | +++ | ++ |
| Biochemistry | | | |
| Parathyroid hormone | ↓ | ↑ | ↓↑ |
| $1,25(OH)_2 D_3$ | ↓ | ↓ | ↓↑ |
| Calcium absorption | ↓ | ↓ | ↓ |
| 1α-Hydroxylase response to parathyroid hormone | ↑ | ↓ | ? |

[a] Adapted from Gallagher (1992) with permission from W.B. Saunders Company.

peak mass. Endocrine reproductive status affects peak bone mass, because early menarche, pregnancy, and the use of oral contraceptive pills are associated with higher bone mass. Sex steroids, estrogen and testosterone, contribute to sexual dimorphism of the skeleton and lead to increased growth velocity at puberty and cessation of linear growth. Increase in bone mass during adolescence results from increases in bone length, bone diameter, cortical bone width, and cancellous bone density. Smoking and alcohol consumption during adolescence and early adult life may have an adverse effect on peak bone mass. Similarly, exercise also modifies bone mass and bone quality through an effect of mechanical loading on the skeleton. Optimum calcium intake and exercise during the early 20s may increase peak bone mass by about 0.5 standard deviations.

## V. MODELING AND REMODELING OF BONE

The adult skeleton is a dynamic tissue that remodels throughout life by a coordinated action of osteoblasts, osteoclasts, and osteocytes. It is composed of 80% cortical (compact) bone and 20% cancellous (trabecular) bone. Bone modeling involves both growth and shaping of bones and occurs during the first 20 years of life in humans. The process of bone modeling involves both bone formation and resorption, the former exceeding the latter. Once the initial modeling and growth of bone are completed, bone is then renewed throughout life by a process known as remodeling. A typical remodeling sequence lasts several months. It involves an initial state of activation of bone resorption lasting several days, creating a cavity. Once the resorption cavity has been completed, osteoblasts immediately enter the cavity and fill in the space with new bone. This process lasts several weeks and further consolidation occurs over a period of several months. This process of remodeling represents the way that older bone is replaced and renewed with new bone (Fig. 1). It may be that the initial impetus for remodeling of bone is the presence of microfractures, which occur as a result of strain and stress.

The first event during bone remodeling is osteoclast activation followed by osteoclast formation, resorption, and ultimately apoptosis. The major systemic hormones involved in osteoclast activity are parathyroid hormone (PTH), 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$], sex steroids, and glucocorticoids. None of these hormones appears to act directly on osteoclasts to stimulate resorption, and, in fact, at the present time only calcitonin has been shown to bind directly to receptors on osteoclasts. These hormones do not directly stimulate the osteoclasts but bring about their effects by causing release of a number of other factors generated by osteoblast-like cells. Local hormones may be more important than systemic hormones for the initiation of bone resorption and for normal bone remodeling. Because remodeling occurs in discrete and distinct packets throughout the skeleton, factors generated locally in the microenvironment of bone govern the cellular events. Some of the local cytokines known to be involved in osteoclast activation are tumor

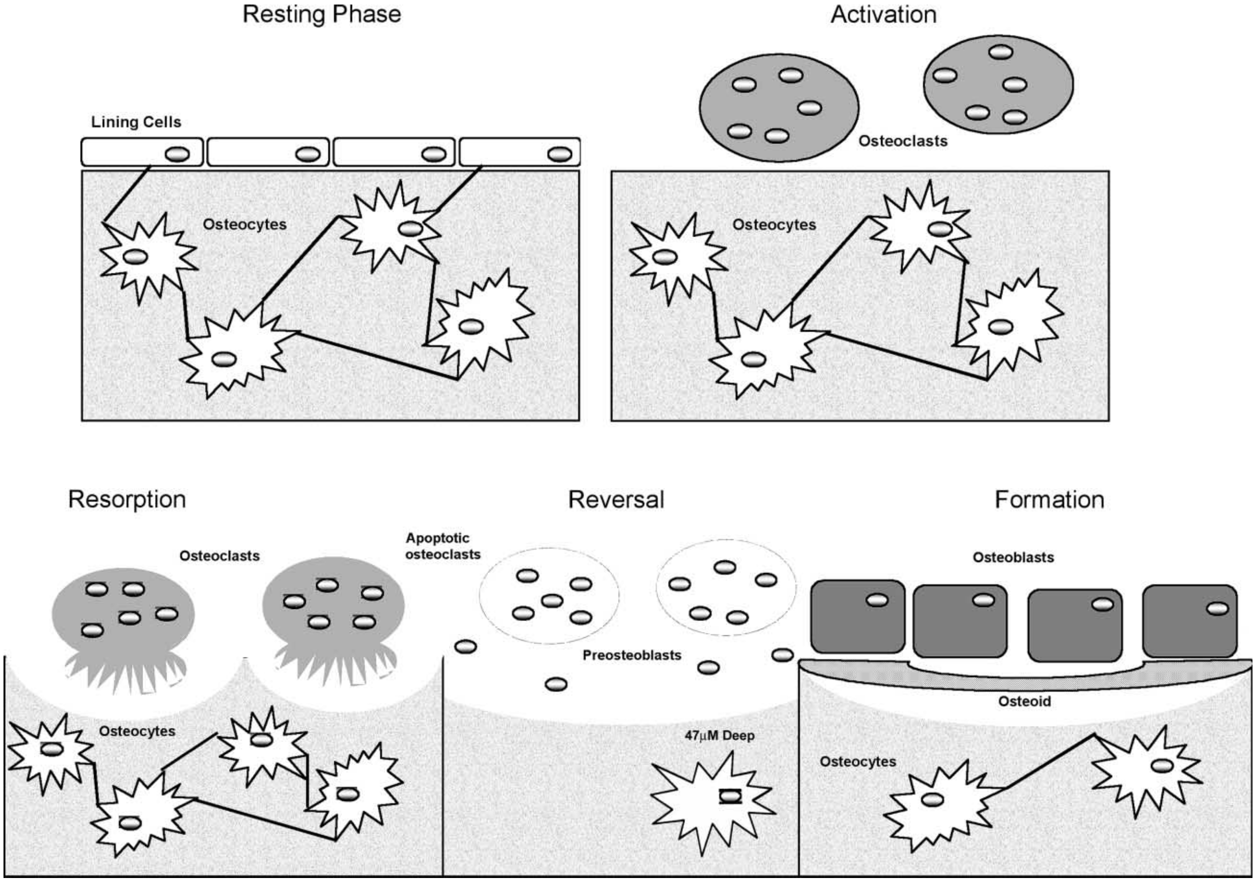

**FIGURE 1** Bone remodeling.

necrosis factor (TNF), interleukins (IL-1, IL-6, and IL-18), interferon γ, TGF-β, and colony-stimulating factor (CSF). Some of these factors stimulate osteoclast activity whereas others inhibit the activity. Stimulation of osteoclast activity involves enhancement of proliferation of osteoclast progenitors, differentiation of committed precursors into mature cells, and activation of mature multinucleated cells to resorb bone. Inhibition of osteoclast activity involves blocking proliferation of precursors, inhibiting the differentiation or fusion, and inactivating the mature multinucleated resorbing cells. Current evidence indicates that most factors that stimulate or inhibit osteoclasts act on at least two of these steps. Three new proteins, which are responsible for the interaction between the cells of osteoblastic and osteoclastic lineages and are considered as the final effectors of osteoclast differentiation, have recently been identified. These proteins belong to the family of tumor necrosis factor receptor and have been referred to by different names by different investigators. It has been recommended by a committee that the ligand (osteoblast-derived paracrine factor) be referred to as receptor activator of nuclear factor κB ligand (RANKL), the receptor on the osteoclast as RANK, and the decoy receptor as osteoprotegerin (OPG). The synonyms for RANK ligand include TNF-related activation-induced cytokine (TRANCE), osteoclast differentiation factor (ODF), and osteoprotegerin ligand (OPGL). The interaction of RANKL and RANK stimulates all aspects of osteoclast function. OPG, produced by cells of osteoblast lineage, acts as a decoy receptor for TRANCE, blocking its interaction with RANK and inhibiting osteoclast formation.

The formation phase of the remodeling sequence involves sequential changes in cells in osteoblast lineage, including osteoblast chemotaxis, proliferation, and differentiation. The formation of mineralized bone follows, and once the new bone is formed, osteoblastic activity ceases. The proliferation and differentiation of osteoblast precursors are also controlled by local osteoblast growth factors. The prominent factors include members of the TGF-β superfamily (TGF-β1 and TGF-β2), platelet-derived growth factor (PDGF), heparin-binding fibroblast growth factor, insulin-like growth factor-I and -II (IGF-I and IGF-II), and bone morphogenetic protein-2 (BMP-2). All of these factors, specifically the TGF-β superfamily members, are important in the coupling that links bone formation to prior bone resorption. The release of local cytokines in the bone remodeling process is another example of autocrine and paracrine control mechanisms.

## VI. BONE LOSS

Peak bone mass throughout the skeleton is reached in the late teens, but there is probably a small amount of further consolidation during the next 5 years, which may increase bone density by 3–5%. However, at the radius, a continuous increase is seen in cortical bone mass up to age 40. At the femur and tibia, and to a lesser extent from the trochanter, bone loss starts immediately after peak bone mass has been achieved and proceeds at a rate of 0.5% per year. This loss, however, varies considerably from person to person, starts at different periods of life, and occurs at different rates throughout the skeleton. About 30% of the decrease in BMD of the femoral neck and Ward's triangle in the proximal femur occurs during premenopausal age. At the time of menopause, the bone loss accelerates for a period of 5 to 7 years, and this early postmenopausal phase bone loss is responsible for about one-third of the total lifetime bone loss. Over a 50- to 60-year age span, this steady loss leads to a reduction of about 40% in the bone density of the femoral neck. In contrast, BMD of the spine shows no significant bone loss prior to menopause. At the time of menopause, there is a rapid loss of bone from the vertebral body and about 50% of the lifetime trabecular bone loss occurs within the first 10 years after menopause. Other sites in the body, such as the radius or the humerus, or the total body, also show predominantly menopausal changes in the pattern of bone loss.

Various regions of the skeleton differ in the proportion of cortical and trabecular bone. The variable rates of bone loss that are seen in different regions of the skeleton can be explained by the fact that the trabecular bone has a large surface area for resorption and shows a greater susceptibility to estrogen deficiency at menopause. In cortical bone, resorption occurs primarily at the endosteal surface. In general, the spine is a more hormone-dependent bone than is the femur, while other aging factors are more important in the pathogenesis of bone loss from the proximal femur (neck and trochanter). Thus, up to age 60, 90% of the bone loss in the spine and 40% of bone loss in the femoral neck are due to estrogen deficiency. By the age 85 years, age-related factors cause 75% of femoral neck bone loss and 50% of spine bone loss, whereas estrogen deficiency causes 25% of femoral neck loss and 50% of spine loss.

## VII. PATHOGENESIS OF BONE LOSS

Bone loss results as a consequence of an imbalance in the remodeling process whereby the resorption of the bone occurs at a higher rate than the formation, leading to a net decrease in bone density. The age-related bone loss of 0.5% per year occurring before menopause is most likely due to less efficient matrix synthesis by osteoblasts. Immediately following menopause, there is a dramatic increase in bone remodeling process due to estrogen deficiency. The biochemical markers of bone resorption and formation, such as urine cross-links, hydroxyproline, plasma tartrate acid phosphatase, and osteocalcin, are elevated twofold, suggesting an increase in the number of remodeling sites. Usually, bone formation and resorption are coupled efficiently so that bone mass is maintained. However, after menopause, the increase in bone formation is not sufficient to match the increased resorption activity, resulting in a rapid net loss of bone during these years. In bone regions that are primarily trabecular in nature, menopause-induced (estrogen-deficient) bone loss is an important cause of bone loss during the first decade after menopause. Overall, the intensive resorption process after menopause initially leads to thinning of the trabelcula elements, but after several years the trabeculae eventually become completely eroded from continuous resorption, leading to trabecular perforation, loss of continuity of bone structure, and structural damage. During the first phase of trabecular thinning, it is possible to stop resorption and return bone mass to normal, but in the second phase, the antiresorptive agents can only prevent further bone loss.

The mechanism by which estrogen deficiency leads to rapid bone loss is beginning to be clearer. Recent work on the physiology of bone remodeling has further clarified the role of estrogen in maintaining bone health and has provided more evidence for the implication of cytokines in postmenopausal bone loss. Available data suggest that IL-1 and TNF are the main causative agents underlying the bone loss induced by estrogen deficiency, mainly by up-regulating osteoclast formation and activation (Fig. 2). Menopause increases the monocytic production of IL-1 and TNF, which stimulates bone marrow

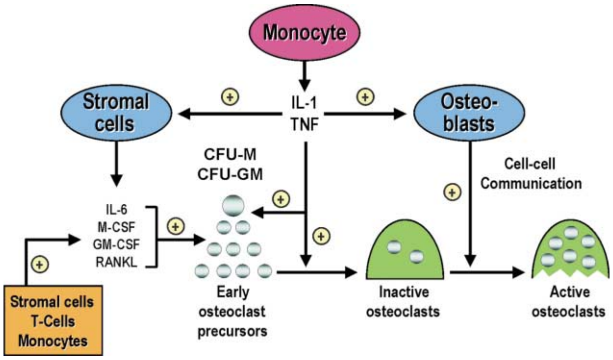
Monocyte
Stromal cells
IL-1
TNF
Osteo-blasts
+
+
CFU-M
CFU-GM
Cell-cell Communication
+
IL-6
M-CSF
GM-CSF
RANKL
+
+
+
+
Stromal cells
T-Cells
Monocytes
Early osteoclast precursors
Inactive osteoclasts
Active osteoclasts

**FIGURE 2** Osteoclastic differentiation and activation in estrogen deficiency. IL-1, Interleukin-1; TNF, tumor necrosis factor; CFU-M and CFU-GM, colony-forming units of monocytes, macrophages, and granulocytes; RANKL, receptor activator of nuclear factor κB ligand. Reproduced from *J. Bone Miner. Res.* **11**, 1043–1051 (1996), with permission of the American Society for Bone and Mineral Research.

stromal cells, or their osteoblast progeny, to release factors [IL-6, IL-11, granulocyte/macrophage colony-stimulating factor (GM-CSF), monocyte/macrophage colony-stimulating factor (M-CSF), and RANKL]. These factors in turn stimulate the proliferation of hematopoietic osteoclast precursor cells originating from cells of granulocyte/macrophage colony-forming units (GM-CFUs) and monocyte/macrophage colony-forming units (M-CFUs) lineage. Osteoclast precursors differentiate into mature inactive osteoclasts in response to IL-1 and TNF. Osteoclast activation is rapidly induced by IL-1, TNF, and RANKL.

Following the initial rapid phase of bone loss after menopause, bone loss continues with age at a slower pace. This late phase of bone loss in women is thought predominantly to be due to age-related factors such as secondary hyperparathyroidism, impaired osteoblast function due to changes in local systemic growth factors or cytokines, and, in some elderly patients, nutrition deficiency of vitamin D. In men, the age-related bone loss is believed to be due to the same age-related factors. Recent evidence suggests that estrogen deficiency is also an important contributor to the late phase of bone loss in both women and men.

## VIII. PATHOPHYSIOLOGY IN RELATION TO TYPE OF OSTEOPOROSIS

### A. Postmenopausal Osteoporosis (Type I)

Type I osteoporosis (Fig. 3) occurs predominantly in women in the mid-60 age group and is presented primarily as crush fractures of the spine and Colle's fractures. It is associated mainly with the bone loss of trabecular rather than cortical bone, which occurs within 5–15 years of menopause. The spine BMD in these patients is usually more than 2 SDs below the

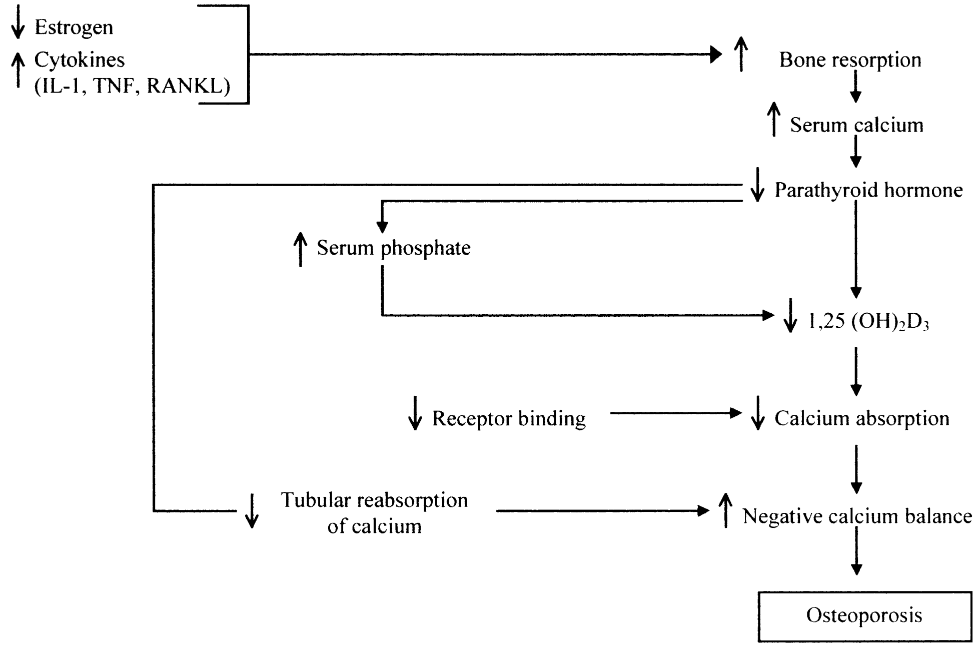
↓ Estrogen
↑ Cytokines (IL-1, TNF, RANKL)
↑ Bone resorption
↑ Serum calcium
↓ Parathyroid hormone
↑ Serum phosphate
↓ 1,25 $(OH)_2D_3$
↓ Receptor binding
↓ Calcium absorption
↓ Tubular reabsorption of calcium
↑ Negative calcium balance
Osteoporosis

**FIGURE 3** Pathogenesis of Type I osteoporosis. IL-1, Interleukin-1; TNF, tumor necrosis factor; RANKL, receptor activator of nuclear factor κB ligand. Adapted from Gallagher (1992) with permission from W.B. Saunders Company.

mean for their age. Histomorphometry of iliac crest biopsies show a reduced bone volume, and about 25% of patients show high bone resorption rates. The rate of bone loss can be anywhere between 1 and 10% per year in the first few years of menopause, and it is likely that women with high rates of bone loss are the ones who develop the fractures due to high-level trabecular bone loss. Another possibility is that these women reach menopause with low peak bone mass, and the menopause-induced bone loss makes them more prone to fractures.

Estrogen deficiency is thought to be the primary factor that underlies Type I osteoporosis. Estrogen deficiency, mediated by changes in cytokines, alleviates bone loss by up-regulating osteoclast formation and activation. The increase in bone resorption is believed to increase serum calcium marginally, which in turn decreases the PTH secretion. The decrease in serum PTH levels down-regulates the production of $1,25(OH)_2\ D_3$ and increases renal calcium excretion. A decrease in circulating $1,25(OH)_2\ D_3$ also results in impaired calcium absorption, which further increases the bone loss.

## B. Senile Osteoporosis (Type II)

Type II (senile) osteoporosis (Fig. 4) occurs both in men and in women who are more than 75 years old. In Type II osteoporosis, there is cortical and trabecular bone loss and the patients present with fractures of the hip, pelvis, humerus, and vertebrae.

Age-related physiological changes contribute to the pathogenesis of Type II osteoporosis. Malabsorption of calcium is quite common in the elderly patient, in part due to low circulating levels of $1,25(OH)_2\ D_3$. However, there is evidence for intestinal resistance to endogenous $1,25(OH)_2\ D_3$, which results in secondary hyperparathyroidism. Vitamin D deficiency due to inadequate exposure to sunlight or to low dietary intakes of vitamin D and calcium is also common in elderly people who are housebound, and this can contribute to malabsorption of calcium, secondary hyperparathyroidism, and osteomalacia once serum $25OH\ D_3$ levels fall below 12 ng/ml (30 nmol/liter). The decreased ability of the aging kidney to produce $1,25(OH)_2\ D_3$ is another factor responsible for decreased calcium absorption. The decrease in calcium absorption leads to negative calcium balance, which stimulates parathyroid function, which in turn promotes further bone loss.

Recent evidence, however, suggests that low circulating levels of estrogens in the elderly are also important in the pathogenesis of Type II osteoporosis. It has been demonstrated that in women 65 or older with undetectable serum estradiol concentrations (<5 pg/ml), there is accelerated bone loss and an increased relative risk of 2.5 for subsequent hip or

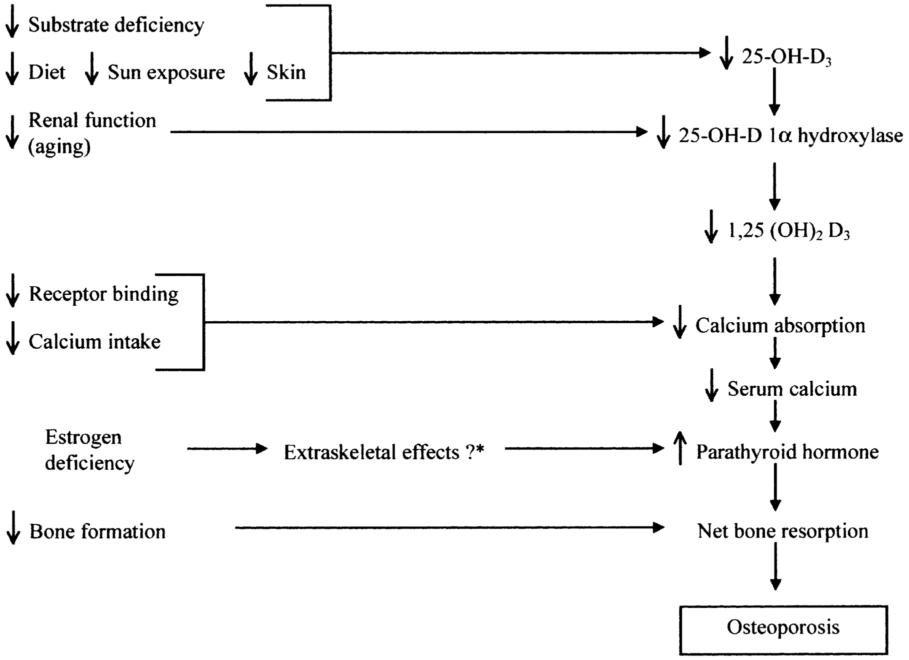

FIGURE 4 Pathogenesis of Type II osteoporosis. The asterisk indicates unitary hypothesis. Adapted from Gallagher (1992) with permission from W.B. Saunders Company.

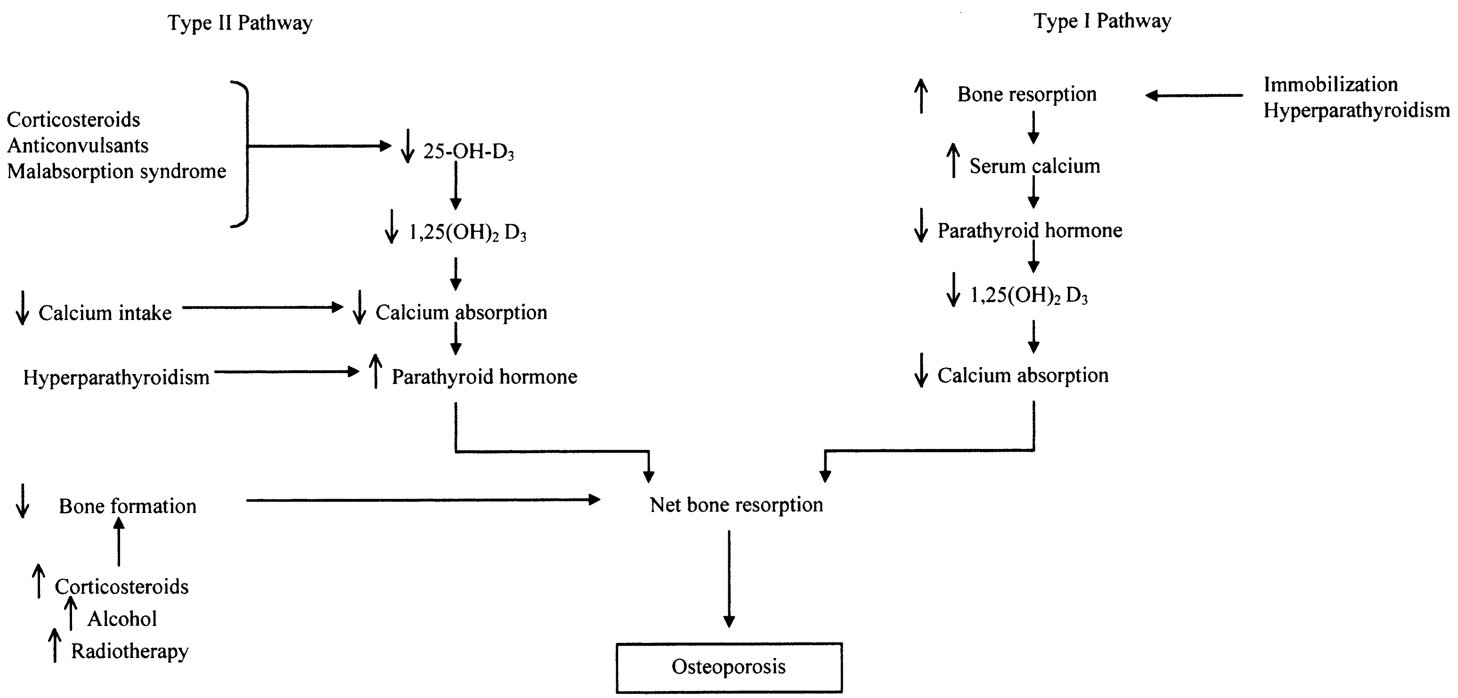
Type II Pathway
Type I Pathway
Corticosteroids
Anticonvulsants
Malabsorption syndrome
↓ 25-OH-$D_3$
↓ 1,25$(OH)_2$ $D_3$
↓ Calcium intake
↓ Calcium absorption
Hyperparathyroidism
↑ Parathyroid hormone
↓ Bone formation
↑ Corticosteroids
↑ Alcohol
↑ Radiotherapy
↑ Bone resorption
Immobilization
Hyperparathyroidism
↑ Serum calcium
↓ Parathyroid hormone
↓ 1,25$(OH)_2$ $D_3$
↓ Calcium absorption
Net bone resorption
Osteoporosis

FIGURE 5 Pathogenesis of Type III osteoporosis. Adapted from Gallagher (1992) with permission from W.B. Saunders Company.

vertebral fractures. High serum concentrations of sex hormone-binding globulin, which binds estradiol and decreases its bioavailability, have also been reported to be associated with increased bone loss and increased risk of hip (relative risk of 2.0) and vertebral (relative risk of 2.3) fractures. Further evidence for the role of endogenous estrogens in age-related bone loss is provided by Heshmati *et al.*, who found an increase in bone resorption markers in older postmenopausal women after reducing the already low levels of serum estrogen to near undetectable levels with an aromatase inhibitor, letrozole. Based on this evidence, a unitary model has been proposed, which suggests that estrogen deficiency is the cause for both Type I and Type II osteoporosis. However, this hypothesis needs to be substantiated further.

### C. Secondary Osteoporosis (Type III)

Secondary osteoporosis (Fig. 5) is used to describe osteoporosis that can be attributed to specific diseases, surgical procedures, and use of certain drugs. As a result of any of these conditions, Type III patients demonstrate accelerated bone loss, leading to vertebral or hip fracture. Type III osteoporosis can occur equally in men and women and at any age. Vertebral fractures in 20–35% of women and 40–55% men are accounted for by secondary osteoporosis. Some of the common conditions that lead to osteoporosis include hormonal imbalances (Cushing syndrome, thyrotoxicosis, and primary hyperparathyroidism), gastrointestinal disorders (primary biliary cirrhosis and malabsorption syndrome), drug therapy (e.g., corticosteroids, cancer chemotherapy, anticonvulsants, and heparin), neoplasms (multiple myeloma and skeletal metastases), alcoholism, chronic renal failure, immobilization, osteogenesis imperfecta, and transplantation (Table 2). The pathogenesis of the bone loss seen in these conditions could be explained by the mechanisms seen in Type I and Type II osteoporosis. However, in some conditions, the pathogenesis still needs elucidating. In general, patients with secondary causes of osteoporosis present with a hip fracture several years earlier than expected.

## IX. THERAPEUTIC CONSIDERATIONS

There are several effective agents for preventing bone loss, and these are grouped together in the category of antiresorptive agents. They include estrogen, selective estrogen receptor modulators (SERMs), bisphosphonates, calcitonin, calcitriol, and, to a lesser degree, calcium supplements that are used as an adjunctive agent. Newer and more potent antiresorptives in the pipeline include osteoprotegerin and RANKL antagonists. However, antiresorptive agents are limited because they only prevent bone loss. For subjects with severe osteoporosis, agents that increase the formation of new bone are needed. Parathyroid hormone is the first of these systemic agents that increase trabecular thickness and continuity of bone. In addition, BMP proteins, which are osteogenic in vivo, are considered to be therapeutic molecules in the treatment of fractures to induce new bone formation at the site of injury. We can expect more anabolic agents to emerge in the next few years as we explore our understanding of the bone remodeling process.

## Glossary

**hyperparathyroidism** Condition defined by excess parathyroid hormone secretion; leads to accelerated bone turnover.

**1,25-dihydroxyvitamin $D_3$** Steroid hormone form of vitamin D; a principal regulator of calcium homeostasis.

**osteoblast** Cell derived from a pluripotent mesenchymal stem cell; principally involved in bone formation and mineralization of bone.

**osteoclast** Large multinucleated cell derived from the hematopoietic precursor of monocyte/macrophage cell lineage; involved in resorption of bone.

## See Also the Following Articles

**Aromatase and Estrogen Insufficiency • Interleukin-1 (IL-1) • Osteoporosis: Hormonal Treatment • Parathyroid Hormone • Parathyroid Hormone-Related Protein (PTHrP) • SERMs (Selective Estrogen Receptor Modulators) • Tumor Necrosis Factor (TNF)**

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# Ovulation

Ariel Hourvitz[*] and Eli Y. Adashi[†]

[*]*Tel Aviv University* • [†]*University of Utah*

I. INTRODUCTION
II. FOLLICULAR GROWTH AND DEVELOPMENT: RECRUITMENT, SELECTION, AND DOMINANCE
III. OVULATION
IV. CORPUS LUTEUM FORMATION AND DEMISE
V. OVARIAN STEROIDOGENESIS
VI. MOLECULAR CHARACTERIZATION OF THE OVULATORY CASCADE
VII. SUMMARY

**Ovulation, a complex process initiated by the surge of LH, constitutes the ultimate step in the maturation of the ovarian follicle and its contained oocyte. Once initiated, a cascade of events transpires that culminates in the disintegration of the follicular wall and the release of a fertilizable oocyte. This complex series of events inevitably involves specific ovarian cell types and diverse signaling pathways. The individual phases of the normal ovarian life cycle are controlled by a highly synchronized and exquisitely timed cascade of gene expression. Appropriate transition from one phase of the ovarian cycle to the next requires the timely expression of a specific gene(s) to ensure the correct continuation of the process.**

## I. INTRODUCTION

The teleologic underpinning of ovarian function draws on the fundamental need to preserve the species. Accordingly, the very existence of the ovary, and for that matter the very existence of the reproductive axis as a whole, is designed to subserve a single central objective: the generation of a mature, fertilizable ovum.

The ovary is the master gland in this process, the function of which is made possible by the contribution of the various other components of the reproductive axis. This article will emphasize the cycle of follicular growth and development: recruitment, selection and dominance, ovulation, and corpus luteum formation and demise. In addition, the major operational characteristics of ovulation will be reviewed.

## II. FOLLICULAR GROWTH AND DEVELOPMENT: RECRUITMENT, SELECTION, AND DOMINANCE

The primordial follicle consists of an oocyte arrested in the diplotene stage of the first meiotic prophase, surrounded by a single layer of granulosa cells. It is first noted by week 16 of intrauterine life, and it is generally accepted that the formation of follicles ends no later than 6 months postpartum. It is quite certain that this stage of follicular development is entirely gonadotropin-independent.

The preantral growth phase is the phase of follicular development in which the primordial follicles (30 μm in diameter) are converted to mature secondary follicles (120 μm in diameter). Initiated during months 5 to 6 of gestation, the process becomes evident when the granulosa cells undergo proliferation and differentiation combined with theca hypertrophy and the growth and differentiation of the oocyte including the acquisition of the zona pellucida. The resulting mature secondary follicles constitute the pool of preantral follicles from which tonic, likely follicle-stimulating hormone (FSH)-dependent recruitment of follicles takes place.

The term recruitment has been used to indicate that a follicle has entered the so-called growth trajectory, that is, the process wherein the follicle departs from the resting pool to begin a well-characterized pattern of growth and development. Recruitment, although obligatory, does not guarantee ovulation. The recruitment process is a "continuum" process beginning at infancy and stopping when the pool of available oocytes is exhausted. It goes on all times and at all ages, uninterrupted by pregnancy or other periods of nonovulation. The stimuli for the recruitment of the follicles are unknown but for the process to continue, a specific hormonal environment is needed. The absence of this environment will cause the atresia of most of those follicles. However, the specific hormonal environment present at the beginning of the cycle, mostly the increase in FSH, will enable the formation of a cohort of developing follicles. Following recruitment, the flattened granulosa cells become cuboidal and small gap junctions are formed between the granulosa cells and the oocyte.

The term selection indicates the final winnowing of the maturing follicular cohort (by atresia) down to a size equal to the species-specific ovulatory quota. Accordingly, selection is complete when the number of healthy follicles (i.e., with ovulatory potential) in the cohort equals the ovulatory quota. Like recruitment, selection does not guarantee ovulation. Given its greater temporal proximity to ovulation, however, selection may, with high probability, be expected to be followed by ovulation in a typical cycle.

The leading follicle can be distinguished from other members of the cohort by its sheer size and the high mitotic index of its granulosa cells. Moreover, only the leading follicle at this point in time displays detectable levels of FSH in its follicular fluid. This same follicle also displays significant follicular levels of estradiol. Indeed, it is generally agreed that the capacity to aromatize androgens efficiently is an important determinant of the chosen follicle. Most important, the follicle destined to ovulate displays a granulosa cell mitotic index that is high enough to ensure that smaller, albeit healthy, follicles are unlikely to catch up.

The term dominance refers to the status of the follicle destined to ovulate given its presumed key role in regulating the size of the ovulatory quota. The selected follicle becomes dominant approximately 1 week before ovulation. Consequently, it must maintain its dominance during the week before ovulation. Stated differently, the follicle selected for ovulation is functionally (not merely morphologically) dominant in that it is presumed to inhibit the development of other competing follicles on both ovaries. Inevitably, and for reasons that are not entirely clear, the dominant follicle (i.e., the sole follicle destined to ovulate) continues to thrive in circumstances that it itself has made inhospitable for others. This dominant follicle has produced relatively more estrogen than the other follicles in its cohort. The dominant follicle thus enjoys an orderly sequence of events wherein FSH and estrogen stimulate growth, antrum formation, and the appearance of luteinizing hormone (LH) receptors. The dramatic increase in estrogen production by the dominant follicle, observable during the second half of the follicular phase, is accompanied by falling levels of FSH. As a result, the nondominant follicles fail to thrive. Apparently, the intrafollicular concentrations of gonadotropins and of steroids are central to the self-amplification process.

Experimental findings are consistent with the possibility that the ovary itself may in fact play a zeitgeber role during the menstrual cycle and that this time-keeping function is subserved by the activities of the cyclic structures of the dominant ovary. The 28-day menstrual cycle is thus the result of the intrinsic life span of the cyclic ovarian dominant structures and is not the result of timed changes dictated by the brain or pituitary. The dominant follicle thus determines the length of the follicular phase, with the corpus luteum determining the length of the luteal phase.

These experiments also suggested that the selection of the follicle destined to ovulate had already occurred by day 8 of the cycle. Indeed, it would appear that no other member of the follicular cohort was competent to serve as a surrogate for the follicle to achieve a timely, midcycle ovulation. Thus, it could be suggested that the dominant follicle itself plays a key role in regulating the size of the ovulatory quota by inhibiting the development of any competing follicles in either ovary. A similar function is subserved by the corpus luteum. Thus, the ovulatory follicle, once it is selected during the midfollicular phase, and the corpus luteum are truly dominant ovarian structures. Accordingly, the next round of follicular growth occurs only after the interference by the cyclic structure is removed either artificially, by experimental intervention, or naturally, after the demise of the corpus luteum. Further insight has been gained from studies wherein progesterone-replaced luteectomized primates were evaluated, revealing progesterone to be the principal luteal hormone responsible for the inhibition of luteal follicular growth. It is critical to note that circulating gonadotropin levels were apparently maintained after follicular or luteal ablation and that follicle recruitment occurred without an attendant increment in circulating gonadotropins. Thus, the inhibition of follicular growth by the cyclic structures of the ovary was not due to a decrement in the circulating levels of gonadotropins. Rather, it appeared to be due to local intraovarian influences. The above notwithstanding, careful re-evaluation of these issues may well be warranted in that careful examination of the data suggests at least a slight, albeit transitory, increase in the circulating levels of FSH after ablation.

Further insight has been derived from experiments revealing that the follicle destined to ovulate attains dominance 5 to 7 days after the demise of the corpus luteum. This conclusion was based on the observation that the levels of estradiol in ovarian venous serum were significantly different between ovaries as early as days 5 to 7 of the cycle. This divergence in estrogen secretion between ovaries provides the earliest hormonal index attesting to the emergence of the dominant follicle.

In the late follicular phase, the intrafollicular concentrations of estradiol are maximal at a time when the circulating estradiol levels surge to a peak. With the ovulatory LH surge, the intrafollicular concentrations of estradiol decrease along with parallel decrements in the intrafollicular concentrations of androstenedione. Concurrently, distinct, progressive increments have been noted for the intrafollicular content of both progesterone and 17α-hydroxyprogesterone, reflecting early granulosa cell luteinization.

## III. OVULATION

As midcycle approaches, there is a dramatic rise in estrogen and a subsequent LH surge and to a lesser extent an FSH surge that trigger the dominant follicle to ovulate. The LH surge rapidly acts on granulosa cells of the preovulatory follicles to terminate the follicular growth and at the same time induce those genes required for the ovulatory process. Ovulation is characterized by various processes leading to follicle rupture, oocyte maturation, and formation of the corpus luteum.

For reasons that are not well understood, but possibly because of unique microenvironmental circumstances, one (rarely, more than one) follicle ovulates and gives rise to a corpus luteum during each menstrual cycle. In the human, both LH and hCG have been shown to stimulate the rupture of mature follicles. In hypophysectomized rats, however, highly purified FSH can serve as the ovulatory hormone after follicular maturation has been stimulated by the administration of FSH and LH. Interestingly, inhibitors of prostaglandin synthesis (introduced systemically or locally into the antrum) have been shown to inhibit ovulation in rats and rabbits. Because LH has been shown to stimulate prostaglandin biosynthesis by ovarian follicles, increased prostaglandin synthesis might mediate the ovulatory stimulus of LH.

Mechanically, ovulation consists of rapid follicular enlargement with subsequent protrusion of the follicle from the surface of the ovarian cortex. Ultimately, rupture of the follicle results in the extrusion of an oocyte–cumulus complex. In the human ovary, this sequence may well begin 5 to 6 days before the onset of the preovulatory LH surge. It is the latter event, however, that marks the end of the follicular phase of the cycle and precedes actual rupture by as much as 36 h. Fortuitous endoscopic visualization of the ovary around the time of ovulation has revealed that elevation of a conical stigma on the surface of the protruding follicle precedes rupture. Rupture of this stigma is accompanied by gentle, rather than explosive, expulsion of the oocyte and antral fluid, suggesting that the latter is not under high pressure. Indeed, direct measurements have demonstrated that intrafollicular pressure is low in preovulatory follicles.

Several hypotheses have been advanced to account for the rapid increase in size and rupture of the follicle. For one, consideration was given to changes in the composition of the antral fluid during the period of rapid preovulatory follicular enlargement. In addition to changes in the steroid hormone content, an increase in colloid osmotic pressure has been noted. Although the granulosa cell-derived proteoglycans undoubtedly play a critical role in regulating the colloid osmotic pressure, little concrete information regarding the nature of their involvement is in fact available. Thus, a cause and effect relationship between the altered composition of antral fluid and the enlargement and rupture of the follicle remains to be established. Alternatively, stigma formation and rupture may reflect the effects of hydrolytic enzymes acting locally on protein substrates in the basal lamina.

In keeping with this notion, instillation of protease inhibitors into the antral fluid inhibits ovulation. One such proteolytic enzyme, plasminogen activator, has been localized in increasing concentrations in the walls of rat ovarian follicle just before ovulation. Plasminogen activator, a serine protease, stimulates the conversion of plasminogen (a follicular fluid constituent) to the proteolytically active enzyme plasmin. The latter is known to activate collagenase, presumably an obligatory element in the dissolution of the basal membrane and the perifollicular stroma in the course of ovulation. It is thus generally presumed that plasminogen activator-mediated conversion of plasminogen to plasmin may contribute to the proteolytic digestion of the follicular wall, a prerequisite of follicular rupture. Consideration is also being given to the possibility that plasminogen activator may be involved in gap junction disruption and thereby in the delicate communication between the oocyte and the surrounding cumulus cells. Although the ultimate physiologic significance of plasminogen activator remains a matter of study, there is little doubt as to the ability of somatic ovarian cells to produce this protease in measurable amounts in a manner subject to tight hormonal regulation. The FSH-dependent production of plasminogen activators by granulosa cells is particularly well documented.

## IV. CORPUS LUTEUM FORMATION AND DEMISE

After ovulation, the dominant follicle reorganizes to become the corpus luteum. Thus, after rupture of the follicle, capillaries and fibroblasts from the surrounding stroma proliferate and penetrate the basal lamina.

Concurrently, the mural granulosa cells undergo morphologic changes collectively referred to as luteinization. These cells, the surrounding theca interstitial cells, and the invading vasculature intermingle to give rise to a corpus luteum.

Clearly, it is this endocrine gland that is the major source of sex steroid hormones secreted by the ovary during the postovulatory phase of the cycle. An important aspect of this phenomenon is the penetration of the follicle basement membrane by blood vessels, thereby providing the granulosa–luteal cells with circulating levels of low-density lipoprotein (LDL). As stated earlier, LDL cholesterol serves as the substrate for corpus luteum progesterone production.

Normally, the functional life span of the corpus luteum is 14 ± 2 days. Thereafter, the corpus luteum spontaneously regresses, to be replaced (unless pregnancy occurs) at least five cycles later by an avascular scar referred to as the corpus albicans. The mechanisms underlying luteolysis remain unclear. Both estrogens and prostaglandins, however, have been suggested as important factors in the promotion of luteal demise. The above notwithstanding, there is little doubt as to the central role of LH in the maintenance of corpus luteum function. Thus, withdrawal of LH support in various experimental circumstances has virtually invariably resulted in luteal demise.

## V. OVARIAN STEROIDOGENESIS

To distinguish steroid hormones secreted by the ovary from those secreted by the adrenal or those produced by peripheral metabolism of precursors, studies have determined and compared the steroid hormone content of ovarian venous effluents and peripheral venous blood. Such studies revealed that the ovaries secrete pregnenolone, progesterone, 17α-hydroxyprogesterone, esterone, dehydroepiandrosterone, androstenedione, testosterone, estrone, and 17β-estradiol. Although such measurements provide significant insight into the steroidogenic pathways under study, they do not identify the specific ovarian cell types involved. In attempts to make these distinctions, steroid hormones have been identified and quantified in medium conditioned by whole (sliced or minced) ovaries, microdissected follicles, or ovarian cell suspensions. It is this combined body of knowledge that underlies our current understanding of adult ovarian steroidogenesis.

Studies using microdissected follicles identified estrone and estradiol as the major products of the follicle. In contrast, progesterone and 17α-hydroxyprocesterone proved to be the major products of the corpus luteum. Studies employing (labeled or unlabeled) C21 and C19 precursors revealed the isolated granulosa cell to be capable of producing mostly progesterone and estrogens along with 17α-hydroxyprogesterone. In contrast, isolated theca cells produced progesterone, 17α-hydroxyprogesterone, and androstenedione.

### A. Estrogen Biosynthesis

Granulosa cells are the cellular source of estradiol and progesterone, the two most important ovarian steroids. Although the granulosa cells and their lutein counterparts are capable of producing progesterone independent of other ovarian cell types, the biosynthesis of estrogens requires cooperation between the granulosa cells and their theca neighbors. The participation of these two cell types and of the two gonadotropins (FSH and LH) in ovarian estrogen biosynthesis underlies the two-cell/two-gonadotropin hypothesis, an integrative process required for ovarian estrogen biosynthesis. According to this view, theca-derived, LH-dependent, aromatizable androgens are acted upon by FSH-inducible granulosa cell aromatase activity. Indeed, in virtually all species ovarian estrone and estradiol derive from the androgen precursors androstenedione and testosterone. A broader view of this concept could and probably should allow its extension to include intercellular exchanges of other steroidogenic substrates. Indeed, recent studies strongly suggest that intercellular exchanges of C21 steroids occur at multiple levels of the steroidogenic cascade.

That follicular estrogen biosynthesis requires both granulosa and theca interstitial cells was first discovered by Falck in 1959 through a series of elegant and now classic experiments. The biochemical basis of this two-cell/two-gonadotropin theory was later provided by Ryan and Petro, whose findings revealed the theca interstitial cells to be the producers of C19 androgens, with the granulosa cells being the primary cellular site of aromatization. Moreover, Ryan *et al.* were able to show that the conversion of acetate to estrogen is substantially enhanced by the co-incubation of granulosa and theca cells. This observation, along with a body of related information, was elegantly summarized by Bjersing in 1967: "C19 precursor steroids are elaborated by theca–interstitial cells and are transferred across the basement membrane of the follicle to the granulosa cells where they are aromatized to estrogens." The above notwithstanding, the two-cell/two-gonadotropin hypothesis has been challenged by *in vivo* studies in the subhuman primate.

In keeping with these conclusions, studies of isolated granulosa cells revealed that FSH, but not LH, stimulates estrogen biosynthesis in a manner contingent on the provision of an exogenous aromatizable androgenic substrate. In contrast, isolated theca cells did not produce significant amounts of estrogens in any experimental circumstances. Indeed, aromatase activity of granulosa cells was estimated to be at least 700 times greater than that of theca cells from large preovulatory follicles. As such, these results are consistent with the hypothesis that granulosa cells are the principal site of estrogen biosynthesis in the dominant preovulatory follicle. These observations suggest that androgen (mainly androstenedione) produced by LH-stimulated theca cells is the main substrate for estrogen biosynthesis by FSH-stimulated granulosa cells. Although estrone may well be the most immediate estrogen produced, it in turn is readily converted to estradiol as a result of the (granulosa cell-based) activity of the steroidogenic enzyme 17β-hydroxysteroid dehydrogenase. The complex aromatization process involves the loss of the angular C19 methyl group and the stereospecific elimination of the 10 and 20 hydrogens of the A ring of the androgen precursor. As such, a total of three hydroxylation reactions are required per mole of estrogen formed.

The hormonal action of both LH and FSH appears to require the intermediacy of the membrane-associated enzyme adenylate cyclase. Indeed, it is generally accepted that gonadotropin-mediated stimulation of adenylate cyclase results in the conversion of intracellular ATP to cyclic AMP. The latter, in turn, is thought to bind to the regulatory subunit of a protein kinase (commonly referred to as A kinase), whereupon the catalytic subunit of the enzyme is activated and dissociated. This in turn phosphorylates key intracellular proteins central to the signal transduction sequence.

## B. Progestin Biosynthesis

The granulosa cell, like the theca interstitial cell, is amply endowed to carry out progestin biosynthesis. Central to this process, however, is the availability of abundant supplies of cholesterol, which serves as the starting material for the steroidogenic cascade. Recent studies have shown that cholesterol used for membrane synthesis and steroid hormone production is derived primarily from circulating serum lipoproteins rather than from *de novo* cellular synthesis from acetate. LDL particles are known to bind to specific membrane receptors, with the LDL–receptor complexes entering the cell by receptor-mediated endocytosis. Thereafter, the endocytotic vesicles are known to fuse to lysosomes wherein LDL cholesterol esters are hydrolyzed to yield free cholesterol. The free cholesterol, in turn, is reesterified and stored in the cytoplasm in lipid droplets. Faced with steroidogenic demands, the cholesterol ester is hydrolyzed and the free cholesterol is transported to mitochondria for standard steroidogenic processing.

The importance of LDL cholesterol for ovarian progesterone secretion is demonstrated by the observation that the presence of LDL is required for maximal progesterone secretion by cultured cells.

High-density lipoprotein does not support human ovarian progesterone biosynthesis. Thus, the very availability of LDL to various ovarian compartments could influence steroid hormone production. For example, the relative avascularity of the granulosa cell layer expectedly limits progestin biosynthetic capacity. In keeping with this observation, human follicular fluid contains little or no LDL, thereby limiting the ability of preovulatory granulosa cells to produce progesterone 67. The above notwithstanding, the intrafollicular concentrations of progesterone do rise after the LH surge (before ovulation), suggesting a diminution in the lipoprotein barrier. Moreover, after ovulation occurs, vascularization of the corpus luteum provides the means by which LDL is delivered to the luteinized granulosa cells, thereby allowing progesterone biosynthesis to begin.

Although lipoproteins clearly constitute the most abundant source of cholesterol, endogenously generated cholesterol may also be employed. Cholesterol itself is in turn converted to pregnenolone through the rate-limiting intermediacy of mitochondrial enzyme cholesterol side chain cleavage. The subsequent conversion of pregnenolone to progesterone occurs relatively readily by virtue of the relative abundance of the cytoplasmic enzymes 3β-hydroxysteroid dehydrogenase/Δ5, Δ4-isomerase. Although the human granulosa cell has been reported to contain low levels of 17α-hydroxylase activity and thus has the ability to convert progesterone to 17α-hydroxyprogesterone, the significance of this finding remains uncertain.

## C. Androgen Biosynthesis

More recent studies of isolated human theca cells have revealed that the theca layer is the major cellular source of follicular androgen and that LH, rather than FSH, stimulates theca androgen production. In contrast, androgen production by isolated cultured human granulosa cells proved negligible with or without added gonadotropins.

The biosynthesis of C19 androgens is the sole domain of the theca interstitial cell. Accordingly, this cell type is amply endowed with the necessary machinery to generate C21 progestational precursors. More important, however, the theca interstitial cell is amply endowed with 17α-hydroxylase/desmolase (17–20) activity and is capable of converting Δ5 and Δ4 progestational precursors (i.e., pregnenolone and progesterone) to C19 products (dehydroepiandrosterone and androstenedione, respectively). Consequently, the presence of 17α-hydroxylase/desmolase (17–20) activity can be viewed as an exclusive feature of the ovarian theca interstitial cell.

## VI. MOLECULAR CHARACTERIZATION OF THE OVULATORY CASCADE

Ovulation, a complex process initiated by the surge of LH, constitutes the ultimate step in the maturation of the ovarian follicle and its enclosed oocyte. Once initiated, a cascade of events transpires, culminating in the disintegration of the follicular wall and the release of a fertilizable oocyte. This complex series of events, inevitably, involves specific ovarian cell types, diverse signaling pathways, and temporally controlled expression of specific genes. To date, a major focus of the research on ovulation has been the analysis of known genes. These studies have led to the establishment of a number of genes as being critical to the murine ovulatory process through the generation of null mutants. Figure 1 depicts those genes

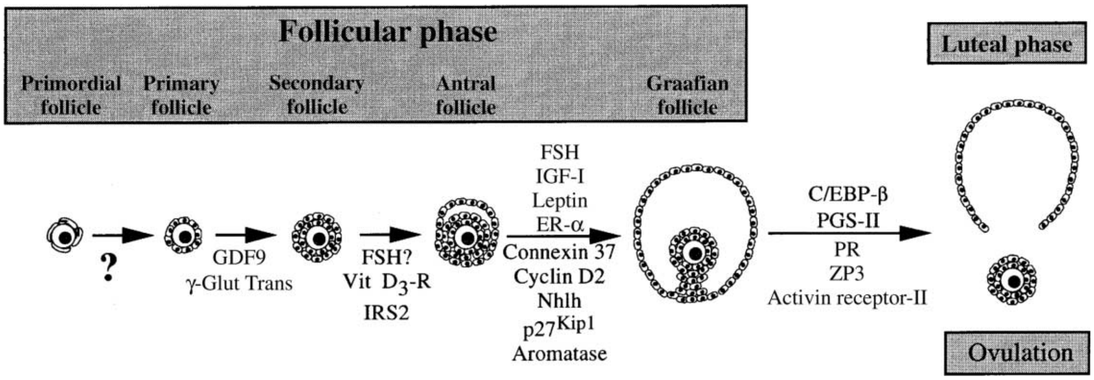

**FIGURE 1** Known molecular determinants of folliculogenesis and ovulation. GDF9, growth differentiation factor 9; γ-Glut Trans, γ-glutamyl transferase; Vit $D_3$-R, vitamin $D_3$ receptor; ER, estrogen receptor; IRS, insulin receptor substrate; PGS-II, prostaglandin synthase II; PR, progesterone receptor; ZP3, zona pellucida protein 3.

**TABLE 1 Gene Mutations Affecting Ovulation and Causing Infertility Due to Ovulation/Fertilization Failure**

| Disrupted gene | Gene symbol | Ovarian phenotype |
|---|---|---|
| Cyclooxygenase type 2 | Cox-2 (PGS-2) | Defective ovulatory process; few eggs recovered, none of which were fertilized; the eggs thus recovered proved developmentally abnormal; ovulation failure even when stimulated with gonadotropins |
| Progesterone receptor | PR | Follicles do not rupture; oocytes become trapped in the ovary |
| Activin receptor II | ActR-II | Absence of corpora lutea, suggesting ovulatory failure |
| CCAAT/enhancer-binding protein β | C/EBPβ | Failure to ovulate; absent corpora lutea |
| Nuclear receptor-interacting protein 1 | Nrip 1 | Complete failure of oocyte release from mature follicles but luteinization appears normal |
| Early growth-response protein 1 | Egr-1 (Krox-24) | Ovulatory failure due to an inhibition in the expression of the LHβ subunit and LH receptor genes |
| $Ca^{2+}$/Calmodulin-dependent protein kinase IV | CaMK-IV | Ovulatory failure with trapped oocytes present in some of the follicles |
| Cyclin-dependent kinase 4 | Cdk4 | Abnormal luteinization with disturbed cellularity, trapped oocytes, and a lack of copora lutea; granulosa cells appear normal; increased proestrous and diestrous phases |
| Cyclin D2 | Cd2 | Inability of ovarian granulosa cells to proliferate normally in response to FSH; failure to release the oocytes; corpora lutea carrying trapped oocytes |
| $p27^{Kip1}$ | $p27^{Kip1}$ | Ovulation is impaired; follicles do not progress to form corpora lutea |

identified thus far as obligatory to the ovarian life cycle inclusive of those implicated in ovulation. The genes targeted were mostly those known to be expressed in the ovary and to play a role in follicular growth. Alternatively, a phenotype inclusive of ovulatory failure may be "stumbled upon" in the context of a study of a previously unknown gene or one not previously recognized as being important in ovarian physiology. Tables 1 and 2 display those genes for which the null deletion resulted in ovulation

**TABLE 2 Gene Mutations Affecting Ovulation and Causing Reduced Ovulatory Efficiency**

| Disrupted gene | Gene symbol | Ovarian phenotype |
|---|---|---|
| Prostaglandin E2 receptor type 2 | EP2 | Reduced ovulatory efficiency; cumulus expansion does not occur |
| Zona pellucida 3 | ZP3 | Reduced ovulatory efficiency; disorganized cumulus granulosa cells |
| Colony-stimulating factor-1 | CSF-1 | Abnormal lengthy estrous cycles; low pregnancy rates; smaller litter sizes |
| Telomerase RNA | TR | Reduced ovulatory efficiency |
| Estrogen receptor-β | ER-β | Reduced fertility with altered ovulatory efficiency; increased number of early atretic follicles and sparse presence of corpora lutea, suggestive of arrested folliculogenesis |
| Nitric oxide synthase | NOS | Reduced fertility with altered ovulatory efficiency; significantly reduced number of ovulated oocytes, longer estrous cycle, and impaired oocyte meiotic maturation |
| Superoxide dismutase-1 | Sod-1 | Infertility or reduced fertility with smaller litter size; primary and small antral follicles but few corpora lutea |
| Urinary trypsin inhibitor | Uti | Severe reduction in fertility; markedly reduced ovulatory efficiency; disorganized corona radiata; large number of retained oocytes |
| Steroid receptor co-activator-3 | Src-3 | Decreased ovulation; lower pregnancy rate; small litter size; longer estrous cycling time |
| c-AMP-specific phosphodiesterase type 4 | PDE4D | Reduced number of ovulated oocytes; degeneration of ovulated oocyte; entrapped oocytes |

failure (Table 1) or reduced ovulatory efficiency (Table 2) and thus in infertility. Understandably, ovulation failure in the different knockout models is usually associated with additional and often considerable ovarian defects.

In the past few years, the use of advanced molecular biology techniques such as differential display reverse transcription-polymerase chain reaction, subtractive suppression hybridization, DNA array technology, and *in silico* methods has led to the identification of new ovulatory genes. Using these different techniques, researchers were able to identify a multitude of novel ovulatory up-regulated genes. These genes induced by LH include among others, carbonyl reductase, 3α-hydroxysteroid dehydrogenase, regulator of G-protein signaling, tumor necrosis factor-induced gene-6, and early growth regulator-1. The exact role of these genes in the ovulatory process is not clear yet, but their diverse functions and spatial expression in the ovary prove the complexity and global effect of the ovulatory process.

## VII. SUMMARY

Recent advances in the human and mouse genome projects, combined with the development of advanced molecular technology such as differential display analysis, subtractive hybridization, and microarray analysis, now provide the opportunity to analyze the expression pattern of thousands of genes. This approach has already led to the identification of new genes that are expressed in the ovary. It will ultimately allow the achievement of a global view of gene expression as it relates to the ovulatory process. Functional studies of these newly discovered genes, based mainly on gene targeting technology, will allow a better understanding of the genetic determinants of the ovulatory cascade and thereby, the endocrine regulation of fertility and its control.

## Glossary

**dominance** The status of the follicle destined to ovulate given its presumed key role in regulating the size of the ovulatory quota.

**LH surge** Midcycle surge of pituitary luteinization hormone initiating the ovulatory cascade.

**luteinization** The ovulatory-related process of formation of the corpus luteum from the ovulatory follicle. The corpus luteum comprises luteal and nonluteal cell types. The luteal cells differentiate from the theca and granulosa cells and have the capacity to produce steroids and peptide hormones.

**ovulation** Release of a fertilizable ovum from the graafian follicle. In its broader sense, the ovulatory response defines the cascade of events following the LH surge and including the resumption of meiosis and oocyte maturation, luteinization, and the rupture of the follicular wall.

**ovulatory cascade** A highly synchronized and exquisitely timed cascade of specific gene(s) expression to ensure the correct process of ovulation.

**recruitment** The process wherein the follicle departs from the resting pool to begin a well-characterized pattern of growth and development. Recruitment, although obligatory, does not guarantee ovulation. Stated differently, recruitment is necessary but not sufficient for ovulation to occur.

**selection** The final winnowing of the maturing follicular cohort by atresia down to a size equal to the species-specific ovulatory quota.

## See Also the Following Articles

**Corpus Luteum in Primates • Corpus Luteum: Regression and Rescue • Decidualization • Endocrine Rhythms: Generation, Regulation, and Integration • Follicle Stimulating Hormone (FSH) • Folliculogenesis • Implantation • *In Vitro* Fertilization • Lipoprotein Receptor Signaling • Luteinizing Hormone (LH) • Oocyte Development and Maturation • Progesterone Action in the Female Reproductive Tract**

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# Oxyntomodulin

Bo Ahrén
*Lund University, Sweden*

I. INTRODUCTION
II. OXYNTOMODULIN BIOCHEMISTRY
III. RECEPTORS FOR OXYNTOMODULIN
IV. SIGNALING PATHWAYS OF OXYNTOMODULIN
V. OXYNTOMODULIN PHYSIOLOGY
VI. OXYNTOMODULIN PATHOPHYSIOLOGY

**Oxyntomodulin is a gut hormone. This article describes the structure and processing of the hormone, the regulation of its expression and secretion, its effects and mechanisms, as well as possible involvement of the hormone in gastrointestinal disorder.**

## I. INTRODUCTION

Oxyntomodulin, a 37-amino-acid peptide, is processed from proglucagon in the intestinal L-cells and is released after food intake. Although the physiological role of oxyntomodulin remains to be established, it inhibits gastric acid secretion, gastric emptying, and pancreatic exocrine secretion and stimulates insulin secretion.

The intestinal proglucagon-derived peptide oxyntomodulin works mainly by activating receptors that are more specific for other proglucagon-derived peptides, through actions involving phosphoinositide hydrolysis and formation of cyclic AMP. Physiologically, the peptide may be an enterogastrone and/or an incretin factor; oxyntomodulin may be involved in gastrointestinal disorders, although this has not been established.

## II. OXYNTOMODULIN BIOCHEMISTRY

### A. Expression of Oxyntomodulin

Oxyntomodulin belongs to the enteroglucagons or the glucagon-like substances in the gut. These substances were initially identified in intestinal mucosa in 1948 by Sutherland and de Duve using bioassay techniques, and in the sixties by Unger and collaborators using radioimmunoassay. Later studies revealed that these substances consist of a variety of several different peptides. All are produced in the gut L-cells, which are of the open type of gut endocrine cells that are located preferentially in the mucosal cell lining of the distal portion of the ileum. All enteroglucagons are encoded by the same glucagon gene, which in humans is located on chromosome 2q36–q37. This gene is also expressed in the pancreatic A-cells and in the brain. It encodes a single proglucagon messenger RNA transcript that is translated into a single 158-amino-acid sequence comprising the proglucagon peptide. Proglucagon, which is identical in all cells expressing the proglucagon gene, is processed through intracellular cleavage, yielding several different peptides (Fig. 1). However, the prohormone processing is different in each cell type expressing proglucagon. In the gut L-cells and in the brain, proglucagon is processed to glicentin, glicentin-related polypeptide (GRPP), oxyntomodulin, glucagon-like peptides (GLP-1 and GLP-2), and intervening peptide-2 (IP-2). In contrast, in the pancreatic A-cells, proglucagon processing gives rise to glucagon, GRPP, major proglucagon factor

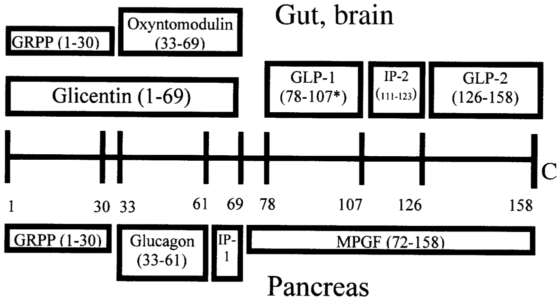

**FIGURE 1** Processing of the 158-amino-acid sequence of the proglucagon molecule in the intestinal L-cells and brain (top) versus the pancreatic A-cells (bottom). Numbers denote amino acids. In the gut and brain, proglucagon is processed to glicentin, glicentin-related polypeptide (GRPP), oxyntomodulin, glucagon-like peptide-1 (GLP-1), intervening peptide-2 (IP-2), and GLP-2, whereas in the pancreas, proglucagon is processed to GRPP, glucagon, IP-1 (sequence 64–69), and major proglucagon factor (MPGF). * Indicates oxidation.

(MPGF), and IP-1. The differences in how glucagon is processed in L- and A-cells are explained by tissue-specific differences in expression of the two prohormone convertases (PCs), PC1 and PC2. Thus, oxyntomodulin is formed in cells expressing PC1, as in the L-cells, whereas glucagon is formed in cells expressing PC2, as in the pancreatic A-cells.

## B. Formation and Structure of Oxyntomodulin

Formation of oxyntomodulin from proglucagon is a two-step process. First, glicentin is formed through cleavage between amino acids 69 and 70 in the proglucagon sequence. Glicentin is then further processed to form the 30-amino-acid sequence of GRPP and the 37-amino acid sequence of oxyntomodulin through a cleavage of a Lys-Arg dibasic sequence. Oxyntomodulin is equivalent to the C-terminal 37 amino acids of glicentin, corresponding to glicentin(33–69). From a quantitative point of view, approximately 20–40% of the glicentin formed is processed to oxyntomodulin, whereas the majority is released as glicentin. Oxyntomodulin also shows a high degree of structural identity to the other proglucagon-derived peptides, glucagon, GLP-1, and GLP-2 (Fig. 2). In fact, the N-terminal 29-amino-acid sequence of oxyntomodulin, i.e., glicentin(33–61), is identical to pancreatic glucagon. The amino acid sequence of oxyntomodulin is conserved through evolution, as is evident by the identical structure of oxyntomodulin in humans and guinea pigs.

## C. Distribution of Oxyntomodulin

Because oxyntomodulin is formed during processing of proglucagon in cells expressing PC1, its cellular distribution is limited to the gut L-cells and certain brain areas. In fact, the rat ileum has a 10-fold higher oxyntomodulin content per gram of tissue, compared to the duodenum, and the content in the colon is approximately double that in the duodenum, which is the same distribution as for L-cells. Studies on oxyntomodulin levels in extracts of various areas of the rat brain show that the most marked expression of oxyntomodulin is in the hypothalamus. Oxyntomodulin is also expressed in the medulla oblongata, although at a 10-fold lower level than in the hypothalamus. In contrast, only trace amounts of oxyntomodulin have been demonstrated in the olfactory bulb, cerebellum, and cortex, and no oxyntomodulin has been detected in the pituitary.

## D. Regulation of Expression of Oxyntomodulin

Nutrient ingestion is the primary stimulus for expression of the proglucagon gene in the gut, and therefore for formation of oxyntomodulin. Increased proglucagon gene expression is seen in rats after feeding, whereas fasting is associated with reduced gene expression. Proglucagon gene expression and proglucagon formation are also increased in rats after intestinal injury or intestinal resection. These findings suggest that proglucagon-derived factors are of importance for prandial processes as well as for response to injury.

## E. Regulation of Secretion of Oxyntomodulin

After its formation, the mature 37-amino-acid oxyntomodulin is stored in the secretory granules and is secreted extracellularly when L-cells are activated. A rapid secretion of the proglucagon-derived peptides from the L-cells is induced by nutrient ingestion, and both intralumenal carbohydrates and triglycerides have been shown to be of relevance. These nutrients stimulate oxyntomodulin release through activation of L-cells from the gut lumenal side. Nutrient ingestion may, however, stimulate L-cell secretion indirectly through activation of intestinal nerves, as illustrated in rats by a study showing that oxyntomodulin released by intraduodenal administration of oleic acid is inhibited via ganglionic blockade by hexamethonium.

| | 1 5 10 15 20 25 30 35 |
|---|---|
| *Oxyntomodulin* | H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T K A N K N N I A |
| *Glucagon* | H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T |
| *GLP-1* | H A E G T F T S D V S S Y L E G Q A A K E F I A W L V K G R * |
| *GLP-2* | H A D G S F S D E M N T I L D N L A A R D F I N W L I Q T K I T D |

**FIGURE 2** Amino acid sequences of oxyntomodulin, glucagon, glucagon-like peptide-1 (GLP-1), and GLP-2. The asterisk indicates amidation.

This is analogous to studies on the potential involvement of nerves in the regulation of GLP-1 secretion, showing that cholinergic nerves and the intestinal neuropeptide gastrin-releasing peptide (GRP) are stimulatory whereas adrenergic nerves are inhibitory.

## F. Circulation of Oxyntomodulin

In plasma, circulating fasting levels of oxyntomodulin are approximately 15 pmol/liter in both humans and rats, and these levels are increased approximately twofold following refeeding. The increase in circulating levels is evident within 30 min after meal ingestion. Similarly, a study on the 24-h profile of oxyntomodulin-like immunoreactivity in humans has shown that plasma levels increase after each meal. Pharmacokinetic studies have shown that oxyntomodulin is rapidly eliminated through a two-phase elimination mechanism. In the pig, a rapid first phase has a half-life of 7 min and a slow second phase has a half-life of 20 min. In humans, the plasma half-life of oxyntomodulin has been estimated to 12 min. However, the metabolic pathways responsible for degradation of oxyntomodulin in plasma are not known. The fate of oxyntomodulin differs from the metabolic fates of the two other hormones from the L-cells, GLP-1 and GLP-2, which are both degraded by removal of the two N-terminal amino acids (His-Ala) by the enzyme dipeptidyl peptidase IV, because this enzyme requires for action either alanine or proline in position 2 of the substrate, which is not the case for oxyntomodulin.

## G. Effects of Oxyntomodulin

The first described effect of oxyntomodulin was a stimulatory action on the formation of cyclic AMP in the acid-secreting fundic portion of rat stomach, which in fact was the basis for Bataille and collaborators to name the peptide oxyntomodulin. They found that oxyntomodulin was approximately 20 times more potent than glucagon in augmenting cAMP in this system, in contrast to having only 10% of the potency of glucagon in stimulating cAMP formation in membranes from the rat liver. This difference was later reproduced in studies using a preparation of highly enriched rat gastric parietal cells, and it was also shown that oxyntomodulin stimulates acid secretion through a cAMP-dependent mechanism.

However, in several other systems, oxyntomodulin has been shown to inhibit gastric acid secretion. Oxyntomodulin inhibits histamine- and pentagastrin-induced gastric acid secretion in rats *in vivo* without affecting basal gastric acid secretion; in humans, oxyntomodulin inhibits pentagastrin-stimulated gastric acid secretion. This inhibitory action on gastric acid secretion *in vivo* is explained by a stimulatory action of oxyntomodulin on somatostatin secretion from D-cells; somatostatin in turn inhibits gastric acid secretion. Oxyntomodulin has also been shown to stimulate smooth muscle contraction in the stomach, as demonstrated by a reduced mean length of isolated rat gastric smooth muscle cells, and the peptide also inhibits ion and water transport through the rat small intestine. Moreover, oxyntomodulin inhibits pancreatic exocrine secretion in rats, both in terms of the volume of juice as well as the bicarbonate and protein output, which has been shown to be an indirect action through activation of the vagal nerves. In humans, oxyntomodulin also delays gastric emptying, inhibits postprandial duodenal motility and exocrine pancreatic enzyme secretion,and inhibits pentagastrin-stimulated gastric acid secretion. Therefore, at least under *in vivo* conditions, oxyntomodulin seems to act as a general inhibitory agent on proximal gastro-entero-pancreatic postprandial events, suggesting that it is involved in the small intestinal inhibitory control of these functions.

Oxyntomodulin also affects processes of relevance for carbohydrate metabolism. Infusion of oxyntomodulin in rats increases glucose absorption in the jejunum and promotes glucose release from isolated hepatocytes. Furthermore, oxyntomodulin stimulates insulin and somatostatin secretion from the pig pancreas and augments insulin secretion in the presence of glucose in the perfused rat pancreas. Infusion of oxyntomodulin increases plasma insulin and C-peptide concentrations in humans. Thus, oxyntomodulin has the ability to increase glucose levels through actions on the gut and liver, but also to reduce glucose levels through its stimulation of insulin secretion.

# III. RECEPTORS FOR OXYNTOMODULIN

In spite of the various effects induced following exogenous administration of oxyntomodulin, no specific oxyntomodulin receptor has been identified or cloned. Instead, oxyntomodulin has been suggested to act through activation of receptors that are more specific for the other proglucagon-derived peptides. For example, oxyntomodulin increases cAMP formation in a cell line transfected with the GLP-1 receptor, and the peptide displaces radiolabeled GLP-1 bound to these cells, although the potency of oxyntomodulin is lower than that of GLP-1. Similarly, oxyntomodulin stimulates the release of somatostatin from a cell line by activating a GLP-1-selective receptor type and displaces radiolabeled GLP-1 from rat parietal cells. This suggests that oxyntomodulin acts at least partially through the GLP-1 receptors. However, oxyntomodulin has also been shown to bind to pig liver glucagon receptors, although with an affinity of only 2% of that of glucagon, suggesting a low-grade cross-reaction with these receptors. In addition, in isolated smooth muscle cells, oxyntomodulin seems to act through a glicentin/oxyntomodulin receptor type that shows high affinity for glicentin and low affinity for oxyntomodulin. Hence, several different receptor subtypes may transduce actions of oxyntomodulin. In a variety of experimental systems, there has been a general finding that the active site of oxyntomodulin resides in its C-terminal portion, because a C-terminal fragment, oxyntomodulin(19–37), is equipotent with the entire peptide.

## IV. SIGNALING PATHWAYS OF OXYNTOMODULIN

Because oxyntomodulin may work through activation of GLP-1 and glucagon receptors, cyclic AMP is a likely second messenger for transducing the effects. This has been confirmed in a few studies of the signaling underlying the effects of oxyntomodulin. It has also been shown, however, that oxyntomodulin may activate other signaling pathways. For example, stimulation by oxyntomodulin of gastric smooth muscle cells is mediated by phosphoinositide hydrolysis, resulting in formation of inositol 1,4,5-trisphosphate ($InsP_3$) and liberation of $Ca^{2+}$ from intracellular stores; this is accompanied by reduced formation of cAMP in a pertussis-toxin-sensitive manner. Oxyntomodulin may therefore activate G-protein-coupled receptors, which are linked to phospholipase C and/or adenylate cyclase.

## V. OXYNTOMODULIN PHYSIOLOGY

The physiological role of oxyntomodulin remains to be established. Release of oxyntomodulin after ingestion of a meal suggests that the hormone is involved in regulation of postprandial nutritional or metabolic events. Its main function, to inhibit gastric acid secretion and pancreatic exocrine secretion, suggests that oxyntomodulin is an enterogastrone candidate. Enterogastrones are prandially released gut-derived factors that inhibit proximal events such as gastric acid secretion, gastric emptying, and pancreatic exocrine secretion, perhaps to prevent excessive acid or exocrine pancreatic secretion. Evidence that oxyntomodulin is an enterogastrone factor includes that it is released after ingestion of each meal and that it inhibits gastric acid secretion and pancreatic exocrine secretion when exogenously administered at dose levels that are equivalent to circulating levels under physiological conditions. Furthermore, one study has shown a negative correlation between circulating levels of oxyntomodulin and gastric acid secretion in humans. On the other hand, the potency of oxyntomodulin to inhibit gastric acid secretion, as demonstrated in dogs, is much lower than the potency of other enterogastrone candidates, such as peptide YY, secretin, and cholecystokinin. Therefore, the role of oxyntomodulin as an enterogastrone remains to be established.

Because oxyntomodulin also stimulates insulin secretion, it may also be an incretin factor. Incretin factors are gut hormones released after meal intake and they stimulate insulin secretion. Again, the evidence that oxyntomodulin may be an incretin factor resides in its release after food intake and its ability to stimulate insulin secretion. However, in comparison with the potency of the two main incretin candidates, GLP-1 and gastric inhibitory polypeptide (GIP), the potency of oxyntomodulin is low. Therefore, although oxyntomodulin is a potential incretin, it remains to be established that such an effect is indeed of physiological relevance.

Although oxyntomodulin is expressed in the brain, particularly in the hypothalamus, no studies so far have shown any effects of the peptide in the central nervous system. For example, oxyntomodulin infused for 7 days in rats has no effect on body weight or food intake. So far, there is no evidence of any role of oxyntomodulin in the regulation of the central nervous system.

## VI. OXYNTOMODULIN PATHOPHYSIOLOGY

The potential involvement of oxyntomodulin in various gastrointestinal disorders has been the matter of a few studies. One study in subjects with duodenal ulcer has shown that circulating oxyntomodulin levels do not differ from those in controls. Another study has shown that children with celiac disease display high circulating levels of oxyntomodulin and that oxyntomodulin levels correlate to markers of malabsorption. Although this may suggest that oxyntomodulin is involved in the development of malabsorption, a more likely explanation is that the peptide is involved in an adaptive mechanism. Expression of the proglucagon gene is known to be stimulated by intestinal injury and after intestinal resection, thus oxyntomodulin levels will increase under such conditions. Whether, however, oxyntomodulin has any role in this adaptation, as has been suggested for GLP-2, is not known. Therefore, the role of oxyntomodulin under pathological conditions is far from established.

## Glossary

**enterogastrones** Hormones from the gut that are released after meal ingestion; inhibit gastric acid secretion and gastric emptying.

**enteroglucagons** Hormones produced in the intestinal glucagon cells (L-cells) through processing of the proglucagon molecule.

**glicentin** Intermediary 69-amino-acid peptide processed from proglucagon and cleaved to form glicentin-related polypeptide and oxyntomodulin.

**glicentin-related polypeptide** A peptide that is processed from the N-terminal end of glicentin in the intestinal L-cells.

**incretins** Hormones from the gut that are released after meal ingestion; stimulate insulin secretion.

**oxyntomodulin** A 37-amino-acid peptide that is processed from proglucagon in the intestinal L-cells and in the brain, but not in the pancreatic A-cells.

## See Also the Following Articles

**Glucagon Action • Glucagon-like Peptides: GLP-1 and GLP-2 • Glucagon Processing • Glucose-Dependent Insulinotropic Polypeptide (GIP)**

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# Oxytocin

John A. Russell and Alison J. Douglas
*University of Edinburgh, United Kingdom*

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**Oxytocin is a nonapeptide ($Cys^1$-$Tyr^2$-$Ileu^3$-$Gln^4$-$Asn^5$-$Cys^6$-$Pro^7$-$Leu^8$-$Gly^9$-$NH_2$) that is produced only in mammals. Synthesis occurs in groups of nerve cells (neurons) and (variably, according to species) in reproductive tissues. Circulating oxytocin is entirely from the posterior pituitary gland. Oxytocin in the brain or cerebrospinal fluid is produced in the brain. Parturition, maternal behavior, and lactation are stimulated by oxytocin release.**

## I. INTRODUCTION

Oxytocin was one of the first hormones to be discovered. At the beginning of the past century, extracts of the posterior pituitary gland were shown to stimulate the contractile activity of the pregnant uterus. This led to the idea that oxytocin had a role in stimulating uterine contractions in parturition, and its name [Gk.: swift (*oxys*) birth (*tokos*)] derives from this action. It was later discovered that posterior pituitary extract is the most effective stimulant of milk let-down or milk ejection in lactating mammals. The other hormonal activity in the posterior pituitary is due to antidiuretic hormone (vasopressin), although it was not until Du Vigneaud characterized and synthesized the vasopressin and oxytocin peptides in the 1950s (for which work a Nobel Prize was awarded) that it was clear these are distinct molecules.

Oxytocin is a nine-amino-acid peptide, comprising a six-member ring formed by sulfide bridges between the two cysteines, and a short tail (~1 kDa). Vasopressin differs from oxytocin by having Phe at position 3 and Arg at position 8. Oxytocin is essentially a mammalian hormone (among other vertebrates it has been found only in the ratfish), and its close chemical similarity to vasopressin indicates that it arose in evolution first, by a duplication of the vasopressin gene (or its forebear), and then by mutations. It is clear, in particular from studies on mice with targeted disruption of the oxytocin gene, that oxytocin is indispensable for milk transfer during suckling; consequently, oxytocin is to be regarded as the hormone without which mammalian reproduction would not be possible. The oxytocin and vasopressin genes remain in close proximity to each other (in humans, on chromosome 20), and in opposite orientation, so that they are transcribed left to right and vice versa. Like other peptide hormones, the RNA transcribed from the oxytocin gene includes transcripts from several (three) exons separated by introns, and this heterogeneous nuclear RNA is processed to an mRNA that codes for the oxytocin peptide and for a much larger (~10 kDa) protein called oxytocin-neurophysin. This acts as a carrier for oxytocin in the cells in which it is produced, and they are secreted together, but as separate molecules; neurophysin has no known hormonal function, but its measurement in the circulation can be used as an indicator of oxytocin secretion. Among the marsupials, the oxytocin hormone produced is either oxytocin or the similar mesotocin (Ile is substituted for Leu at position 8), or both in some species.

Soon after its synthesis in the laboratory was achieved, industrial production began in the mid-1950s so that oxytocin could be used to promote labor in women, replacing use of the relatively impure posterior pituitary extract that had previously been used. Since its introduction into obstetric practice, millions of human births have been aided by the infusion, usually intravenously, of oxytocin. Also, on delivery of the infant, routine intramuscular injection of oxytocin (together with an ergot) to induce strong uterine contractions is a frontline measure to ensure occlusion of spiral arterioles, which are ruptured when the placenta separates, and thus to prevent postpartum hemorrhage. Conversely, the likely involvement of oxytocin in preterm birth has led to the development of oxytocin receptor antagonists to try to prevent this important cause of neonatal morbidity and mortality.

It became clear in the 1950s that oxytocin is also present in neurons projecting within the brain, where it acts as a neurotransmitter or modulator, with stimulatory roles in neural circuits concerned with affiliative and reproductive behaviors (actions that, together with peripheral effects, have earned oxytocin the title “love hormone”). To date, there is no known naturally occurring oxytocin deficiency state, and no known mutations, although both oxytocin peptide and oxytocin receptor “knockout” mice have been generated.

## II. SOURCES OF OXYTOCIN IN THE BODY

### A. The Posterior Pituitary Gland

The posterior pituitary gland contains the greatest amount of oxytocin in the body. About three times more oxytocin is stored in the posterior pituitary at the end of pregnancy than is needed to drive the birth process. However, oxytocin stored in the posterior pituitary is not synthesized there: storage occurs in the many thousands of axon terminals of nerve cells that have their cell bodies in the hypothalamus, where oxytocin is continuously synthesized. These nerve cells are concentrated in two paired groups of neurons, or nuclei, in the hypothalamus: the supraoptic and paraventricular nuclei. The oxytocin nerve cell bodies have the general characteristics of neurons. These include dendritic processes, which, like the cell bodies, have many synapses that mediate inputs from many brain areas, using a wide range of excitatory and inhibitory neurotransmitters. Because the neuronal cell bodies produce relatively large amounts of peptide hormone, to achieve effective concentrations in the systemic circulation, they have abundant mRNA for oxytocin (oxytocin mRNA is the most abundant peptide mRNA species in the hypothalamus) and prominent protein synthetic machinery. This includes the Golgi apparatus, where the oxytocin-neurophysin preprohormone is processed and packaged into membrane-bound vesicles. The cell bodies of the oxytocin neurons are thus larger than other hypothalamic neurons and are visible in histological sections as closely packed "magnocellular" neurons. The similarity between oxytocin and vasopressin has been mentioned already, and this extends to the similarity between the oxytocin neurons and those producing and storing vasopressin. Importantly, magnocellular hypothalamic neurons make either oxytocin or vasopressin, but not both (except for a small percentage of vasopressin neurons that may also express oxytocin when there is a large demand, as in lactation), even though both types of neuron are adjacent to one another in both the supraoptic and the paraventricular nuclei. They can be distinguished from each other with an immunocytochemical technique, using antibodies for oxytocin or vasopressin. These neurons differ in other respects that allow them to respond in the most appropriate ways to the different stimuli that activate them and to generate optimal patterns of hormone secretion (see later). What determines whether a magnocellular neuron develops as an oxytocin or a vasopressin neuron is presently not understood; the intergenomic region that links the oxytocin and vasopressin genes evidently contains important sequences that regulate which gene is expressed.

The vesicles containing oxytocin and neurophysin are actively transported within the axon of each neuron, leaving the cell body and arriving several hours later a few millimeters away in the branching terminals in the posterior pituitary. The stalk of the posterior pituitary gland that connects the gland to the hypothalamus mainly comprises these axons, which are packed with the vesicles containing oxytocin (and its neurophysin). Some of the vesicles containing oxytocin are transported from the cell bodies into the dendrites of the neurons, thus remaining in the brain, in the hypothalamus. The secretion of oxytocin by the nerve terminals in the posterior pituitary gland occurs only when the terminals are depolarized by the arrival of action potentials, conducted along the axons from the cell bodies, at $\sim 1\ \mathrm{m\ s^{-1}}$. These action potentials are generated in the cell bodies of the magnocellular oxytocin neurons in the hypothalamus when they are excited by their synaptic input, or are otherwise depolarized. This mechanism can be simulated by electrical stimulation of the pituitary stalk to study the relationship between the frequency and pattern of action potentials and oxytocin secretion, and the signaling mechanisms in the nerve terminals that couple depolarization to the release of oxytocin.

The key event following arrival of action potentials is the opening of the membrane channels that allow entry of calcium ions; the increase in cytoplasmic calcium ion concentration then triggers the intracellular machinery that moves the oxytocin secretory vesicles to the plasma membrane of the nerve terminal, with which they fuse and release their contents into the extracellular space in the process of exocytosis. The peptide released by this neurosecretory mechanism then passes through the fenestrations in the walls of the adjacent capillaries, to be carried in the systemic circulation to target tissues.

There are two particularly important points about the coupling of the stimulation of the cell bodies of the oxytocin neurons to the secretion of oxytocin from their terminals. The first is that more oxytocin is secreted per action potential if the action potentials are close together (in a "burst"); this is "frequency facilitation" of release. The second is that if all of the oxytocin neurons have a burst of activity at the same time, then the result will be the secretion of a "pulse" of oxytocin into the circulation, achieving a high concentration for a short time. (The importance of this pattern of secretion is explained later.) Once oxytocin is secreted into the circulation it has a half-life of only 2 min or so, being cleared by the tissues on which it acts and by excretion from the kidneys.

## B. Oxytocin Within the Brain

Very little, less than 1%, of the circulating oxytocin is able to enter the brain because the blood–brain barrier prevents entry of this peptide, as for most other peptide hormones. However, oxytocin has well-established functions in the central nervous system, the blood–brain barrier effectively allowing the brain and posterior pituitary oxytocin systems to function independently. There are two sources of oxytocin in the brain, the first being the magnocellular neurons of the paraventricular and supraoptic nuclei that secrete oxytocin from their axon terminals in the posterior pituitary, because they also secrete oxytocin from their dendrites, in a way similar to the mechanism operating in the posterior pituitary. Oxytocin from these dendrites has local and probably more distant actions. Second, in the paraventricular nucleus, there are the separate populations of oxytocin neurons that project their axons only centrally, to other brain or spinal cord regions.

## C. Peripheral Sources of Oxytocin

The hypothalamic magnocellular neuron-posterior pituitary and the central neuron sources of oxytocin are common to all mammals. There are also peripheral sources of oxytocin that show species or class specificity.

### 1. *Uterus*

In pregnancy in humans and in rats, but not in the mouse, cow, or sheep, the oxytocin gene is expressed in the lining of the uterus, placenta, and amnion. Because the adjacent late-pregnant myometrium is a target tissue for oxytocin action, it is possible that oxytocin produced locally acts in this way. However, oxytocin is released not from the side of the cells adjacent to the myometrium, but from the lumenal surface of the uterine epithelium, where there are also oxytocin receptors. It seems unlikely that this source of oxytocin can directly stimulate the myometrium. Instead, this oxytocin is more likely to act by stimulating prostaglandin production by the epithelium or endometrial decidual cells, or in humans by the amnion; in the rat, the amnion lacks the oxytocin receptor.

### 2. *Corpus Luteum*

In ruminants (cattle, deer, and sheep), the corpus luteum, formed in the ovary from the follicle after ovulation, produces oxytocin. This acts, together with oxytocin secreted by the neurohypophysis, on the nonpregnant endometrium and stimulates prostaglandin F2α secretion, provided oxytocin receptors are expressed; estrogen stimulates the expression of the oxytocin receptors in the endometrium. This prostaglandin F2α is then carried back to the corpus luteum in a local countercurrent circulation to stimulate further oxytocin secretion and to initiate luteolysis. The consequent decrease in progesterone secretion permits gonadotropin stimulation of ovarian follicular development and hence ovulation. This mechanism thus brings forward a further opportunity for conception if the previous ovulation fails to result in pregnancy. Obviously, if pregnancy is to be established, then this mechanism must be prevented. This is achieved by suppression of endometrial oxytocin receptor expression as a result of action of interferon τ produced by the conceptus. This mechanism is thus the key factor in the maternal recognition of pregnancy in these species.

In some other species (e.g., pigs) there is a low level of oxytocin expression in the granulosa cells, which may play a role in luteinization. Studies in pregnant mice show that here low levels of circulating oxytocin maintain the corpora lutea, preventing progesterone withdrawal and paradoxically delaying parturition. Consequently, parturition in mice requires loss of luteal oxytocin receptors as well as up-regulation of myometrial receptors.

### 3. *Heart*

In the rat, oxytocin has a natriuretic action. This is consistent with the stimulation of the secretion of both oxytocin and vasopressin by an increase in osmolarity of extracellular fluid in this species. This is usually a result of sodium ion accumulation following food intake or water deprivation; oxytocin promotes sodium excretion, helping to restore normal osmolarity, acting along with vasopressin, which retains water by its actions on the kidney. While oxytocin may act directly on the renal tubules to cause sodium excretion, it now seems that oxytocin also acts on the atria of the heart to stimulate the secretion of atrial natriuretic peptide, which then acts on the kidney to stimulate sodium excretion. It also seems that oxytocin secretion is stimulated by increased blood volume and is important in mediating the subsequent stimulation of atrial natriuretic peptide secretion, which counteracts the volume expansion. Furthermore, the atria evidently produce oxytocin and, if released with atrial distension, as in blood volume expansion, could act locally to stimulate atrial natriuretic peptide secretion in a paracrine fashion.

### 4. Immune System

Oxytocin is synthesized in the thymus gland, particularly in epithelial and nurse cells, although it is not secreted. Nonetheless, oxytocin produced by thymic epithelial cells has been proposed to signal to the differentiating T lymphocytes with which the epithelial cells are in intimate contact. This neuropeptide signaling may be important in the development of immune self-tolerance.

## III. REGULATION OF OXYTOCIN GENE EXPRESSION

Regulation of the massive increase in expression of the oxytocin gene in bovine ovarian follicle granulosa cells at ovulation, as they luteinize, has been extensively studied. As yet the factors regulating gene expression in this system remain unclear.

The store of oxytocin in the posterior pituitary increases by about 50% to the end of pregnancy. This may result from reduced release rather than from increased oxytocin gene expression, in as much as the amount of oxytocin mRNA in the magnocellular neurons has not been consistently reported to increase. The upstream region of the oxytocin gene has variations on the AGGTCA motif, a consensus sequence for nuclear hormone receptor binding, but it is not clear what transcription factors regulate the gene. In view of its reproductive functions, regulation of the production of oxytocin by sex steroids has been investigated by many researchers, but the effects are weak and not consistent. Gene expression is increased at parturition, as a consequence of excitation of the neurons during birth, involving withdrawal of inhibitory actions of progesterone in the presence of estrogen. Oxytocin neurons lack progesterone receptors, but there is a strong inhibitory action of progesterone through its metabolite allopregnanolone (a neurosteroid), which is an allosteric modifier at the inhibitory γ-aminobutyric acid ($\mathrm{GABA_A}$) receptors on the neurons. Withdrawal of progesterone at the end of pregnancy thus facilitates oxytocin neuron excitation. Although estrogen is reputed to have stimulatory actions on oxytocin gene expression, the mechanism is not known. However, inhibitory effects of estrogen on stimulated oxytocin release, and on oxytocin mRNA expression, have also been described. In the rat, oxytocin neurons express estrogen receptor-β (and not estrogen receptor-α, which the neurons express in some species), but any role in mediating estrogen actions on oxytocin expression is not clear. Estrogen may act on estrogen receptors on the cell surface rather than through intracellular receptors.

In the paraventricular nucleus, which contains centrally projecting neurons, oxytocin gene expression in rats is increased by estrogen. Interestingly, this action of estrogen is interfered with by thyroid hormone, raising the issue of the importance of thyroid status in the functioning of oxytocin neurons. In pregnant sheep, oxytocin mRNA expression is increased in these neurons in pregnancy, but sex steroid actions may not be responsible.

In lactation, the store of oxytocin becomes greatly reduced as it is secreted to drive milk ejections. After a week of lactation, there is marked up-regulation of oxytocin mRNA expression in the magnocellular neurons, leading to increased oxytocin synthesis. This increase has been attributed in part to the decline at this time in progesterone secretion, which is increased for several days after the postpartum estrus. Other factors, such as consequences of continual stimulation of the neurons by suckling, are also involved.

## IV. CONTROL OF OXYTOCIN SECRETION

Oxytocin neurons are like other neurons in that they have many synaptic boutons contacting their dendrites and cell bodies. These mediate control of the oxytocin neuron discharge activity by other brain regions and by sensory inputs from the body. These synaptic inputs comprise a rich variety of types of chemical transmitters, such that it is difficult to find a neurotransmitter or neuropeptide that does not act on oxytocin neurons. Thus, there are excitatory amino acid, eicosanoid, monoamine, purine, and peptide actions (e.g., glutamate, noradrenaline, prostaglandin, adenosine, and cholecystokinin), and similarly actions of a range of inhibitory transmitters (e.g., GABA, opioid peptides, and the gas nitric oxide). The effects of these transmitters on oxytocin neurons are, as in other neurons, the result of their depolarizing or hyperpolarizing actions. Again, as for other types of neurons, local modulation of the activity of the synaptic boutons by other transmitters is important in determining the intensity of excitatory or inhibitory barrage. Oxytocin has such a presynaptic action, as do nitric oxide and opioid peptides produced by the oxytocin neurons.

For magnocellular oxytocin neurons, the major inputs in terms of function are those mediating afferent stimuli from the birth canal during parturition and from the mammary glands during suckling (Fig. 1). In the rat, there is an important input from

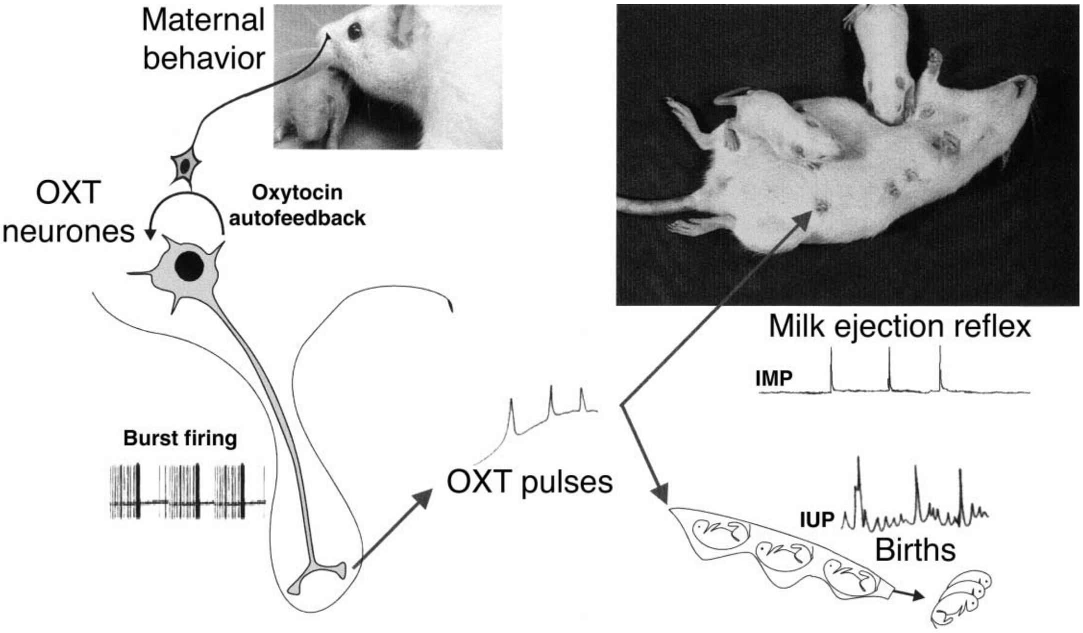

**FIGURE 1** Summary of the peripheral and central effects of oxytocin (OXT) in parturition, maternal behavior, and lactation in rats. During the passage of each fetus through the uterine cervix and vagina, afferent nervous impulses from these structures, as they are stretched, are relayed, via the spinal cord and brain pathways, to oxytocin neurons in the hypothalamus. The hypothalamic neurons are excited and send bursts of action potentials to the posterior pituitary, where oxytocin is released into the circulation. Because all of the neurons send bursts at the same time, the result is secretion of a pulse of oxytocin. This stimulates further uterine contractions, increasing intrauterine pressure (IUP) and expelling the fetus. Oxytocin released in the brain at the same time both further excites the oxytocin neurons and evokes maternal behavior. In lactation, the suckling stimulus has similar effects on oxytocin secretion, and now each pulse of oxytocin causes a milk ejection, seen as a sharp increase in intramammary pressure (IMP).

rostral osmosensitive brain structures involved in regulating oxytocin neurones in the context of natriuretic action. Regulation by all of these inputs involves a balance between local GABA and glutamate inputs. These are modulated by actions of oxytocin and nitric oxide released by the dendrites and perikarya of the oxytocin neurons when they are stimulated. Nitric oxide has a local negative feedback effect. In contrast, oxytocin has a local positive feedback effect, provided there is also stimulation by input from the distended birth canal or suckled mammary glands. The neurochemical signature of these inputs is not certain. Noradrenergic neurons projecting from the brain stem, receiving input from the birth canal, are stimulated during parturition, but they are not alone. Similarly, noradrenaline is one of several candidate transmitters mediating excitation of oxytocin neurons by suckling. It seems that there is redundancy in these essential pathways for mammalian reproduction.

In rats, circulating cholecystokinin stimulates oxytocin secretion. As cholecystokinin is secreted by the small intestine during digestion, the consequent secretion of oxytocin helps excrete ingested salt through its natriuretic action. In contrast with the pathways from the birth canal and mammary glands, the pathway for the action of cholecystokinin is clear. It is mediated by vagus nerve afferents and the area postrema and by noradrenergic neurons in the brain stem (nucleus tractus solitarius) that project to oxytocin neurons.

## A. Terminal Modulation

The principal determinant of oxytocin release from the posterior pituitary is the frequency of action potentials arriving from the cell bodies in the hypothalamus. However, there is an opportunity for preterminal modulation at this level. First, if the action potentials are clustered, then there is a greater rise in [$Ca^{2+}$] in the terminals, and hence greater oxytocin release (a mechanism to increase the amount of oxytocin in a pulse). Second, this is a site of inhibition by κ-opioid peptides, which oxytocin neurons also secrete. This mechanism probably reduces basal secretion and in pregnancy helps to build up a store of oxytocin. At the end of pregnancy, this mechanism is down-regulated, facilitating oxytocin secretion during parturition.

### B. Suspending Oxytocin Secretion and Parturition

Several species slow or stop the progress of parturition in adverse environmental conditions. In the rat and pig, this seems to be primarily a result of inhibition of oxytocin secretion by activation of central mu opioid peptide receptors on oxytocin neurons. This central opioid mechanism emerges during pregnancy and may also function to prevent premature oxytocin secretion. In mice, stopping parturition in response to stress does not involve opioids, but instead β-adrenergic mechanisms are used.

## V. OXYTOCIN RECEPTOR

The actions of oxytocin on target tissues are determined by the level of expression of receptors, their affinity and stability, and their coupling to postreceptor signaling pathways. Both the expression, i.e., the density of oxytocin receptors, and the affinity of the receptors are regulated by steroids. The oxytocin receptor mediates the contractile effects of oxytocin on the myometrial cells of the uterus and the myoepithelial cells of the mammary gland milk-producing alveoli. In marsupials that produce mesotocin rather than oxytocin, the mesotocin receptor seems identical to the oxytocin receptor in other mammals. In the brain, oxytocin receptors mediate the effects of oxytocin on the release of transmitters from nerve terminals and the postsynaptic actions on the electrical activity of neurons.

Like the neurohypophyseal nonapeptides, the oxytocin and vasopressin receptors are also closely related. However, whereas there are three different vasopressin receptors [V1a, V1b (also called V1 and V3, respectively), and V2], each the product of a different gene, there is only one oxytocin receptor gene. The human oxytocin receptor gene is on chromosome 3 and contains four exons and three introns. The oxytocin receptor is a seven-transmembrane domain, class I G-protein-coupled receptor. The second and third extracellular loops and the transmembrane domains of the receptor show remarkable conservation in their amino acid sequences among species, indicating involvement in oxytocin binding. Site-directed mutagenesis studies in expression systems have identified the amino acid residues in the receptor responsible for selectivity. The eighth amino acid (Leu) in oxytocin interacts with several residues in the first and second extracellular loops and Cys-1 then bonds to a Glu residue in the third extracellular loop. The oxytocin ring is then docked in a pocket formed by the transmembrane domains; the interactions between each residue in the oxytocin molecule and residues in the receptor have been derived from computer modeling. The changed configuration of the receptor leads to binding of G-protein to the intracellular domains. Antagonists and oxytocin probably bind to different domains.

The high-affinity state of the receptor in the myometrium has been known for a long time to require $Mg^{2+}$, and more recently cholesterol and progesterone have been identified as allosteric modulators. Progesterone has been found to interfere with the binding of oxytocin to its receptor in rat (but not in human) myometrium, reducing its affinity. Clearly, this action could explain some of the inhibitory actions of progesterone on oxytocin stimulation of the uterus and the need for progesterone to be withdrawn in many species (although not in the guinea pig) before parturition can be initiated. Another modulatory influence on oxytocin receptor function is the requirement for a cholesterol content of ~60% in the membranes in which the receptor is embedded for high-affinity binding. The receptor has a binding site for cholesterol as well as for oxytocin and cholesterol stabilizes the receptor in the high-affinity state. *In situ*, oxytocin receptors are localized in cholesterol-rich caveoli at the surface of myometrial cells, and in this location internalization is delayed following oxytocin exposure. The involvement of oxytocin and its receptor in positive feedback mechanisms (e.g., luteolysis, parturition, and milk ejection) implies that oxytocin-induced down-regulation or desensitization would cause these loops to fail; the pulsatile secretion of oxytocin may help prevent this.

One of the striking features of the oxytocin receptor is the very large change that is seen in the number of receptors in the uterus as pregnancy advances. Early in pregnancy the uterus is rather insensitive to oxytocin, but the myometrium becomes exquisitely sensitive just before parturition. This correlates with a dramatic increase in oxytocin receptor expression and an increase in affinity. In the rat, the increase in expression occurs on the last day of pregnancy. Hence, increasing stimulation of uterine contractions by oxytocin near the end of pregnancy need not rely on increased oxytocin secretion. In contrast, prolonged exposure to oxytocin, as during labor or oxytocin infusion, causes homologous receptor desensitization with marked decreases in receptor binding, and in oxytocin receptor mRNA expression.

Although the expression of the oxytocin receptor in the myometrium is regulated by the sex steroid changes in pregnancy, and, in particular, expression is inhibited by progesterone and up-regulated by estrogen, this does not seem to be by direct actions on the receptor gene. The regulatory elements of the gene lack the palindromic estrogen response element, although there are half-palindromic motifs. The implication is that estrogen acts through other genes, presumably for specific transcription factors, which then regulate the receptor gene. Of particular interest, in view of the proposed role of infection in preterm labor, is the presence of binding sequences for nucleofactor interleukin-6 in the 5′ upstream region. Other regulatory signals may be generated by stretching of the myometrium.

In the brain, even in closely related species there are differences in the distribution of oxytocin receptors. These differences are more striking than changes with reproductive state within a species, except that oxytocin receptor expression in the ventromedial hypothalamic nucleus is exquisitely sensitive to regulation by sex steroids (see later).

### A. Postreceptor Signaling

The intracellular signaling mechanisms mediating actions of oxytocin are similar among the various target tissues. The oxytocin receptor in the myometrium is predominantly coupled via $G_I$ and $G_{\alpha q/11}$ proteins to phospholipase C-β. This pathway leads to liberation of $Ca^{2+}$ from intracellular stores, and there is also entry of $Ca^{2+}$ from outside, including via L-type $Ca^{2+}$ channels. The increased intracellular $[Ca^{2+}]$ forms complexes with calmodulin, and this then activates myosin light chain kinase (MLCK); phosphorylated myosin then interacts with actin, and the shortening actomyosin contracts the cell. The jointly contracting myometrial cells, which are united by strong junctions, generate the expulsive force required for fetal expulsion. The automatic activation of intracellular $Ca^{2+}$ sequestering mechanisms, and active expulsion of $Ca^{2+}$ from the cells, lead to relaxation. Relaxants such as adrenaline activate adenylyl cyclase via $G_{\alpha s}$ and hence a protein kinase A (PKA) pathway. PKA phosphorylates and inactivates phospholipase C-β and MLCK. The amount of $G_{\alpha s}$ increases in pregnancy but is decreased at term. In pregnancy, there is a change in the proportions of the types of adenylyl cyclase isoforms, which have different properties, expressed in myometrial cells. These changes may contribute to the shift from uterine relaxation in pregnancy to greater responsiveness to oxytocin at term.

A similar mechanism involving increased intracellular $[Ca^{2+}]$ mediates oxytocin stimulation of mammary gland myoepithelial cell contractions, which cause milk ejection. An additional signaling pathway, studied in the amnion (of humans and rabbits), activates cytosolic phospholipase $A_2$ and hence releases arachidonic acid for prostaglandin synthesis. Here, the oxytocin receptor couples to $G_q$; the resulting increase in intracellular $[Ca^{2+}]$ contributes to cytosolic phospholipase $A_2$ activation, which is phosphorylated by extracellular signal-regulated kinase (ERK). Prostaglandin E2 synthesis is then stimulated. In the endometrium of ruminants, oxytocin stimulates prostaglandin F2α production via mobilization of intracellular $Ca^{2+}$, which activates a protein kinase C-dependent pathway. As described previously, this mechanism produces a luteolytic signal in the absence of conception. A similar stimulation of prostaglandin F2α production by decidual cells reinforces oxytocin actions on the myometrium in parturition.

The actions of oxytocin on neurons bearing oxytocin receptors involve mechanisms similar to those in other tissues. Hypothalamic magnocellular oxytocin neurons (the source of posterior pituitary oxytocin) are stimulated by oxytocin. Oxytocin induces a rapid and long-lasting increase in intracellular $[Ca^{2+}]$, and most of this is released from intracellular stores. This response has a key role in the driving of oxytocin neuron activity during parturition and lactation.

## VI. OXYTOCIN IN PARTURITION

As mentioned already, no naturally occurring oxytocin deficiency state is known. The phenotype of this state is partly known from studies in transgenic mice with deletion or inactivation of the oxytocin peptide gene; and more recently from studies of mice with inactivation of the oxytocin receptor gene. Such mice get pregnant and deliver their young, evidently at the appropriate time. However, their young do not survive for long because they cannot obtain milk. The young are rescued if the mothers are given injections of oxytocin. The conclusions from these studies are that oxytocin, and its receptor are not essential for ovarian function, reproductive behavior, or parturition. However, they are essential for milk transfer. The conclusion regarding the essential role of oxytocin in milk transfer is clear enough.

It is not appropriate, however, to conclude that oxytocin has no role in, for example, normal parturition. Rather, the performance of parturition in the absence of oxytocin illustrates how other mechanisms, in particular the uterine production of prostaglandins, and possibly vasopressin, can stimulate uterine contractions. This argues for redundancy in the mechanisms that bring about parturition, which might be expected given the crucial importance for the survival of both mother and offspring of the successful completion of this process. If so, then there should be activation of oxytocin secretory mechanisms, and enhanced oxytocin action, at parturition in mice and other species. The large increase in oxytocin receptor expression in the uterus at term and the changes in intracellular signaling mechanisms so that excitatory pathways predominate (see Section V) are strong circumstantial evidence that oxytocin has a role in parturition.

It does seem clear from studies on several species (humans, rats, and guinea pigs) that although parturition can be initiated at term by systemic infusion with oxytocin, the natural initiation of labor is not a result of increased maternal oxytocin secretion. The role of oxytocin in parturition is to promote the strong uterine contractions in the second stage, leading to the passage of the fetus through the dilated uterine cervix and vagina. Oxytocin then stimulates further uterine contractions that cause placental separation and, in polytocous species, the birth of the next fetus. In human deliveries, these further contractions ensure closure of the ruptured spiral arterioles, preventing a potentially fatal postpartum hemorrhage.

The evidence for increased secretion of oxytocin from the maternal posterior pituitary in parturition is manifold. Oxytocin concentration in the circulation increases in the second stage of labor. In marsupials that produce mesotocin, which has actions identical to those of oxytocin, the concentration of mesotocin in the circulation increases at the birth of the fetus. In humans, measurement of oxytocin in the circulation is difficult because the placenta secretes an aminopeptidase that rapidly destroys oxytocin, and secretion is expected to be pulsatile, so occasional blood samples are unlikely to catch a pulse. Nonetheless, increases in circulating oxytocin have been measured in the second stage. Oxytocin antagonist administration is effective in treating preterm labor in humans and delays births in guinea pigs and slows established parturition in rats. The content of oxytocin in the posterior pituitary gland is decreased by ~30% during parturition. Furthermore, oxytocin is released by the dendrites of the magnocellular neurons during parturition, and local oxytocin antagonist disrupts parturition (probably because it interferes with burst firing). Last, magnocellular oxytocin neurons increase their firing rate, express the immediate-early gene product Fos (a sign of activation of the neurons), and increase expression of the oxytocin gene. Most persuasively, in rats, the oxytocin neurons burst-fire as each pup is born and, furthermore, each burst is followed by a pulse of oxytocin that can be measured in the circulation.

So, what stimulates the secretion of oxytocin in parturition? In the 1940s, Ferguson concluded that oxytocin secretion is stimulated by the distension of the birth canal as the fetus is moved through it and stimulates afferent nerve endings. This is a positive feedback mechanism, which, like all such loops, can only end in an explosion—in this case, the birth of the fetus. Subsequent studies have shown that the pathway for this reflex from the birth canal to the oxytocin neurons is via brain stem nucleus tractus solitarius neurons, including noradrenergic neurons, that project directly to the magnocellular oxytocin neurons. There is an increase in noradrenaline release from the terminals of these neurons in the supraoptic nucleus during parturition, and other evidence suggests a role for this input in driving oxytocin neurons.

## VII. OXYTOCIN IN LACTATION

Feeding offspring with milk transferred by suckling is a distinctive feature of mammalian reproduction and is new in evolution, contrasting with parturition that has a long evolutionary connection with egg laying. The production of milk requires stimulation of mammary gland development by sex steroids and the subsequent stimulation by prolactin of milk synthesis and secretion by the epithelial cells of the alveoli. The milk is stored in alveoli (e.g., in rats), in sinuses, which are enlargements of the milk ducts beneath the nipple (e.g., in humans), or in a cistern into which the ducts empty, located beneath the nipple (the udder in cattle and sheep). Oxytocin acts on contractile myoepithelial cells around the alveoli, and the combined action of these cells around the thousands of alveoli causes the movement of milk through the ducts. In species with substantial storage capacity in the sinuses or cistern, this movement comprises milk “let-down.” The suckling young then obtain the milk by the milking action as they suck on the nipple and the subjacent storage sites. Milk ejection from the nipple resulting from the action of oxytocin occurs if more milk is let down than can be stored.

It was shown in electrophysiological studies on lactating rats in the early 1970s that a continual suckling stimulus applied to the nipples is converted to intermittent high-frequency action potential discharge of neurons in the supraoptic and paraventricular nuclei. These bursts were found in about half of the neurons recorded and occurred about 7 min apart during suckling. Each burst preceded a sharp increase in intramammary pressure by the time that it takes oxytocin to travel in the circulation from the posterior pituitary to the mammary gland. Later, paired recordings showed that neurons in the supraoptic or paraventricular nuclei burst-fire almost synchronously. Thus, each episode of synchronized bursting leads to the secretion of a pulse of oxytocin, each of which causes a milk ejection and each comprising about 1 pmol (0.5 mU) of oxytocin.

The suckling stimulus applied to the nipples is carried in afferent nerve fibers to the brain stem, and then, via a multisynaptic pathway that is not fully characterized, to the oxytocin neurons. The synapses on oxytocin neurons contain a multitude of neurotransmitter types, but it is not clear which transmitter is of key importance in signaling the suckling stimulus.

## VIII. IMPORTANCE OF OXYTOCIN IN THE BRAIN

Oxytocin released within the brain has several actions. There are two broad types of oxytocin action. First, oxytocin has local positive feedback actions on the magnocellular neurons that produce it. In this case, the oxytocin is released from the dendrites of the neurons and it acts back on the oxytocin neurons and their local synaptic inputs to excite the neurons during parturition and suckling. These actions are important in generating the pulsatile pattern of oxytocin secretion at these times. Second, oxytocin is released from the axon terminals of centrally projecting neurons (mainly those with cell bodies in the paraventricular nucleus) at several discrete sites in the brain that are important in organizing reproductive behaviors and associated emotion, or autonomic, outputs.

### A. Oxytocin in the Generation of Bursting Activity of Oxytocin Neurons

It is clear that the oxytocin released by dendrites of the oxytocin neurons during the quiet initial period of suckling, before the bursts start, plays an essential role in driving bursts. Oxytocin release in the magnocellular nuclei can be detected by sampling extracellular fluid with a microdialysis probe; bursting is stopped by administration of an oxytocin antagonist into a magnocellular nucleus, and centrally injected oxytocin facilitates bursting.

In addition to actions on the oxytocin neurons, oxytocin also acts presynaptically to suppress excitatory glutamatergic input and to inhibit GABA-ergic input. These actions may lead to periodic increases in local excitatory input. The synchronization of bursting behavior among the oxytocin neurons, without which a pulse of oxytocin could not be secreted, could also follow periodic activation of a local network. Also involved are morphological changes within the magnocellular nuclei due to retractions of glial processes that bring adjacent neurons into more intimate contact, and to the increased density of GABA and glutamate synapses in lactation. Oxytocin release in the bed nucleus of the stria terminalis (a limbic brain region) contributes to facilitation of the bursting activity of magnocellular oxytocin neurons during suckling, and here estrogen has a positive influence. This is one brain area in which oxytocin receptor expression increases in late pregnancy.

### B. Oxytocin and Behavior

Oxytocin in the brain has actions on behavior that are congruent with its peripheral reproductive functions.

#### 1. *Maternal Behavior*

The first observation, in 1979, was that oxytocin injected into the brain in estrogen-treated virgin rats induced the rapid onset of maternal behavior toward foster pups. Without such oxytocin treatment, virgin rats seem fearful of pups. Conversely, oxytocin antagonist infusion into the medial preoptic area or ventral tegmental area, regions known from other studies to be important in maternal behavior, blocked the initiation of maternal behavior postpartum. Part of the action of oxytocin on maternal behavior involves reducing anxiety.

In sheep, oxytocin is released in the brain in response to vagino-cervical stimulation during birth. Bonding of the ewe with its lamb depends on olfactory memory, and this involves oxytocin action in the olfactory bulb. The paraventricular nucleus contains the important population of centrally projecting oxytocin neurons that promote maternal behavior. Oxytocin release from terminals in the brain regions where maternal behavior is organized modulates release of other transmitters that in turn elicit maternal behavior. Once this task is performed immediately postpartum, continued action of oxytocin does not seem to be required to maintain maternal behavior. In contrast, transgenic mice with inactivation of the oxytocin gene show normal maternal behavior, but mice are peculiar in that females show maternal behavior without having been pregnant. Thus, mice do not need the drive provided by oxytocin released in the brain during parturition. Indeed, they lack oxytocin receptors in the forebrain area where oxytocin acts in other species to promote maternal behavior.

#### 2. *Female Reproductive Behavior*

In the rat and hamster, oxytocin from paraventricular nucleus neurons acts in the hypothalamic ventromedial nucleus and medial preoptic area to facilitate female reproductive (lordosis) behavior. The effectiveness of oxytocin depends on estrogen, which induces oxytocin receptors in the ventromedial nucleus, and receptor distribution is expanded by progesterone. This leads to enhanced electrophysiological responses of the neurons to oxytocin. However, estrogen inhibits oxytocin receptor expression in the mouse, and as a corollary the oxytocin knockout female mouse does not show defects in sexual receptivity.

Marked differences in the affiliative behavior of species of voles have been used to uncover roles of brain oxytocin in bonding between sexual partners. Prairie voles are monogamous and live in social groups, but montane voles are not monogamous and live in isolation. Bond formation between a female prairie vole and the male with which she first mates depends on oxytocin released in the brain during mating. In contrast, montane voles do not form bonds after mating, not even if oxytocin is injected into the brain: indeed, they have very low levels of oxytocin receptor expression in brain areas associated with reward, in contrast with prairie voles. These different patterns of receptor expression reflect differences in the upstream region of the gene that confers tissue-specific expression.

A role for oxytocin in the brain in promoting social interaction is supported by finding defects in transgenic mice with inactivation of the oxytocin gene. Unlike normal mice, these transgenic mice fail to learn to recognize conspecifics with which they have become familiar by repeated social exposure. This is attributable to loss of an essential action of oxytocin in the medial amygdala, part of the limbic, emotional, brain.

#### 3. *Ingestive Behavior*

Consonant with its natriuretic action in the rat, oxytocin acts within the brain to inhibit salt ingestion when this is inappropriate. It is also one of several peptides that act in the brain to reduce food intake; though this may reflect a primary role to inhibit intake of the salt in food. Through projections to the dorsal vagal complex in the brain stem, oxytocin neurons inhibit gastric motility, an appropriate accompaniment to inhibition of intake.

## IX. OTHER ACTIONS OF OXYTOCIN/ANTAGONISTS

### A. Oxytocin Actions on the Anterior Pituitary

Oxytocin is present in hypothalamo-hypophyseal portal blood at greater concentrations than in peripheral blood. This oxytocin is secreted from magnocellular neuron axon swellings in the median eminence. Oxytocin acts on V1b receptors on corticotrophs [cells secreting adrenocorticotropic hormone (ACTH), which do not have oxytocin receptors] in the anterior pituitary to augment the action of corticotropin-releasing hormone on ACTH secretion in rats; however, in humans, oxytocin is inhibitory.

The main action of oxytocin on the anterior pituitary is to stimulate prolactin secretion in lactation. This is promoted by a large increase in oxytocin receptor expression in lactotrophs (cells secreting prolactin) at the end of pregnancy, but depends on withdrawal of inhibition by dopamine. However, other factors increase prolactin secretion in lactation. Oxytocin can advance the ovulatory luteinizing hormone surge, though the importance of this is not clear.

### B. Oxytocin in Males

The importance of oxytocin is primarily in the context of female reproduction. In males, oxytocin secretion is increased by sexual intercourse and stimulates seminiferous tubule contractions, so it may aid the transport of sperm from the testis. Produced by Leydig cells in the testis, oxytocin locally influences steroidogenesis; oxytocin produced in the prostate affects prostate androgen metabolism. In the brain, oxytocin acts in the paraventricular nucleus to induce penile erection; this probably activates, through a mechanism involving nitric oxide, oxytocin neurons projecting to autonomic outflow neurons in the brain stem and spinal cord. Transgenic male mice without oxytocin show reduced aggressiveness toward intruder males.

### C. Oxytocin Receptor Antagonists

Several oxytocin receptor antagonists have been synthesized. Most are peptides based on the nonapeptide prototype. One such peptide, Atosiban, is licensed in Europe for use in the management of imminent preterm birth. Nonpeptide antagonists are not in clinical use. The advantage of targeting the oxytocin receptor for tocolysis (uterine relaxation) over other approaches, such as the use of $\beta_2$-adrenergic agonists, is that oxytocin receptors are largely restricted to the pregnant uterus and the mammary gland. Consequently, unwanted actions of selective oxytocin receptor antagonists on other tissues should not be a problem. However, there may be the possibility of actions in the heart, but the main issue is that the antagonists developed so far are also antagonists at V1a receptors. Although this may reinforce tocolytic actions because of the possible involvement of V1a receptors in the stimulation of uterine contractions in parturition, vasopressin stimulation of blood vessel contraction through these receptors will be inhibited. This is a potential problem in the context of defense of blood pressure during hemorrhage. Peptide antagonists are unlikely to enter the brain significantly or to cross the placenta. Atosiban is effective in alleviating the symptoms of dysmenorrhea (pain prior to menstruation), because in this condition vasopressin acting through V1a receptors is responsible for the stimulation of painful uterine contractions.

## Glossary

**amnion** Sac in which the embryo and fetus develop.

**axons** Thin processes of neurons contacting a target (here, either other neurons or blood vessels in the posterior pituitary); rapidly conduct action potentials (waves of depolarization) from the cell body to the terminals; slowly transport peptide to be secreted from the terminals.

**burst** Brief, high-frequency cluster of action potentials generated in a neuron; the basis for secretion of a pulse of oxytocin.

**corpus luteum** Endocrine tissue formed from the ovarian follicle after it ovulates; produces progesterone. Luteolysis is the death of the corpus luteum.

**decidua** Endometrial lining shed at the end of pregnancy.

**dendrites** Thick nerve cell processes that receive synapses (contacts) from other nerve cells; also capable of secreting.

**endometrium** Lining of the human uterus.

**hypothalamus** Brain region concerned with automatic regulation of many body functions, including controlling secretion of hormones from the pituitary gland.

**myoepithelial cells** Contractile cells surrounding the milk-secreting glands in the lactating mammary gland.

**myometrium** Contractile, muscular tissue of the uterus.

**natriuresis** Stimulated sodium excretion by the kidney.

**paraventricular and supraoptic nuclei** Areas in the hypothalamus where most of the nerve cells (neurons) in the brain producing oxytocin are located.

**posterior pituitary gland** Developmentally, a downgrowth from the hypothalamus of the brain. Contains terminals of hypothalamic neurons with stored oxytocin.

**preterm birth** Emergence of the fetus before it is fully prepared for extrauterine life.

## See Also the Following Articles

**Corpus Luteum in Primates • Corpus Luteum: Regression and Rescue • Decidualization • Endometrial Remodeling • Oxytocin/Vasopressin Receptor Signaling • Placental Development • Progesterone Action in the Female Reproductive Tract • Sexual Differentiation of the Brain • Vasopressin (AVP)**

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# Oxytocin/Vasopressin Receptor Signaling

Melvyn S. Soloff and Yow-Jiun Jeng
*University of Texas Medical Branch, Galveston*

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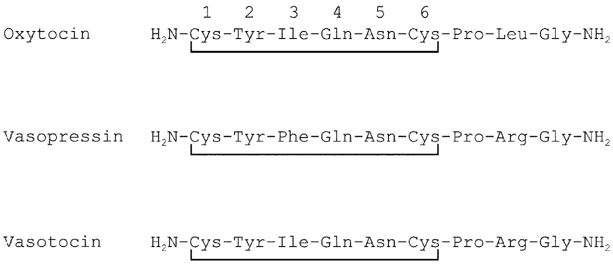
**The neurohypophyseal hormones oxytocin and arginine vasopressin are structurally related and signal through a family of G-protein-coupled receptors. The oxytocin receptor is unique and there are three distinct vasopressin receptors: $V_1$, $V_2$, and $V_3$. Oxytocin and vasopressin $V_1$ and $V_3$ receptors are coupled to $G_{q/11}$ and $G_{i/o}$ heterotrimeric G-proteins, whereas $V_2$ vasopressin receptors act through $G_s$.**

## I. INTRODUCTION

The signal pathways mediating the actions of oxytocin and arginine vasopressin on specific target cells through their unique receptors are highlighted in this article. Although generalizations have been made, some pathways vary with different cell types or species. Even within the same cell type, there may be differences in signaling at different stages of the cell cycle or during development. Exceptions will be noted whenever possible.

## II. OXYTOCIN AND VASOPRESSIN

Oxytocin (OT) and arginine vasopressin (AVP) are produced primarily by the supraoptic and paraventricular nuclei of the hypothalamus; they are transported along axons to cell termini in the posterior pituitary, where they are stored in the form of membrane-bound neurosecretory granules. The two neuropeptides are structurally related to each other and to vasotocin, which occurs in fish, reptiles, amphibians, and birds (Fig. 1). These peptides share a disulfide-linked ring structure of six residues that restricts conformational flexibility, and a flexible three-residue amino-terminal tail. The carboxyl-terminal glycine is amidated in all three peptides. Oxytocin and vasopressin differ only at position 3 of the cyclic peptide portion and position 8 of the C-terminal tripeptide amide region. Duplication of a common ancestral gene likely gave rise to the OT and AVP genes. Both genes reside on the same chromosome and, in humans and mice, are only 12 and 3 kb apart, respectively. Both peptides are synthesized in larger precursor forms and are



1 2 3 4 5 6
Oxytocin $H_2N$-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-$NH_2$

Vasopressin $H_2N$-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$

Vasotocin $H_2N$-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$

**FIGURE 1** Structures of OT, AVP, and vasotocin. In lysine vasopressin, which is found in pigs, a Lys replaces Arg in position 8.

processed to mature hormones in neurosecretory cells. When the nerve cell membrane is depolarized, OT and AVP are released and pass through endothelial cells to enter the circulation. Oxytocin release is caused by vaginal stretch, by suckling, and, in some species, by osmotic stimuli. Higher centers in the brain are also involved. Vasopressin release occurs in response to stimuli that alter the state of fluids in the body, such as increased plasma osmotic pressure and reduced blood volume and/or blood pressure. Oxytocin stimulates mammary myoepithelial cell contraction and is essential for milk ejection in most mammalian species. Oxytocin also elicits contraction of uterine smooth muscle and probably plays a role in labor initiation. Oxytocin targets in the brain elicit responses associated with social, sexual, and maternal behavior. Vasopressin, or antidiuretic hormone (ADH), is a vasoconstrictor, and it also reduces urinary water excretion by increasing the water permeability of the renal collecting duct. Vasopressin is also a costimulator with corticotropin-releasing hormone (CRH) of adrenocorticotropic hormone (ACTH) release from the anterior pituitary.

The similar structures of the neurohypophyseal peptides account for the overlap in agonist activities. Oxytocin has about 1% of the potency of AVP in increasing rat blood pressure or eliciting antidiuresis. Vasopressin has about 15% of the potency of OT in both rat uterus and rabbit mammary gland assays. Generally, these differences are the reflection of reduced affinities for receptor binding sites; but vasopressin has about twice the apparent $K_d$ for OTRs, compared to OT, yet only 15% of the agonist activity. This paradox may be explained by AVP being a partial OT agonist/antagonist that binds to the OTR to a greater extent than is reflected by signal transduction. The selective binding of OT and AVP to their respective receptors is largely determined by the residue at position 8. In vasopressin, a basic residue fills this position; in OT and OT analogues, the residue is neutral and usually aliphatic.

As might be expected from the common ancestry of OT and AVP, the receptors for the neurohypophyseal hormones, which are G-protein-coupled receptors (GPCRs), are more closely related to each other than to any other GPCR family member. The neurohypophyseal hormone receptors comprise a group of four related proteins expressed by separate genes. The OT receptor (OTR) is largely associated with target cells involved in reproductive processes. The $V_2$ receptor is expressed predominantly in the kidney and accounts for the antidiuretic effects of vasopressin. The $V_1$ and $V_3$ receptors can be distinguished by their selectivity for AVP analogues, but the primary difference is in their cellular distribution. Vasopressin $V_1$ receptors are expressed in vascular smooth muscle and other tissues, whereas $V_3$ receptors are largely restricted to the central nervous system. The two extremes in the family, the $V_2R$ and OTR, are identical in 40% of their sequences, with the highest similarity in the transmembrane and extracellular domains.

## III. OXYTOCIN SIGNALING

OTRs are expressed in the lactating mammary gland, uterine smooth muscle (myometrium), uterine epithelium (endometrium or decidua), and the amnion, among other target sites. There have been extensive studies on neural targets for OT, but less information on signaling mechanisms is available. In general, OTRs are highly regulated. In virtually all species studied, the concentration of OTRs in the myometrium is maximal at the time of parturition, rising several 100-fold in women between the beginning and end of pregnancy. The coupling to G-proteins in the rat myometrium is also up-regulated at the end of pregnancy, as determined by gel filtration analysis of solubilized OTR–G-protein complexes. The concentration of OTRs in the endometrium (or decidua) and amnion is also maximal at the end of gestation. Conversely, OTR concentrations in the mammary gland are maximal during lactation, when the concentration of OT in the blood is elevated; the uterine OTRs are down-regulated at this time, so that only mammary myoepithelial cells are stimulated by OT. Although the AVP receptor subtypes are regulated, the magnitude of change is small compared with OTRs.

### A. G-Protein and Effector Coupling to the Oxytocin Receptor

The OTR is functionally coupled to both $G_{q/11}$ and $G_{i/o}$, as demonstrated in several target cell types (Fig. 2). Both classes of G-proteins stimulate phospholipase C-β (PLC-β), which leads to the generation inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG). Inositol trisphosphate triggers $Ca^{2+}$ release from intracellular stores, whereas DAG, acting in conjunction with $Ca^{2+}$, stimulates protein kinase C (PKC). Activation of mitogen-activated protein (MAP) kinase (MEK) in the MAP kinase cascade occurs downstream of PKC phosphorylation.

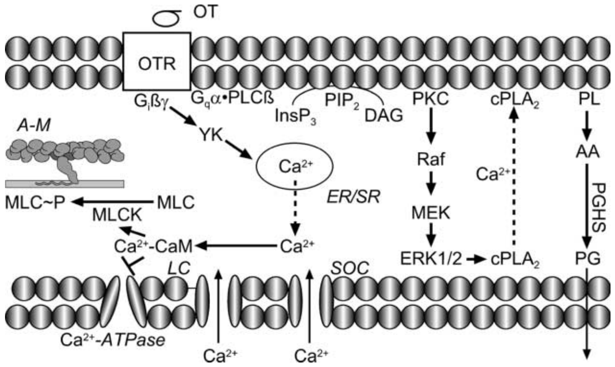


**FIGURE 2** Pathways involved in OT signaling result in an increase in myosin light chain (MLC) phosphorylation and contractile activity and/or the stimulation of prostaglandin (PG) synthesis. Both processes are mediated by an increase in $[Ca^{2+}]_i$ from the endoplasmic/sarcoplasmic reticulum (ER/SR) through $G_{q/11}$/phospholipase C (PLC) and $G_{i/o}$/tyrosine kinase activation. The resultant increase in $[Ca^{2+}]_i$ allows the influx of extracellular $Ca^{2+}$ through store-operated channels (SOCs) and L-type voltage regulated channels (LCs). An increase in the binding of $Ca^{2+}$ to calmodulin (CaM) results in inhibition of a sarcolemmal calcium pump ($Ca^{2+}$-ATPase), resulting in elevated $Ca^{2+}$ levels over a longer time. The $Ca^{2+}$–CaM complex activates MLC kinase (MLCK) to phosphorylate the myosin light chain 20 of myosin II, which promotes the interaction of actin and myosin (A–M) and the contraction of smooth muscle. The increase in $[Ca^{2+}]_i$ also allows translocation of cytosolic phospholipase $A_2$ ($cPLA_2$) to internal membranes. Increased protein kinase C (PKC) activity resulting from $G_{q/11}$ activation also leads to successive extracellular signal-related kinase (ERK2/1) and $cPLA_2$ phosphorylations. The activated $cPLA_2$ catalyzes increases in arachidonic acid (AA) levels from membrane phospholipids (PLs) and greater PG synthesis through prostaglandin H synthase (PGHS) activity. OTR, Oxytocin receptor; $InsP_3$, inositol 1,4,5-trisphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate.

A role for $G_{i/o}$ was initially determined by pertussis toxin (PTX) sensitivity of OT-stimulated signaling pathways (Fig. 2). However, caution is advised in the use of PTX treatment because it elevates intracellular cyclic adenosine monophosphate (cAMP), which inhibits responses to OT that are independent of OTR activation. For example, cAMP-stimulated protein kinase A (PKA) catalyzes the phosphorylation of PLC, resulting in inhibition of OT-stimulated $InsP_3$ production. The inhibition could be reversed by treatment with a selective PKA inhibitor. Thus, at least part of the effects of PTX can be attributed to $G_{i/o}$ that is not coupled to the OTR. Other work, however, has shown that the OTR is associated with $G_{i/o}$; using both coprecipitation with antibody, and assays showing it has been shown that OT stimulation of GTPase activity in intact myometrial membranes is inhibited by PTX. In addition, OT treatment decreases PTX-stimulated ADP-ribosylation of $G_{\alpha i}$ in myometrial membranes. This decrease is consistent with OT activating heterotrimeric $G_i$ by dissociating $G_{\alpha i}$, because ribosylation of the α-subunit occurs only in the heterotrimer. Inhibition of PLC activity by PKA phosphorylation may partially explain the ability of agents that elevate intracellular cAMP to oppose the actions of OT and to maintain uterine smooth muscle in a quiescent state.

Removal of 51 residues from the C-terminus of the rat OTR expressed in stably transfected Chinese hamster ovary (CHO) cells causes the loss of coupling to $G_{q/11}$. PTX-sensitive increases in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), however, are unaffected by receptor truncation. Surprisingly, this signaling is independent of PLC activity; the $G_{i/o}$ pathways leading to increased $[Ca^{2+}]_i$ are yet undefined. Activation of $G_{i/o}$ involves the $G_{\beta\gamma}$ subunits, because transient transfection of the cells expressing truncated OTR with the $G_{\beta\gamma}$ sequestrant βARKct blocks OT-stimulated intracellular $Ca^{2+}$ transients. A generic tyrosine kinase inhibitor also inhibits the effects of OT, suggesting that $G_{\beta\gamma}$ subunits transactivate signaling via tyrosine kinase pathways, as has been shown for other GPCRs. Oxytocin signaling in a mutant lacking 39 residues of the C-terminal tail is indistinguishable from that of the wild-type OTR. Therefore, the region between 39 and 51 residues from the carboxyl terminus appears to be critical for coupling of the OTR to $G_{q/11}$.

## B. Many Effects of Oxytocin are Mediated by Increased Intracellular $Ca^{2+}$ Concentrations

The initial rise in $[Ca^{2+}]_i$ following OT treatment of isolated myometrial cells is due mainly to the $InsP_3$-mediated release of $Ca^{2+}$ from intracellular stores in the endoplasmic or sarcoplasmic reticulum, but $Ca^{2+}$ influx from extracellular sources also occurs (Fig. 2). Studies have indicated that the increased influx of $Ca^{2+}$ is regulated by store-operated or capacitative channels, following the rise in $[Ca^{2+}]_i$ from intracellular sites. Other studies indicate that extracellular $Ca^{2+}$ influx occurs through L-type voltage channels. The large difference in $Ca^{2+}$ concentration between the outside and inside of cells is maintained in part by the unidirectional export of $Ca^{2+}$ by a high-affinity $Ca^{2+}$ extrusion pump in the cell membrane. This process, involving $Ca^{2+}$,$Mg^{2+}$-dependent ATPase activity, occurs in a number of cell types. OT inhibits this ATPase in sarcolemmal (plasma) membranes from the uterine smooth muscle of rabbits, rats, and humans. This process contributes to maintaining elevated $[Ca^{2+}]_i$ in myometrial cells following the increase in $Ca^{2+}$ from intracellular and extracellular sources. The effects of OT are blocked by calmodulin (CaM) inhibition, but the signal pathways require further elaboration.

In uterine smooth muscle cells and mammary myoepithelial cells, which are smooth muscle-like but are derived from ectoderm instead of mesoderm, the rise in $[Ca^{2+}]_i$ leads to $Ca^{2+}$–CaM stimulation of myosin light chain kinase (MLCK) activity. The phosphorylation of myosin light chain (MLC) 20 allows myosin heads to form cross-bridges with actin filaments, leading to contraction. Yet to be elucidated are the molecular actions of OT on neural targets involved in many behavioral patterns, as well as in memory and learning. It seems likely that OT-stimulated rises in $[Ca^{2+}]_i$ control cellular excitability and modulate firing patterns. These processes almost certainly involve stimulation of gene transcription and protein synthesis.

## C. Oxytocin Stimulation of Prostaglandin Synthesis

A second major activity of OT in uterine and other tissues is the stimulation of prostaglandin synthesis. This occurs in endometrial and amnion cells and CHO cells stably transfected with the rat OTR by stimulation of arachidonic acid formation from membrane glycerophospholipids, and the incorporation of arachidonic acid into prostaglandins. OT-induced increases in $[Ca^{2+}]_i$ (described in the preceding paragraph) lead to $Ca^{2+}$ binding to the C-2 domain of cytosolic phospholipase $A_2$ ($cPLA_2$) and its translocation to the nuclear envelope and endoplasmic reticulum, wherein lie glycerophospholipid substrates for $cPLA_2$-catalyzed arachidonic acid formation. The phospholipase is activated by serine phosphorylation by extracellular signal-regulated protein kinases (ERK1/2), which in turn are activated by OT stimulation of the PKC/MEK pathway. Arachidonic acid is converted to prostaglandins (PGs) through the actions of prostaglandin endoperoxide H synthase (PGHS), also referred to as cyclooxygenase (COX). This bifunctional enzyme catalyzes both the oxidation of arachidonic acid to the prostaglandin endoperoxide, $PGG_2$, and its subsequent reduction to $PGH_2$, the precursor for all prostanoids. There are two PGHS isoforms, each expressed by a separate gene. PGHS-1 is constitutively expressed in most cell types, whereas PGHS-2 mRNA is rapidly and transiently induced. Normally, PGHS-2 is undetectable but is induced in the fashion of an immediate-early gene by a variety of agents, including OT, in the endometrium, myometrium, and amnion. Generally, utilization of PGHS-1 is associated with the early phase of prostaglandin synthesis, occurring within several minutes of stimulation, whereas prostaglandin synthesis occurring over several hours coincides with the induction of PGHS-2 expression.

Depending on the cell type and stimulus, PGHS-2 synthesis can be a rate-limiting step with respect to PG synthesis, or it can enhance production of the relatively low levels of PGs synthesized through PGHS-1. The induction of PGHS-2 is necessary for OT-stimulated $PGF_{2\alpha}$ synthesis in bovine uterine endometrium and prostacyclin production by human myometrial cells. In contrast, the levels of PGHS-1 activity in rabbit amnion are constitutively high and the induction of PGHS-2 does not contribute to increased $PGE_2$ production. The role of PGHS-2 in these cells is presently unknown, as are the pathways involved in OT-induction of PGHS-2 expression. In cultured human myometrial cells, this induction is PTX sensitive, as is activation of the MAP kinase pathway.

## D. Down-regulation of Oxytocin Receptors by Endocytosis

Radioligand binding data using intact human myometrial cells have shown that OTR numbers on the cell membrane remain stable for a number of hours after addition of OT. This observation is consistent with physiological observations, because the uterus remains sensitive to OT for a period of time during labor. Human OTRs expressed in human embryonic kidney (HEK) or CHO cell lines, however, are rapidly internalized in response to OT treatment. Internalization is mediated by phosphorylation of two serine clusters in the C-terminal domain of the receptor by PKC, followed by the binding of β-arrestin-2 and uncoupling of the receptor from its G-protein partners. This process results in the termination of OTR signaling. β-Arrestin acts as an adapter protein that links the receptor to components of the endocytic machinery and targets the desensitized receptor to clathrin-coated pits for endocytosis. Like other GPCRs, the internalized OTRs are dephosphorylated in endosomes and recycled back to the cell surface fully resensitized.

It remains to be established whether OTRs in natural target cells, other than myometrial cells, are rapidly internalized. Apart from internalization, the association of receptors with β-arrestin may be involved in initiating and/or regulating signaling pathways. For example, Src recruitment to the $\beta_2$-adrenergic receptor is mediated by β-arrestin, which functions as an adapter protein, binding both c-Src and the agonist-occupied receptor. β-Arrestin also appears to function as a molecular scaffold that organizes and recruits components of the MAPK cascade.

### E. Atypical Oxytocin Target Cells

Functional OTRs have been described in several tissues and a variety of cell types not typically considered targets for OT. Generally, the signal pathways involved in OT action have only been examined in a perfunctory manner. In most cases the cell types were not in primary cultures but were established cell lines; therefore, signaling by OT is not necessarily representative of OT action in untransformed cells.

Oxytocin has insulin-like activity in stimulating glucose oxidation and lipogenesis in rat adipocytes. These effects occur independently of insulin action and are mediated by OTRs, which have been characterized by ligand binding. Unlike insulin, the effects of OT on glucose oxidation in fat cells are not mediated by increased glucose transport. Instead, OT stimulates polyphosphoinositide breakdown and elevated $[Ca^{2+}]_i$ in adipocytes. The molar concentration of OT-stimulating glucose oxidation is about five times greater than that of insulin, and the maximal effect is only about 20% that of insulin. Oxytocin is also less effective than insulin in stimulating lipogenesis; therefore, it is not certain whether there is a physiological role for OT in fat cell metabolism.

Oxytocin receptors are expressed in both undifferentiated and differentiated human trabecular bone cells with osteogenic capacity in primary culture. The addition of OT rapidly increases $[Ca^{2+}]_i$ and stimulates prostaglandin synthesis. Oxytocin receptors have been demonstrated in the aorta, vena cava, and pulmonary vein by reverse transcription polymerase chain reaction (RT-PCR) and ligand binding analyses. Because OT is also expressed in these same tissues, these blood vessels appear to contain an intrinsic OT system, which may be involved in the regulation of vascular tone as well as vascular regrowth and remodeling. It is likely that OTRs are confined to the endothelium of these vessels. Occupancy of these receptor sites mobilizes intracellular $Ca^{2+}$ and causes the release of nitric oxide, which is prevented by chelation of extracellular and intracellular $Ca^{2+}$. Nitric oxide production is associated with vasorelaxation, and presumably OT has a vasodilatory effect on certain blood vessels.

Oxytocin receptors in both atrial and ventricular chambers of the rat heart mediate the OT-stimulated release of atrial natriuretic peptide. This peptide slows the heart and reduces its force of contraction to produce a rapid reduction in circulating blood volume. Oxytocin receptors are found in human breast tumors of epithelial origin, as measured by immunological techniques. There are, however, no clear indications as to whether these receptors are functional. Several breast tumor cell lines have been shown to express OTRs, but the functional role of OT is not clear. Oxytocin receptors have been found in thymocytes, ovarian cells, Leydig cells, and prostate cells, but information regarding signaling pathways in these cell types is still rudimentary.

## IV. LACK OF DISEASES ASSOCIATED WITH IMPROPER EXPRESSION OF OXYTOCIN RECEPTORS

No natural mutations in the OTR gene have been described. Oxytocin-null mice fail to lactate, illustrating the importance of OT for survival of the species. There are no changes in the timing of parturition in these mice, but this process is likely dictated by the marked up-regulation of OTRs before labor. It is possible that AVP may replace OT in stimulating uterine contractions when OTRs are up-regulated.

## V. DIFFERENTIAL BINDING SITES FOR NEUROHYPOPHYSEAL HORMONE AGONISTS AND ANTAGONISTS

Target size estimation, using radiation inactivation analysis, shows that an OT antagonist binds to a site about half the size of the agonist binding site. These findings suggest that the binding sites for agonists and antagonists are distinct. Indeed, by transfer of domains from the OT receptor into the $V_2$ vasopressin receptor, chimeric gain-of-function $V_2$/OT receptors are produced that bind OT agonists with structural requirements distinct from those of antagonists. For agonist binding and selectivity, the first three extracellular receptor domains are the most important. Conversely, the binding site for the OT antagonist $d(CH_2)_5$ [Try(Me)$^2$,Thr$^4$,Orn$^8$,Tyr$^9$-$NH_2$]vasotocin is formed by transmembrane helices 1, 2, and 7, with the upper part of helix 7 contributing to binding affinity. Thus, the antagonist displaces OT by interacting with a distinct binding site, yet behaves as a competitive inhibitor.

## VI. VASOPRESSIN SIGNALING THROUGH THE V$_1$ VASOPRESSIN RECEPTOR

The $V_1$ vasopressin receptor ($V_1R$) mediates a range of physiological processes, such as contraction of vascular smooth muscle cells and cell proliferation. Vasopressin also stimulates hepatic glycogenolysis in the rat but in no other species, and is one of several agonists that stimulate platelets to aggregate and secrete their granular contents. Platelet activation is partly mediated by $InsP_3$ production.

Occupancy of the $V_1R$ leads to activation of $G_{q/11}$–PLC-β production of $InsP_3$ and the subsequent transient increase in $[Ca^{2+}]_i$ in most of the cell lines studied (Fig. 3). Part of the $[Ca^{2+}]_i$ arises from the influx of extracellular $Ca^{2+}$. In rat glomerulosa cells, the influx of extracellular $Ca^{2+}$ occurs through L-type channels in a PTX-sensitive manner. Voltage-operated channels, however, do not mediate the influx of $Ca^{2+}$ by vascular smooth muscle cells. Like OT signaling, increases in $[Ca^{2+}]_i$ lead to $Ca^{2+}$–CaM activation of MLCK and the phosphorylation of MLC. $V_1$ receptor occupancy also results in the release of arachidonic acid by vascular smooth muscle cells and glomerular mesangial cells via activation of $PLA_2$. Arachidonic acid release in rat glomerular mesangial cells is PTX sensitive. Thus, $V_1$ receptors are coupled to both $G_{q/11}$ and $G_{i/o}$ heterotrimeric G proteins, each of which regulates separate pathways.

### A. Phospholipase D

A number of studies have implicated phospholipase D (PLD) in AVP action, but the mechanisms have not been thoroughly characterized. Activating PLD converts phosphatidylcholine to choline and phosphatidic acid, which helps regulate specific cellular functions (Fig. 3). Most agonists that activate PLD also induce phosphatidylinositol 4,5-bisphosphate ($PIP_2$) hydrolysis through the stimulation of PLC. The resulting generation of $InsP_3$ and DAG activates $Ca^{2+}$-sensitive PKC isoenzymes that stimulate PLD. The activation of PLD, however, is not mediated by PKC in all cell types, and it has been suggested that small GTPases of the Rho and ADP ribosylation factor families also activate PLD. In other cases, βγ- and α-subunits of heterotrimeric G-proteins may stimulate PLD upstream from PKC signaling. Although the regulation of PLD by AVP is still poorly characterized, there is evidence that this pathway may be more relevant that PLC-mediated pathways in mediating smooth muscle contraction. For example,

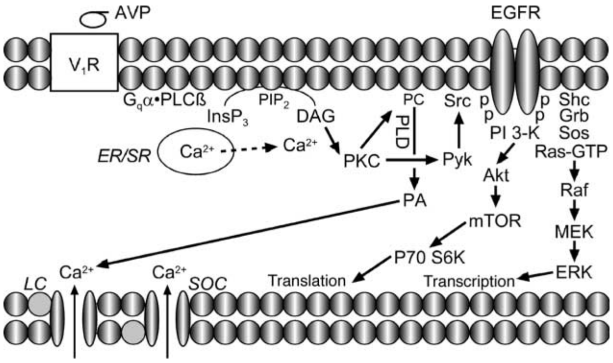


**FIGURE 3** Transactivation of the epidermal growth factor receptor by arginine vasopressin (AVP) signaling through the vasopressin $V_1$ receptor. In addition to signaling by pathways that lead to vascular smooth muscle contraction, as outlined for oxytocin stimulation of myosin light chain phosphorylation in Fig. 2, AVP also stimulates cell proliferation by signaling transcriptional and translational events. These processes are thought to involve transactivation of the epidermal growth factor receptor (EGFR), which serves as a scaffold for signaling proteins. The phosphorylation of a $Ca^{2+}$-dependent tyrosine kinase, Pyk2, by protein kinase C (PKC) creates a ligand for another nonreceptor tyrosine kinase, Src, which is phosphorylated. Src, thus activated, phosphorylates the EGFR, which has phosphotyrosines that interact with proteins containing SH2 domains. Among them, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) is activated to catalyze the formation of phosphatidylinositol 3,4,5-trisphosphate, which serves as an attachment site for the Akt plekstrin homology domain. Phosphatidylinositol 3,4,5-trisphosphate also activates phosphoinositide-dependent kinase, which catalyzes the phosphorylation of Ser and Thr residues on Akt, resulting in activation of Akt. Akt stimulates mTOR, which in turn activates p70 S6 kinase and phosphorylation of the S6 protein of the 40S ribosomal subunit that is involved in translational control of 5′-oligopyrimidine tract mRNAs. Phosphorylation of the EGF receptor also creates docking sites for Shc, which serves a scaffolding function in signaling for a variety of receptor tyrosine kinases. Grb2/Sos binds to phosphorylated Shc, activating the Ras/Raf/MAPK pathway. The end product ERK phosphorylates transcription factors, leading to an increase in transcriptional activity of target genes. Protein kinase activation of phospholipase D (PLD) causes the release of phosphatidic acid, which signals the influx of extracellular $Ca^{2+}$ and $Ca^{2+}$-dependent action potentials, leading to smooth muscle contraction (other abbreviations as in Fig. 2).

in A7r5 vascular smooth muscle cells, 10–500 p*M* AVP stimulates excitation by increasing $Ca^{2+}$ oscillations and $Ca^{2+}$-dependent action potentials via a PLD-mediated pathway. The PLC pathway is activated only at higher concentrations of hormone.

### B. Switching of G-Protein-$V_1$ Vasopressin Receptor Coupling

The coupling of recombinant $V_1$Rs to G-proteins has been examined in transfected CHO cells. When expressed at relatively low levels, $V_1$Rs were coupled solely to $G_q$. At higher expression levels, however, $G_i$ and $G_s$ were associated with $V_1$Rs, reflecting the promiscuity in G-protein coupling under contrived conditions. Changes in G-protein coupling, however, also occur naturally during the cell cycle. In proliferating Swiss 3T3 fibroblasts, AVP-induced increases in $[Ca^{2+}]_i$ are mediated by G-proteins of the $G_q$ family. In quiescent cells ($G_0/G_1$ phase), however, the AVP-induced increase in $[Ca^{2+}]_i$ is partially PTX sensitive, suggesting $G_i$-protein involvement. The blocking effect of PTX pretreatment in $G_0/G_1$ cells was mimicked by microinjection of antisense oligonucleotides, suppressing the expression of the $G_{\alpha i3}$ or $G_{\beta\gamma}$ subunits. The significance of both $G_{q/11}$ and $G_{i3}$ coupling is not clear, because both pathways appear to involve activation of phospholipase C-β. Class-switching mechanisms do not appear to be related to any changes in receptor number or type, or to changes in the relative expression of the G-proteins, but may relate to changes in expression level of proteins that are regulators of G-protein signaling (RGS) during the cell cycle.

### C. Vasopressin Transactivation of Epidermal Growth Factor Receptors

Kidney mesangial cells, important for maintaining the microcirculation of the glomerulus, contract in response to AVP treatment. Vasopressin is also one of several growth factors that stimulate mesangial cells to proliferate in culture. The growth-promoting effects of AVP are mediated by Ras mitogen-activated protein kinase and the phosphatidylinositol 3-kinase (PI3K) signaling pathways. Treatment with rapamycin, an inhibitor of the p70 S6 kinase activator mTOR, inhibits AVP action downstream from PI3K activation. These activities follow AVP transactivation of the epidermal growth factor (EGF) receptor via tyrosine phosphorylation of the $Ca^{2+}$/PKC-dependent nonreceptor tyrosine kinase, Pyk2, leading to Pyk2/c-Src interaction and c-Src activation (Fig. 3). Association of c-Src with EGF receptor results in EGF receptor phosphorylation and creation of docking sites for PI3K and Ras. Thus, like other GPCRs, which transactivate EGF receptor through tyrosine phosphorylation of adapter proteins, the $V_1$R is able to stimulate cell proliferation. Phosphatidylinositol 3-kinase also mediates the growth-promoting effects of AVP on rat fibroblasts.

Vasopressin also stimulates mitogenesis in vascular smooth muscle cells, 3T3 cells, rat hepatocytes, and adrenal glomerulosa cells. All three MAP kinase pathways, ERK1/2, c-Jun N-terminal kinase (JNK), and p38, are phosphorylated after AVP stimulation of vascular smooth muscle cells. JNK or p38 is involved in AVP-stimulated smooth muscle actin transcription.

## VII. VASOPRESSIN $V_2$ RECEPTOR SIGNALING

The $V_2$R is expressed in the kidney medulla and mediates the antidiuretic effects of AVP, which involve water transport across the epithelium of the renal collecting duct. Occupancy of receptor sites on the basolateral plasma membrane of ductal cells leads to activation of adenylyl cyclase type IV via $G_s$ coupling.

### A. Aquaporin-2 Water Channels

Generation of cAMP and activation of PKA cause translocation of intracellular vesicles containing aquaporin-2 (AQP2) water channels to the apical cell surface (Fig. 4). Phosphorylation of AQP2 by PKA occurs on serine residue 256 in the cytoplasmic carboxyl terminus. This step, which is essential for the translocation of AQP2, requires the anchoring of PKA to intracellular vesicles by PKA anchoring proteins. A variety of vesicular-trafficking processes involve localized increases in intracellular $Ca^{2+}$. In renal cells, an increase in $[Ca^{2+}]_i$ results from the AVP-stimulated rise in intracellular cAMP. This process occurs in the absence of activation of the phosphoinositide signaling pathway and involves ryanodine-sensitive $Ca^{2+}$ stores in the endoplasmic reticulum and the type 1 ryanodine receptor. Calmodulin also plays a role in the AVP-stimulated redistribution of AQP2. Introduction of a synthetic peptide corresponding to the C-terminus of the $G_{\alpha i3}$ subunit into permeabilized cells, derived from rabbit cortical collecting duct and stably transfected with rat

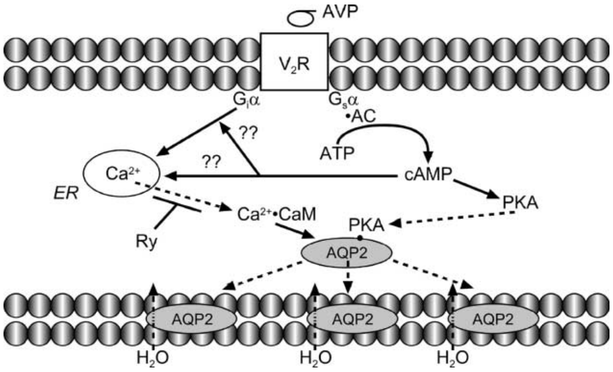


**FIGURE 4** Signaling by occupancy of arginine vasopressin (AVP) $V_2$ receptors ($V_2R$) involves the generation of cyclic adenosine monophosphate (cAMP), which stimulates the phosphorylation of aquaporin-2 (AQP2) via protein kinase A (PKA) and an increase in $Ca^{2+}$-calmodulin (CaM). Both events are involved in the targeting of aquaporin-containing intracellular vesicles to the cell surface. Cyclic AMP stimulates $Ca^{2+}$ release from ryanodine-sensitive (Ry) stores in the endoplasmic reticulum (ER) by mechanisms that may involve $G_{\alpha i}$. Translocation events are indicated by broken arrows.

AQP2 cDNA, inhibits cAMP-induced AQP2 translocation. Thus, a member of the $G_i$ family, most likely $G_{i3}$, is also involved in the cAMP-triggered targeting of AQP2-bearing vesicles to the apical membrane of kidney epithelial cells. The rapid response to AVP involves translocation of AQP2 to the apical cell surface, but longer exposure of cells to AVP (24 h or more) causes an increased abundance of water channels, presumably from increased transcription of the *AQP2* gene.

## B. Vasopressin and Urea Transport

Urea transport in the renal medullary loop of Henle and collecting duct is vital for urine concentration and regulation of renal water excretion. Like water channels, there are distinct transporters that regulate the movement of urea across cell membranes. The urea transporter found in portions of the thin descending limb of the loop of Henle is designated as UT-A, to distinguish it from the UT-B transporter expressed in erythrocytes and endothelial cells. AVP increases the expression of the UT-A2 variant of the UT-A gene (UT-A1 is transcribed from a separate promoter). Chronic treatment of rats with AVP almost doubles the level of expression of a Na–K–2Cl cotransporter in the thick ascending limb of the loop of Henle. Increased cAMP levels presumably mediate AVP-stimulated expression of both the UT-A2 and Na–K–2Cl cotransporter, but the signaling mechanisms are not yet characterized.

## C. Desensitized Response to Vasopressin

The $V_2R$ recruits β-arrestin to the plasma membrane after AVP occupancy and is internalized in a β-arrestin- and clathrin-dependent manner into endosomes. This process is mediated by the persistent phosphorylation of a specific cluster of serine residues in the carboxyl-terminal tail of the internalized $V_2R$, which inhibits recycling of receptors to the plasma membrane and causes a prolonged state of receptor desensitization.

## D. Vasopressin Mutations and Diabetes Insipidus

Diabetes insipidus is a disease characterized by a severe disturbance of antidiuresis caused by a lack of AVP. Patients produce large amounts of dilute urine and must drink large amounts of fluid to replace what is lost. This disease occurs most often as a result of damage to the hypothalamus from trauma or metastatic disease, or it occurs as a primary idiopathic disorder. In rare instances, the disorder is hereditary and generally is transmitted in an autosomal-dominant manner. In these cases, mutations have been identified in the coding sequence of the preprovasopressin–neurophysin II gene. The AVP precursor protein consists of four regions that are cleaved proteolytically during processing. Starting from the amino terminus, they include a 19-amino-acid signal sequence for targeting to the endoplasmic reticulum; the nonapeptide sequence of vasopressin; neurophysin II (NPII), a 93-residue carrier for AVP that is involved in the proper targeting, packaging, and storage of AVP; and a 39-amino-acid glycopeptide of unknown function. The signal sequence is cleaved by signal peptidases on translocation of the precursor into the lumen of the endoplasmic reticulum. After folding and disulfide bond formation, pro-AVP passes through the Golgi apparatus into secretory granules, where mature AVP is formed by proteolytic cleavage. Mutations identified in different kindred are located in the sequences encoding the NPII portion, the signal peptide, and in one case the AVP sequence. Diabetes insipidus usually develops gradually over a period of months or years after birth. Postmortem examination usually indicates degeneration of the hypothalamic magnocellular neurons in which the AVP precursor is synthesized.

Thus, impaired transport and/or processing of the mutant precursor may result in its intracellular accumulation, eventually leading to degeneration of the vasopressinergic neurons and the gradual manifestation of clinical symptoms.

### E. $V_2$ Vasopressin Receptor Mutations and Nephrogenic Diabetes Insipidus

Patients with nephrogenic diabetes insipidus (NDI) are unable to produce concentrated urine despite normal or elevated plasma levels of AVP. The AVP receptor 2 gene is located on the X chromosome (Xq28), and about 90% of patients with congenital NDI are males with the X-linked recessive form of the disease. Over 155 mutations within the gene have been characterized. Mutations have also been identified in the *AQP2* gene on chromosome region 12q13. When expressed in transfected cells, most NDI mutations result in receptors that are trapped intracellularly and are unable to reach the plasma membrane. AQP2 mutant proteins have also been found to be misrouted and cannot be expressed at the apical surface. A few types of mutant $V_2$ vasopressin receptors reach the cell surface but are unable to bind AVP or to properly induce cAMP production. One type of mutation results in perpetual down-regulation of $V_2R$ because of constitutive arrestin-mediated internalization.

### F. Rescue of Misrouted $V_2$ Vasopressin Receptors

Modifying the amino acid sequence of the C-terminus of transfected $V_2Rs$ causes an accumulation of these receptors in the endoplasmic reticulum. Considerable functional activity can be regained, however, by coexpression of the mutant with a C-terminal $V_2R$ peptide (130 amino acid residues) spanning the sequence containing the mutations. In many cases, restoration of receptor activity by the coexpressed receptor peptide is accompanied by a significant increase in receptor numbers on the plasma membrane. In other cases, coexpression allows an increased number of AVP binding sites and stimulation of adenylyl cyclase activity without an increase in surface receptor expression. The mechanisms of this rescue phenomenon are unclear. A physical association between the mutant $V_2R$ proteins and the $V_2$-tail polypeptide has been demonstrated by coimmunoprecipitation, suggesting that functional rescue involves receptor–polypeptide dimerization.

Certain $V_2R$ mutants have been rescued pharmacologically by incubating transfected cells expressing NDI alleles with membrane-permeant receptor antagonists. Of the 15 mutants evaluated, 8 were rescued to the surface and manifested AVP-stimulated cAMP accumulation. A nonpermeant $V_2R$ antagonist was ineffective in receptor rescue, indicating that the effects of the permeant antagonists occur intracellularly, perhaps by inducing a receptor conformation that allows targeting to the cell surface. The expression of partial receptor peptides, by adenovirus-mediated delivery or the use of permeant AVP antagonists that act as pharmacological chaperones, offers an exciting potential treatment modality for NDI.

## VIII. DOMAIN SWAPPING OF $V_2$ VASOPRESSIN AND OXYTOCIN RECEPTORS TO ELUCIDATE LIGAND BINDING AND G-PROTEIN INTERACTIVE DOMAINS

The neurohypophyseal hormone receptor family is ideal for examining the effects of domain swapping, because only the $V_2R$ is coupled to cAMP production. Expression of the $V_1/V_2$ hybrid receptors in COS-7 cells shows that proteins containing the $V_1R$ sequence in the second intracellular loop activate the phosphatidylinositol pathway with high efficiency. Only hybrid receptors containing the $V_2$ receptor sequence in the third intracellular loop are capable of efficiently stimulating cAMP production. These findings indicate that specific, single intracellular receptor domains differentiate between G-proteins.

Chimeric vasopressin $OT/V_2$ receptors have been used to identify receptor regions involved in ligand binding as well as G-protein coupling. A hybrid containing OTR sequences from the N-terminus to the middle of transmembrane region three had high-affinity OT binding sites that were coupled to activation of adenylyl cyclase. In contrast, a hybrid containing OTR sequences extending from transmembrane helix five to the C-terminus preferentially bound AVP but was not coupled to the $V_2$-selective $G_{\alpha s}$. Thus, OT binding requires the N-terminal third of the OTR. Use of $OT/V_2$ receptor chimeras and hybrid hormones (e.g. vasotocin) allows for a detailed analysis of the structural motifs involved in binding. The first two extracellular domains of the OTR are involved in binding to the C-terminal tripeptide of OT. The third extracellular domain of the receptor contacts the cyclic part of OT; the fourth and final outer domain does not appear to be involved in ligand binding.

## IX. $V_3$ VASOPRESSIN RECEPTOR SIGNALING

The $V_3R$ (or $V_{1b}R$) was initially described in corticotroph cells of the anterior pituitary, where AVP potentiates the release of ACTH. Subsequent cloning of the receptor showed that it was a third member of the AVP receptor family. Although studies on the AVP pituitary receptor revealed a different pharmacological profile compared to those of the $V_1R$ and the $V_2R$ subtypes, there are no specific AVP analogues that allow $V_3R$ signaling to be completely distinguished from $V_1R$ signaling. Vasopressin $V_3R$ transcripts have been detected throughout the rat brain by reverse transcription polymerase chain reaction and by *in situ* hybridization. These receptors have also been localized immunohistochemically in fiber networks concentrated mainly in the hypothalamus, amygdala, and cerebellum (particularly in those areas with a leaky blood–brain barrier or close to the circumventricular organs). In peripheral tissues, $V_3R$ transcripts are expressed in the kidney, pancreas, and adrenal medulla. Because of the difficulty in discriminating $V_3$ from $V_1$ receptor activation, most of what is known about $V_3R$ signaling comes from studies in which the receptor has been stably expressed in CHO cells that do not otherwise express AVP receptors. AVP stimulates PLC activity in $V_3R$ transfected cells. In clones expressing high levels of receptors, a portion of PLC stimulation is PTX sensitive, and AVP stimulates cAMP synthesis. These results suggest that the $V_3R$ has a preferential affinity for $G_{q/11}$ but that at higher concentrations of receptor, it can also interact with $G_{i/o}$ and $G_s$, presumably by promiscuous interactions.

Vasopressin occupancy of $V_3Rs$ also stimulates arachidonic acid release by a PTX-sensitive process. Activation of MAP kinases by AVP is dependent on activation of PLC and PKC; both the level and duration of activation are a function of the receptor density. Vasopressin stimulates DNA synthesis in clones expressing medium levels of $V_3R$.

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## Glossary

**amnion** Tough fibrous membrane that surrounds the fetus; has a simple (usually low-cuboidal with microvilli) epithelium facing inward.

**aquaporin-2** Water channel that is expressed in the renal collecting duct; is translocated from intracellular vesicles to the apical membrane in response to an intracellular signaling cascade that is initiated by binding of vasopressin to its receptor.

**endometrium** Comprises the mucosal and glandular-containing submucosal lining of the uterus.

**mesangial cell** Contractile cell of the glomerular mesangium, which is a thin membrane that helps support the capillary loops in a renal glomerulus. Vasopressin stimulates contraction of these cells and stimulates their growth in cell culture.

**myometrium** Uterine smooth muscle.

**pertussis toxin** *Bordetella pertussis* protein that catalyzes the ADP-ribosylation of G-protein $\alpha$-subunits of the $G_i$ family; this modification blocks the receptor-G-protein interaction.

**ryanodine receptors/calcium release channels** $Ca^{2+}$ is released from the endoplasmic and sarcoplasmic reticulum in a wide range of tissues by specialized types of calcium channels, i.e. ryanodine receptors, by the process of $Ca^{2+}$-induced $Ca^{2+}$ release.

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**Oxytocin • Vasopressin (AVP)**

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the pancreas, from which mature PST is secreted, whereas CGA is poorly phosphorylated in the ileum, from which there is no processing to PST.

## III. PANCREASTATIN SYNTHESIS AND SECRETION

Proteolysis of CGA occurs both inside and outside the cell to yield peptides with biological activity. This function of CGA as a precursor of active peptides was first suggested after the isolation of PST, which turned out to have the sequence of a central part of the CGA molecule. This hypothesis was then supported by the observation of pairs of dibasic residues in the sequence of CGA, which usually represent targets for protease cleavage.

Intact CGA is the major component of secretory granules of the adrenal medulla and hypothalamus. Nevertheless, it is generally accepted that degradation products of CGA do exist in chromaffin cells. In the rat pheochromocytoma cell line PC12, intact CGA is present and is processed only at its N-terminus, although mature PST is rare. Chromaffin cells may be the major source of circulating CGA, and they could therefore be the major indirect source of PST in plasma.

The intracellular processing of PST has been fully described for bovine CGA and partially described for rat CGA. The mechanism involves proteases such as prohormone convertase-2 (PC-2) and carboxypeptidase H.

The extracellular processing of CGA may occur as a result of the activities of proteases co-secreted by the chromaffin granule or may be due to exoproteases localized on the extracellular side of the cell plasma membrane. In addition, fragmentation of bovine CGA by plasma kalikrein has also been shown.

The processing of CGA is tissue- and species-specific. Thus, CGA is more fully processed in the stomach, especially in the endocrine cells of the antrum, and in the endocrine pancreas, of which PST is a major product. PST production is higher in insulinoma cells than in primary human islets. In this tissue, PST expression has been found in the beta-, delta-, and alpha-cell populations. The somatostatin-secreting delta-cell line (QGP-1N) also releases PST.

Enterochromaffin-like (ECL) cells, originating from the gastric antrum, represent the best characterized physiological system of PST secretion. These cells respond to gastrin stimulation by increasing both PST secretion and CGA mRNA levels. In the rat *in vivo*, an increase in gastrin levels, either by infusion or by indirect means, leads to the suppression of acid secretion and an increase in PST levels. Conversely, a decrease in gastrin levels by fasting or antrectomy is followed by a decrease in PST levels. Moreover, gastrin receptor antagonists produce a decrease in PST levels. Finally, patients with gastrinomas have increased plasma PST levels, due to an excess of gastrin secreted by the tumor.

Plasma porcine PST-like immunoreactivity levels have been shown to increase 50% (from 100 to 150 pM) in response to a meal. In perfused porcine pancreas, PST-like immunoreactivity is released in parallel with insulin in response to insulinotropic stimuli.

Elevated PST levels have been found in response to a glucose load in type 2 diabetes, in hypertension, and in pregnant women with gestational diabetes, correlating with plasma catecholamine levels.

## IV. BIOLOGICAL EFFECTS OF PANCREASTATIN

Many different biological effects of PST in a variety of tissues have been described. PST may act as an autocrine, paracrine, and/or endocrine peptide in many target cells.

### A. Endocrine Secretion

There is accumulated evidence for the ability of PST to modulate endocrine secretion in a variety of systems.

#### *1. The Endocrine Pancreas*

The first described effect of PST was the inhibition of glucose-stimulated insulin secretion, and especially first-phase insulin secretion, in isolated rat pancreas. PST also potentiates the inhibition of insulin secretion caused by a physiological decrease in glucose concentration. On the other hand, PST has also been shown to prime glucose-stimulated insulin secretion after a second glucose pulse. PST can also inhibit the stimulatory action of other agents, whether these are physiological (arginine), hormonal (GIP, VIP, CCK, glucagon), or even pharmacological (IBMX, sulfonylurea). The inhibitory effect of PST on insulin release has been confirmed *in vivo* in the rat.

On the other hand, other groups have reported no such effect in dogs and pigs, suggesting different effects depending on the species. In general, PST seems to negatively modulate insulin secretion in an autocrine, paracrine, and endocrine manner. Other effects of PST that relate to endocrine pancreatic secretion are the stimulation of glucagon release and the inhibition of pancreatic polypeptide secretion.

However, PST has been shown *in vitro* to be able to modulate the formation of insoluble fibers of amyloid polypeptide. Actually, the formation of amyloid fibers seems to be mediated by an inappropriate balance between the amyloid peptide, insulin, C-peptide, and PST.

### 2. *Parathyroid Secretion*

PST has an inhibitory effect on parathyroid hormone (PTH) secretion. This effect has been observed in porcine and bovine parathyroid cells on stimulation with low (but physiological) concentrations of either calcium or phorbol ester (a nonphysiological agent). This effect was confirmed by the demonstration that PTH secretion increases if parathyroid cells are incubated with anti-PST antibodies. Moreover, PST inhibits not only PTH secretion but also transcription of the PTH and CGA genes; the stabilities of these mRNAs are also decreased. The mechanism by which PST exerts these effects is not known, however.

The parathyroid gland is very rich in CGA, but complete processing of this protein to PST is very rare, due to the lack of prohormone convertases PC2 and PC1/3. Therefore, it cannot be concluded that PST has an autocrine role in parathyroid secretion. Nevertheless, processed PST is present in calcitonin-producing C cells, and in this way PST may be involved in the regulation of the thyroid–parathyroid axis.

### 3. *Adrenal Medulla Secretion*

The inhibition of secretion from the adrenal medulla by CGA-derived peptides (obtained by tryptic proteolysis) suggested that these peptides (including PST) may have some inhibitory action in chromaffin tissue. Consistent with this hypothesis, PST administration in the rat decreases catecholamine levels during surgical stress. It is not yet known whether this is a direct or an indirect effect of PST, however.

### 4. *Atrial Secretion*

PST can also regulate endocrine secretion from the heart. Atrial myocardial cells store and secrete atrial natriuretic peptide. Thus, PST has been shown to stimulate atrial cell secretion by 90%. This result suggests that the PST precursor, CGA, which has been identified in atrial secretory granules, may play an autoregulatory role in atrial secretion.

## B. Exocrine Secretion

### 1. *Exocrine Pancreatic Secretion*

Evidence showing an inhibitory effect of PST on exocrine pancreatic secretion has been accumulating. *In vivo* studies employing physiological stimuli (a meal), CCK-8, and central vagal nerve stimulation have provided convincing data supporting an inhibitory effect of PST on pancreatic exocrine secretion. These effects seem to be mediated by the presynaptic modulation of acetylcholine release from the vagal system. Therefore, PST has been proposed as a new mediator in the islet–acinar axis.

### 2. *Gastric Secretion*

Contradictory *in vitro* versus *in vivo* results regarding the effects of PST on gastric secretion have been reported. Thus, PST inhibits gastric secretion in isolated parietal cells, but *in vivo* seems to enhance gastric acid secretion stimulated by different nutrients.

ECL cells from the gastric antrum are an important source of PST. Therefore, PST may play a role in the paracrine regulation of gastric acid secretion.

## C. Hepatic Glycogen Metabolism

PST activates glycogenolysis in the rat liver. Thus, PST increases glucose release from the liver, resulting in a hyperglycemic effect. This effect can be observed *in vivo*, without even a modification of glucagon or insulin levels, suggesting a direct effect on liver metabolism. This observation was confirmed by studies in isolated hepatocytes, in which PST had a glycogenolytic effect similar to that of glucagon in potency, but was independent of cyclic AMP production and dependent on calcium. In fact, this glycogenolytic effect correlates with the dose-dependent increase in intracellular free calcium produced by a PST challenge.

In addition to its glycogenolytic effect, PST inhibits insulin-stimulated glycogen synthesis, but unlike glucagon it does not affect the rate of insulin-stimulated glycolysis. In this way, even though the glycogenolytic effect of PST is comparable to that of glucagon, the latter produces higher levels of hyperglycemia. Conversely, PST inhibits glucagon-stimulated insulin release and thus enhances the hyperglycemic effect of glucagon.

## D. Metabolic Effects in the Adipocyte

Another target cell for metabolic regulation by PST is the adipocyte. Here, PST has been shown to modulate glucose, lipid, and protein metabolism in isolated adipocytes. PST also inhibits basal and insulin-stimulated glucose transport, lactate production, glycogen synthesis, and lipogenesis in a dose-dependent manner. It therefore opposes the main metabolic action of insulin in adipose tissue. These effects can be observed at a wide range of insulin concentrations, leading to a shift to the right in the dose–response curve. Maximal effects are obtained at 10 nM PST, with an $ED_{50}$ of 0.1 nM. These counterregulatory effects on insulin action suggest that PST may have a role in insulin resistance. Moreover, PST has a dose-dependent lipolytic effect in isolated adipocytes ($ED_{50} = 0.1$ nM). However, this effect is inhibited by 10 nM insulin. On the other hand, PST has a stimulatory effect on protein synthesis and enhances insulin-stimulated protein synthesis in isolated adipocytes.

## E. Insulin Receptor Inhibition

PST signal transduction has been shown to cross-talk with insulin receptor signaling. Thus, PST inhibits insulin receptor tyrosine kinase activity by promoting serine phosphorylation of the insulin receptor β-subunit. Lower tyrosine phosphorylation levels of insulin receptor substrates lead to decreased interactions with the regulatory enzyme phosphatidyl inositol-3 kinase. This is a key enzyme that mediates glucose transport and metabolism in the adipocyte and glycogen synthesis in the hepatocyte. These inhibitory effects of PST on insulin receptor signaling partly mediate the metabolic counterregulatory action of PST and further suggest that PST may have a role in insulin resistance.

Meanwhile, PST stimulates the mitogen-activated protein kinase (MAPK) signaling cascade, potentiating this insulin receptor signaling pathway. In fact, this dual effect on insulin receptor signaling may explain PST's ability to simultaneously inhibit glucose and lipid metabolism and promote protein synthesis in adipocytes.

## F. PST Regulation of Cell Growth

PST has been found to have an inhibitory effect on cell growth in a variety of cell lines (pancreatic and hepatic cells). This inhibitory effect of PST has also been observed *in vivo* in islets transplanted into nude mice. Moreover, PST inhibits basal and CCK-stimulated pancreas growth in mice. Consistent with this finding, PST can also inhibit DNA synthesis in rat fetal islets.

Even though PST stimulates MAPK signaling in HTC (hepatoma cells), it has been found that PST inhibits protein and DNA synthesis. The PST-induced inhibition of cell growth observed in HTC hepatoma cells is mediated by nitric oxide (NO) production. If NO production is blocked, PST stimulates cell growth. This stimulatory effect is mediated by activation of the MAPK pathway. In this way, the final effect of PST on hepatocyte growth may depend on NO availability.

## G. Effects of PST on Central Nervous System

Peripheral administration of PST has been shown to enhance memory retention in mice. In addition, PST can revert the amnesia produced by the cholinergic antagonist scopolamine. This activity in memory retention seems to be a peripheral rather than a central effect, since intraventricular administration produces only a modest relief. Therefore, these effects may be a consequence of the PST-mediated hyperglycemia previously demonstrated in mice, since hyperglycemia is known to increase memory retention. Moreover, intracranial administration of PST elevates blood glucose and free fatty acids in the rat. This action is opposite to that of insulin, further supporting the counterregulatory effects of PST.

PST may also play a role in the formation of senile plaques in Alzheimer's disease, in which a long 13.5 kDa form of PST seems to be present. The physiological or pathophysiological role of PST in Alzheimer's disease is unknown, however.

## H. Immunomodulatory Effect of PST

PST seems to have an immunomodulatory effect. It is able to enhance the mitogenic response of peripheral blood T lymphocytes when they are stimulated by noncognate stimuli (lectins). Therefore, a role for PST as an immunomodulator has been proposed.

# V. PUTATIVE PHYSIOLOGICAL ROLE OF PANCREASTATIN

Although the physiological role of PST has yet to be fully established, a multitude of effects have been ascribed, implicating PST in the modulation of secretion and the control of metabolism.

The variety of PST's effects on the modulation of secretion in glands where this peptide is synthesized and processed has led to the hypothesis that PST may have a role as an autocrine and paracrine regulatory peptide of exocrine and endocrine secretion. Nevertheless, further studies to identify the molecular mechanisms underlying these effects are needed to establish a physiological role in the regulation of secretion.

The metabolic actions of PST have been more thoroughly studied and some of the molecular mechanisms by which PST modulates glucose, lipid, and protein metabolism, with effects that are generally counterregulatory to those of insulin, are already known.

Our current hypothesis is that PST plays a role in the physiology of stress, by regulating the supply of energy to the body (especially the muscle and brain). In fact, PST levels may be increased under stressful conditions, during which CGA is co-secreted with catecholamines. Therefore one would expect that the endocrine actions of PST may actually take place under stressful conditions, when circulating PST levels are high enough to interact with specific receptors in target cells. Consistent with this proposal, specific PST receptors and PST-induced signal transduction have been characterized in liver and adipose tissue, providing a basis for the molecular mechanisms of the metabolic effects of PST.

Moreover, PST can modify the insulin/glucagon ratio by modulating secretion to further increase metabolic anti-insulin effects. In this context, PST could also play a role not only in the physiology of stress, but also in pathophysiological conditions such as insulin-resistant states. In support of this concept, increased levels of PST, which correlate with those of catecholamines, have been found in type 2 diabetes, gestational diabetes, and essential hypertension.

These effects of PST may also be considered complementary to those described for other CGA-derived peptides, which have been found to exert different actions, such as the control of catecholamine secretion, vasodilation, and infection.

In conclusion, PST is a regulatory peptide derived from CGA and, therefore, may be regarded as part of the functional axis controlled by this protein, for which the description of a physiological role has been elusive for so long.

## Glossary

**chromaffin** Tissue of neuroectodermic origin with the specialized function of synthesizing and secreting catecholamines.

**chromogranin A** A glycoprotein that is very abundant in the secretory granules of chromaffin and other neuroendocrine cells.

**insulin resistance** A pathophysiological condition in which the action of insulin is impaired, so that a higher insulin concentration is needed to result in the same effect.

**pancreastatin** A chromogranin A-derived peptide that inhibits secretion and impairs insulin action.

**regulatory peptide** A peptide that is released in response to a stimulus and that exerts specific biological actions. It may function as an autocrine, paracrine, or endocrine agonist or antagonist.

## See Also the Following Articles

**Insulin Actions • Insulin Processing • Insulin Secretion • Pancreatic Polypeptide • Parathyroid Hormone • Stress**

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# Pancreastatin Receptor Signaling

VÍCTOR SÁNCHEZ-MARGALET
*Virgen Macarena University Hospital, Spain*

I. PANCREASTATIN RECEPTOR
II. G-PROTEINS COUPLED TO PANCREASTATIN RECEPTOR
III. EFFECTORS FOR PANCREASTATIN RECEPTOR SIGNALING
IV. CROSSTALK OF PANCREASTATIN RECEPTOR WITH INSULIN RECEPTOR SIGNALING
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**Pancreastatin (PST) receptor signaling has been thoroughly studied in the rat liver and adipose tissue during the past decade, and it is still an active area of research. PST receptor signaling is a paradigmatic example of the signal transduction of a calcium-mobilizing hormone receptor. Even though the PST receptor has not yet been cloned, the signaling triggered in response to PST has the typical pattern of a seven-transmembrane-spanning receptor coupled to hetertrimeric G-proteins in the plasma membrane to exert the metabolic actions observed in the liver and adipose tissue. This article describes the different signaling pathways that have been shown to be activated in response to PST and the possible molecular mechanisms underlying the modulation of the metabolic action exerted by PST in the liver and adipose tissue, as well as the counterregulatory effects on insulin action.**

## I. PANCREASTATIN RECEPTOR

One of the hallmarks confirming the endocrine nature of a peptide is the presence of specific receptors in the plasma membrane. Thus, pancreastatin (PST)-binding sites have been characterized in rat liver, adipose, and heart membranes. Binding data obtained using radioiodinated rat PST in rat liver membranes suggested the presence of specific high-affinity receptors for PST. The PST binding fulfills all the criteria for membrane receptors: it is temperature-, time-, and pH-dependent, and it is saturable and reversible. In addition, the binding of the ligand is very sensitive to the PST sequence, further suggesting the specificity of the receptor. Thus, PSTs from species that share a low level of sequence homology with rat PST, such as human or porcine PST, showed a low level of binding affinity in the studies of radioligand displacement. Analysis of binding data under equilibrium conditions showed similar affinity values in rat liver, HTC rat hepatoma, rat adipose, and heart membranes, indicating the presence of a single site with a $K_d$ ranging from 0.2 to 1 nM. This range of $K_d$ values correlates well with the $ED_{50}$ obtained for the effects of PST in hepatocytes and adipocytes and is in accordance with PST levels found in pig and human plasma. In addition, these values are comparable to those obtained for most peptidic hormone receptors. Therefore, the affinity of the putative PST receptor is consistent with a possible physiological and pathophysiological role for this regulatory peptide.

On the other hand, different concentrations of binding sites have been found depending on the target tissue, from 5 fmol/mg of protein in adipose tissue to 34 fmol/mg of protein in heart membranes, with an intermediate $B_{max}$ of 15 fmol/mg of protein in rat liver membranes. These binding data give an estimate of 1000–5000 binding sites per cell.

Active PST receptors have been solubilized and characterized from rat liver membranes. Molecular analysis of the solubilized receptor by covalent cross-linking and further identification on sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated a single band of 85 kDa. Gel filtration studies of the solubilized receptors confirmed the 80 kDa molecular mass of the PST receptor. In addition, the solubilized receptor is a glycoprotein that can specifically bind to the wheat-germ agglutinin (WGA) lectin.

Taking advantage of the glycoprotein nature of the receptor, a two-step procedure has been employed as a purification strategy. Thus, WGA semipurification followed by affinity purification using a biotinylated PST analogue has led to the purification of PST receptors in homogeneity. The PST receptor can be purified as an 80 kDa monomeric glycoprotein physically associated with a $G_{\alpha q/11}$ protein. The scale-up of the purification process may yield sufficient amounts of receptor proteins to undertake microsequencing in the near future.

## II. G-PROTEINS COUPLED TO PANCREASTATIN RECEPTOR

Heterotrimeric ($\alpha$, $\beta$, $\gamma$) GTP-binding (G) proteins are one of the most important transducers of the signaling from receptors in the plasma membrane to the interior of the cell, coupling the activation of the receptor with the triggering of different effector systems. The coupling of the PST receptor with GTP-binding proteins has been demonstrated by different approaches in rat liver, adipocyte, and heart membranes.

The first evidence for the coupling of the PST receptor with G-proteins was revealed by the sensitivity of PST binding to the presence of guanine nucleotides, especially nonhydrolyzable analogues. These results were then confirmed with direct evidence of physical and functional coupling. Thus, GTPase activity, GTP-binding studies, and photolabeling, in combination with pertussis toxin (PT) pretreatment and blocking antibodies against different $\alpha$-subunits, demonstrated the double coupling of the PST receptor, mainly with a G-protein of the $\alpha_{q/11}$ family and to a lesser extent with a G-protein of the $\alpha_{i1,2}$ family (PT sensitive). Moreover, a physical association was demonstrated by binding studies with radiolabeled PST in anti-$G_{\alpha q/11}$ immunoprecipitates. These results were confirmed by the opposite approach. Thus, $G_{\alpha q/11}$ proteins could be observed along with semipurified and purified PST receptor.

In general, a double system of G-proteins seems to be engaged in PST signaling, a pertussis toxin-sensitive G-protein belonging to the $G_{\alpha i1,2}$ family and a pertussis toxin-insensitive G-protein belonging to the $G_{\alpha q/11}$ family (see Fig. 1). The specific G-protein coupled to the PST receptor has been assessed in rat liver membranes, where $G_{\alpha 11}$ rather than $G_{\alpha q}$ seems to functionally couple to the PST receptor. On the other hand, $G_{\alpha 16}$ (another G-protein of the $\alpha_{q/11}$ family) is the G-protein that couples to the PST receptor in the heart. Since $G_{\alpha 16}$ is not present in liver or adipose tissues, but is present in the heart, the coupling of PST receptor with $G_{\alpha q/11}$ in hepatocytes and adipocytes is compatible with the preferential coupling with $G_{\alpha 16}$ rather than $G_{\alpha q/11}$ in heart membranes.

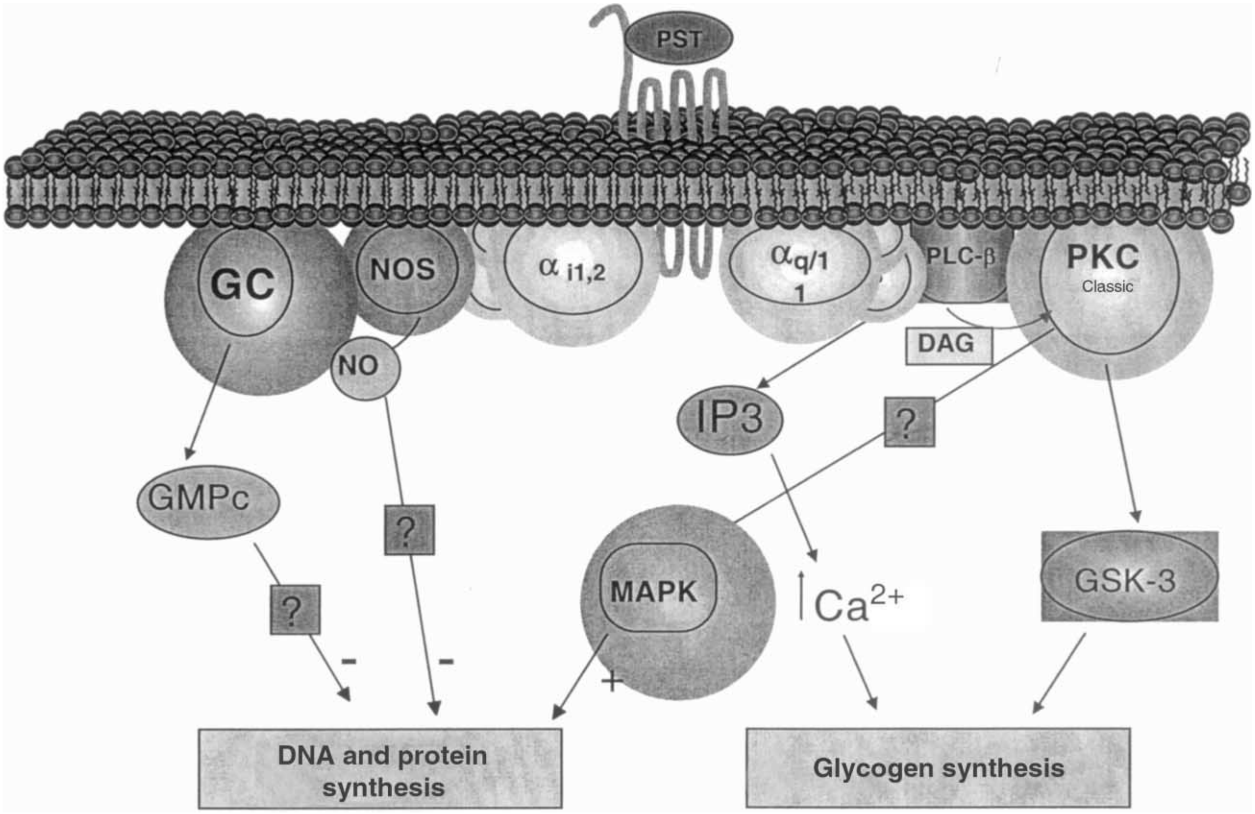

**FIGURE 1** Schematic model of pancreastatin receptor signaling.

## III. EFFECTORS FOR PANCREASTATIN RECEPTOR SIGNALING

### A. Phospholipase C Activity

The polyphosphoinositide-specific phospholipase C (PLC) that is activated by seven-transmembrane receptors by coupling heterotrimeric G-proteins is the membrane-associated PLC-β. PST has been shown to stimulate membrane-associated PLC-β activity in rat liver membranes. Four different PLC-β isoforms (β1–4) have been described, although the expression of PLC-β4 is more limited and cannot be found in hepatic, adipose, or heart tissues. The PLC-β3 isoform seems to be the specific isoform activated by PST receptors in rat liver and adipose membranes, although PLC-β1 is also activated in HTC rat hepatoma cells. The activation of PLC by PST is mediated mainly by $G_{\alpha q/11}$ in liver and adipose membranes, but some activation may be accounted for by βγ released by $\alpha_{i1,2}$ or $\alpha_{q/11}$ activation.

On the other hand, PLC-β2 is the isoform preferentially activated in rat heart membranes by PST, although it can also stimulate some activation of β1 and β3 isoforms. This is not striking when it is taken into account that PST preferentially activates $G_{\alpha 16}$ in heart membranes, and this $G_{\alpha}$ protein preferentially activates PLC-β2, although it can activate β1 and β3 to a lesser extent.

### B. Calcium

The role of calcium in PST action has been studied in the hepatocyte. Thus, PST stimulation of isolated hepatocytes induces a rapid increase in intracellular calcium concentration. Consistent with these results, the glycogenolytic effect of PST was found to be cyclic AMP-independent but very dependent on both extracellular and intracellular calcium. In fact, the dose-dependent glycogenolytic effect of PST correlates with the progressive increase in intracellular calcium concentration. Moreover, PST has been found to increase intracellular free calcium concentration by releasing intracellular stores and increasing the influx of extracellular calcium. These effects are mediated by the production of inositol 1,4,5-triphosphate ($IP_3$) resulting from the activation of PLC-β by $G_{\alpha q/11}$.

### C. Protein Kinase C

The activation of PLC-β activity in the plasma membrane by PST stimulation leads to the production of $IP_3$ as discussed in the previous section, but also produces diacylglycerol (DAG). DAG in addition to calcium is responsible for the activation of protein kinase C (PKC), more precisely, the classical isoforms of PKC. Thus, PST has been found to activate classical isoforms of PKC (α, βI, and βII) by promoting translocation from the cytoplasm to the plasma membrane in HTC hepatoma cells and rat adipocytes. Moreover, the glycogenolytic effect of PST in the hepatocyte and the inhibition of glucose transport in the adipocyte can be prevented by blocking the activation of PKC. Taken together, these results suggest that PKC activity is a very important effector of PST receptor signaling.

### D. Mitogen-Activated Protein Kinase

G-protein-coupled receptors are known to signal to the mitogen-activated protein kinase (MAPK) pathway by two different but complementary mechanisms. Thus, the βγ dimer of heterotrimeric G-proteins can activate the Ras–Raf pathway, whereas the $\alpha_{q/11}$ protein connects with MAPK by activating PKC. Therefore, the reported effect of PST-activating MAPK in hepatoma cells and adipocytes can be explained by this dual mechanism. Thus, PST induces the Ser/Thr phosphorylation of MAPK by activation of MAPK kinase. In fact, this pathway mediates the effect of PST-stimulating protein synthesis in rat adipocytes.

### E. Glycogen Synthase Kinase-3 Activity

PST stimulation is able to activate glycogen synthase kinase-3 (GSK-3) activity in rat adipocytes. The phosphorylation level of GSK-3 is negatively correlated with the activity. Thus, PST inhibited basal phosphorylation of GSK-3. The PST stimulation of GSK-3 activity seems to be mediated by PKC since it can be prevented by a specific PKC inhibitor. This effect of PST on GSK-3 activity results in the inhibition of both basal and insulin-stimulated glycogen synthesis in rat adipocytes. This effect of PST can also be prevented by using a PKC inhibitor. Therefore, PKC activation by PST mediates the activation of GSK-3, which is one of the final effectors of PST receptor signaling to regulate glucose metabolism.

### F. Nitric Oxide and Cyclic GMP

PST was found to increase the production of the second messenger cyclic GMP (cGMP) in rat hepatocytes by a PT-sensitive mechanism, probably involving $G_{\alpha i1,2}$ activation, although this mechanism still needs experimental confirmation to finally define this signaling pathway. In any case, this effect has been proved to be mediated by nitric oxide (NO) production in hepatoma cells. The production of cGMP may eventually lead to down-regulation of the $G_{\alpha q/11}$–PLC-β signaling by activating protein kinase G (PKG), as observed in other systems for other G-protein-coupled receptors. The role of cGMP/PKG in the counterregulation of PST signaling still needs further investigation, however.

On the other hand, NO and cGMP have been shown to inhibit DNA and protein synthesis in hepatoma cells. Therefore, these effectors seem to mediate the antiproliferative effect of PST in HTC rat hepatoma cells. The antiproliferative effect of the NO–cGMP pathway has been found in other systems. In fact, when NO synthase activity is blocked by pharmacological inhibitors, the effect of PST changes to the stimulation of growth and proliferation, due to the activation of the MAPK pathway. Therefore, the final effect of PST on cellular growth and proliferation may depend on the balance between these two signaling pathways, especially the availability of NO.

## IV. CROSSTALK OF PANCREASTATIN RECEPTOR WITH INSULIN RECEPTOR SIGNALING

Crosstalk of the PST receptor with insulin receptor signaling has been studied in hepatoma cells and adipocytes. Similar results have been observed in both systems (Fig. 2).

PST inhibits insulin-mediated autophosphorylation of the insulin receptor β-subunit in a dose-dependent manner. In addition, PST blunts tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2, and p60-70, preventing their association with p85, the regulatory subunit of phosphatidyl inositol 3-kinase (PI3K). This effect results in the inhibition of PI3K activity. Moreover, the insulin activation of the downstream protein kinase B and S6 kinase is also blocked by PST.

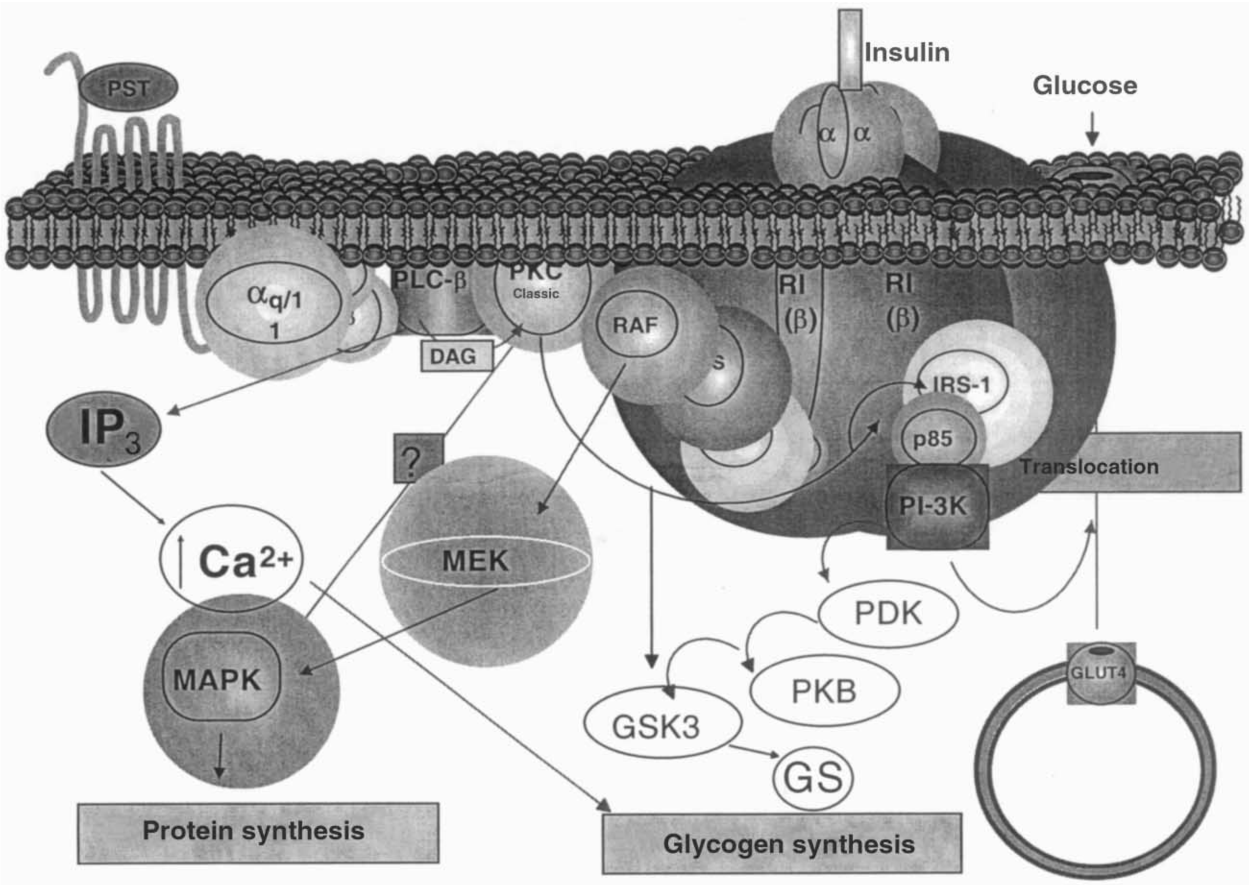

FIGURE 2 Schematic model of the cross talk of pancreastatin with insulin receptor signaling.

These effects of PST in preventing insulin receptor signaling can be fully reversed by blocking protein kinase C activity, strongly suggesting that the PST-induced activation accounts for the crosstalk of both receptors. In fact, PST induces the serine/threonine phosphorylation of insulin receptor β-subunit and IRS-1, which is a well-known mechanism of inhibition of insulin receptor tyrosine kinase activity by counterregulatory hormones. Moreover, the PST-mediated Ser/Thr phosphorylation of the insulin receptor and IRS-1 seems to be caused by the activation of PKC, since this effect can also be prevented by blocking PKC activity. In fact, the PST-mediated activation of PKC has been observed in both the hepatocyte and the adipocyte. PST-inhibited, insulin-stimulated PI3K activity can also be reversed by blocking PKC. In parallel with the signaling results, the PST inhibition of the physiological actions of insulin can also be reversed by preventing PKC activation. Thus, the inhibitory effects of PST on insulin-stimulated glucose transport in adipocytes and glycogen synthesis in hepatocytes are also abrogated by blocking PKC activation.

These findings suggest that PST may exert its anti-insulin effect on the insulin receptor by cross talk with the early signaling events, as a result of PKC-mediated Ser/Thr phosphorylation that inhibits tyrosine phosphorylation in insulin receptor signaling.

## V. CONCLUSIONS

Data from PST signaling studies in hepatocytes and adipocytes point to the presence of a specific receptor of high binding affinity and specificity in the plasma membrane. Signal transduction of the PST receptor engages the activation of a double system of G-proteins. On the one hand, a PT-sensitive $G_{\alpha i1,2}$ pathway may mediate the activation of NO and cGMP production, which then may have a role inhibiting the signaling and mediating the inhibitory effect of PST on cell proliferation. On the other hand, a PT-insensitive $G_{\alpha q/11}$ is activated by the PST receptor and certainly mediates the activation of the PLC-β–PKC–MAPK pathway. The activation of classical isoforms of PKC is a central mediator of the metabolic effects of PST and its crosstalk with the insulin receptor, whose signaling and action are subsequently impaired. The balance of both limbs of signal transduction is also important for the final effect of PST on cell growth and proliferation.

## Glossary

| | |
|---|---|
| **chromogranin A** | A glycoprotein that is very abundant in chromaffin and neuroendocrine secretory granules. This protein may function as a prohormone precursor of biologically active peptides. |
| **cyclic GMP** | Cyclic nucleotide synthesized by guanylate cyclase, an enzyme activated by nitric oxide. |
| **G-protein** | Heterotrimeric protein (α, β, γ) whose α-subunit has GTPase activity. G-proteins couple seven-transmembrane-spanning receptors with different effectors. |
| **nitric oxide synthase (NOS)** | There are three isoforms: inducible, neuronal, and endothelial. The activities of neuronal and endothelial NOS are regulated by extracellular signals and the inducible NOS is regulated at the transcriptional level. |
| **pancreastatin** | Chromogranin A-derived peptide with autocrine, paracrine, and endocrine effects regulating secretion and metabolism. |
| **phospholipase C** | Polyphosphoinositide-specific phospholipase, typically producing inositol 1,4,5-triphosphate and diacylglycerol. The phospholipase C β isoform is located in the plasma membrane. |
| **protein kinase C** | Serine/threonine protein kinase whose activity is dependent on calcium and/or phospholipids. |

## See Also the Following Articles

**Calcium Signaling • Heterotrimeric G-Proteins • Insulin Receptor Signaling • Pancreastatin**

## Further Reading

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# Pancreatic Polypeptide

Ronald E. Chance
*Eli Lilly and Company, Indiana*

I. INTRODUCTION
II. CHEMISTRY
III. BIOLOGY

**Pancreatic polypeptide is a 36-residue peptide hormone with an amidated carboxyl group at the C-terminus. The hormone was discovered fortuitously in 1968 by scientists at Kansas University during research to purify chicken insulin.**

## I. INTRODUCTION

Discoverers Joseph Kimmel, Gail Pollock, and Robert Hazelwood named the persistent contaminant avian pancreatic polypeptide (APP; more recently aPP), fully expecting to rename it later once function was established. Because aPP was indistinguishable from glucagon by polyacrylamide disc gel electrophoresis, the peptide was assayed for glucagon bioactivity by William Bromer (Eli Lilly). Even though no hyperglycemic activity was found by the U.S. Pharmacopeia rabbit assay, continued work revealed that aPP was probably a hormone based primarily on a key structural feature. The C-terminal amino acid was amidated (i.e. tyrosinamide). This prompted the author's laboratory at Eli Lilly to further examine some side fractions that the staff had isolated during studies on the characterization of minor components in crystalline insulins (one of these components was already known to be proinsulin). The bovine, human, ovine, and porcine counterparts of aPP were discovered; furthermore, like aPP, all were 36 amino acids in length and all terminated with tyrosinamide.

Antisera against the avian and mammalian pancreatic polypeptides (PPs) along with the respective hormones were widely distributed to investigators by both laboratories in an effort to better understand the physiological role of this newly recognized pancreatic hormone. Immunohistochemistry studies revealed that PP is the product of a fourth type of islet cell, sometimes referred to in earlier literature as the F cell and now known as the PP cell. In mammals, in particular, the PP cell is located on the periphery of the islet; PP-rich islets are often regionally located in the duodenal portion of the pancreas, as shown in Fig. 1. The so-called uncinate process in the canine pancreas is an especially good source of PP cells. Early findings showed that blood levels of PP rose sharply following meals, especially after protein-rich meals. Insulin-induced hypoglycemia was also found to cause a dramatic rise in PP levels. Further investigations revealed that secretion of PP from the pancreas is mediated by vagal, cholinergic mechanisms, and, for this reason, PP responses have sometimes been used in clinical settings to assess vagal function.

Although PP is found primarily in the pancreas, the hormone is often included in discussions on gut hormones. Indeed, an intestinal peptide that is identical to porcine pancreatic polypeptide has been isolated from pig intestinal extracts. Early studies by Tsung-Min Lin (Eli Lilly) showed that bovine pancreatic polypeptide (bPP) administered to dogs did not affect blood glucose like insulin or glucagon but rather was a potent inhibitor of exocrine pancreatic secretion. Similar results in other laboratories led to speculation about using PP to treat pancreatitis. Meanwhile, aPP was studied extensively in chickens by researchers at the University of Kansas

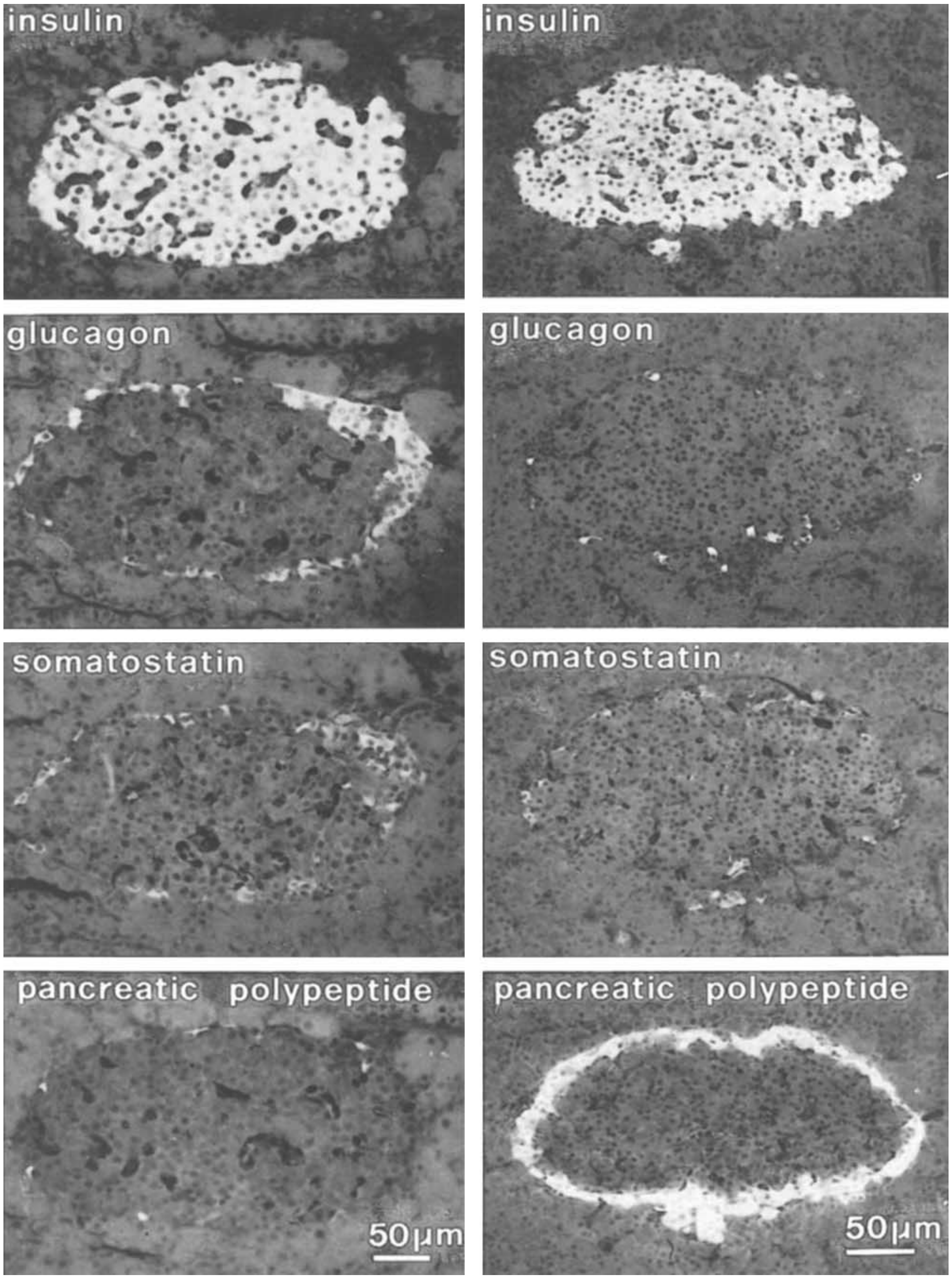
insulin
insulin
glucagon
glucagon
somatostatin
somatostatin
pancreatic polypeptide
pancreatic polypeptide
50μm
50μm

**FIGURE 1** Series of four consecutive sections of a rat pancreatic islet stained, respectively, with anti-insulin, anti-glucagon, anti-somatostatin, and anti-pancreatic polypeptide antisera. The pattern shown in the panels on the left-hand side is characteristic of islets situated in the body and tail of the pancreas (the splenic region). The pattern shown in the panels on the right-hand side is characteristic of islets situated in the lower part of the head of the pancreas. The figure is courtesy of Lelio Orci. Reprinted from Hazelwood (1981), with permission.

in collaboration with Robert Hazelwood at the University of Houston. The avian hormone was found to be a powerful gastric stimulant in chickens.

Because PP was a recognized contaminant in therapeutic insulin preparations prior to the advent of highly purified animal insulins, the PP radioimmunoassay was utilized as a quality control assay on post-1980 purified bovine and porcine insulins. Prior to this, persons with diabetes who received the older traditional and nonchromatographed insulins were sometimes found to have detectable PP antibodies. The assay has also been used in the diagnosis of pancreatic tumors.

## II. CHEMISTRY

The primary structures of aPP and several of the mammalian PPs (bovine, human, porcine, ovine, and canine) were reported in the 1970s. This was followed by the discovery and characterization of two peptide hormones that shared considerable homology with the PPs, yet were distinctly different. Using a more elegant and systematic approach to new hormone discovery, Kazuhiko Tatemoto and Viktor Mutt examined intestinal and brain extracts for peptides possessing amidated carboxyl groups and discovered peptide YY (PYY) and neuropeptide Y (NPY) (see comparison of primary structures in Table 1). Based on X-ray crystal studies of aPP, the hairpin-like fold appears applicable to most if not all members of the so-called PP family, which is now referred to as the PP-fold family.

All of the PP-fold peptides are the result of a precursor–product relationship that has been clarified through the use of molecular biology techniques. Whereas the gene for human NPY is on chromosome 7, the genes for PYY and PP are in close proximity on chromosome 17q21.1, and it is thought that gene duplication of the human peptide YY gene (PYY) generated the pancreatic polypeptide gene (gene symbol PPY). The PPY gene product is a 95-residue protein in which hPP is flanked by a 29-residue signal peptide at the N-terminus and a 30-residue C-terminal extension as shown (hPP sequence in boldface type):

Human Prepropancreatic Polypeptide
$^{1}$MAAARLCLSLLLLSTCVALLLQPLLGAQGly ↓
**APLEPVYPGDNATPEQMAQYA**
**ADLRRYINMLTRPRY**Gly ↓ Lys ↓ Arg ↓ HKED-
TLAFSEWGSPHAAVPArg ↓ ELSPLDL$^{95}$

Single-letter notation is used for all amino acids except those at the processing sites, which are denoted by 3-letter abbreviations and underlined. Prepropancreatic hPP is matured to hPP through the action of several enzymatic steps. After biosynthesis, the preprohormone is translocated from the endoplasmic reticulum to the *trans*-Golgi network with the removal of the signal peptide by signal peptidase. The prohormone is sorted to a regulated transport site and a proprotein convertase cleaves at the COOH-terminus of the Lys-Arg sequence. Arginine and lysine are removed though the action of carboxypeptidase E. Finally, the remaining Gly becomes a substrate for peptidyl glycine α-amidating monooxygenase, resulting in the carboxyamidation of tyrosine at hPP position 36. An icosapeptide and a heptapeptide result from a trypsin-like cleavage at the single arginine residue in the C-terminal extension peptide.

The primary structures of more than 40 PPs have been reported. A partial list is shown in Table 1. Essentially, all are obvious homologues despite species diversity. X-ray crystal structure studies have been limited to aPP (chicken and turkey have identical sequences), although bPP crystals suitable for X-ray analysis have been grown. A solution

TABLE 1 Primary Structures of Some Members of the PP-Fold Family[a]

| | | | | | | | | | | 10 | | | | | | | | | | 20 | | | | | | | | | | 30 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human PP | A | P | L | E | P | V | Y | P | G | D | N | A | T | P | E | Q | M | A | Q | Y | A | A | D | L | R | R | Y | I | N | M | L | T | R | P | R | Y |
| Cat PP | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Dog PP | – | – | – | – | – | – | – | – | – | – | D | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Pig PP | – | – | – | – | – | – | – | – | – | – | D | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Horse PP | – | – | M | – | – | – | – | – | – | – | D | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Cow PP | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Sheep PP | – | S | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | E | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Guinea pig PP | – | – | – | – | – | – | – | – | – | – | D | – | – | – | Q | – | – | – | – | – | – | – | E | M | – | – | – | – | – | – | – | – | – | – | – | – |
| Rabbit PP | – | – | P | – | – | – | – | – | – | – | D | – | – | – | – | – | – | – | E | – | V | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Mouse PP | – | – | – | – | – | M | – | – | – | – | Y | – | – | – | – | – | – | – | – | – | E | T | Q | – | – | – | – | – | – | T | – | – | – | – | – | – |
| Rat PP | – | – | – | – | – | M | – | – | – | – | Y | – | – | H | – | – | R | – | – | – | E | T | Q | – | – | – | – | – | – | T | – | – | – | – | – | – |
| Chicken PP | G | – | S | Q | – | T | – | – | – | – | D | – | P | V | – | D | L | I | R | F | Y | N | – | – | Q | Q | – | L | – | V | V | – | – | H | – | – |
| Human NPY | Y | – | S | K | – | D | N | – | – | E | D | – | P | A | – | D | L | – | R | – | Y | S | A | – | – | H | – | – | – | L | I | – | – | Q | – | – |
| Human PYY | Y | – | I | K | – | E | A | – | – | E | D | – | S | – | – | E | L | N | R | – | Y | – | S | – | – | H | – | L | – | L | V | – | – | Q | – | – |
| Homologous positions | | 2<br>P | | | 5<br>P | | | 8<br>P | 9<br>G | | | 12<br>A | | | | | | | | | | | | | | | 27<br>Y | | 29<br>N | | | 32<br>T | 33<br>R | | 35<br>R | 36<br>Y |

[a] Positions differing from the human PP sequence are indicated. Excluding the sheep PP, there are 11 homologous positions. All tyrosines at position 36 are amidated.

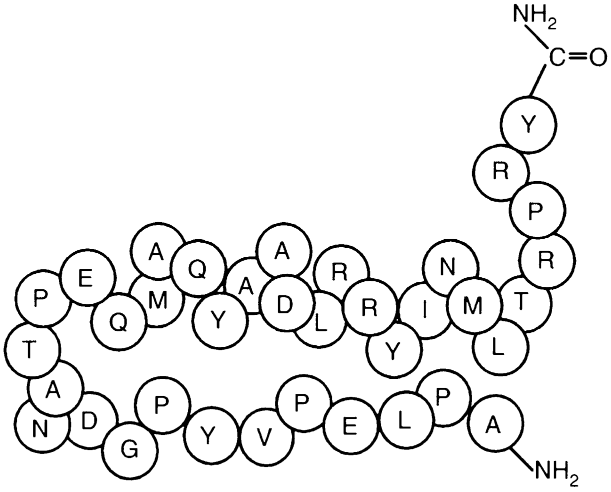

**FIGURE 2** Primary structure of human pancreatic polypeptide (hPP) depicted in the PP-fold configuration. The 36-residue peptide begins with an NH$_2$-terminal alanine and terminates with an amidated tyrosine. See Table 1 for primary structures of several members of the PP-fold family. The computer graphic is courtesy of Don Gehlert (Eli Lilly).

structure of bPP showed a fold remarkably similar to that of aPP. The other PP-family members are considered to fit the so-called PP-fold model, as depicted in Fig. 2. In this model, there is a polyproline type II helix involving residues 2-8, a β-turn, and an α-helical region from residues from ~15 to 32. It is interesting that the prolines at positions 2, 5, and 8 are part of a polyproline-type conformation and interdigitate with the hydrophobic side chains of the helix to form a stable fold.

## III. BIOLOGY

The physiologic function(s) of PP is unknown. The hormone does not have readily apparent actions like the other islet hormones. In both birds and mammals, PP seems to mainly involve regulation of various gastrointestinal activities. For instance, aPP is a powerful gastric stimulant in chickens, and bPP has been found to have a wide spectrum of gastrointestinal actions in dogs, the most notable being the relaxation of the gall bladder and the inhibition of pancreatic exocrine secretion. Results from a few small-scale studies conducted in humans are generally in agreement with the results from the research on dogs. In other studies, PP was administered to 11 patients with chronic pancreatitis with a PP deficiency, as shown by lack of response to a test meal. Results indicated some improvement in abnormal glucose metabolism.

A recurring question concerns the role of PP in satiety. Most animal studies have been inconclusive on this matter. When PP was administered to children with the hyperphagia and obesity of the Prader-Willi syndrome (also shown to have a poor PP response to a protein meal), food intake was reduced slightly. In a 2002 review, Katsuura *et al.* cite recent animal studies showing that peripherally administered PP suppresses food intake and gastric emptying, whereas central administration of PP elicits food intake and gastric emptying. Some of the discrepancies in the literature on this subject may be the result of species specificity for PP. Today, virtually any species of PP can be prepared synthetically with relative ease, whereas older studies were conducted mainly with bovine or porcine PP administered to mice and rats (see Table 1 for PP structural differences). Also, recent transgenic technology is useful in addressing such questions. For example, PP transgenic mice that had overproduction of PP exhibited both decreased food intake and decreased gastric emptying.

The reason that central administration of PP stimulates feeding in several different species of experimental animals may be revealed in the future as PP receptors become better understood. Binding sites for PP have been found in several regions of the rat brain, including sites corresponding to brain regions regulating digestion and autonomic function. Binding sites for PP have been also found in the basolateral membranes of canine small intestinal mucosa as well as the ductus choledochus, duodenum, ileum, adrenal gland, and liver in the rat. In chickens, aPP binding was demonstrated in the spleen, liver, pancreas, gastrointestinal tract, and cerebellum.

Structure–activity studies with PP (bPP) using the canine intestinal mucosa model revealed the importance of the C-terminal tyrosinamide at position 36 for full receptor binding. Removal of this residue abolished receptor binding. Similarly, conversion of the native tyrosinamide to tyrosine also abolished receptor binding. When the carboxyl group was converted to a carboxymethyl form, the receptor binding was restored to 60% receptor recognition. Similar studies confirmed this work using a cloned hPP-preferring receptor, now known as the Y4 receptor, a G-protein-coupled receptor. These studies also demonstrated the importance of the N-terminal portion of PP for full receptor binding.

Future research on the PP-fold family of peptides, and on PP in particular, may reveal the true function of PP.

## Glossary

**neuropeptide Y** A 36-residue neuropeptide hormone with considerable homology to pancreatic polypeptide.

**pancreatic polypeptide** A 36-residue peptide hormone found predominantly in the pancreas.

**peptideYY** A 36-residue peptide hormone found predominantly in the gut; it shows homology to both neuropeptide Y and pancreatic polypeptide.

**PP cell** One of four endocrine cell types in pancreatic islets where pancreatic polypeptide is located. Also known as the F cell.

**PP-fold** Refers to the U-shaped structural fold assumed by members of the pancreatic polypeptide (PP) family (PP, peptide YY, and neuropeptide Y).

## See Also the Following Articles

**Appetite Regulation • Pancreastatin • Pancreastatin Receptor Signaling**

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# Parathyroid Hormone

ROBERT A. NISSENSON

*University of California, San Francisco and Veterans' Affairs Medical Center, San Francisco*

I. INTRODUCTION
II. BIOSYNTHESIS, SECRETION, AND CHEMISTRY OF PTH
III. PHYSIOLOGICAL ACTIONS OF PTH IN BONE
IV. PHYSIOLOGICAL ACTIONS OF PTH IN KIDNEY
V. MECHANISM OF ACTION OF PTH
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**Parathyroid hormone (PTH) is a polypeptide hormone that serves as a major regulator of plasma calcium. Increased circulating levels of PTH result in a compensating increase in ionized calcium in the blood by mobilizing calcium form the body's enormous calcium reserves in bone, by reducing renal calcium**

**loss, and by (indirectly) increasing the efficiency of absorption of dietary calcium by the intestine.**

## I. INTRODUCTION

Parathyroid hormone (PTH) is produced by tiny organs in the region adjacent to the thyroid glands in the neck. Humans generally have four functional parathyroid glands. In evolution, parathyroid glands first appear in terrestrial vertebrates, and their existence is closely linked to the need to maintain adequate levels of ionized calcium in blood. Ionized calcium in the extracellular fluid serves to support a range of essential physiological processes including proper neuromuscular function, exocrine and endocrine secretion, mineralization of bone, and cell growth and differentiation. With the evolution of amphibians, vertebrate life needed to adapt from seawater, which has a very high ambient level of calcium, to land, where calcium must be obtained from dietary sources. A complex endocrine homeostatic system evolved in terrestrial vertebrates in order to ensure maintenance of adequate levels of plasma calcium even under conditions of limited dietary calcium intake. The parathyroid glands play a pivotal role in this homeostatic system, since they are able to detect even small decreases in the level of ionized calcium in the blood and to respond by secreting PTH. This article presents an overview of the current understanding of the biochemistry, physiology, and mechanism of action of PTH as well as a discussion of the clinical diseases associated with abnormalities in this hormonal system.

## II. BIOSYNTHESIS, SECRETION, AND CHEMISTRY OF PTH

As mentioned above, the maintenance of adequate levels of plasma ionized calcium (1.0–1.3 m*M*) is required for normal neuromuscular function, bone mineralization, and many other physiological processes. The parathyroid gland secretes PTH in response to very small decrements in ionized calcium in the blood in order to maintain the normocalcemic state. PTH accomplishes this by promoting bone resorption and releasing calcium from the skeletal reservoir, by inducing renal conservation of calcium and excretion of phosphate, and by indirectly enhancing intestinal calcium absorption by increasing the renal production of the active vitamin D metabolite 1,25$(OH)_2$ vitamin D. The parathyroid gland functions in essence as a "calciostat," sensing the prevailing ionized calcium level in the blood and adjusting the secretion of PTH accordingly (Fig. 1). The relationship between ionized calcium and PTH secretion is a sigmoidal one, allowing significant changes in PTH secretion in response to very small changes in plasma ionized calcium.

In addition to providing acute regulation of PTH secretion, ionized calcium is a primary factor controlling chronic secretion of the hormone. Thus, sustained hypocalcemia promotes increased expression of the PTH gene and results in parathyroid hyperplasia. A common example of the latter is the marked parathyroid hyperplasia (secondary hyperparathyroidism) that frequently accompanies chronic renal failure. 1,25$(OH)_2$ Vitamin D also serves as a negative regulator of PTH gene expression and parathyroid cell hyperplasia. In chronic renal failure, both hypocalcemia and reduced circulating levels of 1,25$(OH)_2$ vitamin D presumably contribute to the progression of secondary hyperparathyroidism.

The plasma membrane of parathyroid cells contains high levels of a calcium-sensing receptor (CaR). Unlike intracellular calcium-binding proteins, which have an affinity for free calcium in the nanomolar range (consistent with intracellular levels of free calcium), the CaR binds calcium in the millimolar range. The receptor is a member of the G-protein-coupled receptor superfamily. It contains calcium-binding elements in its extracellular domain and signaling determinants in its cytoplasmic regions.

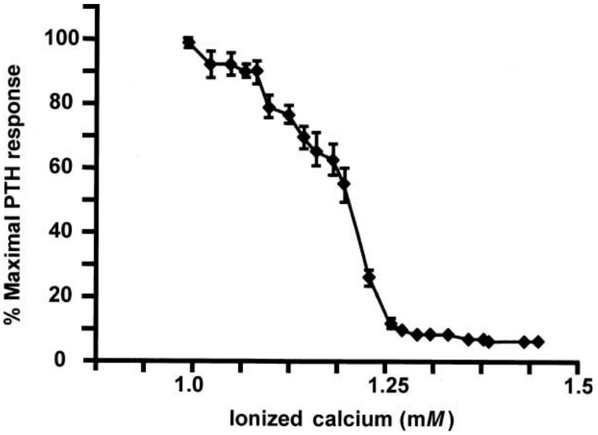

**FIGURE 1** Relationship between plasma levels of ionized calcium and the release of PTH(1–84) in normal humans. Variations in plasma ionized calcium were achieved by the infusion of calcium or EDTA. Note the sigmoidal relationship, ensuring significant changes in PTH secretion with small variations in ionized calcium.

Calcium binding to the receptor triggers activation of G-proteins that are able to suppress the synthesis and secretion of PTH. When ionized calcium in the blood falls, there is less signaling by the CaRs on the parathyroid cell and PTH secretion consequently increases. The essential role of the CaR can best be seen in humans bearing loss-of-function mutations in the CaR gene. In the heterozygous state, such mutations result in familial hypocalciuric hypercalcemia, characterized by inappropriately high levels of PTH secretion in the face of hypercalcemia. These individuals are quantitatively resistant to the suppressive effect of calcium on PTH secretion due to the reduced number of parathyroid CaRs. In the homozygous state, patients display a severe increase in PTH secretion with life-threatening hypercalcemia (neonatal severe primary hyperparathyroidism).

The initial translation product of the PTH gene is prepro-PTH. The N-terminal pre-sequence (signal peptide) of the protein facilitates transport across the membrane of endoplasmic reticulum and into the initial biosynthetic pathway where the pre-sequence is proteolytically removed. The amino-terminal pro-sequence is also required for the proper transport of the protein during biosynthesis, and this 6-amino-acid peptide is cleaved in the Golgi by a furin-like peptidase. The resulting product is the mature, 84-amino-acid secretory product of the parathyroid gland, PTH.

Although the biologically active, mature form of PTH is an 84-amino-acid molecule, the major actions of PTH on bone and kidney can be reproduced with synthetic peptides containing as few as the 34 amino acids of the amino-terminus of the protein. The functional importance of the midregion and carboxyl-terminal region of PTH remains unclear. Truncation of merely a single amino acid from the amino-terminus of PTH results in a dramatic loss of biological activity. Thus, the amino-terminus of the peptide appears to have a central role in the ability of PTH to activate its receptor on target cells in kidney and bone. The 1–34 sequence of PTH has been highly conserved throughout the evolution of terrestrial vertebrates, consistent with the important biological function of this region of the molecule. Interestingly, there is a second gene product that shares sequence similarity with the 1–34 region of PTH and is thus able to activate the PTH receptor. The factor is termed PTH-related protein (PTHrP), and its physiological role is to exert local control over the development and function of a number of tissues. For reasons that are unclear, PTHrP is expressed at very high levels by several types of cancers. With some cancers, circulating levels of PTHrP become high enough to cause excessive bone resorption and increased blood calcium due to the actions of circulating PTHrP on PTH receptors.

Early studies demonstrated that PTH circulates in multiple forms that can be distinguished by radioimmunoassays specific for different regions of the PTH molecule. PTH(1–84) is subject to metabolism within the parathyroid gland, resulting in the secretion of PTH fragments as well as the intact molecule. In addition, PTH(1–84) is metabolized in peripheral tissues. Midregion and carboxyl-terminal fragments of PTH have a much longer half-life in the circulation than does PTH(1–84). As a result, midregion and carboxyl-terminal fragments of PTH circulate at much higher concentrations than does intact PTH(1–84). Rapid plasma clearance of PTH is due primarily to hepatic metabolism, with a lesser contribution by the kidneys. Peripheral metabolism generates midregion and carboxyl-terminal fragments of PTH that resemble those secreted by the parathyroid gland. Midregion and carboxyl-terminal PTH fragments are cleared by renal excretion, and thus circulating levels of these fragments are highly dependent on renal function. Extremely high levels of PTH detected with antibodies against the midregion and carboxyl-terminal region of the hormone in many patients with end-stage renal disease thus reflect a combination of secondary hyperparathyroidism and reduced renal clearance of PTH fragments.

Midregion and carboxyl-terminal PTH fragments lack the amino-terminal 1–34 sequence of the hormone required for binding to PTH/PTHrP receptors and producing the classical effects of PTH on kidney and bone. Metabolism of PTH could produce biologically active, amino-terminal fragments of PTH, but there is little evidence for the presence of significant levels of amino-terminal PTH fragments in the circulation or for significant secretion of such fragments by the parathyroid gland. Presumably, both the parathyroid gland and the peripheral organs contain enzymes that degrade amino-terminal fragments of PTH. This ensures that circulating levels of biologically active PTH are derived exclusively from glandular secretion of PTH(1–84). A few studies have demonstrated potential biological effects of midregion or carboxyl-terminal fragments of PTH, and there is also evidence for the existence of membrane receptors for these fragments. However, the biological role of PTH fragments remains unclear.

## III. PHYSIOLOGICAL ACTIONS OF PTH IN BONE

The major physiological role of PTH is to mobilize calcium from bone in order to maintain an adequate level of plasma ionized calcium. In times of dietary calcium deficiency, blood levels of calcium fall slightly and this serves to increase the secretion of PTH by the parathyroid gland. PTH acts directly on bone to increase the number and activity of osteoclasts—the cells that promote bone resorption. During osteoclastic bone resorption, calcium-rich hydroxyapatite bone mineral is converted to soluble calcium, which is transported into the general extracellular space to support the level of blood calcium. PTH also increases the efficiency of transport of calcium from bone to blood.

PTH receptors have been localized to bone-forming osteoblasts and their precursors, but it is not clear whether osteoclasts possess PTH receptors. Indeed, PTH is not able to activate isolated osteoclasts *in vitro* unless osteoblast-like cells are also present. These findings suggest that PTH may produce its actions on osteoclasts indirectly, perhaps through direct interaction with cells of the osteoblast lineage. Indeed, PTH induces an increase in the expression of a molecule termed RANK ligand (RANKL) on the surface of osteoblasts (Fig. 2). Osteoclasts and their precursors (myeloid lineage cells) express the receptor for RANKL, a molecule termed RANK. When PTH-stimulated osteoblasts come into contact with osteoclasts or their precursors, RANKL interacts with RANK, resulting in signaling events that produce increased formation and activity of osteoclasts. Osteoblasts also produce an inhibitor of RANKL (termed osteoprotogerin or OPG). PTH inhibits the production of OPG, an effect that also promotes increased osteoclast formation and activity.

Although stimulation of bone resorption is the major physiological response of the skeleton to PTH, pharmacological experiments have shown that PTH is also capable of increasing bone formation. Thus, administration of PTH intermittently to animals or humans results in a marked anabolic response of the skeleton. High levels of PTH are known to produce an increase in the number of osteoblasts, which results in part from the coupling between increased osteoclastic resorption and new bone formation. However, intermittent treatment with low doses of PTH produces a direct positive effect on bone formation that is independent of preceding bone resorption. The cellular basis for this action of PTH is not fully understood but could result from an action of PTH to increase the number and/or functional activity of bone-forming osteoblasts. There is currently great interest in exploiting this action of PTH for the purpose of treating individuals with low-bone-mass diseases such as osteoporosis.

## IV. PHYSIOLOGICAL ACTIONS OF PTH IN KIDNEY

PTH produces a series of renal actions that help to ensure that calcium mobilized from bone contributes optimally for the maintenance of plasma ionized calcium levels. The renal actions of PTH include inhibition of renal phosphate reabsorption, stimulation of renal calcium reabsorption, and increased production of 1,25(OH)$_2$ vitamin D. The ability of PTH to inhibit renal phosphate reabsorption has been known for many years, providing the basis for

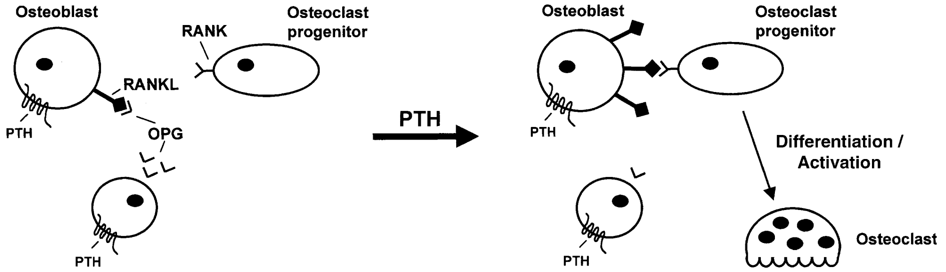

FIGURE 2 Regulation of osteoclast differentiation and activation by PTH. Binding of PTH by receptors on osteoblasts results in increased expression of osteoprotegerin ligand (RANKL) on the cell surface. Activation of PTH receptor can also reduce the secretion of the RANKL inhibitor osteoprotegerin (OPG), which is produced by cells in the bone microenvironment. These effects of PTH promote the action of RANKL on its receptor (RANK) on the surface of osteoclast precursors and mature osteoclasts. RANK signaling, together with the action of macrophage colony-stimulating factor, stimulates the differentiation of osteoclast precursors and promotes the activation of mature osteoclasts.

a clinical test of renal responsiveness to the hormone. Patients with primary hyperparathyroidism display hypophosphatemia and decreased renal tubular reabsorption of phosphate, whereas hypoparathyroid patients are hyperphosphatemic and have increased phosphate reabsorption. Phosphate forms a complex with free calcium in blood. Thus, for a given level of serum calcium, ionized calcium will be reduced as serum phosphate increases. Under conditions of relative hypocalcemia (e.g., during chronic dietary calcium deficiency), PTH secretion is increased, resulting in increased bone resorption. Both calcium and phosphate are released from hydroxyapatite during the process of bone resorption. By promoting renal excretion of phosphate, PTH facilitates a rise in ionized and total plasma calcium. Recently, the molecular basis for PTH-induced inhibition of renal phosphate reabsorption was clarified. PTH inhibits the expression of a specific (type IIa) sodium–phosphate co-transporter in the brush border of proximal renal tubular cells. This results in a reduced $V_{\max}$ for phosphate transport and therefore lower efficiency of proximal phosphate reabsorption.

PTH also acts to increase renal calcium reabsorption, thus ensuring that only small amounts of calcium released during PTH-induced bone resorption are lost via renal excretion. The major sites for this effect of PTH are in the distal convoluted tubule and the thick ascending limb of Henle's loop. Recent evidence indicates that distal renal tubular calcium reabsorption is an active process that requires calcium influx through dihydropyridine-sensitive calcium channels located in the apical plasma membrane. Drugs that inhibit these channels are effective in blocking PTH-induced renal calcium reabsorption. Unlike voltage-sensitive calcium channels in excitable tissues, PTH-responsive calcium channels in the distal nephron are activated by membrane hyperpolarization. PTH appears to open calcium channels by inducing hyperpolarization of the apical plasma membrane. Calcium entering the distal renal tubular cell in this manner is transported into the extracellular compartment via a sodium–calcium exchanger present on the basolateral plasma membrane.

PTH promotes intestinal calcium reabsorption indirectly, through an action to increase circulating levels of $1,25(OH)_2$ vitamin D. This vitamin D metabolite acts directly on intestinal epithelial cells to increase the efficiency of calcium (and phosphate) absorption. Primary hyperparathyroidism is commonly associated with increased circulating levels of $1,25(OH)_2$ vitamin D, whereas reduced levels of this metabolite are present in hypoparathyroidism. PTH produces this effect by increasing the rate of production of $1,25(OH)_2$ vitamin D through activation of the 25(OH) vitamin D-1-hydroxylase enzyme located in the proximal renal tubule. Studies *in vivo* as well as in cultured renal cell lines indicate that PTH increases the expression of the 25(OH) vitamin D-1-hydroxylase gene through a transcriptional mechanism.

The actions of PTH to promote increased bone resorption, reduced calcium excretion, and (indirectly) increased intestinal calcium absorption all help to maintain adequate levels of plasma calcium even under conditions of dietary calcium deficiency.

## V. MECHANISM OF ACTION OF PTH

PTH action is initiated by the binding of the hormone to a G-protein-coupled receptor on the surface of target cells in kidney and bone (Fig. 3). The major G-protein activated by the PTH receptor is $G_s$, the G-protein coupled to activation of adenylyl cyclase and production of cyclic AMP. Indeed, shortly after the discovery of the cyclic AMP signaling pathway, it was found that PTH is capable of increasing the levels of cyclic AMP in target cells through activation of the adenylyl cyclase. Cyclic AMP is a second messenger in the cellular action of a wide variety of hormones and other extracellular regulatory molecules. It activates cyclic AMP-dependent protein kinase A (PKA), which in turns phosphorylates and thereby regulates key proteins that participate in physiological responses. Very little is known about the identity of substrates of PKA that are phosphorylated in response to PTH receptor activation. These presumably include transcription factors, ion channels, transporters, and enzymes involved in cellular metabolism.

The important role of the cyclic AMP pathway in mediating PTH action is underscored by the ability of cyclic AMP analogues (or drugs that directly activate adenylyl cyclase such as forskolin) to reproduce many of the biological effects of PTH. For example, cyclic AMP analogues and forskolin are capable of reducing the expression of the renal type IIa sodium–phosphate co-transporter. The also promote an increase in the expression of RANKL in osteoblasts. Furthermore, genetic deficiency in the α-subunit of $G_s$ (as seen in the human disease pseudohypoparathyrodism type Ia) results in renal and skeletal resistance to PTH action.

PTH receptors also couple to the G-protein $G_q$, which activates phospholipase C, an enzyme that

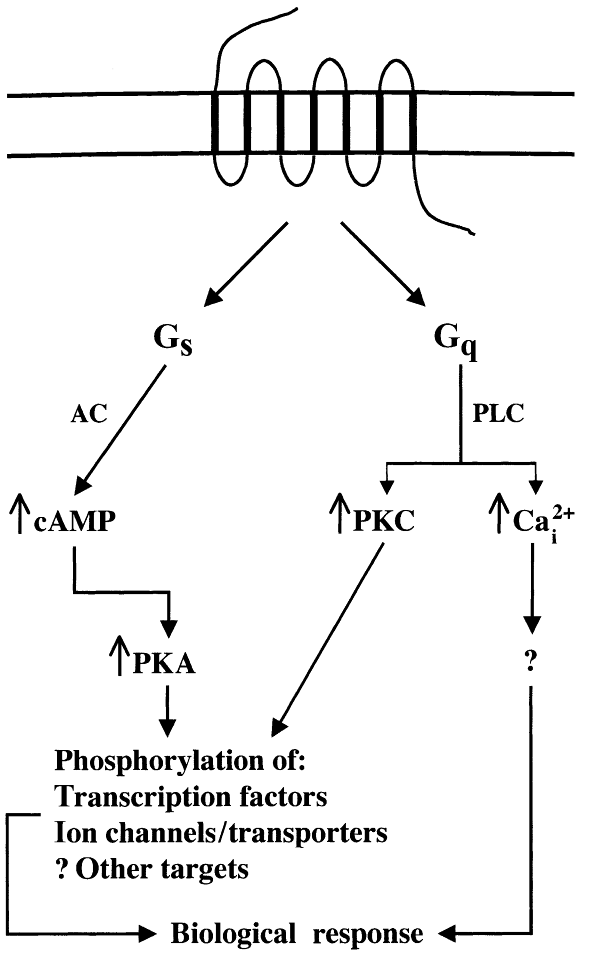

FIGURE 3 Signal transduction by the PTH/PTHrP receptor. PTH and PTHrP bind to determinants in the extracellular domain and in the body of the receptor. This leads to conformational changes in the transmembrane helices and consequent structural changes in the cytoplasmic domain. The latter permit productive interactions between the receptor and the G-proteins $G_s$ and $G_q$, activating the adenylyl cyclase (AC) and phospholipase C (PLC) signaling pathways, respectively. These pathways are thought to cooperate in determining the cellular response to the receptor activation. Most available evidence supports a primary role of the cyclic AMP/protein kinase A (PKA) pathway in mediating the biological effects of PTH/PTHrP receptor activation, with the PLC pathway playing a modulatory role. PKC, protein kinase C.

hydrolyzes the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol (DAG) and soluble inositol 1,4,5-trisphosphate ($IP_3$). DAG and $IP_3$ function as second messengers—the former by activating protein kinase C and the latter by binding to and opening calcium channels on the membrane of the endoplasmic reticulum, thereby increasing cytosolic free calcium. There is currently great interest in understanding how this pathway contributes to the physiological effects of PTH in bone and kidney.

There has been great interest in understanding how the structure of the PTH receptor contributes to ligand binding and signal transduction. The PTH receptor is a member of the large superfamily of G-protein-coupled receptors. All members are intrinsic plasma membrane proteins with seven membrane-spanning segments that line a central cavity. Ligands (PTH and PTHrP) bind to the large N-terminal extracellular domain of the receptor as well as to the extracellular loops. This facilitates the interaction of the ligand with the transmembrane cavity of the receptor, producing a conformational change in the receptor. In the ligand-bound state, the receptor interacts with and activates its cognate G-proteins on the cytoplasmic surface of the plasma membrane. Many features of this activation mechanism are shared by other (so-called class II) G-protein-coupled receptors that are related to the PTH receptor (e.g., receptors for glucagon, calcitonin, and secretin).

As with most G-protein-coupled receptors, signal transduction by the PTH receptor is under strict regulatory control. After an initial burst of signaling, agonist-occupied PTH receptors lose their signaling capacity. This desensitization results from the phosphorylation of the active PTH receptor by a G-protein-coupled receptor kinase. This phosphorylation event promotes the interaction of the receptor with a cytoplasmic protein called arrestin. When arrestin binds to the PTH receptor, interaction with G-proteins is sterically inhibited and thus signaling is prevented. The desensitized PTH receptor is internalized via clathrin-coated pits and is recycled back to the plasma membrane. It is likely that dephosphorylation of the PTH receptor occurs within endocytic vesicles, allowing resensitization of the receptor prior to its reinsertion in the plasma membrane.

## VI. DISEASE STATES

The most common diseases of the PTH endocrine system result from excessive production of ligands for the PTH receptor. Primary hyperparathyroidism most commonly results from a benign tumor affecting one of the four parathyroid glands. Occasionally, all four glands are enlarged. Parathyroid cancer can also occur but is extremely rare. Excessive secretion of PTH results in increased bone resorption, increased renal reabsorption of calcium, and increased production of $1,25(OH)_2$ vitamin D (and thus increased intestinal calcium absorption). These combined actions result in hypercalcemia and (over time) bone loss due to hyperresorption. A similar syndrome occurs in patients with malignant (nonparathyroid) tumors that secrete large amounts of PTHrP. Optimal treatment for these disorders is the removal of the parathyroid tumor or malignancy responsible for producing excessive PTH or PTHrP, respectively. When surgical treatment is not possible, the hypercalcemia can be treated by the administration of drugs that inhibit bone resorption such as bisphosphonate compounds.

Secondary hyperparathyroidism can occur as a result of conditions that produce chronic hypocalcemia such as chronic renal failure. Hypocalcemia has both a direct stimulatory effect on PTH secretion and an indirect effect by promoting the increased proliferation of parathyroid cells. Over time, chronic hypocalcemia produces a massive increase in the size of the parathyroid glands due to hyperplasia. This results in very high circulating levels of PTH and excessive bone resorption, often resulting in bone pain and fragility. Treatment of this disorder is targeted toward restoring the plasma calcium level and thereby removing the stimulus for further proliferation of parathyroid cells. $1,25(OH)_2$ vitamin D is often used therapeutically, since it promotes an increase in intestinal absorption of calcium and thus helps to suppress PTH secretion and parathyroid cell growth.

Although much less common, genetic mutations in components of the PTH receptor signaling pathway are also known to be associated with human diseases. Certain mutations in the PTH receptor are known to result in constitutive (i.e., hormone-independent) receptor signaling. Individuals harboring such a mutation display evidence of PTH receptor hyperfunction, including hypercalcemia and increased bone resorption. They also display premature cartilage differentiation reflecting an exaggeration in the normal physiological action of PTHrP (acting through the PTH receptor) on chondrogenesis. This disorder is known as Jansen's metaphyseal chondrodysplasia. Complete loss of the PTH receptor results in Blomstrand's lethal chondrodysplasia due to the generalized failure in the proper development of endochondral bones. Finally, partial loss of the function of the G-protein ($G_s$) that couples receptors to adenylyl cyclase results in a disorder known as pseudohypoparathyroidism type Ia. These individuals show partial resistance to a number of hormones that utilize the $G_s$/adenylyl cyclase signaling pathway. Resistance to PTH action is often particularly severe due to genetic imprinting of the $G_{s\alpha}$ gene in PTH-responsive tissue. These individuals also have a developmental phenotype (Albright's hereditary osteodystrophy) as a consequence of a deficiency in $G_s$ signaling during embryogenesis and early development.

## Glossary

**adenylyl cyclase** The enzyme responsible for converting ATP to the intracellular second messenger cyclic AMP.

**bone mineralization** The process by which calcium and phosphate are deposited onto the extracellular matrix of bone, resulting in the formation of hydroxyapatite.

**G-proteins** A family of peripheral membrane proteins that couple membrane receptors to specific effector molecules such as adenylyl cyclase.

**hyperplasia** Increase in the size of an organ due to excessive proliferation of the constituent cells.

**osteoblasts** Cells responsible for promoting bone formation by secreting bone matrix proteins and by facilitating bone mineralization.

**osteoclasts** Multinucleated cells that attach to the mineralized surface of bone and secrete acid and proteases, resulting in the breakdown of bone (bone resorption).

**osteoporosis** A disorder of bone characterized by decreased bone mass and decreased strength.

## See Also the Following Articles

**Calcium Signaling • GPCR (G-Protein-Coupled Receptor) Structure • Humoral Hypercalcemia of Malignancy • Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Parathyroid Hormone-Related Protein (PTHrP) • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone**

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# Parathyroid Hormone-Related Protein (PTHrP)

Joshua N. Van Houten and John J. Wysolmerski
*Yale University School of Medicine*

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**Parathyroid hormone-related protein (PTHrP) is a peptide with the ability to act as a systemic hormone or a local growth factor. It is involved in a wide variety of seemingly disparate functions in development and physiology and it is a key contributor to the pathophysiology of human cancer. In this article, the structures of PTHrP and its gene are characterized and the roles that PTHrP plays in cancer, embryonic development, and reproduction are examined.**

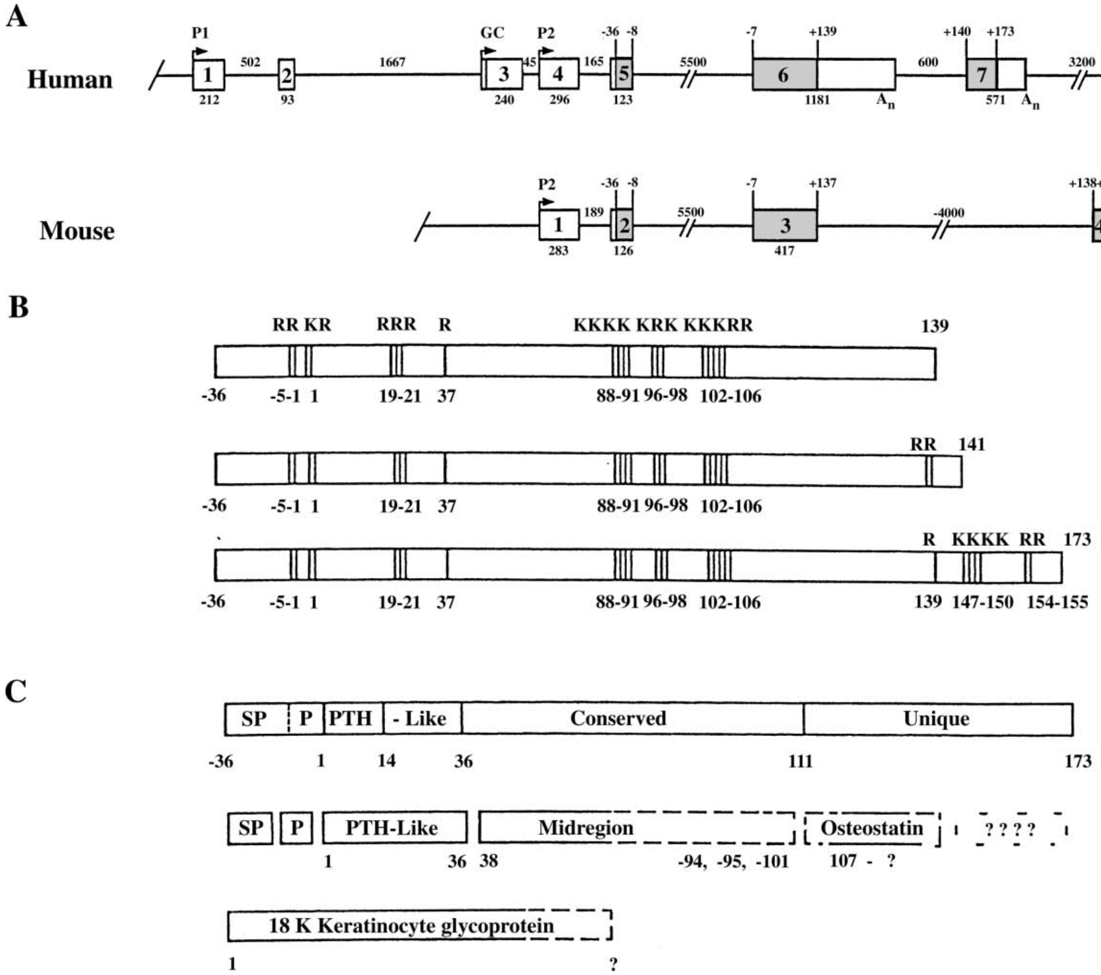
## I. INTRODUCTION

The isolation of parathyroid hormone-related protein (PTHrP) was the fruit of over 30 years of efforts aimed at understanding the mechanisms underlying the humoral form of hypercalcemia in patients with malignancy. In the 1940s, Fuller Albright predicted that the cause of hypercalcemia in these patients would be a tumor-derived factor related to the calciotropic peptide, parathyroid hormone (PTH). This prediction proved correct when, in the 1980s, PTHrP was isolated concurrently in three laboratories. By the 1990s, the human, mouse, rat, and chicken PTHrP genes had been cloned, and since then the PTHrP gene has also been isolated from a teleost (*Fugu rubripes*) and the sea bream, *Sparus aurata*. Over the past decade, our understanding of the biology of PTHrP has exploded through the study of a variety of transgenic and knockout mouse models. This article will first summarize key aspects of the structure of PTHrP and its gene. Then PTHrP's role in cancer will be discussed. Finally, our new understanding of the biology and comparative physiology of this remarkably versatile protein will be examined.

## II. THE PTHrP GENE

As its name suggests, PTHrP is related to parathyroid hormone. The modern versions of these two genes arose from a common ancestor as the result of a tetraploidization event some 200 to 300 million years ago. Several lines of evidence now suggest that, in fact, the PTH gene was derived from an ancestral PTHrP gene, perhaps in response to the new demands of calcium metabolism related to the development of a bony skeleton in fish or to the adaptation of amphibians to a terrestrial environment. Although the two genes have diverged considerably, they share common locations on paired chromosomes, a common genomic organization, and amino-terminal sequence homology, all of which point to their common ancestry. For practical purposes, the last feature is the most important shared trait, for the similar amino-termini of both peptides allows them to bind to and signal through a shared PTH/PTHrP receptor. This unusual arrangement is responsible for the clinical syndrome that led to the discovery of PTHrP (see below).

The human PTHrP gene is located on the short arm of chromosome 12 and is flanked by the genes for lactate dehydrogenase B and the K-ras proto-oncogene. It spans more than 15 kilobases and contains eight exons. It has three different promoters, one that is GC-rich and two that contain typical TATA sequences. There are four noncoding exons at the 5′-end of the gene (Fig. 1) and there is considerable alternative splicing to generate a series of mRNAs with different 5′-ends. In addition, there is alternative splicing at the 3′-end of the gene that gives rise to three distinct classes of transcripts encoding peptides of different lengths [139, 141, or 173 amino acids (aa)]. Thus, there are a variety of different mRNAs for PTHrP that differ in both their 5′- and 3′-ends. However, the physiological significance of these different mRNA species remains obscure. This complexity is a relatively recent event in the evolution of the gene, as the rodent genes are much simpler and contain only one promoter and use only one 3′-terminus. Transcriptional control of the PTHrP gene will not be discussed in this article. Interested readers are referred to recent comprehensive reviews.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1 36 38 -94, -95, -101 107 - ?
18 K Keratinocyte glycoprotein
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**FIGURE 1** Schematic of the human and mouse PTHrP genes. (A) Numbered boxes represent exons, with distances indicated in base pairs. Arrows denote promoters, and amino acid residues are numbered relative to the start of the mature peptide. Polyadenylation sites are marked $A_n$. Potential multibasic cleavage sites in the three human PTHrP isoforms. (B) Arginine (R) and lysine (K) residues are marked. Functional domains and sequence homologies in PTHrP. (C) Intracellular posttranslational processing removes the signal peptide (SP) and the propeptide (P). The first 36 amino acids constitute the N-terminal PTH-like peptide, reflecting the strong sequence similarity in amino acids 1–13 between PTH and PTHrP. The region between residues 36 and 111 is evolutionarily conserved, whereas the least conserved region of the peptide is the C-terminus (amino acids 111 to 139 or 141). The extreme C-terminus, residues beyond 141, has been found only in humans. Secreted products of PTHrP include the amino-terminal PTH-like fragment (1–36), the glycosylated amino-terminal fragment found in keratinocytes, and midregion peptides. C-terminal species remain ill-defined.

## III. PTHrP STRUCTURE AND POSTTRANSLATIONAL PROCESSING

The primary transcript of PTHrP represents a polyprotein analogous to the primary transcript of the proopiomelanocortin gene. Therefore, most cells expressing the PTHrP gene actually secrete several different PTHrP peptides. The primary transcript has a typical prepro sequence encompassing amino acid residues $-36$ to $-1$, which confers the ability for secretion from the cell. The mature peptide is very well conserved across species with residues 1–111 being 98% homologous in chickens compared with humans. This area has several stretches of basic amino acids that allow proteolyic cleavage of the primary transcript by prohormone convertases within the Golgi and secretory granules to generate a series of peptides encompassing the amino-terminus, the midregion, and the carboxy-terminus of PTHrP.

The amino-terminus of PTHrP is the portion that is homologous to PTH. Of the first 13 aa of the two proteins, 8 are identical and 3 represent conservative changes. Furthermore, there is considerable similarity in the predicted conformation of the two peptides through residue 34. There are several species containing this amino-terminal region that appear to be secreted by various cells. Most cells produce PTHrP 1–36, which is equipotent with PTH 1–34 at binding and activating the type I PTH/PTHrP receptor (PTH1R). In addition, an "intact" PTHrP molecule that includes at least the first 74 aa circulates in patients with HHM. Finally, keratinocytes have been reported to make a glycosylated amino-terminal version of PTHrP with a molecular weight of 18 kDa.

Various cells have also been shown to produce midregion peptides that begin at Ala-38 and stretch to amino acids 94, 95, or 101. These peptides have been shown to circulate and their secretion appears to be regulated. This portion of PTHrP appears to function to facilitate placental calcium transport from mother to fetus. However, the receptor for this portion of PTHrP has not been identified. The midregion of the molecule also contains nuclear localization sequences (NLSs) and there is a growing body of literature that suggests that nuclear-targeted PTHrP may have effects on cellular proliferation, differentiation, and apoptosis. At this point, it is unclear whether the midregion portions that are secreted versus targeted to the nucleus are the same or represent distinct peptides, as there are potential processing sites between Ala-38 and the NLS between amino acids 87 and 106. Interestingly, in this regard, recent data have suggested that initiation of PTHrP translation may occur downstream of the signal peptide to generate a nonsecreted form of PTHrP. Other evidence suggests that longer peptides containing both the amino-terminal and the NLS sequences may be imported to the nucleus after binding to cell surface PTH1R. Obviously, there is much information concerning this portion of the molecule that remains to be elucidated.

Finally, carboxy-terminal portions of PTHrP also appear to circulate and have been detected in the urine of normal patients and in the serum of patients with renal failure. Peptides encompassing residues 107–111 and 107–139 have been shown to inhibit bone resorption *in vitro* and this portion of the molecule has been dubbed "osteostatin." However, not all groups have demonstrated this effect and the biological significance of carboxy-terminal PTHrP remains undefined.

## IV. PTHrP RECEPTORS

As noted previously, the amino-terminal portion of PTHrP can use the type I PTH/PTHrP receptor (PTH1R) for signal transduction. This receptor does not discriminate between PTHrP and PTH, and both peptides are equipotent at binding and activating signaling. The PTH1R is a seven-transmembrane-spanning, G-protein-coupled receptor that signals via the cyclic AMP (cAMP)/protein kinase A and protein kinase C/calcium transient pathways. The ability of PTH and PTHrP to use the same receptor is the reason that patients with cancers that secrete PTHrP into the circulation develop hypercalcemia. Under physiologic conditions, PTHrP is excluded from the circulation and does not interact with PTH1Rs in bone and kidney meant for PTH. PTHrP target tissues appear to have a lower receptor density and rely on high local concentrations of PTHrP for activation of signaling. The exceptions to this rule may be in the setting of tooth eruption and during lactation. In these cases, PTHrP appears to be involved in the physiologic regulation of bone resorption and thus its action mimics that of PTH. These functions may represent the evolutionary pressure that has preserved the ability of both peptides to use the same receptor. With the exception of placental calcium transport, all the currently well-defined biological effects of PTHrP are mediated through the PTH1R. There are undoubtedly other receptors for the non-amino-terminal portions of PTHrP. However, they have not yet been characterized.

## V. PTHrP IN CANCER

### A. Humoral Hypercalcemia of Malignancy

Humoral hypercalcemia of malignancy (HHM) is a paraneoplastic syndrome defined by elevated calcium levels in patients with malignancy, but no bone metastases. It is the most common metabolic complication of cancer and results from increased bone resorption and decreased calcium excretion by the kidneys. As noted above, PTHrP was isolated from tumors causing this syndrome and several lines of evidence have established that tumor-derived PTHrP is the cause of the perturbations in calcium metabolism in this syndrome. For example, removal of the tumor or administration of neutralizing antibodies against PTHrP have been shown to reverse the syndrome, whereas infusion of PTHrP into rodents or humans reproduces the syndrome. Since PTH and PTHrP share a common receptor, this syndrome is the result of tumor-derived PTHrP acting on the population of PTH1R in bone and kidney normally reserved for the actions of PTH. Therefore, in HHM the physiological autocrine/paracrine signaling molecule, PTHrP, becomes an endocrine hormone and mimics the pathologic effects of PTH as seen in hyperparathyroidism. Although many types of solid tumors and hematologic malignancies are associated with HHM, squamous cell carcinomas and urothelial malignancies are the most frequent cause.

### B. Bone Metastasis

In addition to its role in HHM, PTHrP appears to be important in the development of bone metastases in breast cancer. Several studies have found that, in breast cancer patients, PTHrP is expressed more frequently in bone metastases than in metastases to other sites. Initially, it was thought that PTHrP might be involved in targeting cancer cells to bone and, therefore, that PTHrP expression by a primary tumor might predict the occurrence of bone metastases. However, a recent study of transgenic overexpression of PTHrP in a rodent model of breast cancer did not support this idea. Furthermore, in the only large prospective study performed to date, PTHrP expression within the primary breast tumor did not correlate with the presence of bone metastases. However, there is ample experimental evidence demonstrating that manipulation of PTHrP expression within breast cancer cells can alter the development of osteolytic bone lesions. Currently, it is thought that although PTHrP production in the primary tumor may not predict bone metastases, the ability of breast cancer cells to up-regulate PTHrP production in the bone microenvironment is critical. Guise and colleagues have suggested that tumor-derived PTHrP production stimulates bone resorption, which liberates growth factors from the bone matrix that stimulate tumor cell proliferation, further PTHrP production, and increase bone resorption. It appears that bone matrix-derived transforming growth factor-β (TGF-β) is particularly important in up-regulating PTHrP production by breast cancer cells in bone. If this construct proves to be correct, the TGF-β/PTHrP/PTH1R loop may become an attractive therapeutic target for the treatment or prevention of bone metastases in breast cancer.

### C. Proliferation

Another proposed role for PTHrP in tumorigenesis is the regulation of proliferation. PTHrP has been reported to have variable effects on the proliferation and/or differentiation of a variety of cultured cell lines. These effects have generally been modest and there is no existing evidence to suggest that PTHrP has a dominant role in regulating the proliferation of cancer cells. However, it was recently found that transgenic overexpression of PTHrP in the mammary gland resulted in a shorter latency to tumor development and a higher overall incidence of mammary tumor development in mice exposed to a chemical carcinogen. Interestingly, recent experiments have also suggested that the intracellular trafficking of PTHrP may determine whether it regulates the growth of breast cancer cells negatively or positively. For example, overexpression of PTHrP in the MCF-7 breast cancer cell line was reported to be mitogenic, whereas adding PTHrP to the extracellular medium inhibited growth. Another group of investigators has reported that neutralization of the endogenous PTHrP produced by MCF-7 cells led to an acceleration of their growth rate in culture. More data, especially from experiments *in vivo*, will be needed to determine whether PTHrP participates in the regulation of breast tumor growth in a meaningful manner.

## VI. PTHrP IN EMBRYONIC DEVELOPMENT

PTHrP is expressed early in development in all three embryonic cell lineages as well as the amnion and the trophoblast. In fact, it has been reported that PTHrP and the PTH1R are the earliest hormone/receptor pair to appear, being first detectable at the morula stage. During the later stages of development, the distribution of PTHrP expression is even broader than in the adult, suggesting its importance as a developmental regulatory molecule. Functionally, in early development, PTHrP appears to participate in the regulation of the differentiation of parietal endoderm from primitive endoderm. During organogenesis, paracrine signaling between PTHrP and the PTH1R appears to be involved in inductive tissue interactions in several organs. The role that PTHrP plays in skeletal development and mammary gland development is discussed below because these systems have been well characterized. However, PTHrP is also necessary for tooth eruption and may be important in the development of the skin, the lung, the heart, and other organs.

## A. Skeletal Development

Genetic ablation of PTHrP in mice causes perinatal lethality and short-limbed dwarfism. Upon histological examination, PTHrP null mice exhibit a form of chondrodysplasia in which chondrocytes within the growth plates of endochondral bones differentiate prematurely, leading to early mineralization of endochondral bones and premature loss of linear growth capacity. In contrast, PTHrP overexpression targeted to chondrocytes with a collagen II promoter slows chondrocyte differentiation and inhibits apoptosis of hypertrophic chondrocytes, leading to a delay in the formation of "mature" bone. Thus, the overall effect of PTHrP on endochondral bone formation is to inhibit the program of chondrocyte differentiation and to preserve the proliferative (growth) capacity of the growth plate. These effects are mediated through the PTH1R, since PPR1 null mice phenocopy PTHrP null mice. In humans, Blomstrand's chondrodysplasia, a short-limbed dwarfism similar to that seen in PTHrP and PTH1R null mice, is caused by loss-of-function mutations in the PTH1R gene. Another human disorder, Jansen's metaphyseal chondrodysplasia, results from activating mutations in the PTH1R gene and is similar to the phenotype observed in the collagen II–PTHrP transgenic mice.

The regulation of chondrocyte proliferation and differentiation within the growth plate is complicated and relies on the concerted actions of several families of growth factors and their receptors. It is now known that PTHrP acts together with at least one other of these growth factors, Indian hedgehog (IHH), in a feedback loop to regulate the differentiation of chondrocytes (Fig. 2). PTHrP is expressed in the periarticular region, and the PTH1R is expressed in prehypertrophic chondrocytes. IHH is expressed in the transition zone where proliferating chondrocytes become prehypertrophic chondrocytes. Like PTHrP, IHH activity slows the differentiation of chondrocytes in bone explants from wild-type mice, but not in explants from PTH1R null mice. Therefore, IHH from prehypertrophic chondrocytes is thought to act on the perichondrium (possibly through smoothened, patched, and/or hedgehog-interacting protein), to stimulate PTHrP production in the periarticular region, which, in turn, inhibits further procession of the chondrocytes down the differentiation pathway toward hypertrophy and apoptosis.

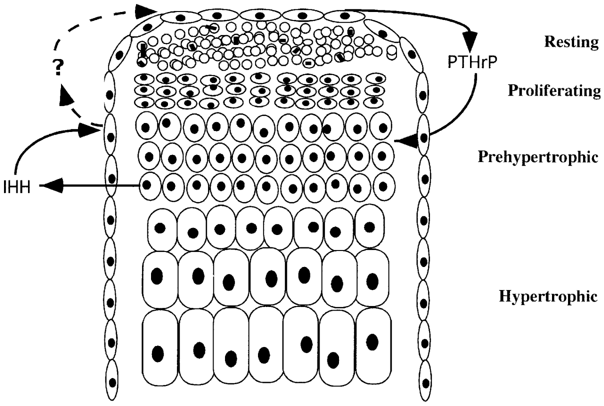

FIGURE 2 Schematic representation of the proposed interactions of PTHrP and Indian hedgehog (IHH) during chondrocyte differentiation in a growth plate. IHH produced by prehypertrophic chondrocytes in the lower zone acts on the adjacent perichondrium to communicate with the PTHrP-producing cells. PTHrP, in turn, acts on PTHR1-expressing prehypertrophic chondrocytes to impair their further differentiation by up-regulating Bcl-2 expression.

## B. Mammary Gland Development

PTHrP signaling is necessary for the formation of the mammary gland in mouse and human embryos. PTHrP and PTH1R knockout mice, as well as human fetuses with Blomstrand's chondrodysplasia, lack mammary glands and nipples. The first step in the formation of embryonic mammary glands is the formation of an epithelial bud, which is surrounded by a specialized stroma or mesenchyme. During the development of the mammary buds in mice and humans, PTHrP is produced by epithelial cells within the mammary bud, and the PTH1R is expressed by the surrounding mesenchymal cells. Like many developing organs, the mammary glands rely on a constant conversation between epithelial and mesenchymal cells to coordinate proper morphogenesis.

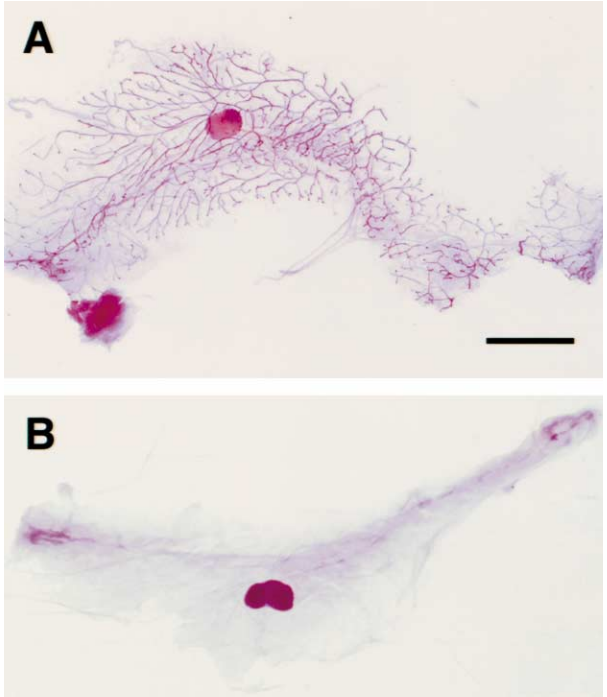

FIGURE 3 Whole-mount analysis of mammary glands from PTHrP null mice rescued from perinatal lethality by targeted expression of PTHrP in chondrocytes (col II-PTHrP/PTHrP null mice) and normal littermates. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In contrast, the col II-PTHrP/PTHrP null gland (B) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. These results show that PTHrP is required for the development of mammary epithelium. Scale bar represents 5 mm.

PTHrP participates in this conversation by acting as a message from the mammary epithelial cells that triggers mammary-specific differentiation of the mesenchymal cells. If this message is not received, as in the PTHrP or PTH1R knockout mice, the mesenchyme becomes dermis instead of mammary stroma, the mammary epithelial cells regress, and no further breast development occurs (Fig. 3).

## VII. PTHrP IN REPRODUCTION

### A. Uterus

In the rat uterus, myometrial PTHrP mRNA levels rise during pregnancy from very low levels in virgin rats to a peak right around the time of birth. Levels then decrease significantly within 4 h after parturition, and by 4 days postpartum, PTHrP mRNA levels have returned to baseline virgin levels. It appears that this profile of expression is related to stretching of the myometrium and not to hormonal changes of pregnancy, as it can be reproduced in virgin rats using mechanical stimuli. However, it has also been reported that uterine PTHrP mRNA expression is also up-regulated by estrogen. Functionally, PTHrP and PTH have been shown to induce uterine smooth muscle relaxation, indicating that this effect is mediated by PPR1 signaling. Therefore, PTHrP expression induced and maintained by some combination of mechanical stretch and estrogen during gestation could serve to prevent premature parturition by inducing uterine relaxation or by antagonizing contractions, allowing the uterus to expand and accommodate the growing fetus. Similar observations in the bladder, stomach, and egg-laying gland of the chicken have suggested that PTHrP might play a more general role in regulating the expansion of hollow, muscular organs.

### B. Placenta

There is an uphill gradient of calcium from mother (approximately 9.5 mg/dl) to fetus (approximately 13 mg/dl) that is maintained through the actions of a placental calcium ATPase. Recent data have demonstrated that PTHrP acts to facilitate the placental transport of calcium from mother to fetus. Interestingly, this function of PTHrP appears to be mediated by the midregion portion of the peptide and not by the N-terminal region. In a fetal parathyroidectomized lamb model, midregion PTHrP, but not amino-terminal PTHrP or PTH, was shown to facilitate placental calcium transport. In mice, the maternal–fetal calcium gradient depends upon an intact PTHrP gene, and midregion PTHrP stimulates placental calcium transfer. Thus, there is a considerable body of evidence that now supports a role for midregion PTHrP in regulating placental calcium transport from mother to fetus. However, the nature of the receptor for this portion of PTHrP and the mechanisms by which it stimulates calcium transport remain obscure.

### C. Mammary Gland—Lactation

Not long after PTHrP was discovered, several laboratories found it to be expressed in lactating mammary tissue and secreted into milk in large quantities. In fact, milk is the most abundant source of PTHrP in nature. The expression of the PTHrP gene is up-regulated at the beginning of lactation, and PTHrP mRNA is localized to the epithelial cells of the lobuloalveolar units. Expression levels during lactation have been shown to be modulated by systemic prolactin and local signals initiated by suckling.

Great quantities of calcium are secreted in milk each day and large increases in bone resorption and decreases in bone mass occur during lactation, presumably to mobilize calcium from the maternal skeleton to meet the demands for milk production. Given PTHrP's ability to mimic the actions of PTH on calcium metabolism, it has been suggested that PTHrP might participate in this mobilization of skeletal calcium stores. In order for this to be the case, PTHrP from the breast would need to enter the systemic circulation and act on the maternal skeleton. Although not all studies have concurred, the weight of evidence suggests that circulating PTHrP levels are elevated during lactation. Furthermore, it has been reported that circulating PTHrP levels during lactation correlate with increases in rates of bone turnover and decreases in bone density. Case reports suggest that elevated PTHrP may compensate for the lack of PTH in lactating women with hypoparathyroidism. In addition, studies in rats and cows have correlated the suckling-induced increase in mammary PTHrP with a rise in cAMP and phosphate in urine, possibly indicating systemic effects of PTHrP produced in the mammary gland. However, in the only study that attempted to manipulate PTHrP levels during lactation, passive immunization with anti-PTHrP antibodies did not alter calcium metabolism in lactating mice. Thus, although there is considerable evidence to support an endocrine role for mammary gland-derived PTHrP in lactation, the data are not conclusive. Alternative roles for PTHrP produced by the lactating mammary gland include the regulation of blood flow to the mammary gland or the regulation of gut development or calcium homeostasis in neonates.

## VIII. OTHER ACTIONS OF PTHrP

### A. Pancreas

PTHrP is expressed in all pancreatic islet cell types and in pancreatic ductular epithelium, but not in adult exocrine cells. Although the PTH1R has not been demonstrated on beta cells, N-terminal PTHrP (or PTH) acts on cultured beta cells to increase intracellular free calcium. This might indicate an alternative PTH/PTHrP receptor. The precise role of PTHrP in the pancreas is not well understood; however, data from transgenic mice expressing PTHrP under control of the rat insulin II promoter (RIP–PTHrP mice) suggest that PTHrP might regulate beta-cell mass. The RIP–PTHrP mice demonstrate islet hyperplasia, due to both an increase in the number of islets and their size. As a result, these mice are hyperinsulinemic and hypoglycemic, conditions that become acutely symptomatic upon fasting. Interestingly, RIP–PTHrP mice are also resistant to the beta-cell-toxic effect of streptozotocin. How these findings relate to the physiologic role of PTHrP in the pancreas is unknown.

### B. Cardiovascular System

As mentioned previously, PTHrP may have a general function in the regulation of smooth muscle cell contractility. In tissues such as the uterus, the urinary bladder, and the hen oviduct, PTHrP expression in smooth muscle cells is induced by stretch, with the level of induction proportional to the degree of stretch (or filling). Furthermore, PTHrP acts on smooth muscle cells to cause relaxation. In addition to the organs mentioned above, PTHrP is expressed in the muscularis layer of most vascular beds. In vascular smooth muscle cells, vasoconstrictors, such as angiotensin II, serotonin, endothelin, norepinephrine, bradykinin, and thrombin, induce a potent, but transient, up-regulation of PTHrP expression. As before, mechanical stimulation (stretch) also induces PTHrP expression in vascular smooth muscle cells, but probably through a different pathway than vasoconstrictors. N-terminal PTHrP is a vasorelaxant in many vascular beds, including those of the mammary gland, the placenta, the aorta, the portal vein, the renal artery, and the coronary artery. The ability of PTHrP to relax smooth muscle cells appears to be mediated by a decrease in intracellular free calcium caused by the inhibition of the activity of L-type voltage-sensitive calcium channels (L-VSCC). In addition to its effects on contractility, PTHrP has been reported to modulate vascular smooth muscle cell proliferation. When added exogenously, PTHrP inhibits smooth muscle cell proliferation by inducing the cyclin-dependent kinase inhibitor $p27^{kip1}$. However, when PTHrP is overexpressed in the A10 smooth muscle cell line, proliferation was increased. This occurred only when the cells expressed PTHrP with an intact nuclear localization signal, suggesting that this was an intracrine effect of PTHrP. These effects are identical to the aforementioned studies with MCF-7 cells, again demonstrating that PTHrP can have opposing effects on the same cells, depending on the trafficking of the protein. Finally, exogenous PTHrP also inhibits migration of vascular smooth muscle cells in response to platelet-derived growth factor. The effects of PTHrP on vascular smooth muscle cell contractility, proliferation, and migration have all generated interest in the potential role of this peptide in atherosclerosis.

In addition to blood vessels, PTHrP has direct effects on the heart. N-terminal PTHrP has been shown to exert positive chronotropic and ionotropic effects in *ex vivo* perfused hearts. It also increases the rate of cardiac myocyte contraction *in vitro*. Although some of the tachycardia caused by systemic infusion of PTHrP is likely related to its ability to cause vasodilation and reduce blood pressure, these experiments also suggest that it may have direct effects on pacemaker function.

### C. Skin

The first nonmalignant cell type that was shown to produce PTHrP was the keratinocyte. During development, PTHrP participates in the epithelial–mesenchymal interactions necessary for proper skin appendage formation. In addition, PTHrP may also play a role in the epithelial mesenchymal interactions in the skin of adult animals. Keratinocytes express PTHrP but not the PTH1R, and dermal fibroblasts express the PTH1R but not PTHrP. One likely function of PTHrP in the skin is to regulate the hair growth cycle. Systemic antagonists of the PTH1R have been shown to shorten telogen, lengthen anagen, and inhibit catagen. This suggests that PTHrP may promote the resting or catagen/telogen stage of the hair cycle, thereby causing reduced hair follicle growth. However, somewhat paradoxically, mice expressing no PTHrP in their skin undergo coat thinning over time, rather than the expected increase in hair growth. In addition to any effects on the hair cycle, evidence from mice expressing no PTHrP in their skin and mice overexpressing PTHrP in their skin suggests that PTHrP may also modulate keratinocyte differentiation. It remains unknown whether the effects of PTHrP on keratinocyte differentiation and hair follicle growth result from paracrine signaling to dermal fibroblasts or from autocrine or intracrine pathways. However, current data support the notion that PTHrP participates in the regulation of these processes.

### D. Central Nervous System

Both PTHrP and the PTH1R are expressed within the brain. Interestingly, recent studies have demonstrated that other members of the PTH receptor family as well as other potential ligands for these receptors are expressed within the CNS. Therefore, PTH1R signaling is likely to have important yet complex functions in the nervous system. As with vascular smooth muscle cells, PTHrP, acting via this receptor, has been shown to affect the function of L-VSCC in neurons. In response to depolarization-induced L-VSCC calcium influx, PTHrP expression is up-regulated in cerebellar granule cells in culture. In turn, it appears that PTHrP can inhibit calcium entry through L-VSCC. Since calcium entry through these channels can be toxic to neurons (a phenomenon known as excitotoxicity), it has been suggested that PTHrP might participate in a putative neuroprotective short-feedback loop. However, in contrast to its effects on granule cells, PTHrP has been shown to increase L-VSCC activity in PC-12 cells, an effect that leads to an increase in dopamine secretion.

## IX. COMPARATIVE BIOLOGY OF PTHrP

### A. PTHrP, a Calciotropic Factor in Fish

Fish face a different set of problems than terrestrial mammals in achieving mineral homeostasis. The main challenges to marine fishes are to eliminate excess salts and prevent dehydration. The PTHrP sequence is most similar between fishes and tetrapods at the N-terminus, suggesting that regulation of calcium metabolism could be a conserved function. In support of this hypothesis, PTHrP expression co-localizes with the main sites of active calcium transport in fish (gills, skin, intestine, and opercular epithelium). Furthermore, in sea bream larvae, PTHrP increases the uptake of calcium and decreases total calcium efflux. PTHrP may be a classical endocrine hormone in fish, where it is secreted from the pituitary gland. In elasmobranchs, circulating PTHrP levels correlate positively with potassium and calcium and negatively with sodium, chloride, and urea, suggesting that PTHrP regulates ion transport and/or osmolality.

### B. PTHrP in the Avian Oviduct

In the hen, PTHrP is expressed in the immature oviduct of growing animals, but is not highly expressed in the nulliparous adult oviduct. During the egg-laying cycle, expression of PTHrP is greatly up-regulated in the oviduct. The localization of PTHrP mRNA changes with the position of the egg as it descends. When the egg moves through the magnum, PTHrP is expressed most highly in the isthmus, and when the egg moves through the isthmus, PTHrP expression is up-regulated in the shell gland. Finally, when the egg passes out of the shell gland, PTHrP message is rapidly down-regulated. This sequence of events is strikingly similar to what happens in the mammalian uterus, where PTHrP expression is up-regulated until the time of birth and then declines. One possibility is that PTHrP regulates the passage of the egg through the oviduct by relaxing the smooth muscle "ahead" of the egg so that the contracted muscle "behind" the egg will push it forward. PTHrP can, in fact, relax strips of oviduct smooth muscle *in vitro*. Another possibility is that PTHrP regulates calcium transfer from the hen to the egg for shell formation. Interesting parallels can be raised between a function in egg shell formation and the putative roles of PTHrP in regulating placental calcium transfer and calcium transfer to the milk during lactation. Finally, PTHrP could regulate blood flow to the oviduct through its actions on vascular smooth muscle.

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## Glossary

**endochondral bone formation** Bone development that occurs at growth plates by mineralization of a cartilaginous framework.

**humoral hypercalcemia of malignancy** A syndrome characterized by elevated calcium levels in cancer patients without metastases.

**intracrine** A mechanism of signaling in which a hormone produced by a cell is rapidly transported to the nucleus of that same cell where it exerts its effects.

**parathyroid hormone/parathyroid hormone-related protein receptor type 1** A seven-membrane-spanning receptor that can be activated by parathyroid hormone or N-terminal parathyroid hormone-related protein peptides.

**polyprotein prohormone** A hormone precursor that is posttranslationally processed into multiple active peptide hormones.

## See Also the Following Articles

**Calcium Signaling • Humoral Hypercalcemia of Malignancy • Parathyroid Hormone**

## Further Reading

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# PCOS

See ***Polycystic Ovary Syndrome***

# PDGF

See ***Platelet-Derived Growth Factor (PDGF)***

# Peptide YY

THOMAS E. ADRIAN
*Northwestern University Medical School*

I. CHEMISTRY AND MOLECULAR BIOLOGY OF PYY
II. TISSUE DISTRIBUTION AND ONTOGENY
III. PYY SECRETION AND RELEASE MECHANISM
IV. PYY RECEPTORS AND SECOND-MESSENGER SYSTEM
V. BIOLOGICAL EFFECTS OF PYY

VI. PHYSIOLOGICAL EFFECTS OF PYY
VII. PATHOPHYSIOLOGY OF PYY
VIII. THERAPEUTIC POTENTIAL

**Peptide YY, a hormonal peptide produced by endocrine cells in the intestinal mucosa, was first isolated from porcine small intestine using a novel chemical assay to detect peptides with a carboxy-terminal amide group, a structural feature shared by many regulatory peptides. PYY inhibits cholecystokinin- and secretin-stimulated pancreatic secretions, suggesting a hormonal role. In this article, the biological effects of PYY in the gastrointestinal tract are described and the clinical ramifications of these effects are explored.**

## I. CHEMISTRY AND MOLECULAR BIOLOGY OF PYY

Peptide YY is a straight-chained 36-amino-acid polypeptide that is structurally related to pancreatic polypeptide (PP) and neuropeptide Y (NPY). All three of these biologically active peptides have both C-terminal and N-amino-terminal tyrosine residues, which is the source of the name assigned to PYY (in the single-letter-code peptide nomenclature, Y represents tyrosine). Human PYY differs from porcine peptide at three residues, whereas the sequences of rat, dog, and pig PYY are identical. The amino acid sequences of the human peptide family are shown in Table 1. Truncated forms of human and canine PYY with 34 residues (PYY 3–36) have been isolated from colonic mucosa. The differences in the biological effects of the truncated and full-length forms of PYY relate to the PYY receptor subtypes (see later). Another posttranslational modification of PYY is phosphorylation of the serine residue in position 13 of the peptide. This phosphorylated form retains biological activity but is slightly less potent than the nonphosphorylated form.

The human and rat PYY genes have been cloned and sequenced. In both cases, the gene is composed of four exons and three introns spanning approximately 1.2 kbp. The human gene resides on chromosome 17q21.1 in a cluster with the PP gene. Two mRNA species are derived from the PYY gene by alternative splicing. The PYY precursor is a 98-amino-acid protein residue in which the PYY sequence is preceded by a signal peptide and followed by the cleavage and amidation sequence of Gly-Lys-Arg, which is followed by a cryptic peptide of another 31 amino acids. Thus, the precursor is similar in structure to that of pancreatic polypeptide and neuropeptide Y, and all are derived from a common ancestor by gene duplication. Indeed, the most recent gene duplication event has led to production on the 17q21 chromosome of a second cluster called the PYY2–PPY2 gene cluster, which has been detected in the cow, baboon, and human genomes. Despite the 92% sequence identity of the two gene clusters, a few specific mutations have resulted in significantly altered peptide sequences. These structural alterations have led to acquisition of new functions. The cow PYY2 gene codes for seminal plasmin, which is involved in reproductive function rather than gastrointestinal physiology.

A peptide with strong sequence identity with PYY has been isolated from the skin of the South American arboreal frog, *Phyllomedusa bicolor.* This peptide, called skin peptide tyrosine-tyrosine (SPYY), inhibits melanotropin release from frog neurointermediate lobes in a manner similar to that of NPY.

## II. TISSUE DISTRIBUTION AND ONTOGENY

Cells containing PYY are found in the mucosa throughout the small and large bowel in humans. PYY concentrations increase distally through the gut, reaching peak concentration in the rectal mucosa. Similar gut distributions have been reported in the rat, mouse, dog, and monkey, with concentrations in the colon generally 100- to 200-fold higher than in the duodenum. PYY is colocalized with the glucagon gene products in the intestinal L cells. Interestingly, this

TABLE 1 Amino Acid Sequences of Mammalian PYY, NPY, and PP

| Peptide[a] | Sequence |
|---|---|
| PYY (porcine[b]) | YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY-$NH_2$ |
| PYY (human) | YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-$NH_2$ |
| NPY (human) | YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-$NH_2$ |
| PP (human) | APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-$NH_2$ |

[a] PYY, Peptide YY; NPY, neuropeptide Y; PP, pancreatic polypeptide.
[b] Rat and canine PYY sequences are identical to the porcine sequence.

colocalization is also seen in the glucagon-producing pancreatic islet alpha cells in mice, rats, guinea pigs, cats, dogs, pigs, and cows. These cells expressing glucagon and PYY are the earliest islet cells identified in the fetus and presumably represent the islet stem cells that have differentiated from the pancreatic endoderm. At this early stage, the products of the glucagon gene represent those expressed in the adult intestine (glicentin and the glucagon-related peptides, rather than glucagon), and because of this difference in posttranslational processing, the metabolic hormone glucagon is not made. In the developing mouse pancreas, PYY is transiently co-expressed with insulin, PP, and somatostatin, when the cell types expressing these peptides first appear. PYY expression may thus serve as a marker for islet differentiation. Production of PYY peaks just before term and ceases in the human pancreas shortly after parturition, although PYY continues to be co-expressed with glucagon in the rodent pancreas. In the mouse pancreas, the immunoreactive PYY is mostly confined to a major subpopulation of alpha cells in the splenic lobe. The PYY-expressing cells are also the earliest endocrine cells to appear in the fetal intestine. Once again, co-expression of PYY with other hormones such as secretin, cholecystokinin, neurotensin, gastrin, and somatostatin is seen when these hormones are first expressed. These findings suggest that PYY plays an important role in endocrine cell differentiation in the gut and the pancreas and support the existence of a common endocrine cell progenitor in these tissues.

Peptide YY is found not only in endocrine cells of the gut but also in nerve fibers in the myenteric plexus in several species, including rats, cats, ferrets, pigs, and dogs. In the rat, these are restricted to the stomach, but in cats, ferrets, and dogs, numerous fibers are also found in the upper small intestine. In the pig, only a few fibers are seen in these myenteric plexus locations and no fibers are seen in human gastrointestinal smooth muscle tissues.

## III. PYY SECRETION AND RELEASE MECHANISM

As would be expected with a gastrointestinal hormone, circulating concentrations of PYY in humans change with food ingestion. Circulating concentrations rise in response to food intake and the magnitude of the increase reflects the total caloric load ingested. It should be emphasized that the increase in response to a small meal in healthy subjects is small. Ingested fat and protein, in particular, elicit a PYY response in humans. Similar increases in circulating PYY in response to feeding have been noted in a number of species, including rats, pigs, and dogs. In dogs, fat, rather than proteins and carbohydrates, appears to be responsible for PYY release.

The observed rapid PYY response to feeding led to early speculation that the foregut played a role in release of the peptide. It was seen that intraduodenal oleic acid releases PYY into the circulation even when the flow of chyme is prevented, whereas ileocolectomy abolishes PYY release in response to duodenal oleate. Infusion of the upper intestinal hormone, cholecystokinin (CCK), causes concentration-dependent release of PYY into the circulation. Other studies in the dog have shown that response to a fatty meal as well as to CCK infusion is abolished by a specific CCK-A-type receptor antagonist, underlining the importance of CCK-stimulated PYY release in this species. Concentrations of PYY in cord blood are substantially greater than those in normal fasting adults, and the response to feeding in neonates is massive, suggesting a role for the peptide in early adaptations to extrauterine life. Concentrations in neonatal animals, such as the pig, are also high.

Secretion has also been investigated in isolated intestinal endocrine cells cultured *in vitro*. Sodium oleate concentration dependently stimulates PYY release, as does bombesin, epinephrine, and forskolin, but not carbamyl choline. Studies in isolated perfused rabbit left colon have revealed that bile salts, rather than fatty acids per se, are responsible for release of the peptide following colonic stimulation. In contrast, high concentrations of amino acids are more effective at releasing PYY from the dog colon than is fat, glucose, or protein. Bile salts selectively and potently released PYY from the human colon. Although added oleic acid does not increase the PYY response to deoxycholate, this fatty acid causes a concentration-dependent increase in release of glucagon gene products. Because both peptides appear to be secreted from the same cell type, either there must be a difference in the hormone content of granules within the responding endocrine cells or a subpopulation of enteroglucagon secreting cells must be stimulated. Short-chain fatty acids also release PYY from the rabbit colon, a mechanism that could be highly significant in this species, which largely relies on short-chain fatty acids from colonic fermentation for its caloric intake.

Secretion studies in rats show a different spectrum of PYY release in response to nutrients, compared to the other mammalian species just mentioned.

Intraduodenal isocaloric amounts of glucose and amino acids release as much PYY as does a meal; oleic acid in place of a meal is less effective. This is likely to reflect the high tonicity of these solutions, because hyperosmolar saline also releases PYY but isotonic solutions have little effect. Studies in the vascularly perfused isolated rat colon show relatively weak stimulation of PYY by CCK and secretin, but a marked increase in PYY secretion in response to glucose-dependent insulinotropic peptide (GIP). Similarly, blockade of CCK and bombesin receptors is without effect on PYY release in anesthetized rats. With regard to intralumenal stimulation of PYY release in isolated perfused rat intestine, only supraphysiologic concentrations of glucose and amino acids are able to elicit a response. In contrast, some fibers, including pectin and gum arabic, but not cellulose, stimulate marked PYY secretion. The β-adrenergic agonist isoproteranol, the cholinergic agonist bethanechol, and calcitonin gene-related peptide all increase PYY secretion in this model. The effects of these agents are not affected by tetrodotoxin, suggesting direct effects on the PYY cells rather than mediation through the enteric nervous system.

There is some controversy regarding the extrinsic neural release of PYY. Several groups have shown a PYY response to two peptide transmitters, vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), or to the amphibian analogue to GRP, bombesin. Studies in the pig reveal that electrical stimulation of the vagus supplying the pig distal intestine results in PYY release, whereas splanchnic stimulation has no effect. In keeping with this observation, atropine and hexamethonium significantly inhibit the PYY response to food. In contrast, total extrinsic jejunoileal denervation results in an increased PYY response to intralumenal fat stimulation in the dog. Interestingly, the CCK responses to fat are abolished in this denervation model, indicating that the increased PYY responses to fat are not mediated by CCK. Similarly, the PYY responses to food are enhanced by truncal vagotomy. Taken together, these findings suggest that food-induced PYY release is influenced by both an atropine-blockable postganglionic, parasympathetic pathway and a tonic inhibition, probably through a vagal cholinergic pathway.

A variety of other hormones and growth factors can influence the production and secretion of PYY. Pharmacologic inhibition of gastric acid secretion elevates circulating gastrin levels and concomitantly reduces both transcription and secretion of PYY. This inhibition is prevented by the gastrin receptor antagonist LY365,260. This control mechanism may relate to enterogastrone effects of PYY (see later). Insulin-like growth factor stimulates both expression of PYY mRNA and the peptide as well as its secretion into the circulation. A similar effect on tissue and plasma PYY levels, but not its mRNA content, is seen in transgenic mice overexpressing transforming growth factor-α. Somatostatin has been shown to inhibit PYY release in all species studied. This effect is mediated via type 5 somatostatin receptors in the rat and type 2 somatostatin receptors in humans.

## IV. PYY RECEPTORS AND SECOND-MESSENGER SYSTEM

The PYY peptide family shares several different receptor (Y receptor) subtypes. There are currently six recognized members of this family, although only five of these (excluding Y3) have been cloned, i.e., Y1, Y2, Y4, Y5, and y6 (it should be noted that the y6 receptor is a pseudo-receptor in humans and pigs, hence the designation y6). The y6 receptor is absent in the rat but is apparently functional in the rabbit and mouse. All of the Y receptors are members of the G-protein-coupled seven-transmembrane-domain "serpentine" superfamily of receptors, which are coupled to inhibition of adenylate cyclase. The G-proteins involved are members of the $G_o$ and $G_i$ family and the effect of receptor activation is generally pertussis-toxin-sensitive, although exceptions in presynaptic receptors have been reported. This is, however, likely to arise from the inability of pertussis toxin to penetrate and activate the G-proteins rather than from a distinct signaling mechanism.

Structure–affinity and structure–activity studies with peptide analogues, together with studies based on site-directed mutagenesis and antireceptor antibodies, have given insight into the individual characterization of each receptor subtype relative to its interaction with ligand, as well as to its biological function. The characteristics of the different Y receptor subtypes, including binding characteristics and examples of locations, functions, and selective agonists and antagonists are summarized in Table 2. This summary is simplified because there are species differences as well as differences in reported activities from different laboratories. PYY binds to all the active subtypes, although it is less potent than NPY on Y1 receptors and much less potent than PP on Y4 receptors. Y2 receptors strongly bind PYY 3–36, a naturally occurring product of PYY produced in the small intestine.

TABLE 2 Subtypes of Receptors for PYY and Other Family Members

| Subtype | Binding characteristics | Example locations | Example functions | Selective agonists | Selective antagonists |
|---|---|---|---|---|---|
| Y1 | NPY > PYY ≫ PP | Gut, brain, heart, kidney | Vasoconstriction, anxiolysis; antisecretory in intestine | [Pro34]PYY, [Pro34]NYY | BIBP 3226, GR231118[a] |
| Y2 | PYY = NPY ≫ PP strongly binds PYY 3-36-NH$_3$ | Brain, low levels in peripheral tissues | Presynaptic inhibition of neurotransmitter release | PYY 3-36, PYY 13-36 | BIIE0246 |
| Y3[b] | NPY ≫ PYY | Nucleus tractus solitarius, colon, and adrenal | Regulation visceral afferents | None available | None available |
| Y4 | PP > PYY = NPY[c] | Small intestine, colon, prostate, and CNS | Inhibition of pancreatic secretion | PP | GR231118 |
| Y5 | PYY = NPY = PP | Brain areas involved in food intake | Increased food intake | Ala-31-Aib$_{32}$ NPY and PP/NPY chimera, [Dtrp32]NPY | None available |
| Y6 | Pseudogene in human and pig brain, absent in rat, functional in rabbit and mouse | Small intestine and adrenal (mouse) | Not known | None available | None available |

[a] Putative receptor, not yet cloned.

[b] GR31118 has high affinity for Y1 and Y4 receptors; selectivity toward Y5 is not established.

[c] PYY activates the human but not the rat Y4 receptor.

## V. BIOLOGICAL EFFECTS OF PYY

With the considerable overlap between the PP-fold peptides for the various receptor subtypes, it is not surprising that the biological effects of the PYY family overlap, particularly the effects of PYY and NPY. This does not mean that all of these biological effects are important physiological functions of the respective peptides. It is necessary to deliver the appropriate concentration of peptide to receptor, which, in the case of PYY, is via the circulation, with, perhaps, some local effects in the intestine. For NPY, the delivery is made to synaptic receptors in the peripheral and central nervous systems.

The broad range of biological effects of PYY includes gastrointestinal effects, such as slowing of gastric emptying and intestinal transit and inhibition of gastric pancreatic and small intestinal secretions. In peripheral tissues, PYY is also a vasoconstrictor and cardiac stimulant. PYY can reach some brain receptors via the circulation. The central effects of PYY include simulation of food intake as well as effects on circadian rhythm, anxiety, and memory. Interestingly, PYY 3-36 has been shown to inhibit food intake and to reduce weight gain through the NPY Y2 receptor. PYY 3-36 is released from the intestine postprandially directly in proportion to the amount of calories consumed. The motor and secretory effects on the gastrointestinal tract are seen at circulating concentrations that are within the range seen after physiological stimuli, and are, therefore, undoubtedly physiologically relevant. The physiological importance of the other biological effects of PYY is uncertain.

## VI. PHYSIOLOGICAL EFFECTS OF PYY

### A. Motor Effects of PYY in the Gastrointestinal Tract

Early investigations of PYY showed inhibitory effects on jejunal and colonic motility together with intestinal vasoconstriction. Infusion studies in humans revealed marked inhibitory effects on gastric emptying and intestinal transit. It is now clear from the work of several groups that, in humans and experimental animals, PYY is responsible for the upper gut inhibitory motor effects that are seen in response to ileal infusion of lipids, known as the "ileal brake." Ileal or colonic infusion of lipid lengthens the interval between migrating complexes and also slows gastric emptying through the stimulation of PYY secretion. These motor effects of PYY appear to be mediated through vagovagal pathways.

There are apparently paradoxical effects of PYY in the stomach. Several studies show that PYY inhibits the ileal brake through the dorsal vagal center. However, under some circumstances, PYY can stimulate gastric motility. Y2 agonists inhibit thyrotropin-releasing hormone (TRH)-stimulated motility, mimicking the suppressive effect of PYY, but have no effect on basal acid secretion. In contrast, Y1 agonists stimulate motor function under basal conditions. This difference is probably due to the localization of Y1 and Y2 receptors in the dorsal vagal complex and the rapid conversion of PYY to a Y2 agonist by the ubiquitous dipeptidyl aminopeptidase.

The role of PYY in gallbladder function is controversial. One group has reported marked relaxation and increased filling of the prairie dog gallbladder in response to modest increases in PYY concentrations. In contrast, studies in the dog have revealed no effect of PYY on gallbladder function.

## B. Role of PYY in Gastric Secretion

There is a consensus that PYY is a physiological enterogastrone in several species, including dogs and humans. PYY inhibits gastric acid and pepsin secretion stimulated by pentagastrin, cholinergic agonists, vagal activity, and histamine. The point that is somewhat controversial is the site of action. Most evidence points to inhibitory effects on vagal neurons. However, one group has provided evidence in support of a vagally independent mechanism. It is possible that, in view of the high concentrations of gastrin that are seen in newborns, the very high concentrations of PYY in neonates are important for inhibiting gastric acid secretion, i.e., allowing the tropic effects of gastrin to occur in the gastric mucosa without hyperstimulation of acid secretion.

## C. Effects of PYY on Pancreatic Secretion

Multiple studies in dogs and rats have demonstrated that PYY inhibits pancreatic secretion stimulated by CCK, secretin, food, and intraduodenal amino acids, or vagal stimulation by infusion of 2-deoxyglucose. In contrast, a single study in humans showed no effect on CCK- and secretin-stimulated pancreatic secretion. Although there is complete agreement with regard to the effect of PYY in dogs and rodents, the site of action appears to be different in these two species. In dogs, the inhibitory effect of PYY on pancreatic secretion is independent of the vagus, and PYY inhibits secretion from the denervated canine pancreas even in the presence of atropine. Results from another study on dogs suggest that PYY acts on the intrapancreatic cholinergic nerves rather than on extrinsic pathways. Other explanations from groups working with dogs are that PYY acts, at least partially, by blocking CCK release and reducing blood flow.

In one rat study, PYY predominantly inhibited neurally stimulated secretion and this inhibition was blocked by coinfusion of a cholinergic agonist. Results from a more recent study lead to the conclusion that the effect of PYY in the rat is predominantly mediated through the CCK pathway at a site proximal to convergence with the neural pathway. Immunoneutralization studies in the rat have confirmed that PYY is a physiologically important regulator of food-stimulated pancreatic secretion.

## D. Effects of PYY on Small Intestinal and Colonic Secretion

Early studies using Ussing chambers demonstrated that PYY has antisecretory effects in the small intestine and colon. PYY reduces basal and VIP-stimulated increases in short-circuit current as well as VIP-stimulated increases in cAMP production and chloride secretion. PYY also abolished the secretory responses to cholera toxin and forskolin but not to 8-bromo-cAMP, indicating that the effect is mediated through inhibition of receptor-coupled adenylate cyclase.

*In vivo*, secretion and absorption occur simultaneously and all regions beyond the duodenum normally show net absorption. When infused at concentrations that mimic the postprandial response, PYY causes a substantial increase in net water and electrolyte absorption in Thiry–Vella fistula dogs. Other studies have revealed that infusion of PYY can augment the increase in net absorption that occurs postprandially, and that immunoneutralization with a monoclonal PYY antibody blocks the meal-induced increase in absorption. Whether the proabsorptive effect acts directly on the enterocyte or is mediated indirectly is not clear. One study has shown that the effects on short-circuit current and chloride secretion are blocked by hexamethonium, suggesting neural mediation, whereas another study has concluded that the effects are independent of neural blockade.

### E. Growth and Differentiation-Inducing Effects of PYY in the Gut

Some studies in rats and mice have shown that administration of PYY can increase the weight and DNA content of the duodenum, ileum, and colon. However, other infusion studies in the rat have shown no effect on mucosal weight or crypt cell production rate, a very sensitive indicator of intestinal hyperplasia. In Caco-2 intestinal epithelial cells, PYY induces expression of the brush border enzyme alkaline phosphatase and decreases the specific activity of dipeptidyl dipeptidase. These findings suggest that PYY promotes differentiation of Caco-2 cells to a more colonocyte phenotype.

The effects of PYY on preventing the gut hypoplasia that accompanies total parenteral nutrition (TPN) have been assessed after 7 days of TPN in tumor-bearing rats. PYY was coinfused with clenbuterol, an anabolic β-adrenergic agonist. The combination resulted in significant savings in small intestinal weight and protein content, but had no effect in sparing the colon. Histologic analysis of the ileal tissues suggested that the effects of PYY were mostly mucosal, whereas clenbuterol mainly influenced the muscle. The combination treatment also resulted in a significant saving of protein content in the gastrocnemius muscle, suggesting a reduction in the cachectic response. Studies in breast (MCF7 and ZR-75) and pancreatic cancer (MIA PaCa-2) cells have shown significant growth-inhibitory effects of high concentrations of PYY.

### F. Other Effects of PYY on the Gastrointestinal Tract and Metabolism

As already mentioned, PYY has vasoconstrictor effects on several vascular beds and, in particular, it reduces intestinal blood flow. Infusion of PYY in humans is accompanied by a small but significant increase in both systolic and diastolic blood pressure. Indeed, it is quite possible that inhibitory effects on secretion and motor activity may be mediated, at least in part, through effects on the splanchnic vasculature.

PYY increases expression of intestinal fatty acid-binding protein, suggesting that PYY plays a role in the trafficking of free fatty acids in the enterocyte. Similarly, physiologically relevant concentrations of PYY increase production of apolipoprotein AIV in the intestinal mucosa. PYY has also been shown to stimulate mucin secretion in the rat colon. PYY has been shown to inhibit the secretion of several gastrointestinal hormones, including motilin and pancreatic polypeptide in humans and cholecystokinin in rats and dogs. In contrast, PYY has no effect on circulating concentrations of glucagon, gastrin, glucose-dependent insulinotropic peptide, neurotensin, or enteroglucagon. A study in dogs has shown that PYY inhibits the insulinotropic effects of GIP, suggesting a role for the peptide in the negative feedback regulation of insulin secretion. Studies in humans show no effect on basal or glucose-stimulated insulin secretion or on the glucose elimination rate after intravenous glucose. Furthermore, PYY has no significant effect on circulating concentrations of glucose, lactate, glycerol, or free fatty acids. It remains to be seen whether PYY influences the enteroinsular axis in humans.

PYY infused at concentrations similar to those seen after a meal had significant effects on renal function in a group of male subjects. The effects included a decrease in glomerular filtration rate, plasma renin activity, and aldosterone levels. Infusion of PYY at a higher dose, which reproduced plasma levels seen in diarrheal illness, resulted in similar changes in function but also reduced renal plasma flow. These findings suggest that PYY may be an important mediator of the postprandial natriuretic response.

### G. Orexigenic Effects of PYY

Lateral ventricular injections of PYY and NPY in hamsters induces a dose-dependent increase in food intake but causes a marked reduction in lordosis duration. In rats, injection of these peptides into the paraventricular nucleus causes a dramatic dose-dependent increase in food intake as well as a small increase in water intake. When the animals are able to select from different macronutrient diets, they overwhelmingly select carbohydrate over diets rich in either fat or protein. A recent study showed that infusion of PYY 3-36-$NH_2$ at physiological concentrations has marked inhibitory effects on food intake in humans.

## VII. PATHOPHYSIOLOGY OF PYY

In endocrinology, a lot can be learned about the physiological role of a hormone by studying syndromes associated with excessive hormonal secretion and lack of hormonal production. This is the case with PYY, which appears to play an adaptive role in several intestinal disorders and after small bowel resection.

## A. PYY in Gastrointestinal Diseases

Circulating basal and postprandial PYY concentrations are grossly elevated in patients with celiac sprue and in patients with postinfective tropical malabsorption (tropical sprue). Patients with these disorders have severe malabsorption resulting from small intestinal mucosal atrophy; in celiac patients this is due to sensitivity to the gliadin fraction of wheat flour and in tropical sprue it is caused by an unknown infective agent. In both conditions, PYY levels normalize with successful treatment with a gluten-free diet or antibiotics, respectively. The increased secretion of PYY is entirely appropriate in these conditions, given the inhibitory roles of PYY on gastrointestinal secretion and motility. PYY is presumably released in response to unabsorbed nutrients that reach the PYY-containing cells in the distal ileum and colon. This contrasts with the normal situation in which nutrients are almost totally absorbed in the proximal small bowel. High concentrations will reduce secretions and therefore the intralumenal volume in the gut and will also slow gastric emptying and intestinal transit. All of these physiological changes tend to enhance absorption by increasing the contact time with the small intestinal mucosa and by increasing local nutrient concentrations.

Smaller elevations of PYY are seen in patients with inflammatory bowel diseases (Crohn’s disease and ulcerative colitis) and chronic pancreatitis and in patients recovering from a severe, acute, infective diarrhea. These conditions are associated with a degree of malabsorption, although direct effects of intestinal inflammation may also play a role in enhancing secretion of the hormone in inflammatory bowel disease and diarrhea. Once again, the elevation in diarrhea is appropriate, because PYY will slow nutrient transit and enhance intestinal absorption.

PYY concentrations are normal in meal studies performed in patients with other gastrointestinal disorders, including peptic ulcer, diverticular disease, and irritable bowel syndrome.

## B. PYY Abnormalities Following Alimentary Surgery

Peptide YY secretion changes in response to a variety of surgical procedures, which, once again, could be considered to be appropriate adaptive changes. First, a proportion of patients who undergo gastric resection will suffer from “dumping” syndrome. In these patients, rapid emptying of hypertonic chyme into the upper small intestine causes water to pass from the vascular space into the intestinal lumen. This results in a shortness of breath, rapid heart rate, pain, bloating, nausea, vomiting, and even diarrhea. Furthermore, rapid absorption of glucose results in an exaggerated insulin response that can cause a reactive hypoglycemia, with symptoms including weakness, cold sweating, confusion, and headache. PYY levels are markedly increased following oral glucose ingestion in patients with dumping syndrome.

The pattern of secretion after massive small bowel resection is similar to that seen in sprue. This is not surprising, because unabsorbed nutrients rapidly reach the distal bowel, triggering release of the peptide YY. Generally, the more small intestine that is resected, the greater the elevation of PYY levels. Similar increases of PYY secretion are seen in dogs and rats after small bowel resection. In dogs and humans, infusion of PYY at concentrations that raise circulating levels to those seen after resection markedly slows intestinal nutrient transit, inhibits gastrointestinal secretions, and enhances net water and electrolyte absorption throughout the small intestine. As previously mentioned, PYY does not seem to be responsible for the ileal resection-induced adaptive mucosal hyperplasia and hypertrophy that are seen in the remaining bowel in continuity. Indeed, it is now clear that glucagon-like peptide-2 (GLP-2), a product of the same cell but not of the PYY gene, is responsible for the adaptive intestinotropic response to bowel resection. Studies on the expression of the GLP-2 and PYY genes have revealed that their respective mRNA contents are already markedly increased within 6 h after enterectomy, even before the animals are fed. Plasma PYY concentrations are also increased within 24 h. One can only speculate on this early endocrine response to small bowel resection. In the absence of food, it is possible that biliopancreatic secretions reaching the distal intestine are responsible. A modest increase in PYY response accompanies orthotopic jejunoileal autotransplantation in the dog. This is nothing like the magnitude of the increase seen after small bowel resection and is likely to be the result of motor changes in the denervated gut.

In contrast to the effect of massive small bowel resection, colonic resection is associated with low PYY levels. Here the postprandial response reflects the postsurgical anatomy. Levels are very low in patients with an ileostomy; this is not surprising because all of the PYY-containing colonic mucosa is out of the intestinal stream. Responses are a little greater in patients with an ileoanal anastomosis, but there is no difference between straight ileoanal anastomosis and patients who have a J-pouch fecal reservoir. Interestingly, in patients with ileoanal pouches, fecal retention time is related to the plasma PYY response as well as to mouth-to-pouch transit time. This finding suggests that it would be advantageous to maintain PYY responses in patients who have undergone colonic resection. A recent report of a new pouch procedure, the 9-pouch with a recycle segment, reveals that this more physiological approach, with attention to the effects of ileal peristalsis, results in PYY responses that are not different from those of healthy controls and offers a marked improvement in the functional outcome in these patients. The time-course of changes in PYY function has been carefully studied in the dog and rat after colonic resection and ileoanal anastomosis. Both the number of PYY positively stained cells and the PYY content of the ileal J-pouch mucosa are greater than is seen in control ileum and mucosa from straight ileoanal anastomoses. Furthermore, PYY responses increase with time after resection, suggesting that these changes represent an adaptive response that is improving the functional outcome.

PYY levels are modestly increased after total pancreatectomy. This pattern of response is very similar to that in chronic pancreatitis with exocrine deficiency, undoubtedly reflecting the malabsorption.

### C. PYY and Tumors of the Intestine

Peptide YY, glucagon gene products, and PP are all common constituents of rectal carcinoid tumors, but PYY is not generally seen in endocrine tumors from other regions of the gut, such as the pancreas. An interesting report of a patient with an ovarian carcinoid revealed that excessive PYY production was associated with severe constipation that resolved after tumor resection. Low concentrations of PYY are seen in colonic cancer tissues and adenomatous polyps, compared with normal mucosa. Extremely low concentrations appear to reflect the malignant potential of these lesions, but this is probably related to the differentiation of the tumors, because concentrations are highest in tubular polyps, lower in villus polyps, and lowest in carcinomas. This is consistent with epithelial dysplasia and incomplete formation of mucosal endocrine cells.

### D. PYY in Eating Disorders

A recent report on the measurement of PYY in the cerebrospinal fluid (CSF) in patients with eating disorders showed a dramatic increase in CSF PYY levels in bulimics who had abstained from bingeing for 30 days, compared with controls or their own levels when actively bingeing. No differences were seen in anorexia nervosa patients. It is possible that bulimic behavior corrects a central nervous system abnormality in PYY. Because PYY can profoundly increase food intake, these results are very interesting and worthy of further investigation.

## VIII. THERAPEUTIC POTENTIAL

The spectrum of actions of PYY, including slowing of gastrointestinal nutrient transit and inhibition of secretions, make this peptide an ideal candidate for treating a number of different conditions, including acute or chronic diarrheal diseases and patients who have undergone colonic resection. In addition, PYY alleviates experimental pancreatitis, presumably because of its inhibitory effects on pancreatic secretion, so it also has potential for treating this condition.

Problems with the therapeutic use of PYY, however, are that the peptide has a short half-life (about 10 min) in the circulation and is large, making synthesis expensive. It is possible that there will be further development of long-acting analogues of PYY or some of the small-molecule agonists that target the PYY receptors that are responsible for PYY secretory and motor functions. Such compounds would have widespread clinical use. Because of the profound inhibitory effects of PYY on intestinal motor function, it is possible that a PYY antagonist could be useful in the treatment of postoperative ileus.

## Glossary

**enterogastrone** Putative inhibitory hormone from the small intestinal mucosa; inhibits gastric acid secretion.

**enteroinsular axis** Stimulation of insulin secretion by hormonal signals from the gut; triggered by ingested sugars and amino acids.

**ileal brake** Inhibitory effects on upper gastrointestinal motor activity; triggered by a distal intestinal hormone that is released in response to intralumenal fat.

**ileoanal anastomosis** Surgical end-to-end connection between the terminal ileum and distal rectum after total colonic resection.

**ileostomy** Terminal ileum exteriorized through the abdominal wall; allows intestinal waste to be collected in a bag.

**Thiry–Vella fistula** Loop of intestine with ends exteriorized through the abdominal wall; allows easy access to intestinal contents. The loop retains its blood and neural supply through intact mesentery.

**Ussing chamber** Small glass chamber in two halves that sandwich a small piece of bowel or mucosa; used to study ion fluxes. A voltage clamp across the tissue allows measurement of short-circuit currents.

**vagovagal reflex** Signal transmitted through afferent vagal fibers to the dorsal vagal complex and then back to an abdominal target through efferent vagal fibers.

## See Also the Following Articles

**Bombesin-like Peptides • Cholecystokinin (CCK) • Gastrin • Gastrointestinal Hormone-Releasing Peptides • Neuropeptide Y (NPY) • Secretin • Somatostatin • Vagal Regulation of Gastric Functions by Brain Neuropeptides • Vasoactive Intestinal Peptide (VIP)**

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# Peptidomimetics

Mark T. Goulet and Ralph T. Mosley

*Merck & Company, Inc., New Jersey*

I. INTRODUCTION
II. "NATURAL" PEPTIDOMIMETICS
III. STRATEGIES FOR PEPTIDOMIMETIC DISCOVERY AND DESIGN
IV. SUMMARY

**Many G-protein-coupled receptors can utilize peptides or proteins as their natural ligands for effecting signal transduction. Nonpeptide ligands, or peptidomimetics, that can either reproduce the activity of a peptide agonist or act as an antagonist of that system have been discovered for many of these receptors. Rational peptidomimetic designs rely on structural information derived from the ligand in either the receptor-bound or the solution state. Peptidomimetic ligands can serve as useful tools for the study of receptor signaling pathways and as important pharmacological agents for the treatment of disease.**

## I. INTRODUCTION

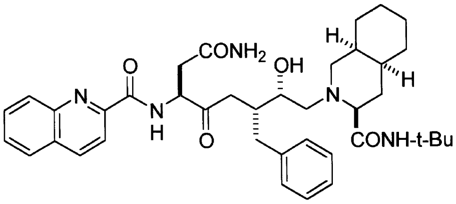
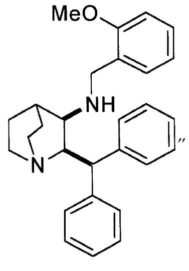
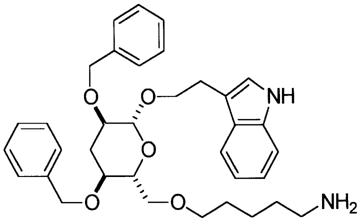
Approximately 60% of known G-protein-coupled receptors (GPCRs) utilize peptides or proteins as their natural ligand for effecting signal transduction. For many of these receptors, non-peptide ligands, or peptidomimetics, have been discovered that can either reproduce the activity of a peptide agonist or act as an antagonist of that system. Natural products derived from the secondary metabolism of lower organism occasionally function as peptidomimetic ligands for GPCRs, but a majority of peptidomimetic ligands are the outcome of a premeditated design as a means of obtaining biologically active receptor modulators with properties distinct from those of peptides. This effort is driven primarily by research in the pharmaceutical industry, because nonpeptides have demonstrated improvements over peptides with respect to oral bioavailability, metabolic stability, and tissue distribution. Many modern drugs are peptidomimetic ligands for receptors that either cause or ameliorate disease. Along with the discovery of peptidomimetics has come an increased understanding of the principles for their design and the nature of the nonpeptide–protein interaction.

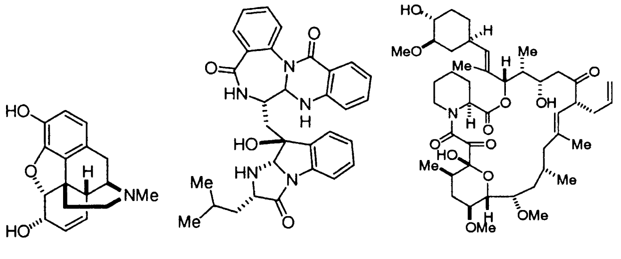
For the purposes of classification, three types of peptidomimetics have been described, based primarily on the nature of their interaction with macromolecules. A type I mimetic incorporates an amide bond surrogate, or "isostere," that is intended to mimic the local topography about an amide bond. Often type I mimetics are designed as atom-for-atom replacements of the amide bond and are utilized predominantly in the construction of enzyme (e.g., protease) inhibitors. Type II mimetics are functionally analogous to a given peptide agonist or antagonist, but their mode of binding to the receptor is distinct from that of the original ligand. Thus, type II mimetics do not necessarily mimic the structure of the parent hormone. The type III mimetics possess the necessary groups, arrayed about a nonpeptide core, to mimic the parent ligand topographically. Often these structures bear no obvious resemblance to the original hormone, but because their mode of binding is predicted to be the same as the parent they have been termed "ideal" peptidomimetics. Representatives of the three types of peptidomimetics are shown in Table 1.

## II. "NATURAL" PEPTIDOMIMETICS

Evaluation of the biological activity of natural products, isolated primarily from plants and

TABLE 1 **Classification of Peptidomimetics**

| Type | Structure | Peptide | Receptor |
|---|---|---|---|
| Type I |  CONH$_2$<br>OH<br>H<br>H<br>N<br>O<br>N<br>H<br>O<br>N<br>CONH-t-Bu<br>Saquinavir | ≀-$\underline{\mathbf{L}}$ $\underline{\mathbf{N}}\underline{\mathbf{F}}$· $\underline{\mathbf{P}}\underline{\mathbf{I}}$-≀<br>(gag-pol) | HIV protease<br>(enzyme) |
| Type II |  <br>CP-96345 | R P K P Q Q F F G L M-NH$_2$<br>(Substance P) | Neurokinin-1<br>receptor |
| Type III |  | A G C K N $\underline{\mathrm{F}}$ $\underline{\mathrm{W}}$ $\underline{\mathrm{K}}$ T $\underline{\mathrm{F}}$ T S C-OH<br>(Somatostatin) | Somatostatin<br>receptors 1–5 |



Morphine Asperlicin FK-506

FIGURE 1 Natural product peptidomimetics.

microorganisms, has revealed a number of compounds fitting the description of peptidomimetics. Some representative "natural" peptidomimetics are shown in Fig. 1. Perhaps the most well-known example is the plant-derived alkaloid morphine. Morphine and related structures are agonists of the opioid receptor family and act by mimicking the N-terminal Tyr-Gly-Gly-Phe of the endorphin neuropeptides. Morphine, as well as its synthetic and semisynthetic analogues, has been used extensively for the study of receptor–ligand interactions and subsequent GPCR signaling. Asperlicin is a natural product that acts as an antagonist of the neurotransmitter peptide cholecystokinin (CCK). Although only weakly active as a CCK antagonist ($IC_{50} = 1\ \mu M$), asperlicin has served as the structural basis for the design of potent and subtype-selective CCKA and CCKB receptor antagonists. The macrocyclic bacterial metabolite FK-506 (Tacrolimus) is an interesting peptidomimetic of clinical relevance to the field of immunosuppression. Through studies relating to the pharmacological mechanism of action, it was discovered that the amidopyranose portion of FK-506 functions as a peptidomimetic ligand for the peptidyl-prolyl isomerase FKBP-12. This complex, in turn, binds and inhibits the serine/threonine phosphatase PP2B (calcineurin) to block signal transduction in activated T cells. It is thought that portions of the polyketide-derived backbone act as a peptidomimetic ligand for PP2B during this second binding event.

## III. STRATEGIES FOR PEPTIDOMIMETIC DISCOVERY AND DESIGN

### A. Privileged Structures

Certain nonpeptide units that exhibit a propensity for binding to GPCRs and other macromolecules have been termed "privileged structures." In general, these are hydrophobic assemblages that often contain aromatic rings and are constructed in a manner so as to maintain a defined shape even in an aqueous environment, thereby avoiding what is known as hydrophobic collapse. Several common privileged structures are depicted in Fig. 2. A successful strategy for discovering peptidomimetics is to identify a privileged structure or derivative thereof that exhibits affinity for the desired receptor, then modify this structure by addition of appropriate groups to optimize potency and selectivity for that receptor. At times, the same privileged structure can be used to produce potent peptidomimetics for a variety of receptor types. For example, the biphenyltetrazole privileged structure is found in the angiotensin II antagonist prodrug losartan and in the Merck growth hormone secretagogue (Fig. 3). Preparation of chemical libraries based on a privileged structure has proved to be a successful method for both generation and optimization of pharmacologically active peptidomimetics.

### B. Benign Scaffolds

Privileged structures within a peptidomimetic both bind to the macromolecule and provide a site(s) for additional chemical modification. Another strategy for peptidomimetic design is the use of a rigid template, onto which groups that can interact with a receptor are appended. These "benign scaffolds" are not intended to participate directly in the binding event but rather to connect and display the pharmacophore elements in a manner appropriate for binding. One example of a benign scaffold is the sugar core of the type III peptidomimetic shown in Fig. 1. Another is the thyrotropin-releasing hormone (TRH) agonist RO 24-9975, wherein elements of the three amino acid side chains present in TRH are displayed about a 1,3,5-trisubstituted cyclohexane ring (Fig. 4). Computer algorithms that utilize

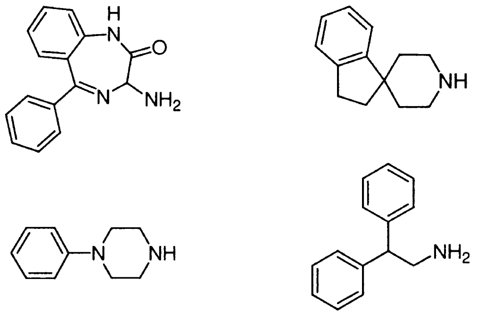


FIGURE 2 Some common privileged structures.

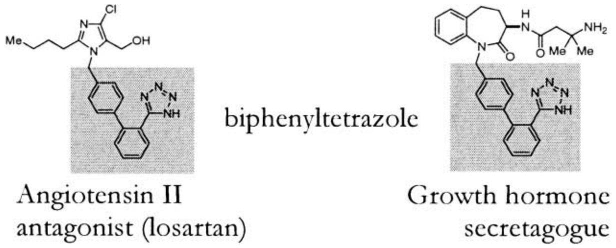

biphenyltetrazole
Angiotensin II antagonist (losartan)
Growth hormone secretagogue

**FIGURE 3** An angiotensin II antagonist and growth hormone secretagogue that share a common privileged structure (shaded area).

knowledge of the bound conformation to predict useful benign scaffolds have enhanced the success of this approach to peptidomimetic design.

## C. Using Structural Information

The regular nature of α-amino acid amide linkages has facilitated study through biophysical and theoretical methods such that it is possible to predict secondary structure from primary sequence and, in limited cases, to propose reasonable tertiary structures. This amount and type of information also permits the discovery of materials that can mimic large structural motifs, such as entire β-strands and α-helices (which are beyond the scope of further discussion here), as well as smaller structural features, particularly β and γ turns. And, although it can be shown that widely dispersed residues of a peptide hormone make contributive interactions with a receptor for binding and function, it is often the case that small regions of the hormone, comprising three to four amino acids, critical to receptor activation can be identified. This is typically accomplished through amino acid scans (sequential replacement of each residue in the polypeptide strand by an unobtrusive amino acid, typically alanine) and peptide truncation. Oftentimes, these critical residues are found within a turn region of the peptide. Analysis in this manner results in the development of a conceptual pharmacophore and begins the peptidomimetic design process. Having identified the requisite residues for activity, additional analytical and computational tools, such as X-ray crystallography, nuclear magnetic resonance (NMR), and molecular modeling, are often applied to further refine the structural requirements for peptidomimetic design and optimization.

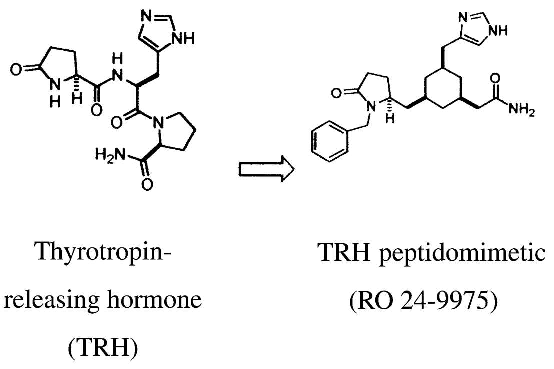

Thyrotropin-releasing hormone (TRH)
TRH peptidomimetic (RO 24-9975)

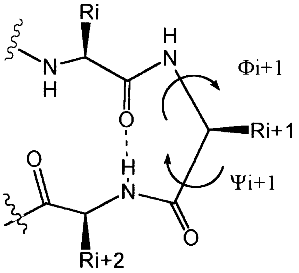
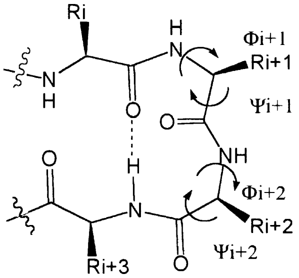
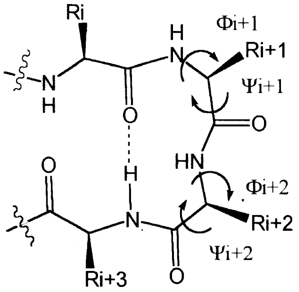
**FIGURE 4** Thyrotropin-releasing hormone mimetic derived from a benign, cyclohexane scaffold.

The conformation of the receptor-bound peptide hormone and, by extension, the placement of the peptide side chain moieties would be the most useful information for peptidomimetic design. At present, this information is largely gathered by inference from peptide structure–activity relationships (SARs), receptor mutagenesis, and studies with related peptidomimetics. Alternatively, it might be assumed that the conformation that can be confirmed in the solution phase is relevant, because it is that conformation that is initially "sensed" by the receptor and from which the bound conformation is derived.

The clearest pieces of structural data from solution-state NMR studies include strong nuclear Overhauser effects (NOEs), which typically indicate stable and close proximity (2–2.5 Å) between two protons, small temperature coefficients for amide protons (indicating lack of solvent accessibility and, by extension, involvement in an internal hydrogen bond), up-field chemical shifts of proton resonances (indicating a deshielding environment typically that is found near an aromatic ring system), and coupling constants between vicinal protons (indicating torsion angles between them). These types of information can be used in conjunction with molecular modeling techniques to generate energy-minimized conformations that meet the conditions stipulated by the NMR experiments. The two most prevalent ways to generate conformations are via distance geometry followed by energy minimization or by molecular dynamics (MD) simulations. The NMR-generated data can be used to restrain the calculation, usually the case during MD, or as a postprocessing filter. The resultant peptide conformations can be clustered on the basis of the backbone conformation and energetics to propose the prevalent bioactive conformation.

If NMR information is unavailable, small peptides can be modeled as just described and the resultant low-energy conformers can be examined for any proclivity toward a particular turn type as determined by the Φ–Ψ angles of the main-chain

TABLE 2 **Commonly Mimicked Reverse Turns**

| Turn structure | Turn type | $\Phi_{i+1}$ | $\Psi_{i+1}$ | $\Phi_{i+2}$ | $\Psi_{i+2}$ | Amino acid turn inducers |
|---|---|---|---|---|---|---|
|  Ri<br>H<br>N<br>H<br>N<br>Φi+1<br>O<br>Ri+1<br>O<br>H<br>N<br>Ψi+1<br>O<br>Ri+2 | γ classical | 70 to 85 | − 60 to − 70 | — | | — |
| | γ inverse | − 70 to − 85 | 60 to 70 | — | | — |
|  Ri<br>H<br>N<br>Φi+1<br>Ri+1<br>N<br>H<br>O<br>O<br>Ψi+1<br>O<br>H<br>NH<br>Φi+2<br>N<br>Ri+2<br>Ri+3<br>O<br>Ψi+2 | βI | − 60 | − 30 | − 90 | 0 | *i*: D, N, S, C<br>*i* + 1: P<br>*i* + 2: no P<br>*i* + 3: G |
| | BI′ | 60 | 30 | 90 | 0 | *i* + 1: G<br>*i* + 2: G |
|  Ri<br>H<br>Φi+1<br>N<br>Ri+1<br>N<br>H<br>Ψi+1<br>O<br>O<br>O<br>HN<br>H<br>Φi+2<br>N<br>Ri+2<br>Ψi+2<br>Ri+3<br>O | βII | − 60 | 120 | 80 | 0 | *i* + 1: P<br>*i* + 2: G, N |
| | βII′ | 60 | − 120 | − 80 | 0 | *i* + 1: G |

atoms. Identification of a turn type can be further advanced by examination of the peptide SAR (e.g., increases in potency by use of amino acids known to induce reverse turns in the suspected loop region). Table 2 contains the typical Φ−Ψ angles for the most common reverse turns, as well as some of the amino acids known to contribute to their stabilization.

## D. Design Strategies

With this data, there are now several approaches that can be followed to maintain the shape of the peptide while removing much of the peptide character. Which approach is taken depends on the derived SAR. A constrained amino acid via Cα−Cβ cyclization, Cα or Cβ substitution, or N-side chain cyclization can fix the conformation of the side chain (usually measured as the $\chi$ angle: N-Cα−Cβ−Cγ) and sometimes the entire turn region. If maintaining the β-strand motif and main-chain atoms is important, the side chains of residues at the $i + 1$ and $i + 2$ positions in a β-turn can be replaced with a cyclic structure to maintain the β-strand structural motif, a so-called "external scaffold." Finally, if the side chains provide the important contacts, the main chain of these residues can be replaced with some cyclic system, or an "internal scaffold." These different routes of structure stabilization are schematically depicted in Fig. 5.

Choosing to internally cyclize the γ or β reverse turn is equivalent to replacing the hydrogen bond to create a 7-membered or 10-membered ring, respectively. In the latter case, this typically takes the form of a fused bicyclic system. At this point, modeling of the proposed peptidomimetic is typically conducted to ensure that the three-dimensional realization of the design is in agreement with the target. Modeling studies of some turn mimetics have indicated that there can be several conformational families present or that the turn mimetic even fails to meet the desired conformation.

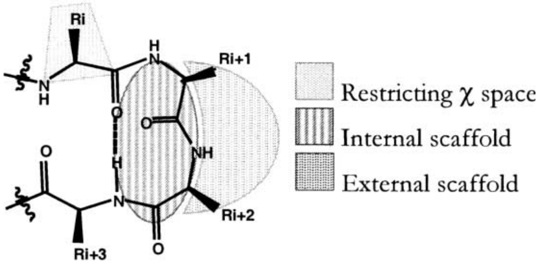


**FIGURE 5** Schematic of a β-turn depicting various peptidomimetic approaches.

Several conclusions from work in the peptidomimetic field can be proposed:

1. The more sequentially posited the amino acids to be mimicked, the greater the probability for success.
2. Determining the conformation of both the peptide backbone and side chain, particularly for the critical residues, is important and becomes more so as the critical amino acids become less contiguous in the sequence.
3. Identification of a nonpeptide lead through screening approaches (e.g., binding assays), which can then be further developed as a peptidomimetic, rather than *de novo* design based on peptide sequence, is another frequently used method of discovery.

Ultimately, the goal is to imitate or antagonize the function of the peptide ligand, and mimicking the conformation of the peptide is merely one potential means to that end.

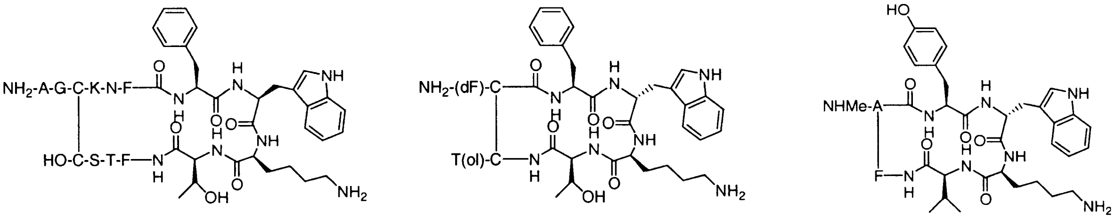
## E. Database Search Approaches

Rather than design the scaffold, various searching methods can be used to identify scaffolds to replace the main-chain atoms. One approach is to use three-dimensional substructure searches in which the Cα–Cβ bond vectors of the modeled peptide hormone are used as the probe to identify scaffolds that contain similar bond vectors, such as is done in the program CAVEAT. Or, one might use three-dimensional similarity searches to identify replacements for the main chain directly.

Designing and synthesizing a scaffold or identifying one through a database search still requires that care then be taken to incorporate the relevant side chains into the design process. Rather than take this intermediary step, a number of tools exist that can be used to search for replacements of the requisite amino acid side chains directly, either in the form of three-dimensional substructure or three-dimensional similarity searches. An example of this is discussed in the following case study for the somatostatin peptidomimetics. Finally, database searching technologies exist that allow one to forgo the use of three-dimensional information entirely. Markush queries can be readily constructed for two-dimensional substructure searches to identify molecules that contain the side chain moieties of interest. Topological similarity searches are more problematic in that a peptide probe tends to identify other peptides; the recurrence of the peptide bond creates a "signal" that overwhelms that of the side chains. Even here, methods have been developed that circumvent this to identify peptidomimetics successfully.

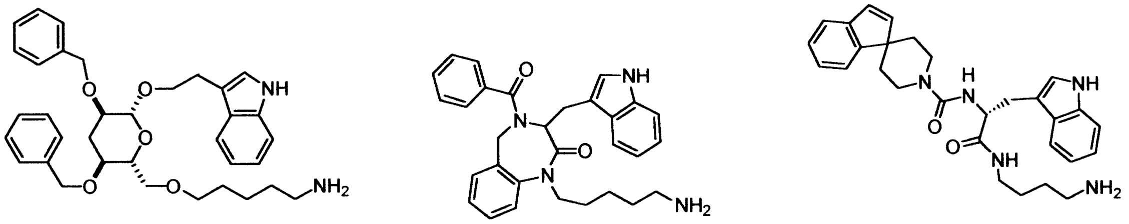
## F. Case Study: Somatostatin Peptidomimetics

Somatostatin is a neuropeptide known to modulate the secretion of growth hormone, insulin, and glucagon, among others; somatostatin agonist drugs could lead to treatments for diabetes, cancer, and acromegaly. Somatostatin occurs in both a 28- and 14-residue peptide form and binds to five distinct somatostatin receptor (SSTR) subtypes (1-5), which are members of the G-protein-coupled receptor family A (rhodopsin-like). Early SARs showed the importance of Trp-8 and Lys-9 to activity; peptides cyclized via a Cys–Cys disulfide linkage, e.g., the octapeptide Sandostatin, or via regular peptide bonds, e.g., the hexapeptide seglitide (MK-678), are highly potent compounds (Fig. 6). NMR-derived data such as NOEs, vicinal proton coupling constants,



Somatostatin (SRIF-14) Octreotide (Sandostatin) Seglitide (MK-678)

**FIGURE 6** Somatostatin and related constrained peptide analogues.



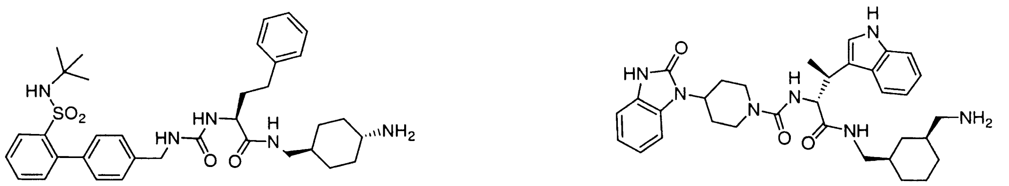
Glucose scaffold Benzodiazepine core Merck database lead

FIGURE 7 Somatostatin peptidomimetics.

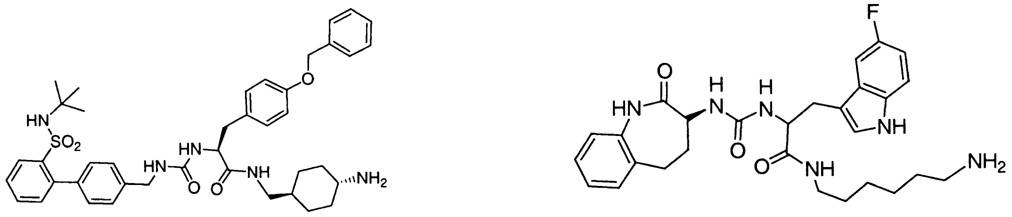
and small-temperature coefficients indicate the occurrence of a βII′-turn at the DTrp-8–Lys9 of these peptides. Upfield chemical shifts in the NMR spectrum of methylene protons in the Lys side chain indicate proximity to an aromatic system, presumably the indole of DTrp8. Additionally, it is known that the backbone atoms of the peptide are unnecessary for SSTR activation.

As an alternative to the backbone cyclization strategy just described, several attempts have been made to mimic the side chain positions by supporting their equivalents from a central scaffold, such as a sugar or benzodiazepine core (Fig. 7). Typically, this has resulted in compounds with modest agonist activity. A different approach is to use the three-dimensional structure derived from NMR and SAR results to query a database containing three-dimensional models of molecules and thereby identify new leads without predilection for a particular scaffold or other structural constraint to be used as a synthetic starting point. Using a three-dimensional model of one of the cyclic peptides, the probe can be constructed to replicate the SAR using only side chain atoms for Phe-7–DTrp-8–Lys 9 in which the side chains of DTrp-8 and Lys-9 are near each other. Interestingly, the most potent compounds identified using this search method at Merck Research Laboratories do not contain a central scaffold, but rather mimic the basic amine of the Lys and the aromatics of the Trp and Phe in a linear manner, presumably while maintaining the three-dimensional relationship of the pharmacophoric elements. This is accomplished, in part, by the incorporation of a "privileged structure" unit (Fig. 7).

The Merck database lead was amenable to optimization by both traditional medicinal chemistry and combinatorial chemistry and became the basis for the design and synthesis of analogues with a 10,000-fold increase in binding potency and 1000-fold increase in selectivity for the various receptor subtypes compared to the original lead. The Merck database lead is composed of three units, a diamine to mimic Lys, an amino acid to mimic Trp, and a "privileged structure" amine to mimic Phe. These units are combined in sequence by forming amide and urea bonds between the fragments. In order to facilitate biological screening of what could potentially be a



hSSTR1 Selective hSSTR2 Selective



hSSTR4 Selective hSSTR5 Selective

FIGURE 8 Selective somatostatin peptidomimetics from a combinatorial approach.

very large chemical library, it was decided to synthesize mixtures of compounds, which could then be quickly and selectively deconvoluted if a particular mixture showed activity in the assay. The library is synthesized by first coupling the diamine to a resin. A protected amino acid is then added to create the amide bond, the amine protecting group is removed, and the urea link to the terminal amine is formed. The products are then cleaved from the resin. Some of the resin is archived after each addition of a subunit to facilitate later deconvolution and identification of active components. Theoretically, 131,670 compounds were prepared in this library of 79 mixtures, when stereo- and regioisomers for the 20 diamines, 20 amino acids, and 79 amines are taken into account.

The design process for peptidomimetics was used in the selection of the library components. The most important consideration was to ensure that the pharmacophoric elements identified for somatostatin were present in the selected starting materials. Most of the amino acids used contained an aromatic group, as did the final amine fragment. Conformational restraints in the form of rings and branching groups were used in all components to further restrict flexibility, with the "privileged structure" amine biased toward piperidines and piperazines to further mimic the database lead. Biological screening followed by iterative deconvolution of components in the active mixtures led to the identification of compounds that were individually selective as agonists for somatostatin receptor subtypes 1, 2, 4, and 5 (Fig. 8).

## IV. SUMMARY

Peptidomimetic ligands can serve as useful tools for the study of receptor signaling pathways and as important pharmacological agents for the treatment of disease. One method for their discovery involves activity screening of natural products and other compound collections. Use of "benign scaffolds" and chemical modification of "privileged structures" are two tactics for further refinement of the potency and selectivity of a peptidomimetic lead structure. Principles for rational design have also been put forth that rely on structural information derived from the ligand in either the receptor-bound or the solution state. Application of computational methods has greatly assisted this design process.

## Glossary

**chemical library** A group of chemical compounds generally related by structure and method of preparation.

**isostere** A chemical equivalence in which an undesired chemical moiety is replaced by an atom or groups of atoms that retain the desired properties of the moiety being replaced.

**Markush query** Chemical database search tool in which substituents on the core structure do not need to be precisely enumerated.

**peptidomimetic** Nonpeptidic compound that, when bound to a receptor, can either imitate or block the action of a peptide ligand at the receptor level.

**pharmacophore** A conceptualization of features, both steric and electronic, minimally required in a molecule in order to elicit some desired biological response.

**privileged structure** Fragment or group often used in peptidomimetic design; contains elements of both pharmacophore (typically hydrophobic/aromatic) and scaffold.

**topography** Representation or "mapping" out of three-dimensional features in two-dimensional space.

## See Also the Following Articles

**Angiotensins • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Ligand Modification to Produce Pharmacologic Agents • Multiple G-Protein Coupling Systems • Somatostatin • Thyrotropin-Releasing Hormone Receptor Signaling**

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# Peroxisome Proliferator-Activated Receptors

Karen L. MacNaul and David E. Moller
*Merck Research Laboratories, New Jersey*

I. INTRODUCTION
II. STRUCTURE OF PPARs
III. PPAR-α AND DISEASE TARGETS
IV. PPAR-γ AND DISEASE TARGETS
V. PPAR-δ AND DISEASE TARGETS
VI. SUMMARY

**Peroxisome proliferator-activated receptors regulate key genes involved in glucose and lipid metabolism. In addition, these receptors are involved in many diverse biological functions, including development of the central nervous system, inflammation, cancer, and fertility. Development of specific, potent ligands for these receptors offers the potential for therapeutic intervention in many debilitating diseases.**

## I. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of a large superfamily of ligand-activated nuclear receptors. Three human isoforms, encoded by separate genes, have been identified in the past decade: PPAR-α [NR1C1], PPAR-δ [NR1C2], and PPAR-γ [NR1C3]. The subtypes share many structural similarities typical of nuclear receptors. Physiologically, PPARs are known to regulate key genes involved in both glucose and lipid metabolism. Synthetic PPAR agonists have been found to be efficacious in decreasing serum glucose, insulin, and lipids. By lowering these parameters in humans, metabolic disorders such as type 2 diabetes and dyslipidemia are improved. With the prevalence of type 2 diabetes and related metabolic disorders such as dyslipidemia and atherosclerosis reaching epidemic proportions in countries consuming a high-fat Western diet, the need for effective, safe therapies is critical.

## II. STRUCTURE OF PPARs

PPARs have been identified and cloned from numerous species, including *Xenopus*, rodents, and humans. Like other nuclear receptors, they share characteristic functional domains. The amino-terminal region contains a variable "A/B" domain, which is poorly conserved between the three isoforms and contains a ligand-independent activation function (AF-1) domain. The highly conserved central "C" region contains the DNA-binding domain. This region contains two zinc finger structures that bind to PPAR responsive elements (PPREs) in target genes. Comparing the human isoforms, there is greater than 80% amino acid identity in this region. A flexible hinge region, "D," separates the DNA-binding domain (DBD) from the ligand-binding domain (LBD). The LBD "E" region is also highly conserved, with greater than 60% amino acid identity between human isoforms. The carboxy terminus of the LBD contains a ligand-dependent activation function domain, AF-2 (Fig. 1). The crystal structures of all three PPAR LBDs have been solved, both as apoproteins and co-crystallized with ligands. PPARs are ligand-dependent transcription factors that switch from an inactive form to an active form on binding agonist. The LBDs contain 13 α-helices and a small four-stranded β-sheets (Fig. 2). This structure contains a relatively large hydrophobic ligand-binding pocket in comparison to other nuclear receptors.

As with many nuclear receptors, all three PPAR subtypes are known to heterodimerize with the 9-*cis*-retinoid X receptors (RXRs) (Fig. 3). PPAR:RXR heterodimers are "permissive" in that they can be activated by either PPAR- or RXR-selective agonists. These heterodimers bind to direct repeats (DR-1) of a hexameric nucleotide sequence with the consensus sequence AGGTCA separated by one nucleotide. PPREs are located in the promoter of PPAR target genes. After agonists are bound, the LBD undergoes conformational changes, co-activators are recruited, and transcriptional activation is initiated.

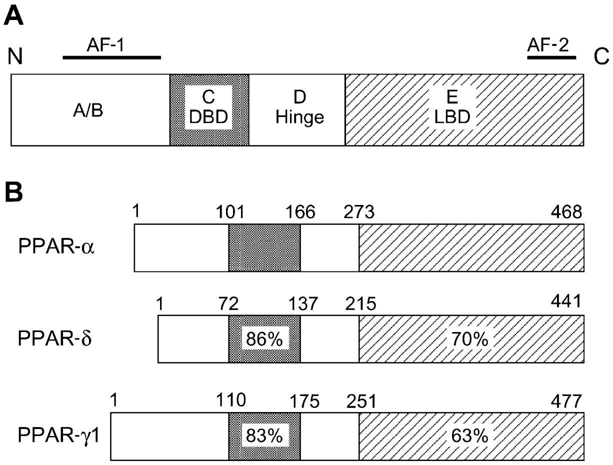

**FIGURE 1** Functional domains of the PPARs. (A) The N-terminal A/B domain contains the ligand-independent activation function domain (AF-1). Domain C contains the DNA-binding domain (DBD), connected by hinge region "D" to the "E" region containing the ligand-binding domain (LBD). The C-terminus of the LBD contains a ligand-dependent activation function domain, AF-2. (B) The amino acid identities of DBD and LBD regions of the three human subtypes are compared; the percent identity relative to PPAR-α is indicated within the domains. The numbers above the individual receptors indicate the number of amino acid residues from the N-terminus for the regions.

PPARs can complex with a number of co-activator or corepressor proteins to regulate transcriptional activation or repression, respectively, of PPAR target genes. Binding of co-activators usually occurs at the AF-2 region, which has been located on helix 12 of the LBD. Several PPAR co-activators have been identified to date and can be grouped into three general categories based on their mode of regulation of transcriptional activation. Two prototypes are steroid receptor co-activator-1 (SRC-1) and cyclic adenosine monophosphate response element binding (CREB) protein p300 (CBP/p300); both have histone acetylase activity that is involved in the remodeling of chromatin structure to allow transcription factors access to DNA binding sites. Another class of co-activators that includes members of the vitamin D-interacting protein/thyroid hormone receptor-associated protein (DRIP/TRAP) complex augments basal transcriptional activity by interacting with the basal transcriptional complex. Several additional co-activators, including the PPAR-γ co-activator (PGC-1), receptor-interacting protein-140 (RIP140), and androgen receptor 70-kDa co-activator (ARA70), have been implicated as regulators of PPAR action, although their mechanisms of action are not well understood.

## III. PPAR-α AND DISEASE TARGETS

### A. Characterization and Ligands

PPAR-α was the first isoform to be identified and cloned from a murine liver cDNA library in 1990. PPAR-α has since been cloned from several other rodent species, *Xenopus*, and humans. The human PPAR-α gene has been mapped to chromosome 22q12–q13. PPAR-α is expressed at highest levels in tissues involved in fatty acid catabolism, such as liver, kidney, heart, skeletal muscle, and cells of the arterial wall. In these tissues, PPAR-α plays a key role in lipid metabolism by regulating genes involved in the β-oxidation of long-chain fatty acids, including acyl-CoA oxidase, enoyl-CoA hydratase/dehydrogenase multifunctional enzyme, keto-acyl-CoA thiolase, CYP4A cytochrome P450 enzymes, and fatty acid binding protein. Fatty acid transport protein (FATP) and fatty acid translocase (FAT, also known as CD36), which are involved in transport of fatty acids across the cell membrane, are up-regulated by

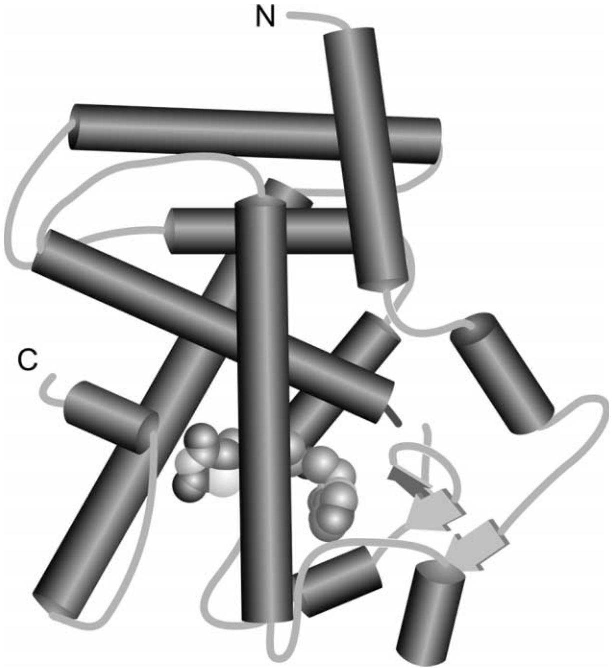

**FIGURE 2** Overall structure of the human PPAR-γ ligand-binding domain complexed with rosiglitazone. The α-helices are represented by tubes and the small, four-stranded β-sheet is denoted by arrows. Within the large binding pocket is the thiazolidinedione rosiglitazone, represented by the ball structure, which binds in a U-shaped conformation and occupies only ~40% of the pocket. The AF-2 domain located on the C-terminal α-helix is important for cofactor binding and transcriptional activation.

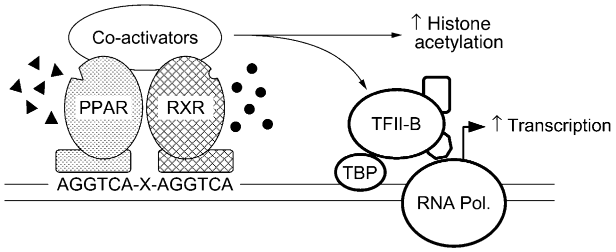

**FIGURE 3** The peroxisome proliferator-activated receptor: retinoic acid X receptor (PPAR:RXR) transcriptional complex. PPARs heterodimerize with RXR and bind to specific DNA response elements in the regulatory regions of target genes. PPAR agonists (▲) and RXR agonists (•) bind their respective receptors and undergo conformational changes and recruitment of co-activator proteins. This illustration depicts binding of a co-activator with histone acetylase activity that is involved in remodeling the chromatin structure to allow interaction with basal transcriptional factors (such as TFII-B, TBP, and RNA polymerase).

PPAR-α activation; apoproteins A-I and A-II (apoA-I and apoA-II), the major constituents in high-density lipoproteins, are up-regulated by PPAR-α as well.

Natural ligands for PPAR-α present in human serum are composed of many saturated and unsaturated fatty acids, including palmitic acid, oleic acid, linoleic acid, and arachidonic acid (Fig. 4). These fatty acids bind to PPAR-α with micromolar affinities. However, it is not known if all of these ligands are present in sufficiently high concentrations to be physiologically relevant. Higher affinity naturally occurring PPAR-α activators have been identified, including the lipoxygenase 8(*S*)-hydroxyeicosatetraenoic acid [8(*S*)-HETE] and leukotriene B4 (LTB4). However, these ligands also may not be present at sufficient levels in relevant tissues to be considered key endogenous ligands.

PPAR-α can be activated by a diverse range of synthetic compounds, including industrial plasticizers, pesticides, and the fibrate class of drugs prescribed for hypertriglyceridemia. Among the fibrates are clofibrate, gemfibrozil, and fenofibrate. These compounds are relatively weak agonists (30–50 $\mu M$) and require large doses clinically in humans for efficacy in hyperlipidemia. In rodents, PPAR-α agonists cause an increase in the size and number of liver peroxisomes, subcellular organelles containing enzymes involved in long-chain fatty acid oxidation. Peroxisome proliferation in rodents progresses to hepatomegaly and hepatocarcinoma. In humans, however, significant hepatic peroxisome proliferation has not been observed on exposure to PPAR-α activators, as evidenced by the long-term clinical experience with fibrates. Hence, the name “peroxisome proliferator-activated receptors” is a misnomer for this class of human nuclear receptors. It is therefore currently believed that activation of PPAR-α in humans does not warrant concerns for hepatotoxicity. The reason for this species-to-species difference may be because there is 10-fold lower expression of hepatic PPAR-α mRNA in humans, compare to mice. In addition, 30–50% of the PPAR-α expressed in human liver is a truncated splice variant lacking exon 6, which encodes for the ligand-binding domain. This further reduces the amount of functional receptor compared to mice.

## B. Dyslipidemia and Atherosclerosis

The increased prevalence of a high-fat Western diet in many societies, coupled with increasingly sedentary lifestyles, has resulted in an increased incidence of metabolic disorders, including type 2 diabetes. Common comorbid conditions are dyslipidemia and progression to atherosclerosis, the leading cause of death among patients with type 2 diabetes. Dyslipidemia, an inappropriate alteration in lipid chemistry, is generally characterized by elevations in serum triglycerides (TGs) and very low-density lipoproteins (VLDLs) and a decrease in high-density lipoproteins (HDLs). PPAR-α activation by fibrates results in the up-regulation of lipoprotein lipase, which enhances

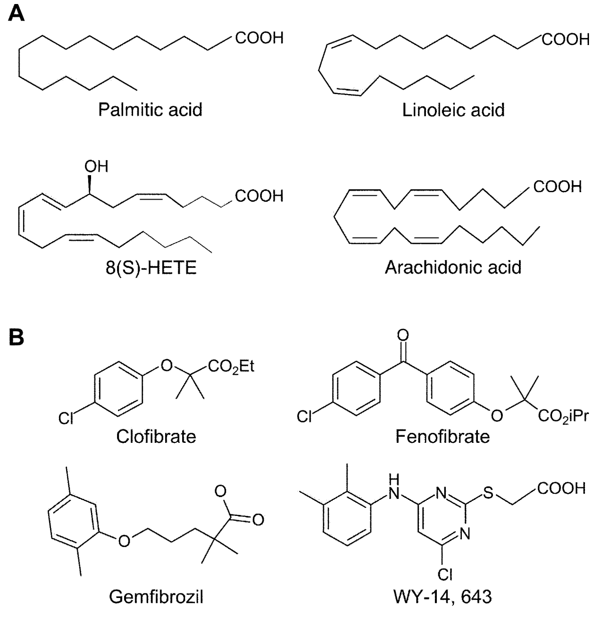

**FIGURE 4** The structures of various natural (A) and synthetic (B) PPAR-α ligands. 8(*S*)-HETE, 8(*S*)-Hydroxyeicosatetraenoic acid.

the lipolytic activity of triglyceride-rich VLDL particles, and the suppression of hepatic apoC-III gene expression, which increases VLDL clearance. Fibrates, prescribed since the 1960s, have been investigated in humans in several large clinical trials; they have been found to be efficacious in lowering serum TGs and produce a modest increase in HDL levels. Moreover, fibrates have the proved benefit of reducing the risk of atherosclerosis.

PPAR-α null mice have been generated and exhibit a phenotype of significantly higher levels of total serum cholesterol, HDL cholesterol, and apoA-I mRNA. After 6 months of age, these mice exhibit a phenotype of increased body fat and increased hepatic fatty acids, visualized as lipid vesicles in the parenchyma. Unlike wild-type mice, the PPAR-α null animals do not respond to fibrate challenge with an induction of enzymes responsible for fatty acid oxidation. Neither is there a reduction in serum TGs or total serum cholesterol, further illustrating the role of PPAR-α in lipid homeostasis.

The formation of atherosclerotic lesions involves the recruitment of monocytes and their activation to macrophages, followed by their incorporation into the arterial wall via the vascular cell adhesion molecule 1 (VCAM-1). As the lesion progresses, macrophages become laden with cholesterol esters (foam cells) and a fibrous cap is formed from vascular cells and cell debris. Concurrent with this process is a local inflammatory response involving plasma cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα), as well as the proinflammatory eicosanoid LTB4. As atherosclerotic plaques mature, they protrude into the arterial lumen, restricting circulation. Rupture of these plaques results in thrombosis, which can cause myocardial infarctions and/or stroke.

PPAR-α is expressed in human monocytes, macrophages, endothelial cells, and vascular smooth muscle cells of atherosclerotic lesions. PPAR-α agonists can improve the outcome of atherosclerosis in a number of ways. One mechanism is the down-regulation VCAM-1 expression in human vascular endothelial cells, thus reducing the formation of lesions. Numerous *in vitro* studies have also shown that PPAR-α activators can inhibit the expression of the inflammatory cytokines IL-6 and IL-1β in a PPAR-α-dependent manner. In addition, arachidonic acid-induced ear-swelling in mice shows that the inflammatory response is prolonged in PPAR-α null mice compared to wild-type mice. However, the most relevant data for humans come from clinical trials in which coronary artery disease (CAD) patients treated with fibrates for several years show reduced prevalence of recurrent myocardial infarction.

## IV. PPAR-γ AND DISEASE TARGETS

### A. Characterization and Ligands

PPAR-γ, the most extensively studied PPAR isoform, has been cloned from a wide variety of species, including *Xenopus*, mice, hamsters, rhesus monkeys, and humans. The level of amino acid conservation across species is highest (95% identity between murine and human receptors) for this isoform. The human PPAR-γ gene has been mapped to chromosome 3p25. PPAR-γ is expressed at high levels in adipose tissue and is a key regulator of fatty acid metabolism and adipocyte differentiation. Recently, PPAR-γ was found to be expressed in foam cells of atherosclerotic plaques, implying a role for this receptor in cardiovascular disease. The three PPAR-γ mRNA isoforms that have been identified in humans, γ1, γ2, and γ3, differ by alternate promoter usage and splice variants. PPAR-γ1 and PPAR-γ3 encode the same protein whereas PPAR-γ2, a splice variant of PPAR-γ1, has an additional 30 amino-terminal amino acids encoded by one exon. The expression pattern of these three isoforms is regulated in a tissue-specific manner. PPAR-γ1 is expressed in a wide range of tissues, including skeletal muscle, heart, colon, intestines, kidney, pancreas, and spleen. PPAR-γ2 is expressed primarily in adipose tissue, where it is the predominant isoform. PPAR-γ3 expression has been detected only in adipose tissue, macrophages, and colon epithelia. Presently, the functional significance of the two protein isoforms and their differential tissue expression is unclear.

PPAR-γ can be activated at micromolar affinity by a range of natural ligands (Fig. 5), including the fatty acids linoleic acid, arachidonic acid, and eicosapentaenoic acid; the 9- and 13-hydroxyoctadecadienoic acids (9-HODE and 13-HODE) that are generated from the conversion of linoleate by 15-lipoxygenase are micromolar PPAR-γ agonists. Additionally, the prostaglandin (PG) derivatives, most notably 15-deoxy-$\delta^{12,14}$-PGJ2, PGH1, and PGH2, have activation affinities of $<10\ \mu M$ on the PPAR-γ receptor. The affinities of these natural ligands are within the range of levels normally seen in human serum. However, the physiological level, chemical form, or specificity of these ligands in target cells is not known. Recently, the most potent natural ligand for PPAR-γ, the oxidized alkyl phospholipid, hexadecyl azelaoyl phosphatidylcholine (azPC), was identified and

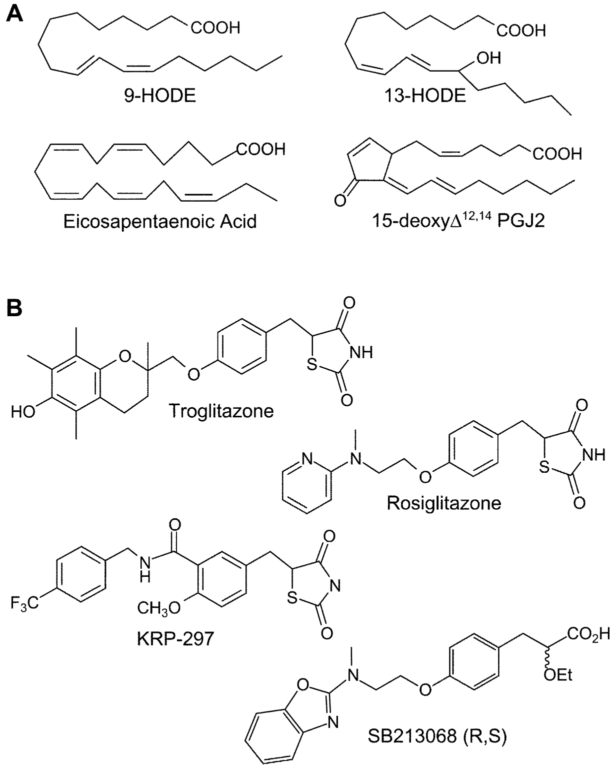

**FIGURE 5** The structures of various natural (A) and synthetic (B) PPAR-γ ligands. 9- and 13-HODE, 9- and 13-Hydroxyoctadecadienoic acids; 15-deoxy-$\Delta^{12,14}$-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2.

characterized as a ~40 n*M* activator. Hexadecyl azPC is formed by oxidation of low-density lipoprotein (LDL) phospholipid particles, which are believed to initiate and maintain vascular inflammation during atherogenesis, linking the expression of PPAR-γ in this disease process to a relevant ligand.

Numerous potent synthetic ligands spanning a range of structural classes have been identified as PPAR-γ agonists. The thiazolidinediones (TZDs), also termed "glitazones," were the first class reported. TZDs were identified through empirical compound screening in insulin-resistant, diabetic rodents and have since been developed as insulin-sensitizing agents in humans. Compounds included in this class are troglitazone, pioglitazone, and rosiglitazone, which have affinities ranging from 20 to 100 n*M* for the human and murine receptors. These TZDs are relatively selective for PPAR-γ, but a newer TZD, KRP-297, binds potently to both PPAR-γ and PPAR-α. Recently, another compound class has been identified, the α-alkoxy-β-phenylpropionates, represented by SB 236636 and SB 213068, which are potent PPAR-γ agonists with significant PPAR-α activity. Several nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and fenoprofen have also been found to activate PPAR-γ and PPAR-α at micromolar concentrations and are able to induce adipocytic differentiation *in vitro*.

## B. Type 2 Diabetes

The most extensive use for PPAR-γ agonists is in the treatment of type 2 diabetes in humans. The synthetic TZDs were the first class of compounds shown to have a direct correlation between their *in vitro* potency and their efficacy as insulin sensitizers in diabetic animal models and humans. TZDs increase the sensitivity of target tissues (adipose tissue, liver, and muscle) to the action of insulin. Thus, they improve glucose disposal and inhibit hepatic glucose production, thereby reducing plasma glucose levels. Since 1995, several TZDs have been approved for treatment of patients with type 2 diabetes (troglitazone, rosiglitazone, and pioglitazone), resulting in a major therapeutic advancement in treatment for a metabolic disorder that affects approximately 28 million people worldwide. In March 2000, troglitazone was removed from the market due to idiosyncratic hepatotoxicity, severe enough in some patients to require liver transplants or to cause death. These severe adverse effects have not been seen with rosiglitazone or pioglitazone.

PPAR-γ is expressed at highest levels in adipose tissue, where it has been studied extensively. However, very few PPAR-γ target genes have been identified to explain adequately a mechanism for insulin-sensitizing effects of TZDs. In isolated human adipocytes, rosiglitazone increases expression of p85-α phosphatidylinositol 3-kinase (p85-α PI3K) mRNA, a component of the insulin signaling pathway. Insulin receptor substrate-2 (IRS-2), a key intracellular substrate in the insulin signaling pathway, has also been demonstrated to be increased in cultured adipocytes and human adipose tissue treated with PPAR-γ agonists.

In adipose tissue, PPAR-γ regulates the transcription of several PPAR-responsive genes encoding lipogenic proteins and proteins involved in the differentiation of adipocytes. These genes include the adipocyte fatty acid-binding protein (aP2), phosphoenolpyruvate carboxykinase (PEPCK), lipoprotein lipase (LPL), and the fatty acid transporters FATP and CD36. Currently, it is speculated that altering the expression of fat cell genes may, through a secondary mechanism, result in insulin sensitization of muscle and liver. Confirming this hypothesis, an insulin-resistant, hyperglycemic transgenic mouse model in which adipose tissue has been ablated fails to show significant glucose lowering when treated with a TZD.

PAR-γ null mice have been generated. However, they demonstrate an embryonic lethal phenotype at approximately day 10 of gestation due to severe defects in placental development and myocardial thinning. Studies performed using PPAR-γ-deficient stem cells to generate chimeric mice have shown that PPAR-γ is required for differentiation of adipose tissue. Surprisingly, PPAR-γ heterozygous mice fed a high-fat diet show decreased fat mass, smaller adipocytes, and improved insulin sensitivity relative to wild-type mice. In humans, three individuals with severe insulin resistance have been found to have two different heterozygous loss-of-function (and dominant-negative) mutations in helix 12 of the PPAR-γ ligand-binding domain (P467L and V290M). These mutations also result in the development of type 2 diabetes at an early age.

## C. Atherosclerosis

Monocytes and macrophages are key cell types involved in the immune response. They act by releasing inflammatory cytokines such as IL-1β, IL-6, and TNF α, along with inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of a potent oxidant, nitric oxide (NO). These cell types are also important early mediators in the pathogenesis of atherosclerosis. PPAR-γ has been shown to be significantly up-regulated during the differentiation of monocytes into macrophages, and has been found to be expressed in cholesterol-laden, macrophage-derived foam cells within atherosclerotic lesions. Current evidence shows that PPAR-γ agonists may exert both pro- and anti-atherogenic effects via the expression of macrophage genes involved in atherosclerosis. Treatment of macrophages with TZDs and an RXR agonist *in vitro* increases the expression of the class B scavenger receptors, CD36 and SR-B1, involved in the transport of oxidized LDL (Ox-LDL) cholesterol particles into foam cells. In addition, the Ox-LDL-derived products 9- and 13-HODE are PPAR-γ activators, suggesting a positive feedback loop for a pro-atherogenic effect. On the other hand, TZDs have variably been reported to down-regulate the expression of potentially pro-atherogenic proteins, including matrix metalloproteinase-9 (MMP-9; also named gelatinase B, an enzyme implicated in atherosclerotic plaque destabilization) and scavenger receptor class A (SR-A), along with vascular cell adhesion molecule-1(in vascular cells. Patients treated with troglitazone show a reduction in plasminogen activator inhibitor type 1 (PAI-1), a serine protease inhibitor that promotes thrombosis. These data suggest an anti-atherogenic effect of PPAR-γ agonists. Given that the net effects of PPAR-γ agonist treatment in mouse models of atherosclerosis are to reduce lesion formation, it is hoped that chronic treatment of humans will produce similar effects. However, current clinical data have not yet substantiated this hypothesis.

## D. Cancer

PPAR-γ is expressed in a variety of human tumors, including lung carcinoma, breast adenocarcinoma, prostate carcinoma, liposarcoma, renal cell carcinoma, and colorectal carcinomas, in addition to a wide range of human cancer cell lines. PPAR-γ activators have been shown to inhibit the proliferation of fibroblasts *in vitro* during differentiation into normal preadipocytes, leading to the hypothesis that PPAR-γ agonists play a role in inhibiting the growth of adipose tissue-derived carcinoma. This has been demonstrated in metastatic human liposarcoma, a soft tissue malignancy that responds poorly to conventional therapies. Terminal differentiation of this malignant growth cell type has been achieved *in vitro* by treatment with the PPAR-γ activator pioglitazone and an RXRα-specific agonist. In addition, clinical troglitazone treatment of patients suffering from advanced liposarcomas results in inhibition of cell proliferation and terminal differentiation of the solid tumor.

PPAR-γ activators have also been shown to inhibit proliferation of human prostate cells *in vitro*, and patients with advanced prostate cancer treated with troglitazone have a decrease in prostate-specific antigen, a marker for prostate cancer. In human mammary adenocarcinomas, PPAR-γ is also expressed at significant levels and agonists have been reported to inhibit growth and induce terminal differentiation of malignant breast epithelial cells. Although PPAR-γ is expressed in human colonic polyps, primary colon tumors, and colon cancer cell lines, conflicting data as to the benefits vs proneoplastic effects of agonism have been generated from various animal and cellular models of this disease. Further investigation is needed to elucidate the role PPAR-γ may have in cancer and the mechanism by which TZDs exert their antiproliferative effects.

## V. PPAR-δ AND DISEASE TARGETS

### A. Characterization and Ligands

PPAR-δ, first cloned in 1992 from *Xenopus*, was initially given the name PPAR-β. Subsequently, in various labs it was cloned from humans and mice and was named NUC1 and PPAR-δ, FAAR, or NUC1, respectively. The current accepted nomenclature for all species is now PPAR-δ. The amino acid homology between human and rodent PPAR homologues is 90% in the LBD. The human PPAR-δ gene has been mapped to chromosome 6p21.1–p21.2. PPAR-δ is ubiquitously expressed in adult rat tissues. It has been reported to be expressed in human liver, intestine, kidney, skeletal muscle, brain, abdominal adipose, and skin. Presently, no specific target genes for PPAR-δ have been identified and studies to elucidate its physiological role have just recently begun to emerge.

PPAR-δ binds naturally occurring saturated and unsaturated fatty acids, as well as to their metabolites, including dihomo-γ-linolenic acid, eicosapentaenoic acid, and arachidonic acid, with affinities in the low micromolar range (Fig. 6). Synthetic ligands include L-165041, a compound with 10- to 30-fold selectivity for human PPAR-δ vs PPAR-γ. Recently, GW501516 has been identified as a potent PPAR-δ agonist. This compound is selective for the human, rhesus monkey, and mouse PPAR-δ subtypes vs other nuclear receptors.

### B. Dyslipidemia

In *db/db* mice, which serve as a model of type 2 diabetes (increased serum TGs, glucose, and insulin), L-165041 causes a modest increase in HDL at a dose that does not cause TG or glucose lowering. GW501516 has been studied in a population of obese rhesus monkeys with metabolic parameters similar to those in humans with metabolic syndrome (dyslipidemia, insulin resistance, hyperinsulinemia, and hypertension). In this model, GW501516 induces a significant increase in HDL-cholesterol levels along with a reduction in TG. Elevated levels of plasma insulin are also suppressed by GW501516 treatment, whereas serum glucose levels are unaffected. These results suggest that PPAR-δ agonists may prove useful in the treatment of dyslipidemia, but it is important to note that this was a limited study with respect to species and compounds. The role of PPAR-δ in dyslipidemia requires further investigation.

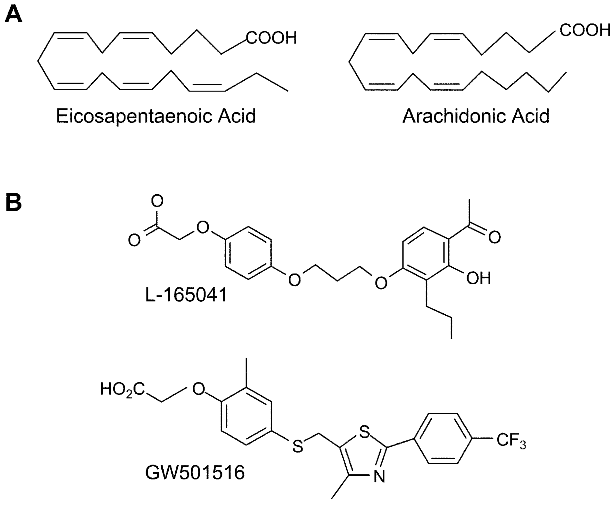

**FIGURE 6** The structures of various natural (A) and synthetic (B) PPAR-δ ligands.

### C. Fertility

In mice, PPAR-δ is the only PPAR subtype expressed at the site of blastocyst implantation. Expression is induced in the stromal layer surrounding the implanting blastocyst and in the decidual layer following implantation. The same expression pattern is seen for cyclooxygenase 2 (COX-2). COX-2 is the rate-limiting enzyme involved in the synthesis of prostaglandins. Several prostaglandins generated from the COX-2 biosynthetic pathway, such as prostacyclin, are PPAR-δ ligands. COX-2 null mice have defects resulting in reduced implantation and decidualization. Treatment of COX-2 null mice with a natural PPAR-δ agonist, carbaprostacyclin, or the synthetic PPAR-δ agonist L-165041, restores implantation to normal in these animals. When PPAR-δ heterozygous null mice are bred, fewer than expected null offspring are produced, indicating that although PPAR-δ may play a role in implantation and fertility, it is not essential.

### D. Cancer

PPAR-δ has been recently identified as a downstream target in a pathway involved in colorectal carcinogenesis. PPAR-δ mRNA expression is up-regulated in many human and rodent colon carcinomas. During the development of colorectal carcinoma, an inactivation mutation in the adenomatous polyposis coli (APC) gene occurs. This mutation causes an increase in β-catenin, a cytoplasmic protein involved in cellular adhesion and development. β-Catenin forms complexes with a protein called T-cell factor 4 (TCF-4), which binds DNA and induces genes involved in cellular proliferation. β-Catenin/TCF-4 complexes can bind and activate the PPAR-δ promoter and have been shown to up-regulate the expression of PPAR-δ in colon carcinoma cells. A human colorectal cancer cell line, HCT116, in which the PPAR-δ gene is deleted, was established and injected as xenografts into nude mice. In this model, the PPAR-δ null cells result in fewer tumors with slower progression compared to xenografts of wild-type HCT116 cells. Taken together, these data suggest that inhibition of PPAR-δ expression or function by antagonists may result in colon tumor suppression.

### E. Central Nervous System

The expression of PPARs during rat embryonic development, assessed by *in situ* hybridization, shows that PPAR-δ is expressed ubiquitously and earlier in development than PPAR-α or PPAR-γ. Interestingly, PPAR-δ is expressed in embryonic tissues at much higher levels compared to the other isoforms, in sharp contrast to expression levels in adult tissues. Peak expression of PPAR-δ occurs at embryonic day 13.5 (E13.5) in tissues of the developing nervous system, suggesting an important role for this subtype in differentiation of cells within the CNS. *In vitro*, PPAR-δ mRNA is expressed in immature murine oligodendrocytes. These are the major lipid-producing cells in the CNS that differentiate into cells with myelin sheets. Treatment of neonatal primary glial cell cultures, which contain immature oligodendrocytes, with the PPAR-δ selective agonist L-165041 results in accelerated differentiation of oligodendrocytes, increased processes, and the formation of membrane sheets. *In vivo*, PPAR-δ null mice have alterations in the extent of myelination in the corpus collosum compared to wild-type controls. This subregion normally expresses high levels of PPAR-δ.

## VI. SUMMARY

Data have emerged over the past decade showing the involvement of the three PPAR isoforms as sensors for fatty acids and lipid metabolites in metabolically active tissues. In these tissues, PPARs transcriptionally regulate target genes that play critical roles in lipid metabolism. Therapeutic use of the fibrates in humans has validated the efficacy of PPAR-α agonists in improving hypertriglyceridemia and reducing the risk for atherosclerosis and subsequent myocardial infarctions. Both the TZD and the more recently identified non-TZD PPAR-γ agonists have been shown to improve insulin sensitivity and lower serum glucose in patients with type 2 diabetes, a metabolic disorder of epidemic proportions in westernized cultures. In addition, PPAR-γ agonists have demonstrated provocative effects in animal models, implying that there is great potential for their use in treating or preventing atherosclerosis in humans. The role of PPAR-γ in cancer is just emerging. Further investigation is needed to elucidate the mechanisms of action by which this receptor positively impacts on these disease states. PPAR-δ, the isoform for which only limited data have emerged, may have therapeutic utilities in such diverse disorders as dyslipidemia, fertility, cancer, and demyelinating CNS diseases. Specific, potent ligands are being identified that will allow a deeper understanding of the pleiotropic effects of the PPARs.

## Glossary

**adipocytes** Cells that store energy in the form of triglycerides and release energy as free fatty acids during periods of nutritional deprivation.

**atherosclerosis** Condition caused by progressive thickening and hardening of the walls of medium-sized and large arteries as a result of extensive fat and cellular deposits on their inner lining, ultimately leading to arterial occlusion.

**fatty acid** Long-chain aliphatic carboxylic acid found in fats, oils, membrane phospholipids, and glycolipids.

**hormone receptor** Protein in or on the surface of target cells; functions as a sensor for the hormone by binding the hormone and initiating a cellular response.

**ligand** Small molecule that binds specifically to a larger one; for example, a hormone is the ligand for its specific protein receptor.

**metabolic syndrome** Condition defined by insulin resistance, hyperinsulinemia, and dyslipidemia (generally elevated serum triglycerides and decreased high-density lipoprotein cholesterol).

**peroxisome** Subcellular organelles in the cytoplasm of eukaryotic cells; involved in the β-oxidation of long-chain fatty acids.

**type 2 diabetes** One of the two major types of diabetes, in which the beta cells of the pancreas produce insulin but the body is unable to use it effectively because the target cells have become resistant to the action of insulin.

## See Also the Following Articles

**Diabetes Type 2 • Ligand Modification to Produce Pharmacologic Agents • Lipoprotein Receptor Signaling**

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# Pheromone Production in Insects

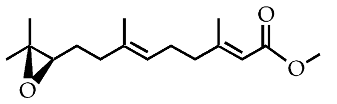
Gary J. Blomquist
*University of Nevada*

I. INTRODUCTION
II. PBAN REGULATION IN LEPIDOPTERA
III. JUVENILE HORMONE REGULATION IN COLEOPTERA
IV. JUVENILE HORMONE REGULATION IN BLATTODEA
V. ECDYSTEROID REGULATION IN DIPTERA
VI. CONCLUDING REMARKS

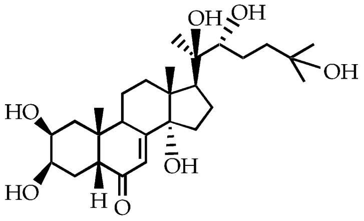
**The production of pheromones can be regulated by hormones from the brain, ovary, and corpora allata. In insects, at least three different hormones have been shown to regulate pheromone production: pheromone biosynthesis-activating neuropeptide, juvenile hormone, and ecdysteroids. This article discusses several model insect systems of endocrine regulation of pheromone biosynthesis.**

## I. INTRODUCTION

The observation that females of certain species have repeated reproductive cycles and that mating occurs only during defined periods of each cycle led to the proposal that pheromone production might be under hormonal control. Studies on a limited number of species demonstrate that pheromone production can be under the regulation of products of the brain, ovary, and corpora allata (CA). At least three distinct hormones have been shown to regulate pheromone production in insects. In many female moths (Lepidoptera), pheromone biosynthesis is regulated by a 33- or 34-amino-acid pheromone biosynthesis-activating neuropeptide (PBAN) (Fig. 1). PBAN alters enzyme activity at one or more steps during or subsequent to fatty acid synthesis during pheromone production. In some species of Coleoptera, Blattodea, and Lepidoptera, juvenile hormone (JH) (Fig. 1) induces pheromone production. In the female housefly, ovarian produced ecdysteroids (Fig. 1) regulate



**Juvenile hormone III**



**20-Hydroxyecdysone**

1 5 10 15
Hez-PBAN **Leu**-Ser- Asp-**Asp-Met-Pro-Ala-Thr**-Pro-**Ala-Asp-Gln-Glu**-Met-**Tyr**-Arg-Gln-
Bom-PBAN **Leu**- Ser- Glu-**Asp-Met-Pro-Ala-Thr**-Pro-**Ala-Asp-Gln-Glu**-Met-**Tyr**-Gln-Pro-
Lyd-PBAN **Leu**-Ala-Asp-**Asp-Met-Pro-Ala-Thr**-Met-**Ala-Asp-Gln-Glu**-Val-**Try**-Arg-Pro-

**PBAN**

20 25 30
Asp-**Pro-Glu**-Gln- Ile- Asp-**Ser-Arg**-Thr-Lys-**Tyr-Phe-Ser-Pro-Arg-Leu**-$NH_2$
Asp-**Pro-Glu**-Glu-Met-Glu-**Ser-Arg**-Thr-Arg-**Tyr-Phe-Ser-Pro-Arg-Leu**-$NH_2$
Glu-**Pro-Glu**-Gln- Ile- Asp-**Ser-Arg**-Asn-Lys-**Tyr-Phe-Ser-Pro-Arg-Leu**-$NH_2$

**Amidated C-terminal pentapeptide**

FIGURE 1 Structures of juvenile hormone, 20-hydroxyecdysone, and PBAN.

the chain length of cuticular alkenes so that the pheromone component (*Z*)-9-tricosene (muscalure) becomes a major product. It appears that JH induces key regulatory enzymes, whereas ecdysteroids repress specific enzymes to result in pheromone production. Discussed below is work from a limited number of model insect systems in which endocrine regulation of pheromone production has been most extensively studied. It is not by any means an exhaustive list, and excellent work in other insects has been done. Studies on the endocrine regulation of pheromone production to date have utilized relatively few species, and large gaps in our knowledge are readily apparent.

## II. PBAN REGULATION IN LEPIDOPTERA

A pheromone biosynthesis-activating neuropeptide was first purified and sequenced from *Heliothis zea* in 1989 by Ashok Raina and co-workers. PBANs from other lepidopterans, including *Bombyx mori*, *Lymantria dispar*, *Agrotis ipsilon*, and *Helicoverpa assulta*, have subsequently been isolated and identified. All of the PBANs examined to date are 33- or 34-amino-acid peptides that share approximately 80% homology and an amidated C-terminus. The minimum sequence necessary for biological activity is the C-terminal pentapeptide (Phe-Ser-Pro-Arg-Leu-$NH_2$), although the activity of this pentapeptide is an order of magnitude or so lower than that of the intact peptide.

It is becoming clear that in most moths PBAN is released into the hemolymph and acts directly on the pheromone gland to stimulate pheromone biosynthesis. There is evidence, however, that in some species PBAN undergoes neural transport. Regardless, PBAN is produced in the subesophageal ganglia and transported to the corpora cardiaca (CC). In most species, PBAN is then released from the CC (a neurohemal organ) and transported through the hemolymph to the pheromone gland.

The proposed direct mechanism of PBAN involves the binding of PBAN to a specific pheromone gland receptor (Fig. 2). This triggers second messengers, including $Ca^{2+}$ and cyclic AMP, and may involve phosphatidyl inositol. The steps in pheromone biosynthesis that are regulated by PBAN have been examined, and in some species it appears that PBAN increases acetyl coenzyme A (acetyl CoA) carboxylase activity and in others it may affect the reduction of fatty acyl groups to aldehydes and alcohols or other steps in pheromone biosynthesis.

Although PBAN and PBAN-like peptides have been found in all Lepidoptera examined to date as

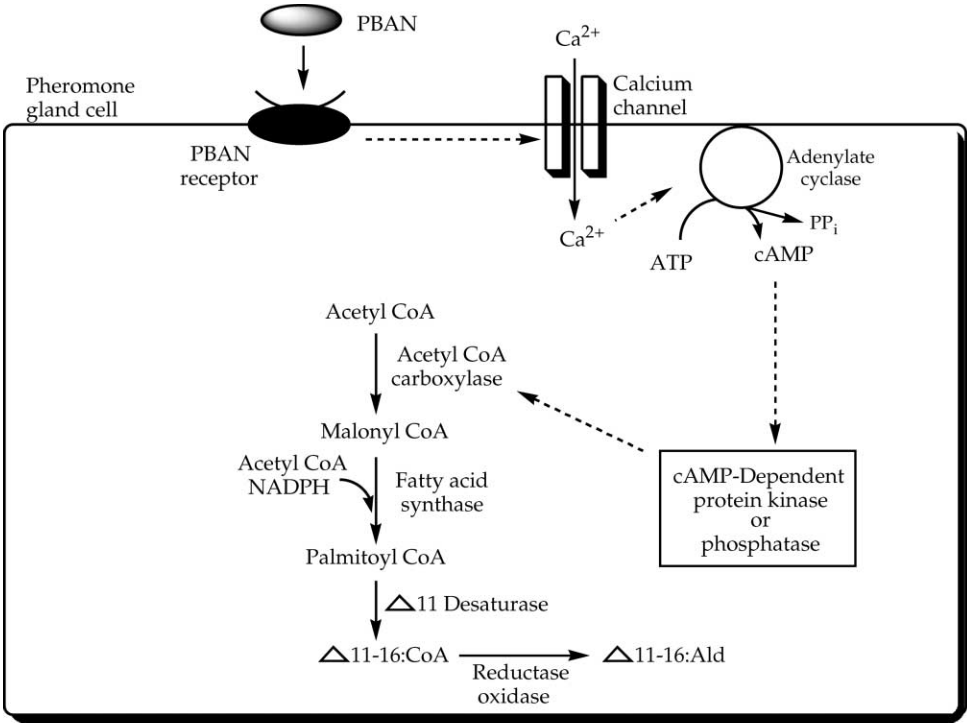

PBAN
Pheromone gland cell
PBAN receptor
$Ca^{2+}$
Calcium channel
$Ca^{2+}$
Adenylate cyclase
$PP_i$
ATP
cAMP
Acetyl CoA
Acetyl CoA carboxylase
Malonyl CoA
Acetyl CoA NADPH
Fatty acid synthase
Palmitoyl CoA
△11 Desaturase
△11-16:CoA
Reductase oxidase
△11-16:Ald
cAMP-Dependent protein kinase or phosphatase

**FIGURE 2** Proposed mode of action of PBAN on pheromone production in *Helicoverpa zea*.

well as insects from other orders, in some species they do not appear to regulate pheromone production. For example, in *Trichoplusia ni*, the pheromone gland becomes competent to produce pheromone at adult eclosion and pheromone production continues unregulated for the duration of the life of the female. In some Lepidoptera, JH and ecdysteroids are involved in regulating pheromone production, in some cases apparently regulating PBAN release.

## III. JUVENILE HORMONE REGULATION IN COLEOPTERA

Coleoptera (beetles) produce and/or emit pheromones in response to various environmental or physiological factors, with JH often being involved. In many insect species, JH induces vitellogenin production and coordinates other reproductive events, so it is not unexpected that it can also affect pheromone production. A key question for certain species, especially bark beetles, is whether JH stimulates the conversion of host precursors to pheromone or whether it stimulates *de novo* pheromone synthesis. Recent findings, primarily with the pine engraver, *Ips pini*, have demonstrated that JH III can directly induce *de novo* pheromone production.

Perhaps the most complete picture of JH regulation of pheromone production occurs in *I. pini*. Radiotracer studies with male *I. pini* monitoring *in vivo* incorporation of radiolabeled acetate into ipsdienol showed that incorporation increased with increasing topical JH III dose. The *in vivo* incorporation of radiolabeled mevalonolactone into ipsdienol by male *I. pini* was not affected by increasing JH III dose, indicating that the JH regulation of isoprenoid pheromone production occurred at steps between acetyl CoA and mevalonate (Fig. 3). Subsequent studies showed that feeding or topical application of JH III induces the mRNA transcript for 3-hydroxy-3-methylglutaryl CoA reductase (HMG-R) 20- to 30-fold. The emerging picture for this insect is that feeding stimulates JH III production, which in turn induces HMG-R transcript and enzyme activity, resulting in a large increase in pheromone production (Fig. 3). HMG CoA synthase transcript was increased only several fold by JH III, indicating that the main regulatory step is HMG CoA reductase.

Using a mRNA probe to HMG-R, *in situ* hybridization on exposed whole mounts of JH-stimulated male *I. pini* demonstrated that the site of pheromone synthesis is midgut tissue. Furthermore,

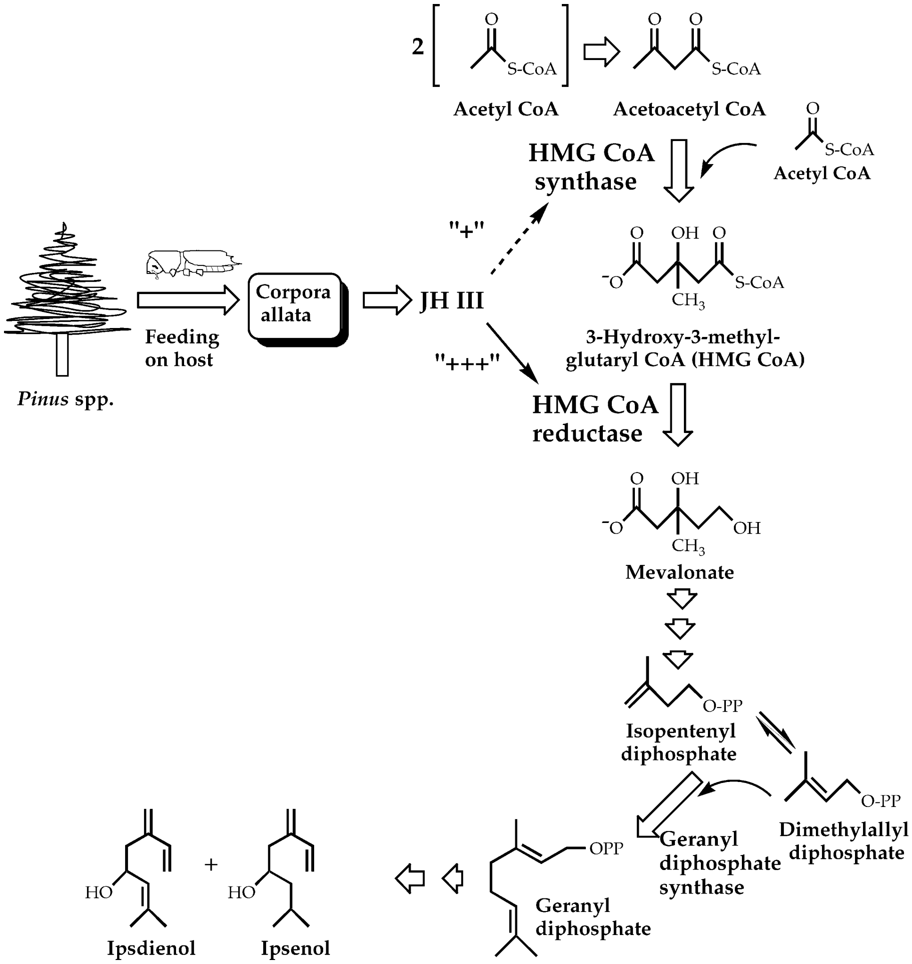

2
S-CoA
Acetyl CoA
S-CoA
Acetoacetyl CoA
S-CoA
Acetyl CoA
HMG CoA
synthase
"+"
Pinus spp.
Feeding
on host
Corpora
allata
JH III
"+++"
S-CoA
CH3
3-Hydroxy-3-methyl-
glutaryl CoA (HMG CoA)
HMG CoA
reductase
OH
CH3
Mevalonate
O-PP
Isopentenyl
diphosphate
O-PP
Dimethylallyl
diphosphate
Geranyl
diphosphate
synthase
OPP
Geranyl
diphosphate
HO
HO
Ipsdienol
Ipsenol

FIGURE 3 Current understanding of the ipsdienol and ipsenol biosynthesis in *I. pini* showing the proposed mode of action of JH III. The major regulatory step induced by JH III is HMG CoA reductase.

isolated midgut tissue incorporated labeled acetate into ipsdienol.

The molecular details of how JH affects transcription and ultimately, coleopteran pheromone biosynthesis, remain to be elucidated. Following release from the CA, lipophilic JH binds to a specific protein (the JH-binding protein) for transport through the aqueous hemolymph and protection from degradative enzymes. Once at the target tissue for pheromone biosynthesis, it would most likely cross the cell membrane and bind to intracellular receptors, although JH has been shown to act at the membrane level in some systems. The isolation and identification of an intracellular receptor for JH remain elusive and controversial. Although it is tempting to extend the intracellular molecular mode of action for steroidal hormones to JH, the diverse physiological phenomena regulated by JH may suggest a more complex interplay involving an ensemble of regulatory proteins or transcription factors with the putative JH receptor. The large induction of HMG-R transcript by JH apparently acting alone (without the involvement of ecdysteroids as occurs during development) makes the *I. pini* system an attractive model for elucidating the mode of action of JH.

## IV. JUVENILE HORMONE REGULATION IN BLATTODEA

By far the best studied and most understood example of JH regulation of pheromone production in the Blattodea was done by Schal and collaborators in female German cockroaches, *Blattella germanica*. *In vivo* synthesis of the contact sex pheromone, 3,11-dimethylnonacosan-2-one (3,11-DMN:Ke) and its accumulation on the cuticle are correlated with the *in vitro* synthesis of JH III by the CA and oocyte development, suggesting common JH regulation of sex pheromone production as well as other reproductive events. Comparison of the patterns of pheromone and hydrocarbon production in starved, allatectomized, and head-ligated females, as well as in females rescued with hormone replacement therapy, suggests two mechanisms of regulation of sex pheromone production: (1) hormonal, a JH-induced conversion of the hydrocarbon precursor to the oxygenated sex pheromone that is related to the CA cycle and oocyte development, and (2) nonhormonal, a JH-independent process, probably related to feeding, that supplies precursors for hydrocarbon (pheromone) biosynthesis.

Dependence of pheromone synthesis on JH levels in female *B. germanica* is supported by the following findings: (1) the pattern of accumulation of 3,11-DMN:Ke and 3,11-dimethylheptacosan-2-one (minor pheromone component) on the cuticle correlates with the pattern of JH synthesis through two gonotropic cycles; (2) the rates of synthesis of methyl ketones, using labeled propionate, correspond to rates of JH synthesis; and (3) pheromone production declines in allatectomized females or females with experimentally inhibited CA (e.g., starved, protein-deprived, ootheca implanted), whereas juvenile hormone analogue (JHA) treatment restores pheromone production in these females.

Whereas pheromone production is completely suppressed in individuals of other allatectomized cockroach species, allatectomized female *B. germanica* produce a small quantity of pheromone. Because JHAs are also less effective inducers of pheromone production in unfed female *B. germanica*, it was hypothesized that feeding might indirectly influence pheromone production by influencing the availability of pheromone precursor (hydrocarbon), and results from recent studies support this hypothesis.

## V. ECDYSTEROID REGULATION IN DIPTERA

In several species of Diptera, ecdysteroids have been shown to regulate the female reproductive process of vitellogenesis. Ecdysteroids also regulate sex pheromone production in the female housefly, *Musca domestica*. Pheromone biosynthesis normally begins approximately 2 days after emergence to adult in female *M. domestica*. However, ovariectomy of newly emerged females prevents pheromone biosynthesis. Pheromone production can be restored in ovariectomized females by implantation of ovaries or injection of 20-hydroxyecdysone (20-HE). These and other studies have led to the conclusion that sex pheromone production in female *M. domestica* is regulated by ecdysteroids.

Male houseflies normally do not produce significant amounts of (*Z*)-9-tricosene or its oxygenated products. However, implantation of ovaries or treatment with 20-HE induces the production of the female sex pheromone. Immature females and males of all ages normally produce cuticular alkenes of 27 carbons or more. Since 20-HE appears to be a product of the mature ovary in adult houseflies, adult males normally are not exposed to 20-HE. Treatment with 20-HE induces pheromone synthesis, indicating that the male has the biosynthetic machinery to produce pheromone.

An in-depth study of the biochemistry of pheromone production in the housefly led to an understanding of the enzyme activity affected by 20-HE. The endocrine-mediated induction of sex pheromone biosynthesis in the housefly involves a change in the fate of tetracosenoyl CoA (*Z*15-24:CoA) from one of elongation to one of reduction to the aldehyde and then decarboxylation to the main sex pheromone component, (*Z*)-9-tricosene (*Z*9-23:Hy) (Fig. 4). The two most likely regulatory points where 20-HE could exert its effect were (1) the fatty acyl CoA elongation step(s) and/or (2) the reductive conversion of fatty acyl CoA to hydrocarbon formation step(s). *In vitro* enzyme assays demonstrate that ecdysone predominantly affects the elongation enzyme(s) rather than the enzymes functioning in the conversion of *Z*15-24:CoA to *Z*9-23:Hy (Fig. 4). It appears that 20-HE exerts its effect primarily if not exclusively by repressing the fatty acyl CoA elongase that converts 24:1-CoA to longer chain fatty acids, resulting in the build up of 24:1-CoA, which is then reduced to the aldehyde and decarboxylated to (*Z*)-9-tricosene (muscalure). A portion of the (*Z*)-9-tricosene is then converted to epoxide and ketone, both of which function in the female sex pheromone (Fig. 4).

As is the case with JH III and coleopteran pheromone biosynthesis, the molecular details of how 20-HE influences the enzymatic reactions in dipteran pheromone biosynthesis remain to be elucidated. By analogy to steroidal hormones in other systems, it is reasonable to hypothesize that gene expression would be regulated for key biosynthetic enzymes, in this case repression of a specific fatty acyl CoA elongase. In general, steroid hormones such as 20-HE are thought to diffuse freely through the cell membrane into the cytoplasm and/or nucleus to bind specific intracellular hormone receptors. A dimerized

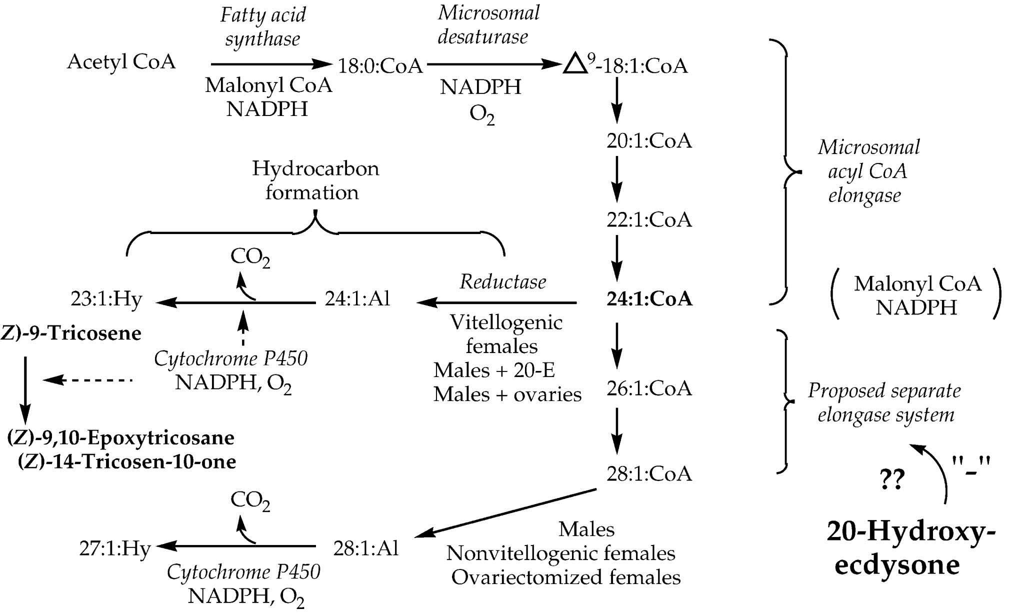

Acetyl CoA
Fatty acid synthase
Malonyl CoA
NADPH
18:0:CoA
Microsomal desaturase
NADPH
$O_2$
$\Delta^9$-18:1:CoA
20:1:CoA
22:1:CoA
24:1:CoA
26:1:CoA
28:1:CoA
Microsomal acyl CoA elongase
Malonyl CoA NADPH
Proposed separate elongase system
??
"-"
20-Hydroxy-ecdysone
Hydrocarbon formation
$CO_2$
23:1:Hy
(Z)-9-Tricosene
24:1:Al
Cytochrome P450
NADPH, $O_2$
Reductase
Vitellogenic females
Males + 20-E
Males + ovaries
(Z)-9,10-Epoxytricosane
(Z)-14-Tricosen-10-one
$CO_2$
27:1:Hy
28:1:Al
Cytochrome P450
NADPH, $O_2$
Males
Nonvitellogenic females
Ovariectomized females

**FIGURE 4** Biosynthetic pathway for the housefly pheromone showing the proposed site of action of 20-HE. Reprinted from Tillman *et al.* (1999), with permission from Elsevier Science.

form of the receptor–ligand complex then binds to specific DNA sequences called hormone-responsive elements to affect gene expression. Although the motivation for their study has been insect morphogenesis, ecdysteroid receptors have been isolated from species of Diptera, Lepidoptera, and Coleoptera. These receptors occur in a surprising variety of isoforms and this may have implications for the regulation of pheromone biosynthesis in the Diptera.

## VI. CONCLUDING REMARKS

In no model pheromone biosynthetic system are the molecular mechanisms of hormonal regulation completely understood, and studies to address these obvious gaps in our understanding are being pursued in representative species. It is now clear that ecdysone and JH regulate pheromone production by inducing or repressing specific enzymes, but the molecular basis for these changes is not understood. A better understanding of the PBAN receptor and the biochemical steps regulated in pheromone production is needed. In no system are the exact enzymatic steps affected by the pheromone biosynthesis-regulating hormone known with certainty and completeness. Ultimately, just as behavioral chemicals themselves have been extended to pest management, research on pheromone biosynthesis and its hormonal regulation may be directed toward application and ultimately used in insect control.

## Glossary

**corpora allata** Paired ganglia-like bodies often located just posterior to the corpora cardiaca. They synthesize and secrete juvenile hormone.

**corpora cardiaca** Paired neurohemal organs located behind the brain and often near the corpora allata. They release neurosecretory material from the brain, including pheromone biosynthesis-activating neuropeptide.

**ecdysteroids** Polyhydroxy steroids that induce molting during development in insects and that are also involved in reproductive processes in some adult insects, including Diptera.

**juvenile hormones** Sesquiterpenoids with epoxide and methyl ester functional groups produced by the corpora allata. They serve to keep the insect in the immature state during development and to coordinate reproductive events in many adult insects.

**pheromone** A chemical (or blend of chemicals) that is released by an organism and causes specific behavioral or physiological reaction(s) a in one or more conspecific individuals.

**pheromone biosynthesis-activating neuropeptide** A 33- or 34-amino-acid peptide produced in the brain that triggers pheromone production in many species of Lepidoptera.

**subesophageal ganglion** A large nerve center consisting of the fused ganglia of the original mandibular, maxillary, and labial segments, situated in the head, beneath the esophagus.

## See Also the Following Articles

**Ecdysteroid Action in Insect Reproduction • Ecdysteroids, Overview • Insect Endocrine System • Juvenile Hormone Action in Insect Development • Juvenile Hormone Action in Insect Reproduction • Juvenile Hormone Biosynthesis • Juvenile Hormones, Chemistry of • Neuropeptides: Roles in Regulation of Juvenile Hormone Production**

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# Phytoestrogens

Alice L. Murkies[*] and Mark Frydenberg[†]

[*]*Jean Hailes Centre for Women, Australia* • [†]*Monash University, Monash Medical Centre, Australia*

I. INTRODUCTION
II. CLASSIFICATION AND METABOLISM
III. FOOD SOURCES AND SAFETY
IV. POTENCY AND BIOLOGICAL ACTIVITY
V. EFFECTS IN HUMANS
VI. PHYTOESTROGENS IN SPECIFIC DISEASES
VII. SUMMARY

**Phytoestrogens are plant compounds with binding affinity for the estrogen receptor and consequently have estrogen-like biological activity. Phytoestrogens have physiological effects in humans that vary depending on the phytoestrogens and tissue end-points examined. The most supportive data of the beneficial effects of phytoestrogens in humans are from the effect of soy protein on lipids, lipid profiles, and blood pressure. The active moiety appears to be present in whole foods, which have a greater physiological effect than subfractions or supplements. This article examines the classification and metabolism of phytoestrogens and discusses their biological activities in humans.**

## I. INTRODUCTION

Historically, estrogenic activity in plants has been referred to in folk medicine. The pomegranate is associated with fertility, and hops were used by German clergy in the Middle Ages to lower fertility.

In 1923, the Allen Doisy bioassay for estrogens was published, and in 1926, plant extracts were reported to exhibit estrogenic activity. By 1975, several hundred plants were reported to have estrogenic activity on bioassay or to contain estrogenically active compounds. Phytoestrogens gained importance in the 1940s due to infertility in sheep grazing on pastures rich in subterranean clover in Western Australia later known as "clover disease."

Setchell reported measurement of urinary phytoestrogens in nonhuman primates in 1979 and in humans in 1982. Based on epidemiological studies, Adlercreutz has suggested that a diet rich in phytoestrogens as consumed by individuals in Asiatic and Mediterranean nations is associated with reduced risk of the so-called Western diseases such as breast and prostate cancer and cardiovascular disease.

Estrogenic activity has been reported in compounds produced by animals, plants, and microorganisms. Industrially manufactured chemicals, including plastics, household products, food packaging, and pesticides, such as DDT, contain weak estrogen-like compounds and are classified as xenoestrogens. These include organochlorine pesticides, polychlorinated biphenyls (PCBs), phenolic compounds, and phthlate esters. Deleterious effects of xenoestrogens have been hypothesized but not substantiated. Of concern are the long-half-life of these compounds and the persistence in fat tissue for many years. Adverse effects have been documented in wildlife but no direct evidence to show a cause and effect relationship has been reported in humans. The estrogenic potency of industrially derived estrogenic compounds is very limited, but the estrogenic potency of phytoestrogens is significant and the classification, metabolism, and biological effects in men and women and disease states will be the essence of this article.

## II. CLASSIFICATION AND METABOLISM

There are three main classes of phytoestrogens found in plants or their seeds. Isoflavones and coumestans, also known as isoflavonoids, are synthesized within the plant itself. One plant can contain more than one category of phytoestrogen. The soybean contains predominantly isoflavones and the soy sprout is a rich source of coumestrol, the major coumestan. Lignans are formed from the action of gut microflora on the lignan precursors found in the plant wall. The resorcyclic acid lactones exhibit estrogenic activity and the active metabolites, zearalenone and zearalenol, are produced by molds that contaminate cereal crops and are better described as mycoestrogens (Fig. 1).

The major isoflavones, genistein and daidzein, exist as inactive glucosides genistin and daidzin. They are also derived from their respective methyl ethers biochanin A and formononetin and after breakdown by intestinal glucosidases are converted to genistein and daidzein. Daidzein can be further metabolized to equol and *O*-desmethylangiolensin (O-DMA).

The estrogenically active lignans enterodiol and enterolactone are derived from secoisolariciresinol and matairesinol plant precursors found in the aleuronic layer of the grain close to the fiber layer. Enterodiol can be oxidized to enterolactone.

In humans, after consumption of plant isoflavones and lignans, complex enzymatic metabolic conversions occur in the gastrointestinal tract by the microflora, resulting in the formation of heterocyclic polyphenols similar in structure to estrogen (Fig. 2). The absorbed phytoestrogen metabolites undergo enterohepatic circulation and may be excreted in the bile, deconjugated by intestinal flora, reabsorbed, reconjugated by the liver, and excreted in the urine.

Phytoestrogen metabolites have been measured predominantly in urine, in trace amounts in feces, and also in semen, bile, saliva, and breast milk. Measurement of urinary phytoestrogen excretion is considered a reliable indication of dietary phytoestrogen intake. Concentrations of metabolites can vary within individuals even in a controlled setting of isoflavone and lignan supplements. This is attributed to the variation in metabolism determined by gastrointestinal flora, antibiotic use, bowel disease, and concomitant dietary intake of fiber, fruit, and vegetables. Similarly, dietary fat, fiber, protein, alcohol, and micronutrients may affect endogenous estrogen metabolism.

Little is known about the metabolism of coumestans in humans.

## III. FOOD SOURCES AND SAFETY

The isoflavones that exhibit estrogenic activity occur almost exclusively in legumes and beans, predominantly the soybean, and in derivative products that contain most or all of the bean.

Second-generation soy foods are produced by adding soy to other manufactured foods and thereby reducing the original isoflavone content, for example, soy yogurt and soy noodles. Lignans are widely

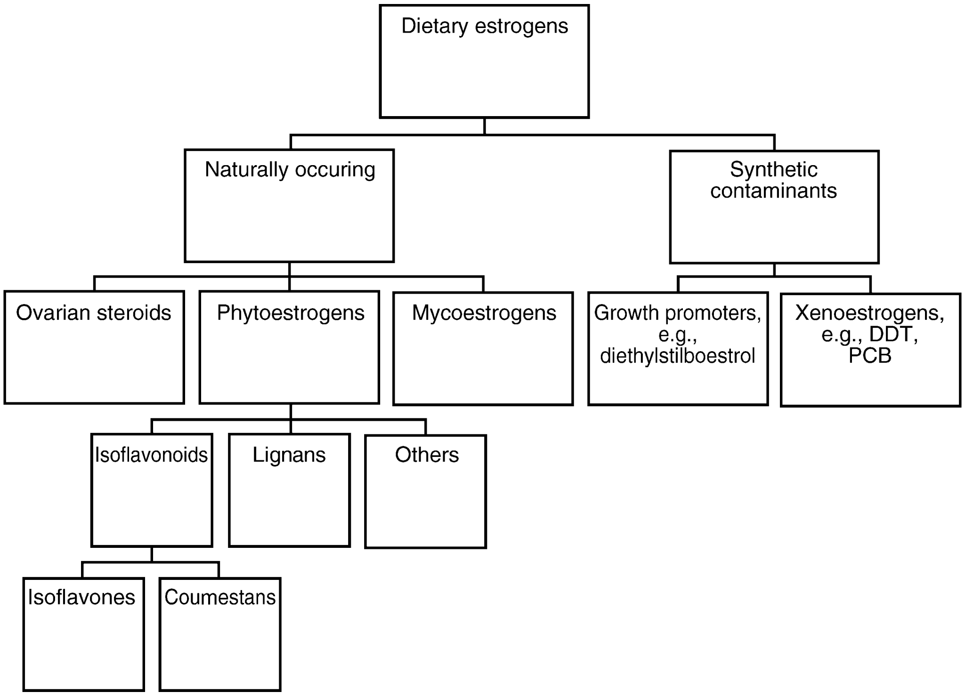

**FIGURE 1** Sources and classification of dietary estrogens. Reprinted from Murkies, A.L., Wilcox, G., and Davis, S.R. Clinical review 92: Phytoestrogens. *J. Clin. Endocrinol. Metab.* 83, 297–303, 1998, with permission. Copyright The Endocrine Society.

distributed in cereals, fruit, vegetables, and seeds, with the highest concentration in flaxseed.

Coumestans occur predominantly with germination, for example, in sprouting beans and fodder crops.

Resorcyclic acid lactone metabolites are produced by *Fusarium* species of mold found growing on crops stored under damp conditions. The compounds are concentrated in the seed hull and are predominantly removed in food processing.

Variation in phytoestrogen content in food can occur due to seasonal variation, genetic differences in plants, infection with fungal diseases, processing, and as part of the plant's defense mechanism.

Promotion of foodstuffs reported to contain a fixed phytoestrogen concentration cannot be substantiated because isoflavone content varies widely between strains in different soybean varieties and between locations of grown soybeans. Soy-enriched breads display a wide range of isoflavone content, from two- to threefold, within and between breads. Due to the natural variation of phytoestrogen concentration, it is probably not prudent for consumption of these foods to be based on isoflavone content alone in the belief that these are constant doses. Products derived from the whole bean such as soy flour, tofu, and soy milk have the highest concentration of isoflavones.

Phytoestrogens are considered beneficial rather than harmful to humans despite evidence from a number of animal species that phytoestrogen consumption can interfere with reproductive development and function. Observations of Asian societies that have consumed a phytoestrogen-rich diet for centuries and appear not to have any deleterious effects have led to a presumption that phytoestrogens may be safe. Consideration of the safety of phytoestrogens introduced into the adult diet is not unreasonable as up to 60% of processed foods may contain soy derivatives.

Macrobiotics and other vegetarians have the highest excretion values of lignans. Asian populations ares estimated to consume 20–150 mg/day of isoflavones, with a mean of 40 mg from tofu and miso. This can be achieved in Western diets with consumption of modest quantities of soy foods. Consumption of isoflavones in the United States has been estimated at <1 mg/day. It has been shown that 50 mg isoflavone consumption is sufficient to have endocrine effects in females and it appears that the dose,

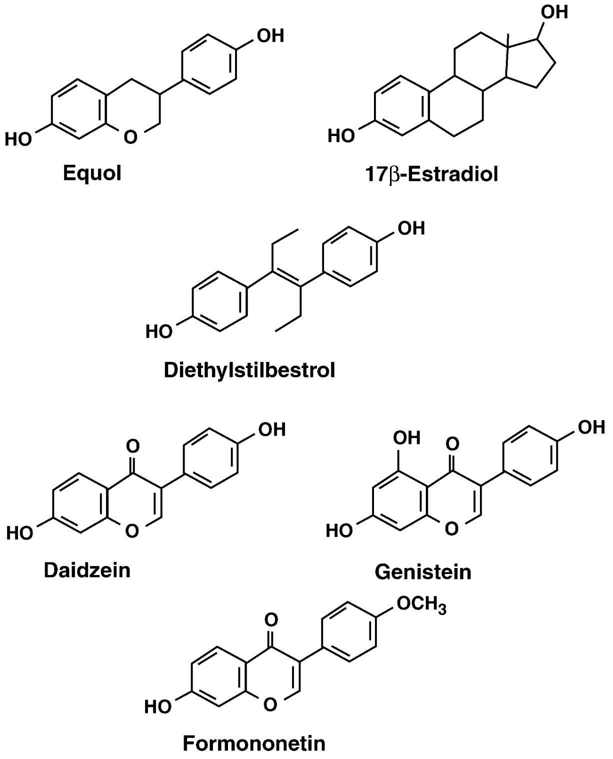

FIGURE 2 A comparison of the chemical structure of the phytoestrogen equol, genistein and daidzein, which are formed in the gastrointestinal tract of humans and animals, 17β-estradiol, and diethylstilbestrol. Reprinted from Setchell, K. D. *et al.* (1984). Non-steroidal estrogens of dietary origin: Possible roles in hormone dependent disease. *Am. J. Clin. Nutr.* 40, 569–578, Reproduced with permission by the *American Journal of Clinical Nutrition* © Am. J. Clin. Nutr. American Society for Clinical Nutrition.

duration, and timing of exposure of intake will influence clinical and biological outcomes.

## IV. POTENCY AND BIOLOGICAL ACTIVITY

The biological potencies of phytoestrogens vary greatly. The majority of the compounds are nonsteroidal in structure and vastly less potent than synthetic estrogens ($10^{-2}$ to $10^{-5}$). The potency determined by human cell culture bioassays varies with species, routes of administration, and tissue end-points used. With estradiol arbitrarily given the value of 100, the relative potencies of estrogenic isoflavonoids are as follows: coumestrol 0.202; genistein 0.084; equol 0.061; daidzein 0.013; and formononetin 0.0006.

More recently, the relative binding affinity of phytoestrogens to estrogen receptor-α (ER-α) and ER-β has been determined with the relative binding affinity of estradiol arbitrarily assigned at 100. Observations are comparable with the human cell culture bioassays, with coumestrol being the most potent. Relative binding affinities to ER-α and ER-β, respectively, are as follows: coumestrol 94 and 185; genistein 5 and 36, with a stronger binding affinity of phytoestrogens to ER-β.

### A. Estrogenic Activity, Anti-estrogenic Activity, and Other Biological Properties

Varying physiological effects of individual phytoestrogens highlight the complexity of response of the estrogen receptor. Ligands for ER-α and ER-β can act as estrogen agonists, as antagonists, or as partial or selective agonists/antagonists, depending on the tissue receptors, co-regulators, and the interaction on estrogen-regulated genes. Knowledge of the effects of individual phytoestrogens is limited. Genistein is reported to bind in a manner similar to that observed for 17β-estradiol within the three-dimensional structure of the estrogen receptor-β ligand-binding domain. However, the ER-β–genistein complex induces a distinct orientation in the transactivation helix that is not agonist but partial agonist so that the transcriptional response to certain ligands is attenuated. A diagrammatic representation of the receptor sites for ER-α and ER-β in the human body is seen in Fig. 3.

Adverse estrogenic effects have been observed in animals. Reproductive disturbances observed in sheep in 1946 were attributed to the estrogenic effect of the fodder crop. In some but not all studies, estrogenic effects are seen in laboratory animals, with isoflavones stimulating uterine growth. In contrast, genistein administered with estradiol functions as an anti-estrogen, decreasing uterine estradiol uptake in animal models.

Genistein *in vitro* has been shown to exert both proliferative (estrogenic)and anti-proliferative (anti-estrogenic) effects in human cell lines. In the human $ER^+$ MCF-7 breast cancer cell line, the effects are biphasic with stimulation at low concentrations of genistein ($10^{-5}$ to $10^{-8}$ M) and inhibition at higher concentrations ($10^{-4}$ to $10^{-5}$ M), the latter probably not mediated via the estrogen receptor. At low concentrations, genistein competes with estradiol for binding to the ER with a 50% inhibition concentration of $5 \times 10^{-7}$ M, and it stimulates the expression of pS2mRNA, a specific marker of ER-mediated estrogen-like activity. Similar stimulatory effects have been shown with daidzein, equol, and enterolactone.

Most studies to date indicate induction of DNA synthesis in tumor cell lines at concentrations close to

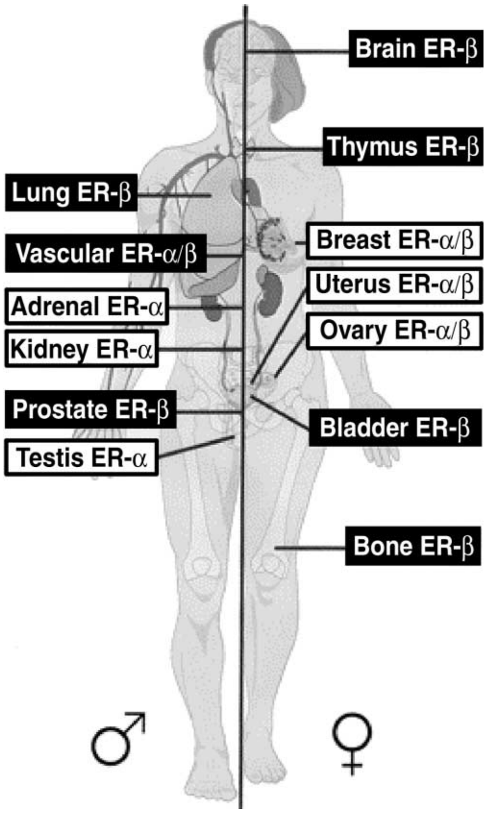

FIGURE 3 Simplified diagram illustrating the anatomical distribution of the newly described estrogen receptor ER-β to sites that are specific targets where classical estrogen replacement is beneficial. Reprinted from Setchell, K.D.R., and Cassidy, A. (1999). Dietary isoflavones: Biological effects and relevance to human health. *J. Nutr.* **129**, S758–S767, with permission from the American Society for Nutritional Sciences.

probable plasma levels in humans, achieved when consuming a high isoflavone intake, and inhibition only at very high concentrations, not achievable by diet.

Isoflavones exhibit anti-carcinogenic activity *in vivo*. Laboratory animals fed soy-fortified diets have less breast tumor proliferation after stimulation with direct and indirect tumor-inducing agents, *N*-methyl-*N*-nitrosurea and dimethylbenz(a)anthracene, respectively.

Anti-proliferative effects are demonstrated *in vitro* and appear to be independent of the estrogen receptor. Genistein and possibly other phytoestrogens interfere with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinases that are essential for transduction of mitogenic signals and hence inhibit tumor growth. Genistein inhibits epidermal growth factor-receptor (EGF-R) tyrosine autophosphorylation or alternatively exerts inhibitory effects downstream from EGF-R tyrosine autophosphorylation by some other mechanism.

Genistein has been reported to inhibit topoisomerase II, an essential nuclear enzyme involved in DNA replication, and this may result in cell cycle arrest.

Isoflavones exhibit other biological properties *in vitro* and inhibit angiogenesis, cell cycle progression, and aromatase enzyme activity, stimulate sex hormone-binding globulin (SHBG) synthesis, may decrease bioavailable endogenous estrogen, and have antioxidant properties and digitalis-like activity.

## V. EFFECTS IN HUMANS

### A. Infants

Soy infant formulas contain significantly high levels of daidzein and genistein. Large quantities of phytoestrogens were identified in the plasma and urine in human neonates, raising concerns about possible effects on neuroendocrine developmental processes. This probably arises as neonates have reduced intestinal degradation of the ingested isoflavones and frequent feeds. Cows' milk has been shown to contain isoflavones at low concentrations. A retrospective cohort study examining the association between infant exposure and health in adult life has found that exposure to soy does not appear to lead to different general or reproductive outcomes than cows' milk. This is reassuring about the safety of infant soy formulas.

### B. Premenopausal Women

A cyclic pattern of lignan excretion was observed in the menstrual cycle of the vervet monkey and also in humans. Controlled intervention studies with dietary phytoestrogens have shown endocrine-modulating effects with increased cycle length in premenopausal women. Sixty grams of soy protein daily has been reported to increase follicular phase length and to suppress the midcycle follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surges. Thirty-six ounces (1000 ml) of soy milk daily has been reported to decrease serum 17β-estradiol and luteal-phase serum progesterone in young ovulating women. Daily intake of flaxseed at 10 g per day increases luteal-phase length with no difference in follicular phase in normally cycling women. SHBG was not increased in these studies. Supplementation with 60 g soy/day increases breast epithelium proliferation in premenopausal women.

## C. Postmenopausal Women

Epidemiological studies comparing Asian and Western populations have suggested that consuming a phytoestrogen-rich diet may ameliorate the symptoms of estrogen deficiency, such as hot flashes at menopause. However, intervention studies have shown no benefit from phytoestrogens as an alternative to hormone therapy in postmenopausal women.

In 1990, mild estrogenic effects in the vaginal-cell maturation index of postmenopausal women were first reported after supplementation with 45 g soy flour for 6 weeks. Subsequent studies have failed to demonstrate a correlation between vaginal-cell estrogenic response and vasomotor symptoms. Also, a strong placebo effect has been noted. Some studies have reported a reduction in hot flashes upon supplementation with soy versus placebo but the effects have not been dramatic. A controlled trial of wheat versus soy unexpectedly found that wheat-supplemented women showed a significant reduction in hot flashes after 3 months and had low urinary isoflavones. These studies highlight the difficulties in treating hot flashes over time due to their natural resolution. The differences in response could be attributed to varying soy products used, study design, and individual variability in response. Specific dosages and formulations cannot be recommended.

Data on the effect of phytoestrogens on serum estradiol FSH, and LH concentrations are also variable. Isoflavone supplements have no benefit beyond placebo for reduction in hot flashes and no effect on plasma hormones including SHBG and FSH.

Phytoestrogens have no effect on the other symptoms of menopause, such as arthralgia, myalgia, headaches, and anxiety, which comprise part of the Kupperman index.

## D. Men

Exposure to compounds with estrogenic activity has been postulated to be responsible for adverse changes in male reproductive health, including declining sperm counts, increase in testicular cancer, and testicular genital malformations although currently there are no definitive studies. Data are limited; however, intervention studies in males fed textured vegetable protein or soy protein isolate report no change in serum testosterone.

# VI. PHYTOESTROGENS IN SPECIFIC DISEASES

## A. Cardiovascular Disease

Vegetarians and individuals in Asian countries have a reduced risk of cardiovascular disease compared with individuals in Western nations, and Adlercreutz suggested that this may be partly attributable to phytoestrogen consumption. Dietary intake of vegetable protein, particularly soy, is associated with decreased cardiovascular disease risk.

### *1. Lipid Profiles*

Intervention studies with soy-supplemented diets indicate that cardiovascular disease benefits may result from improved lipoprotein profiles. It is not known whether the effects are derived from the soy protein isoflavone or other nonphytoestrogen components of soy such as saponins, from fiber, or from a combination of the two. Limited data suggest that soy subfractions are not as effective at decreasing cardiovascular disease risk factors.

Soy in which substantial portions of the isoflavones and saponins are extracted by alcohol is described as isoflavone deplete (soy − ) and has been used in primate models and compared with soy protein with isoflavones intact (soy + ), to help identify the active moiety in soy protein. Studies in primates and humans overall report a beneficial effect in lipid profile of soy + supplementation comparable with studies of estrogen. Since 1977, soy has been reported as a treatment for hypercholesterolemia. A meta-analysis of 38 published controlled clinical trials of soy protein consumption in humans that averaged 47 g/day and serum lipoprotein concentrations found that soy protein consumption was significantly associated with mean reductions in total cholesterol [9.3% (−0.6 mmol/liter) decrease, 95% CI 0.35–0.85 mmol/liter], LDL cholesterol [12.9% (−0.5 mmol/liter) decrease, 95% CI 0.30–0.82 mmol/liter], and triglycerides [10.5% (−0.15 mmol/liter) decrease, 95% CI 0.003–0.29 mmol/liter], with little change in HDL concentration. The degree of cholesterol reduction is similar to that observed with dietary intervention of plant-based foods such as oat bran and garlic. The hypocholesterolemic effect is more profound in men and women with elevated cholesterol, with only marginal benefit for those with a normal baseline lipid profile.

For example, a soybean protein diet in subjects with type II hyperlipoproteinemia may lower cholesterol on average by 20%. Ingestion of 60 g/day of soy protein isolate by normocholesterolemic men resulted in no change in plasma lipids. Ingestion of 40 g soy supplementation in normocholesterolemic men and women resulted in decreases in triglycerides and an increase in lipoprotein Lp(a) by 15%.

Although results from individual studies are variable and dependent on the original serum cholesterol levels, the Food and Drug Administration approval for labeling of soy foods states "included in the daily diet, they may reduce heart disease."

#### *2. Blood Pressure*

Soy supplementation is reported to lower blood pressure in normotensive individuals. In a study of normotensive men and postmenopausal women, 3 months of 40 g daily soy supplementation significantly reduced systolic (2.4 mm Hg lower), diastolic (3.9 mm Hg lower), and mean blood pressure (BP) (4.2 mm Hg lower) compared with the casein-fed placebo group. These changes are greater than reported with dietary intervention alone. Soy supplementation in nonhypercholesterolemic, nonhypertensive, perimenopausal women resulted in a significant decline in diastolic BP (5 mm Hg lower) in the 20 g twice daily soy diet compared with the placebo diet. In contrast, concentrated phytoestrogen supplements did not alter BP in normotensive subjects. This highlights the discrepancy of phytoestrogen doses, the consumption of soy protein in preference to extracts, and the possibility of the hypotensive effect being mediated by nonestrogenic mechanisms.

#### *3. Vascular Compliance and Endothelial Function*

ER-β has been shown to be the primary estrogen receptor in the vessel wall and is up-regulated in response to vascular injury. Interest has focused on whether soy has vascular effects similar to those of estrogen. Studies of rhesus monkeys support a reduction in atheromatous plaque with isoflavone-intact soy supplementation and enhanced dilator responses of atherosclerotic coronary arteries to acetylcholine in female monkeys, improving blood vessel dilation and consequent blood flow.

However, a large study of both men and women consuming soy protein reported no beneficial effect on arterial compliance. Two small studies in peri- and postmenopausal women, with no male subjects included, have reported improved systemic arterial compliance of 26% in concentrated phytoestrogen supplements.

Brachial flow-mediated vasodilation (FMD) is a strong predictor of coronary endothelial dysfunction. In the larger previous study, soy supplementation had no effect on FMD in healthy postmenopausal women and unexpectedly reduced FMD in males. In comparison, estrogen administration does not improve FMD in healthy adult males or improve endothelial-dependent vasodilation in the male monkey model.

A small study of postmenopausal women supplemented with an 80 mg phytoestrogen tablet similarly reported no improvement of FMD.

Cross-sectional data from postmenopausal women indicated that women with high genistein intake had a significantly lower body mass index ($P$-trend = 0.05), waist circumference ($P$-trend = 0.05), and fasting insulin level ($P$-trend = 0.07) than those with no daily genistein consumption. Similarly, a population-based cohort of midlife women found that women with a high intake of isoflavones, >40 mg/day, derived from diet were more likely to exercise, were less likely to smoke, had a lower mean body mass index, and observed lifestyle factors that were associated with reduced cardiovascular disease risk.

Dietary soy phytoestrogens may provide cardioprotection via direct lipid effects; however, the response pattern does not follow that of estrogen with respect to BP responses, lipoprotein changes, and vascular function effects and not all effects are beneficial, in particular, elevated lipoprotein Lp(a) and reduced FMD in males. The mechanisms of action are still uncertain. Isoflavones, especially genistein, *in vitro* are reported to have anti-atherogenic properties and inhibit the process of coagulation, a key promoter of plaque formation, by inhibition of growth factors such as platelet-derived growth factor and inhibition of tyrosine kinase activity, an enzyme central to thrombin formation and inflammation in general. Genistein is the most potent antioxidant of the isoflavones in soy protein.

In summary, phytoestrogens have beneficial effects for cardiovascular disease risk via lipid reduction, particularly in hypercholesterolemic men and women, and reduction in blood pressure in normotensive men and women with no overall beneficial effect on arterial compliance or endothelial function in adult males. Individuals that consume phytoestrogens have healthy lifestyle factors that are preventative of cardiovascular disease.

### B. Cancer

The incidence of hormone-dependent cancers is lower among vegetarians and in Asia and Eastern Europe than in Western countries. Breast, ovarian, prostate, and colon cancers show a negative correlation with cereal and phytoestrogen intake when cancer mortality rates and food availability data between countries are compared.

### *1. Breast Cancer*

Some epidemiological studies have suggested an association between high phytoestrogen intake and reduced breast cancer risk. Studies of urinary phytoestrogen excretion in women immediately after diagnosis of breast cancer have shown lower equol, enterolactone, and daidzein excretion in women with breast cancer than in controls, despite similar dietary patterns.

Japanese immigrants to North America have a higher incidence of breast cancer than their counterparts in Japan, with a higher incidence of cancer the younger the age of migration. Such findings support the role of environmental factors in the etiology of breast cancer.

Studies have shown conflicting results. A significant graded inverse association in Japanese women has been reported between risk of breast cancer and consumption of miso (soybean paste soup). A diet high in soy products conferred a low risk of breast cancer in premenopausal women in Singapore, with no effect observed in postmenopausal women. A Chinese study reported no association between soy and breast cancer but did find an inverse correlation between fiber and other nutrient intake and breast cancer rates. Similarly, a progressive reduction in relative risk of breast cancer with each quintile of increasing fiber intake for women has been reported in Australian women. Lower levels of $ER\text{-}\alpha^+$ cells have been shown in normal breast tissue in Japanese women, 9% compared with white Australian women ($>17\%$ for those over 50 years and 12% for those $<50$ years), and this may contribute to ethnic differences in breast cancer rates.

Isoflavones have been reported to have protective effects in animal models of experimentally induced breast cancer, measured by tumor number, incidence, metastasis, and latency. It may be that prepubertal exposure may confer greater protection by precocious maturation of breast terminal end buds. Breast cancer cell line studies have shown that at low concentrations (equivalent to levels measured in humans consuming a 40 mg isoflavone supplement) genistein stimulates $ER^+$ cells with no effect on $ER^-$ cells. Consistent with these findings, soy supplementation stimulates breast epithelium proliferation in premenopausal women. Genistein at high concentrations, beyond that achieved by diet, inhibits the growth of $ER^+$ and $ER^-$ cell lines. Genistein in cell studies has been shown to antagonize the anti-estrogenic effect of tamoxifen, and caution is advised regarding supplements in women with breast cancer.

Phytoestrogens have estrogenic effects on the breast and larger long-term studies are needed before concentrated supplements can be safely recommended.

### *2. Prostate Cancer*

Men in Asia were found to have a higher concentration of isoflavones, equol, and daidzein in plasma and prostatic fluid than their counterparts in Europe. This led to the speculation that phytoestrogens may be protective of prostate cancer. Epidemiological studies have reported an association between consumption of lentils, peas, tomatoes, and dried fruits and decreased prostate cancer in 14,000 Seventh Day Adventist men. A study of Japanese immigrants to North America has shown an increased incidence in prostate cancer, the younger the age at migration, supporting the hypothesis of environmental factors, including diet, having an impact on cancer risk.

In the rodent model, an isoflavone-rich diet reduced the incidence of prostate-related cancer and prolonged the disease-free interval by 27%. The effects were observed only when the rodents were fed soy prior to the exposure of cancer, indicating that early exposure to soy supplementation may protect against prostate cancer in later life.

It is plausible that the effects of genistein are mediated via ER-β, in view of the greater affinity of genistein to ER-β versus ER-α and the high levels of ER-β found in the prostate. In human prostate cancer cell lines, high concentrations of genistein and biochanin A inhibit the growth of androgen-dependent cells. *In vitro* high levels of genistein inhibit 5α-reductase and 17β-hydroxysteroid dehydrogenase in genital skin fibroblasts and prostatic tissue. These two enzymes are involved in androgen and estrogen synthesis. Genistein inhibits the growth of prostatic cells from benign prostatic hypertrophy and prostate cancer cells in histoculture in a dose-dependent manner. In addition, genistein inhibits tyrosine kinase and topoisomerase, which are crucial to cellular proliferation.

Phytoestrogen supplementation with a 160 mg (4 × 40 mg) isoflavone equivalent, made from red clover, was taken for 7 days prior to surgery for an infiltrating low-grade adenocarcinoma of the prostate in one subject. At surgery, the prostatectomy specimen showed a moderately high-grade adenocarcinoma with patchy microvacuolation and prominent apoptosis typical of a response to prior estrogen therapy. Decreased serum testosterone, decreased serum prostate-specific antigen, and altered sexual behavior have been demonstrated in men who were administered an herbal remedy containing phytoestrogens used as treatment for prostate cancer. Further intervention studies are currently being conducted.

### C. Osteoporosis

Osteoporosis is related to a number of factors including genetics, aging, hormone deficiency, and diet. Epidemiological studies have shown reduced rates of osteoporosis in Asian women compared with Western women and phytoestrogens have been postulated as a protective factor although many other factors could account for these findings. *In vitro* cell culture has shown that phytoestrogens inhibit osteoclasts and may stimulate osteoblasts. *In vivo* animal studies suggest that in high enough doses, dietary soybean prevents bone loss in ovariectomized rats.

ER-β has been identified in bone and its ligand specificity toward phytoestrogens has been reported. Isoflavones appear to have an effect on osteoblasts via the ER, whereas the effects on osteoclast appear to be non-ER-mediated, such as growth factor activity and cytokine activity. There are limited human studies. Postmenopausal women supplemented with casein, soy protein, or soy protein fortified with isoflavores for 6 months demonstrated increased bone mineral content and density in the spine in the soy fortified group alone compared with controls. Forty-five grams of soy-enriched bread improved bone mineral content in postmenopausal women compared with controls. Long-term data on the efficacy and safety of ipriflavone for prevention of postmenopausal bone loss have been conflicting. Ipriflavone (7-isopropoxy-isoflavone), a synthetic flavonoid, inhibits osteoclast function and 600 mg/day has been reported to prevent bone loss at the distal radius in postmenopausal women. More recently, a 4-year study of 474 postmenopausal women randomly assigned ipriflavone at 200 mg three times per day or placebo found no difference in bone loss prevention and in biochemical markers of bone metabolism. Long-term studies are needed before the role of phytoestrogens in the prevention of osteoporosis can be supported.

## VII. SUMMARY

Cancer research is still in its infancy. The epidemiological and animal data suggest that phytoestrogens may play a beneficial role in breast and prostate cancer. It may be that prepubertal exposure is of importance and ingestion needs to be lifelong. Extrapolations from cell line and animal studies need to be viewed with caution. It is probably naive to attribute many health outcomes to one food, and other lifestyle factors, such as exercise, substance abuse, and the diet as a whole, may be relevant.

In contrast to animal studies in which deleterious effects were observed with consumption of a phytoestrogen diet, in humans few adverse effects are observed. In humans, a genetic tolerance may have evolved in nations with a high-soy-intake diet or the varied human diet may be protective of one food group alone dominating and causing adverse effects. The limited studies to date have confirmed that diet can have significant hormonal effects and these may be of benefit in preventing some of the common diseases. Global nutrition is an increasing problem and further knowledge from scientific studies of plant-based foods is warranted.

## Glossary

**coumestans** A potent subgroup of phytoestrogens found in fodder crops.

**daidzein** An isoflavone metabolite derived from daidzin by gastrointestinal bacteria.

**enterodiol** A lignan metabolite derived by gastrointestinal bacteria.

**enterolactone** A lignan metabolite derived by gastrointestinal bacteria.

**genistein** An isoflavone metabolite derived by gastrointestinal bacteria from genistin.

**isoflavones** A subgroup of phytoestrogens derived from legumes, e.g., soybean.

**lignans** A subgroup of phytoestrogens found in vegetables, fruits, nuts, and seeds.

**phytoestrogen** A plant compound with binding affinity for the estrogen receptor.

## See Also the Following Articles

**Estrogen and Progesterone Receptors in Breast Cancer • Estrogen in the Male • Estrogen Receptor Actions through Other Transcription Factor Sites • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Follicle Stimulating Hormone (FSH) • Lipoprotein Receptor Signaling • Luteinizing Hormone (LH) • Sex Hormone-Binding Globulin (SHBG)**

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# Pilosebaceous Unit (PSU)

DIANNE DEPLEWSKI
*University of Chicago*

I. INTRODUCTION
II. POSTNATAL GROWTH AND DEVELOPMENT OF THE PSU
III. ANDROGEN ACTION IN THE PSU
IV. ROLE OF NONANDROGENIC HORMONES IN THE PSU
V. DISORDERS OF THE PSU: HIRSUTISM, PATTERN ALOPECIA, AND ACNE VULGARIS

**The pilosebaceous unit (PSU) consists of a piliary component and a sebaceous component. Each PSU has the capacity to differentiate into either a terminal hair follicle (in which a large medullated hair becomes the prominent structure) or a sebaceous follicle (in which the sebaceous gland becomes prominent and the hair remains vellus). This article provides an introduction to the role of androgens and other hormones in normal PSU development and in PSU disorders.**

## I. INTRODUCTION

Androgens play a key role in the development of the pilosebaceous (PSU) in most areas of the body. In androgen-sensitive areas before puberty, the hair is vellus and the sebaceous glands are small. In response to increasing levels of androgens, PSUs become large terminal hair follicles (sexual hairs) in sexual hair areas or they become sebaceous follicles (sebaceous glands) in sebaceous areas. Androgens play a role in PSU disorders, namely, hirsutism, pattern alopecia, and acne vulgaris. However, it is clear that the pathogenesis of these disorders involves more than androgen.

## II. POSTNATAL GROWTH AND DEVELOPMENT OF THE PSU

### A. Hair Follicle

Hair follicles vary considerably in size and shape depending on their localization in the body. Approximately 5 million hair follicles cover the human body, and this number is established before birth. New follicles are not formed after birth; however, the size of the follicles and hairs can change with time, primarily under the control of androgen. The difference in the apparent density of sexual hair between men and women is due to a different density of terminal hairs rather than a difference in the number of PSUs. Racial differences also affect various features of hair, such as shape and medullation.

Normal development and cycling of the hair follicle depend on the interaction of the follicular epithelium with the dermal papilla, which consists of specialized fibroblasts located at the base of the hair follicle. Hair grows cyclically by reverting from the anagen (growth) phase, through the catagen (shortening) phase, to the telogen (resting) phase (Fig. 1). The dynamics of the hair growth cycle vary between species, between different body sites in the same species, and between different follicle types at the same body site. It is likely that hair follicles have an intrinsic rhythmic behavior that is modulated by multiple growth factors. The duration of the anagen stage is the major determinant of the length to which a hair grows, and it varies with the location of the hair follicle. For example, scalp hairs are in anagen for a long time (between 2 and 8 years) and produce long hairs, whereas mustache hairs stay in the anagen stage for only 4 months and thus produce short hairs. Other factors influencing hair growth in various areas of the body include the linear growth rate of the hair fiber, as well as the diameter and density of the terminal hairs. During the catagen phase, hair follicles go through a highly controlled process of regression and involution, caused by apoptosis (programmed cell death). This is the shortest phase of the hair growth cycle and lasts from 2 to 3 weeks. During the telogen phase, a club hair develops and is eventually shed from the follicle to make room for new hair growth. This phase lasts for 3 to 4 months. Whereas shaving does not induce hair growth, plucking a resting (telogen) hair causes an advancement in the onset of anagen and thus induces hair regrowth.

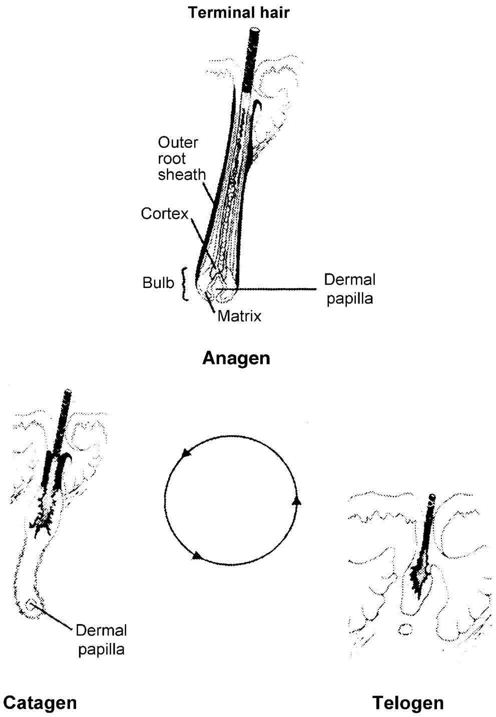

**FIGURE 1** The hair growth cycle. Hair follicles progress through repetitive cycles of growth, from anagen (active phase of growth), through catagen (shortening of the hair follicle), to telogen (resting phase of the hair cycle), after which the club hair is shed, and the follicle begins a new hair cycle again. Modified from D. Deplewski and R. L. Rosenfield, Role of hormones in pilosebaceous unit development. *Endocr. Rev.* **21**, 363–392, 2000, with permission. Copyright 2000, The Endocrine Society.

## B. Sebaceous Gland

The life cycle of sebaceous cells (sebocytes) begins at the periphery of the gland in the highly mitotic basal layer. As sebaceous cells differentiate, they accumulate increasing amounts of lipid and migrate toward the central duct. Eventually, the most mature sebocytes burst and their lipid is extruded into the ducts of the sebaceous gland as the holocrine secretion sebum. The cells of sebaceous glands turn over more rapidly than those of hairs, as they are normally completely renewed every month.

## III. ANDROGEN ACTION IN THE PSU

Before puberty, the androgen-dependent PSU consists of a prepubertal vellus follicle, which consists of a virtually invisible hair and a tiny sebaceous gland component (Fig. 2). Under the influence of androgens produced at adrenarche and then puberty, these PSUs differentiate in a distinctive pattern that depends on their location. In the sexual hair areas, a terminal hair follicle develops and the sebaceous gland develops only moderately. In acne-prone areas, androgen causes the prepubertal vellus follicle to develop into a sebaceous follicle in which the hair remains vellus and the sebaceous gland enlarges tremendously. In the balding-prone area of the scalp, PSUs respond to androgen in yet a different manner in individuals predisposed to pattern alopecia. Terminal hair follicles that previously grew without androgen gradually change with each growth cycle to an intermediate kind of follicle in which the hair component reverts to the vellus state, leaving an adult vellus follicle. Hair follicles are still present and cycling, even in

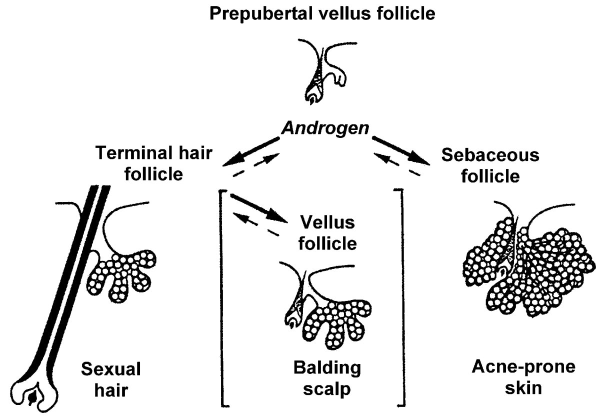

FIGURE 2 Role of androgen in the development of the pilosebaceous unit. Solid lines indicate the effects of androgen; dashed lines indicate the effects of anti-androgens. Hairs are depicted only in the anagen (growing) phase of the growth cycle. In balding scalp (bracketed area) terminal hairs not previously dependent on androgen regress to vellus hairs under the influence of androgen. Reprinted from *Am. J. Med.* 98, R. L. Rosenfield and D. Deplewski, Role of androgens in the developmental biology of the pilosebaceous unit. pp. 80S–88S. Copyright 1995, with permission from Excerpta Medica Inc.

bald scalp. These phenomena are reversed by anti-androgens: both types of androgen-dependent PSUs revert toward the prepubertal state. The fact that individual PSUs can respond differently to the same circulating hormones illustrates the complexity of the response of the PSU to androgen.

The most direct evidence that androgens are the principal hormones controlling sexual hair growth is that androgens stimulate hair growth in eunuchs and castration reduces hair growth. The latter classic observation illustrates the pliable nature of the PSU response to androgens, i.e., the reversion from terminal to vellus follicles. The sensitivity of PSUs to androgen is determined by their pattern of distribution and generally wanes from pubis to head (Fig. 3). Thus, rising androgen levels recruit an increasing proportion of PSUs in a given area to initiate the growth of terminal hair follicles, each in accordance with its preset genetic sensitivity to androgen. The apparent dose–response curve to androgen is fairly steep, with a mustache typically appearing at plasma testosterone levels just slightly above the upper limits of normal for women and the beard requiring 10-fold higher levels for full growth. There is considerable individual variability. Androgens are thought to control hair growth by influencing the synthesis and release of growth factors from dermal papilla cells that act in a paracrine fashion on the other cells of the hair follicle.

The sensitivity of sebaceous glands to androgens seems to follow a different dose–response curve than the hair follicle, with most sebaceous glands being highly and similarly sensitive to testosterone. Sebum production is at its nadir at approximately 4 years of age and begins to increase between 8 and 11 years of age. Microcomedones (1 mm or less in diameter), which form when desquamated cornified cells of the upper canal of the sebaceous follicle become abnormally adherent and form a plug in the follicular canal, make their appearance in approximately 40% of 8- to 10-year-olds. Thus, sebaceous gland function begins approximately coincident with adrenarche, before true puberty, at levels of androgen below those ordinarily required for the initiation of pubic hair growth (Fig. 4). Seventy-five percent of the normal male amount of sebaceous gland function is achieved at androgen levels normal for women. There is considerable individual variability in the degree of sebum production to a given level of androgen.

It is not yet clear what controls the nature of the response of a PSU to androgen; however, the variability in PSU responsiveness may be related in part to variations in androgen metabolism. Enzymes important in androgen metabolism in skin are shown in Fig. 5. Two forms of 5α-reductase, which are differentially expressed in various tissues, exist. The type 2 isozyme is important for most androgen actions in sexual organs; however, the type 1 isozyme is the major form of 5α-reductase in skin. The activity of 5α-reductase has been found to be higher in

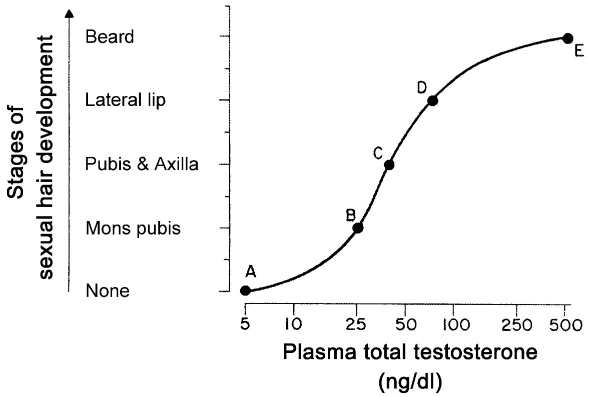

FIGURE 3 Relationship of stages of sexual hair development to testosterone as a representative plasma androgen. Note logarithmic scale for testosterone. (A) Prepubertal; (B) stage 3 public hair: (C) stage 5 public hair: (D) moderate hirsutism; (E) adult male. Reprinted from Rosenfield, R. L. (1986). Pilosebaceous physiology in relation to hirsutism and acne. *Clin. Endocrinol. Metab.* 15, 341–362, with permission.

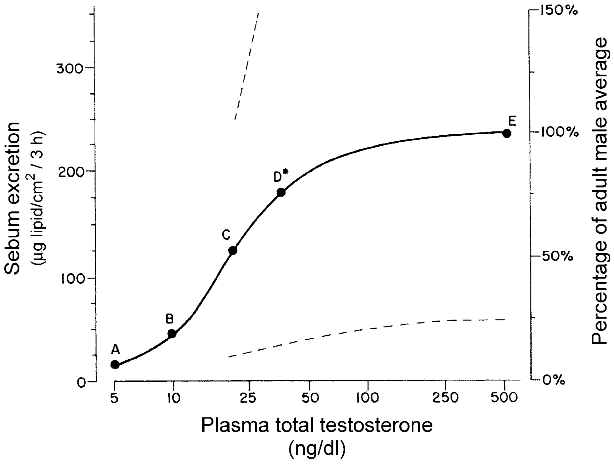

FIGURE 4 Relationship between sebum output and testosterone as a representative plasma androgen. Note the logarithmic scale for testosterone. Dashed lines show the normal range of sebum excretion. (A) 4-year-old children; (B) 7- to 11-year-old prepubertal children; (C) castrated man; (D) 20- to 40-year-old normal adult women; (E) 20- to 40-year-old normal adult men. *Average sebum level of normal 15- to 19-year-old boys and girls. Reprinted from Rosenfield, R. L. (1986). Pilosebaceous physiology in relation to hirsutism and acne. *Clin. Endocrinol. Metab.* **15**, 341–362, with permission.

sebaceous glands of the scalp and facial skin (acne-prone skin) than in other skin areas. However, no obvious difference in type 1 isozyme expression has been found between balding and nonbalding areas of adult scalp. In contrast, greater 3β-hydroxysteroid dehydrogenase activity has been found in sebaceous glands from balding scalp than in those from nonbalding scalp. Table 1 depicts the characteristic pattern of androgen metabolism in skin. A summary of the localization of the mediators of androgen signal transduction in the PSU is provided in Table 2.

## IV. ROLE OF NONANDROGENIC HORMONES IN THE PSU

In addition to androgen, other hormones including estrogen, growth hormone, insulin-like growth factor-I, insulin, glucocorticoids, prolactin, thyroid hormone, retinoids, and others are well recognized to play roles in PSU growth and development, and these are summarized in Table 3.

$$\mathbf{DHEA} \xrightarrow{\mathbf{3\beta\text{-HSD}}} \mathbf{AD} \overset{\mathbf{17\beta\text{-HSD}}}{\longleftrightarrow} \mathbf{T} \xrightarrow{\mathbf{5\alpha\text{-R}}} \mathbf{DHT}$$

FIGURE 5 Androgen metabolism in skin. The weak androgen dehydroepiandrosterone (DHEA) is metabolized to the more potent androgen dihydrotestosterone (DHT) by specific enzymes as depicted. AD, Androstenedione; T, testosterone; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 5α-R, 5α-reductase.

## V. DISORDERS OF THE PSU: HIRSUTISM, PATTERN ALOPECIA, AND ACNE VULGARIS

### A. Hirsutism

Hirsutism is typically defined as excessive male-pattern hair growth in women. This definition distinguishes hirsutism from hypertrichosis, which is the term reserved to describe the androgen-independent growth of body hair that is vellus, prominent in nonsexual areas, and most commonly familial or caused by metabolic disorders (e.g., thyroid disturbances, anorexia nervosa, or porphyria) or medications (e.g., phenytoin, minoxidil, or cyclosporine).

### B. Pattern Alopecia

Pattern alopecia is the androgen-dependent thinning of hair that occurs progressively with advancing age in genetically susceptible men and women. However, it can begin as soon as the early teenage years. The process is mainly the result of miniaturization of terminal to vellus hair follicles (Fig. 2). The androgen-dependency of pattern alopecia was initially deduced on the basis of eunuchs not suffering from male pattern hair loss unless they are given replacement testosterone. Pattern alopecia is generally thought to be distinct from the diffuse thinning of scalp hair associated with aging. However, it remains possible that pattern alopecia may partially be due to an accentuation of the normal process of hair loss associated with aging. In men, pattern alopecia typically presents as temporo-occipital pattern (male-pattern) balding. In female-pattern alopecia, the thinning typically begins with involvement of the crown of the scalp (rather than the vertex and bifrontal areas as in men) and may become fairly diffuse. Pattern alopecia can be psychologically devastating in both sexes. The genetic predisposition to pattern alopecia is still poorly understood. However, the pattern of inheritance is considered to be polygenic with variable penetrance. It is likely that the penetrance is greatly determined by the height of the plasma androgen level.

Minoxidil and finasteride are hormonal treatments for pattern alopecia. Minoxidil induces and prolongs the anagen stage and converts vellus follicles to terminal follicles. Finasteride inhibits type 2 5α-reductase, which converts testosterone to DHT.

TABLE 1 **Pattern of Androgen Metabolism within Skin Organelles of Axilla and Scalp**

| | Relative enzyme activity (percentage of total) | | |
|---|---|---|---|
| | 5$\alpha$-Reductase ($n = 8$–$10$) | 17$\beta$-HSD ($n = 6$–$16$) | 3$\beta$-HSD ($n = 2$) |
| Sebaceous gland | 17 | 21 | 50 |
| Hair follicle | 8 | 15 | |
| Sweat gland | 60 | 47 | 40 |
| Dermis | 9 | 6 | 8 |
| Epidermis | 6 | 11 | 2 |
| Total | 100 | 100 | 100 |

*Note*. Regional differences in enzyme activity between axilla and scalp may exist. 17$\beta$-HSD, 17$\beta$-hydroxysteroid dehydrogenase; 3$\beta$-HSD, 3$\beta$-hydroxysteroid dehydrogenase. Reprinted from D. Deplewski and R. L. Rosenfield, Role of hormones in pilosebaceous unit development, *Endocr. Rev.* **21**, 363–392, 2000, with permission. Copyright 2000, The Endocrine Society.

Finasteride inhibits miniaturization of hairs, converts vellus follicles to terminal follicles, and prolongs the anagen stage in androgen-dependent scalp follicles. Typically, 9 to 12 months of treatment is needed to judge the efficacy of a given treatment on hair growth, because of the long duration of the hair growth cycle. Unfortunately, simply removing androgens does not usually cause a significant conversion of miniaturized vellus follicles to terminal follicles; thus, current treatments for pattern alopecia are less than optimal.

## C. Acne Vulgaris

Sebum, the holocrine secretion of sebaceous glands, plays a central role in the pathogenesis of acne vulgaris. Acne occurs at the onset of puberty when plasma androgen levels rise, peaks at midpuberty, and usually resolves by the mid-20s. Virtually all adolescents have at least a few open and closed comedones, which are noninflammatory enlarged sebaceous follicular ducts known as blackheads and whiteheads, respectively. Androgens are an incitant of acne vulgaris since they are necessary for the growth and differentiation of sebaceous glands.

Dehydroepiandrosterone sulfate (DHEAS) plays a role in acne through its conversion to more potent androgens that stimulate sebum production. Plasma DHEAS is likely the most important androgen for the initiation of comedonal acne in early puberty, as it rises first. Excessive DHT formation in skin has also been implicated in the pathogenesis of acne vulgaris, suggesting that the activity of 5$\alpha$-reductase may also play an important role.

There is more to acne than sebaceous gland growth and sebum production: abnormal sebaceous duct keratinization, bacterial colonization with *Propionibacterium acnes*, and host immune response factors are also important. The pathogenesis of acne is thought by most to commence with plugging of the outlet of the sebaceous gland with desquamated cornified cells of the upper canal of the follicle. The more severe stages of acne are the consequences of obstruction and impaction, with bacterial secondary infection of static sebum occurring in an anaerobic environment. A closed comedone takes 2 months to form from its precursor lesion, the microcomedone. Inflammatory acne, consisting of papules, pustules, nodules, and cysts, is a later phenomenon that develops from comedonal acne.

TABLE 2 **Androgen Mechanism of Action in the Pilosebaceous Unit**

| | Sebaceous gland | | Sexual hair | |
|---|---|---|---|---|
| Parameter | Stroma | Sebocytes | Dermal papilla | Hair epithelium |
| 3$\beta$-HSD | ? | +++ | ? | ± |
| 17$\beta$-HSD | ? | +++ | Type 3 | Type 2 |
| 5$\alpha$-Reductase | ? | Type 1 | Type 2 | Type 1 |
| Androgen receptor | + | ++++ | ++++ | ± |

*Note*. 3$\beta$-HSD, 3$\beta$-hydroxysteroid dehydrogenase; 17$\beta$-HSD, 17$\beta$-hydroxysteroid dehydrogenase. Reprinted from D. Deplewski and R. L. Rosenfield, Role of hormones in pilosebaceous unit development, *Endocr. Rev.* **21**, 363–392, 2000, with permission. Copyright 2000, The Endocrine Society.

TABLE 3 Hormone Action on the Pilosebaceous Unit

| Hormone | Action on PSU |
|---|---|
| Androgen | • Has diverse effects on hair follicles depending on their location in body; for example, sexual hairs grow only in certain areas of the body, whereas hair on the scalp undergo regression from a terminal to a vellus type in genetically susceptible individuals<br>• Induces sebaceous gland development in acne-prone areas |
| Estrogen | • Prolongs the anagen phase of hair growth<br>• Rapid growth postpartum causes telogen effluvium (loss of a large number of hairs due to simultaneous advancement into telogen)<br>• Directly suppresses sebaceous gland function |
| Insulin | • Hair growth retarded in diabetes mellitus and accelerated with insulin treatment<br>• Essential for hair follicle growth and sebaceous cell growth *in vitro*<br>• May act as an IGF-I surrogate |
| Growth hormone | • Augments androgen effects on hair growth and sebocyte differentiation<br>• Important for sebocyte growth and development<br>• GH excess of acromegaly is associated with excess output of sebum from the sebaceous gland (seborrhea)<br>• GH receptor found in hair follicles and sebaceous gland |
| Insulin-like growth factor | • Prevents hair follicles from entering catagen stage<br>• May mediate some of the androgen effects on PSUs—induces the up-regulation of 5α-reductase by DHT in genital skin fibroblasts<br>• Found in both hair follicles and sebaceous glands |
| Glucocorticoids | • Hypertrichosis (diffuse excessive hair growth) is present in Cushing's syndrome<br>• Glucocorticoid therapy aggravates acne |
| Prolactin | • Hyperprolactinemia can cause hirsutism and seborrhea<br>• Receptors localized in dermal papilla and sebaceous glands |
| Thyroid hormone | • Low or high levels can cause telogen effluvium<br>• Low level cause scalp hair to become dull or brittle<br>• Can stimulate sebum production<br>• Receptors found in PSUs |
| PPARs | • Likely important in sebaceous gland development |
| Retinoids | • Important in hair follicle formation and patterning<br>• Prolong anagen stage and decrease telogen stage of hair growth cycle<br>• Trace amounts promote sebocyte growth and differentiation; larger doses cause atrophy of sebaceous glands and a decrease in sebum production |
| Catecholamines | • May be involved in the aggravation of acne by stress |
| Vitamin D receptor | • Mutations associated with alopecia in humans |
| Melanocortin-5 receptor | • Targeted disruption in mice causes a decrease in sebaceous lipid production |
| Parathyroid hormone | • Inhibits hair growth |

*Note*. DHT, dihydrotestosterone; GH, growth hormone; IGF-I, insulin-like growth factor-I; PPARs, peroxisome proliferator-activated receptors; PSU, pilosebaceous unit.

Topical agents and retinoids play an important role in the treatment of acne, and 2 to 3 months may be needed to see the full effect of treatment on acne. Whereas trace amounts of retinoids promote sebocyte growth and differentiation, larger doses cause atrophy of sebaceous glands and a decrease in sebum secretion in both animals and humans. Retinoids have been postulated to inhibit lipid synthesis in sebocytes either directly, through an inhibition of lipogenic enzymes, or indirectly, by decreased cell proliferation. Retinoids have been used for the treatment of acne vulgaris for a long time although the precise mechanism for their efficacy has not been completely elucidated.

## Glossary

**acne vulgaris** A disorder of the sebaceous gland; characteristic lesions include open (blackhead) and closed (whitehead) comedones, papules, pustules, and nodules.

**hirsutism** Excessive male-pattern hair growth in women.

**pattern alopecia** The androgen-dependent thinning of hair that occurs progressively with advancing age in genetically susceptible men and women; the process is mainly the result of miniaturization of terminal to vellus hair follicles.

**pilosebaceous unit** A skin appendage consisting of a hair follicle, a hair shaft, and a sebaceous gland.

**sebaceous gland** A small sacculated organ within the dermis; composed of acini, which are attached to a common excretory duct that is continuous with the wall of the piliary canal and, indirectly, with the surface of the epidermis.

**terminal hair** Thick, long, pigmented hair on scalp and body.

**vellus hair** Thin, short, usually nonpigmented hair.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor-Related Pathology • Androgens: Pharmacological Use and Abuse • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology**

## Further Reading

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# Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Its Receptor

Joseph R. Pisegna
*VA Greater Los Angeles Healthcare System and UCLA School of Medicine*

I. INTRODUCTION
II. PACAP HORMONE
III. THE PAC1 RECEPTOR
IV. PHYSIOLOGY OF PACAP AND ITS RECEPTOR, PAC1
V. CONCLUSIONS

**This article provides an overview of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor, PAC1, which were discovered more than a decade ago. The current understanding of the structure and function of the PACAP hormone and an in-depth understanding of the structure and physiology of the PAC1 receptor are presented. The important roles that the PACAP hormone and receptor play in the regulation of physiological actions in the body are examined. Since the receptor and hormone are localized in both the central nervous system and the periphery, their important effects in both systems are described.**

## I. INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is the most recently discovered neuropeptide in the vasoactive intestinal polypeptide (VIP), secretin, and glucagon family of peptide hormones. It was designated PACAP because it stimulated adenylyl cyclase in rat anterior pituitary cells in culture and was a polypeptide of 38 amino acids. Since their discovery, PACAP hormone and its receptor, PAC1, have been identified in numerous tissues by immunohistochemistry and radioimmunoassay. Although the exact role of PACAP in the brain has not been determined, its broad distribution and presence during development suggest that it acts as a neurotransmitter or neuromodulatory peptide. Therefore, PACAP appears to play an important role in the growth and development of the brain. Its role as a neuromodulatory hormone will be discussed in more detail below. PACAP is also present in a number of peripheral tissues, including

TABLE 1 Alignment of the Amino Acid Sequences for PACAP and Related Hormones

| | |
|---|---|
| PACAP-38 | **HSDGIFTDSYSRYRKQMAVKKLAAVLGKRYKQRVKNK-NH**$_2$ |
| PACAP-27 | **HSDGIFTDSYSRYRKQMAVKKLAAVLG-NH**$_2$ |
| VIP | **HSD**AV**FTD**N**Y**T**R**L**RKQMAVKKL**NSILNK**-NH**$_2$ |
| Secretin | **HSDGTFT**SEL**SR**L**R**EGARLQRLLQGLV**G-NH**$_2$ |
| Helodermin | **HSD**A**IFTYS**KLLARL**A**LQ**KYLA**S**ILG**S**R**TSPPP-NH$_2$ |
| Glucagon | **HS**Q**G**T**FT**SD**YS**K**Y**LDSRRAQDFVQWLMNT-NH$_2$ |
| GRF | YA**D**A**IFT**N**SYS**KVLG**Q**LSAR**K**LLQDIMSRQQGESNQERGARARL-NH$_2$ |

*Note*. Boldface type indicate amino acids with complete identity at those positions of the PACAP-38 hormone.

the gastrointestinal tract, adrenal gland, and testis. Another important action of PACAP is its ability to stimulate cellular growth and differentiation. PACAP appears to regulate the growth of a number of tumors and may have important actions in cancer biology.

## II. PACAP HORMONE

### A. Discovery

Arimura and colleagues isolated fractions of ovine hypothalamic extracts that stimulated adenylyl cyclase activity in anterior pituitary cells. The hormone pituitary adenylate cyclase-activating polypeptide was so named for this activity. The hormone PACAP was shown to contain 38 amino acids, and later a 27-amino-acid form was identified. Since both a 27-amino-acid form and a 38-amino-acid form are biologically active, they are designated PACAP-27 and PACAP-38, respectively. There is significant interspecies conservation of the amino acid structure of the PACAP hormone.

### B. Structure

The primary sequence of the PACAP hormone is 68% identical to its closest hormone relative, VIP. These hormones belong to a broader category of hormones including PACAP, VIP, secretin, glucagon, and GRF. As demonstrated in Table 1, there is significant homology of these peptides. The peptides most closely related to PACAP are grouped closest to PACAP at the top of Table 1. The three-dimensional structures of the hormones in this family show close similarity. Minor differences in the α-helix conformation may account for receptor specificity of PACAP and VIP.

### C. PACAP Hormone Gene

The PACAP hormone gene has been cloned in mice and humans. The human gene is composed of five exons with a structure that is similar to other members of this family of peptides. This close similarity suggests that all of the members of this family of peptides may originate from a similar ancestral gene through duplication.

### D. PACAP Hormone Distribution

The greatest concentration of PACAP is detected in the central nervous system of mammals. The site of greatest activity in the rat is the paraventricular and supraoptic nuclei in the hypothalamus. It is thought that the hormone is transported to the pituitary gland, where the hormone has activity in the anterior pituitary. PACAP-38 immunoreactivity is also present in extrahypothalamic sites, such as the substantia nigra, cerebellum, pons, and the paraventricular nuclei of the thalamus. The spinal cord also contains PACAP that is localized mainly in the dorsal root ganglia and dorsal horn. PACAP has been identified in the gastrointestinal enteric neural plexus, where it is an important mediator of gastric acid secretion and intestinal motility. In the adrenal gland, PACAP is present in the adrenal medulla, where it appears to be a potent stimulator of catecholamine release.

## III. THE PAC1 RECEPTOR

### A. Cloning and Pharmacological Characterization

There are three receptors with high affinity for PACAP hormone that have been identified and cloned. The first of these receptors to be cloned was the classical VIP receptor (VPAC1). Subsequently, a receptor with affinity for only PACAP was cloned, the type I PACAP receptor (PAC1). The last receptor to be cloned in this family was the VIP2 receptor (VPAC1). These receptors can be differentiated pharmacologically based on their relative affinities for the ligands, as shown in Table 2. PAC1 has affinity for only PACAP-38 and PACAP-27, whereas the

TABLE 2 **Relative Affinities of the Three PACAP and VIP Receptors for the Ligands PACAP-27, PACAP-38, VIP, and Helodermin**

| IUPHAR nomenclature | Relative affinities |
|---|---|
| PAC1 | PACAP-27 = PACAP-38 ≫ VIP > Helodermin |
| VPAC1 | PACAP-27 = PACAP-38 = VIP ≫ Helodermin |
| VPAC2 | Helodermin > PACAP-27 = PACAP-38 = VIP |

*Note*. Classification of the receptors in the PACAP superfamily based on relative affinities to the related peptides. Adapted from Harmar *et al.* (1998), with permission.

VPAC receptors have nearly identical affinities for the ligands PACAP and VIP.

Cloning of the rat PAC1 cDNA identified it as a member of the VIP and secretin family of peptide receptors. The receptor cDNA encoded a putative protein of approximately 50 kDa and 495 amino acids. Similar to the VIP and secretin receptors, the PAC1 receptor contained seven hydrophobic domains, conserved cysteines in the extracellular domains, and several N-linked glycosylation sites. Cloning of the PAC1 gene indicated that the receptor could exist as four major splice variants, which were subsequently identified. As shown in Fig. 1, the four potential splice variants differed in the length of the third intracellular domain and were identified by Spengler and colleagues as hip, hop, hip-hop, and null. More importantly, these investigators identified variations in signal transduction coupling to phospholipase C and differences in the tissue distribution of the splice variants. The PAC1 cDNA was also cloned in a number of other species such as rat, mouse, and bovine. Subsequently, all of the human PAC1 receptor cDNA splice variants were cloned. The cloning of human PAC1 revealed a high level of homology with the rat and mouse receptor and a similar gene organization. However, unlike the rat splice variants, differences in signal transduction coupling were not observed in humans. Instead, a higher efficacy for the hop variant, an intermediate coupling for the hip-hop splice variant, and a lower level of coupling for the hip splice variant for coupling to phospholipase C were observed. Similar to the human receptor gene, the rat PAC1 receptor gene has been shown to be large (~50 kb); however, unlike the human gene, which is localized to chromosome 7, the rat gene is localized to chromosome 4.

## B. Signal Transduction Characteristics

The PAC1 receptor is coupled to a dual signal transduction pathway. This was first demonstrated in PC12 cells. With the cloning of the PAC1 receptor, the specificity of PACAP-38 and PACAP-27 was shown. A fourth transmembrane splice variant was shown not to couple to either adenylyl cyclase or phospholipase C, yet it does couple to an L-type $Ca^{2+}$ channel. The region of PAC1 shown to be responsible for signal transduction coupling is the COOH-terminus. Two critical amino acids in this region of the receptor, Ser and Arg, were shown by mutagenesis studies to be coupled to signal transduction intermediates.

## C. Localization of PAC1 Receptors

The greatest density of receptors occurs in the hypothalamus, for instance, in the supraoptic nucleus, periventricular nucleus, and lateral hypothalamus. Other areas include the olfactory bulb and regions of the thalamus and cerebellum. In the rat brain, the predominant splice variant is the null variant. PAC1 has been detected by immunohistochemistry as well as electron microscopy in the retina, where it appears to be distributed in the inner plexiform layer.

In peripheral tissues, PAC1 receptors have now been identified in a large number of organs. The greatest density has been identified in the adrenal medulla where the predominant receptor splice variant appears to be of the hop type. Similarly, PAC1 receptors are expressed at high levels in the anterior pituitary gland, where, again, the predominant splice

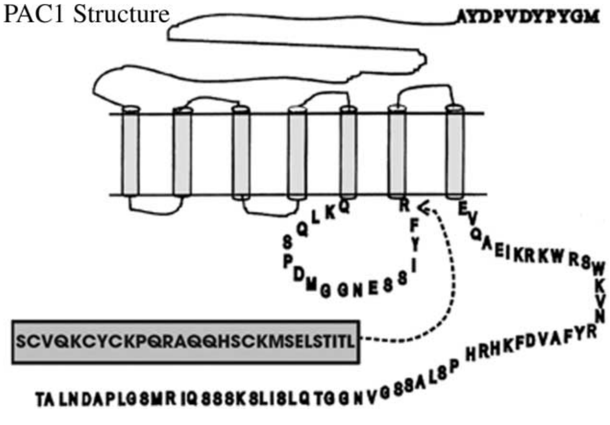

FIGURE 1 Structure of the heptahelical receptor for PACAP, PAC1. The receptor has seven transmembrane domains indicated by the cylinders and a long third intracellular loop. Splice variants are indicated in the shaded rectangle.

variant is the hop type and to a lesser extent the null variant. The human prostate gland also contains PAC1 receptors that are present in conditions such as benign prostatic hyperplasia. PAC1 receptors are also present on germ cells and spermatogonia as well as Sertoli and Leydig cells. The respiratory system contains predominantly VPAC1 receptors with little to no PAC1 receptor expressed, whereas the cardiovascular system contains all three receptor types. The PAC1 and VPAC 1 receptors have been identified in the gastrointestinal tract with the PAC1 receptor expressed on the gastric enterochromaffin-like (ECL) cells and the VPAC1 expressed on the somatostatin-containing D cells and chief cells of the stomach. The liver appears to contain predominantly VPAC1 receptors, although careful studies examining PAC1 receptor expression in this organ have not been performed. The smooth muscles of the gastrointestinal tract contain VPAC1 and PAC1 receptors with the PAC1 receptor well described in the rat taenia coli. Another system that has received much scrutiny recently is the immune system. The PAC1 receptor has been described in macrophages, whereas other PACAP receptor types have been described in a number of tumor cell lines. The cloning of rat PAC1 from the rat pancreatic acinar carcinoma cell line AR42J underscores this observation. PAC1 receptor expression has been described in human lung and breast cancer cell lines. Differences in the expression of splice variants of PAC1 receptors in pituitary tumors have also been shown previously. The rat pheochromocytoma cell line PC12 was one of the classical cell systems in which the signal transduction cascade for PAC1 receptors was reported.

## IV. PHYSIOLOGY OF PACAP AND ITS RECEPTOR, PAC1

### A. Central Nervous System

PACAP appears to exert a multitude of effects within the CNS. Exogenously administered PACAP increases the activity level as well as amount of vasopressin released. The predominant signal transduction pathway involved in the stimulus and release is the protein kinase A pathway. Intracisternal administration of PACAP has been shown to regulate the release of a number of hormones such as gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), prolactin, somatostatin, and the dopamine analogue DOPAC. PAC1 receptors have been shown to be expressed at relatively high levels within the pineal gland and appear to stimulate melatonin secretion. Intracerebral injection of PACAP increases rapid eye movement sleep in sleep-deprived rats. Another potential action of PACAP is in the control of appetite as suggested by its distribution in key areas of the hypothalamus. In cultured cell systems, PACAP has been shown to activate the c-fos, c-Jun, and mitogen-activated protein kinase signaling system, indicating its role in regulating the proliferation of cells. PACAP has been shown to reduce the cytopathic effects of the human immunodeficiency virus envelope protein gp120 in cultured neuroblasts, again supporting a role for this hormone as a neuroprotective factor. In cerebellar granule cells, PACAP appears to reduce apoptosis.

### B. Endocrine Organs

PACAP stimulates gonadotropes, somatotropes, lactotropes, and thyrotropes. Somatotropic cells that release growth hormone are stimulated by PACAP and appear to be additive to the effects of GRF. PACAP activates both adenylyl cyclase and phospholipase C in cultured pituitary cells. PACAP appears to be a major regulator of anterior pituitary function by stimulating the release of growth hormone, LH, follicle-stimulating hormone (FSH), prolactin, and the adrenocorticotropic hormone (ACTH). PACAP may act in a synergistic manner with GnRH to stimulate the release of LH and FSH. In cultured lactotropes, PACAP appears to directly stimulate the release of intracellular $Ca^{2+}$. Unlike the somatotropes, gonadotropes, and lactotropes, the effect of PACAP on corticotropes appears to be indirect, by stimulating corticotropin-releasing factor, the major stimulus for ACTH release. Similarly, no direct effect of PACAP on the thyrotroic cells of the pituitary has been shown.

The second major endocrine site of physiological activity is in the male and female reproductive tract. PACAP has been localized to the smooth muscles of the female reproductive tract, where it may be involved in muscle relaxation. PACAP has also been localized in the placenta. VPAC2 receptors have also been identified in placental tissue, which is the site of initial cloning of the VPAC2 receptor. The ovary also contains PACAP in the granulosa zone. PACAP appears to stimulate an increase in progesterone production in the preovulatory phase. As described earlier, PACAP and PAC1 receptors have been localized to the male gonadal germ cells. PACAP has been shown to stimulate testosterone release. PACAP has been localized to the epididymis and may be an important trigger for sperm release. A reduction in PACAP may be an important mechanism in penile erection and thus may have a clinical role in male impotence. The adrenal gland contains the highest concentration of PACAP outside of the CNS. It has been shown that PACAP is the most potent stimulator of catecholamine release from the adrenal gland.

## C. Respiratory Organs

The major effect of PACAP in the respiratory tree is bronchodilation, an effect that is mediated primarily through the VPAC1 receptor and the activation of cyclic AMP (cAMP). With the discovery that PACAP and VIP may also activate smooth muscle nitric oxide synthesis, this may represent another important action of these hormones. Given the potent actions of PACAP on bronchodilation in humans, the development of potent agonists may be clinically useful.

## D. Gastrointestinal Tract

PACAP-containing enteric nerve fibers have been described in the stomach and co-localized with PAC1 receptors on the surface of enterochromaffin-like cells. In the stomach, PACAP appears to be the major neural pathway involved in gastric acid secretion and may account for the observed nocturnal increase in gastric acid secretion. Through its activity on the VPAC1 receptor expressed on the surface of the D cell, PACAP not only activates gastric acid secretion but also, along with galanin, inhibits acid secretion through the release of somatostatin from the gastric D cell (Fig. 2). Another important effect of PACAP is in the regulation of intestinal motility. The action of PACAP is mainly that of relaxation, being mediated through the VPAC1 receptor, but in rat colon, PACAP appears to stimulate apamin-sensitive $K^+$ channels. The major colonic peristaltic reflex is mediated through VIP, whereas the descending relaxation phase of intestinal peristalsis appears to be regulated by PACAP through its actions on the VPAC1 receptor. Another novel mechanism that has been discovered is the interplay between the hormones VIP and PACAP in nitric oxide synthesis.

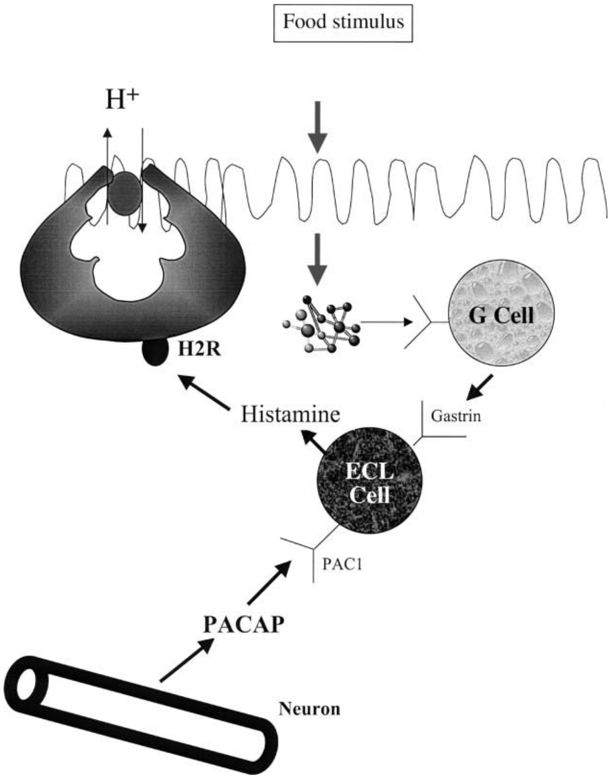

FIGURE 2 Model for the regulation of gastric acid secretion by the hormone PACAP. Released by neurons of the gastrointestinal enteric neural plexus, PACAP binds to the PAC1 receptors of nearby enterochromaffin-like (ECL) cells. PACAP stimulation of ECL cells triggers the release of histamine. Liberated histamine then binds to histamine-2 receptors (H2R) located on the surface of stomach parietal cells, thereby regulating gastric acid secretion.

## E. Cardiovascular System

As shown in both the respiratory and the digestive systems, the primary effect of PACAP is the relaxation of smooth muscles through the activation of cAMP and protein kinase A. Similarly, in the cardiovascular system, PACAP relaxes the smooth muscle in vessels, resulting in an overall reduction in blood pressure. In whole animal studies, intravenous administration of PACAP results in a biphasic effect with initial vasodilation and a subsequent catecholamine release reflex causing an increase in blood pressure. In cardiac myocytes, PACAP produces a positive inotropic and chronotropic effect and a later reduction in ionotropic effect that appears to be mediated by the resulting vagally released acetylcholine.

## F. Immune System

PACAP and VIP have effects on a wide range of immune cells. The role of PACAP in the regulation of cell-mediated immunity has not been thoroughly investigated. In the mouse, PACAP has been shown to activate murine macrophages that would then be able to stimulate T-cell proliferation through specific VPAC1 receptors with a consequent release of interleukin-10 (IL-10) and inhibition of IL-6 and IL-12 production. VIP and PACAP have been shown

TABLE 3 Nomenclature for PACAP and Related VIP Receptors

| Receptor type IUPHAR nomenclature | Selective Agonists |  | Selective antagonists | Fluorescent agonists | Selective antagonist |
|---|---|---|---|---|---|
|  | Previous nomenclature |  |  |  |  |
| PAC1 | PACAP type I<br>PVR1 | PACAP-38<br>PACAP-27<br>Maxadilan? | PACAP 6–38<br>PACAP 6–27 | Fluor-PACAP | PACAP(6–38) |
| VPAC1 | VIP | [Arg$^{16}$] chicken secretin | Ro- | Fluor-VIP | [Ac-His$^{1}$, D-Phe$^{2}$, Lys$^{15}$, Arg$^{16}$] |
|  | VIP1 | [Lys$^{15}$Arg$^{16}$Leu$^{27}$]VIP (1–7)GRF(8–27)-NH$_2$ |  |  | VIP(3–7)GRF (8–27)-NH$_2$ |
|  | PACAP type II<br>PVR2<br>VIP/PACAP1 |  |  |  |  |
| VPAC2 | VIP2<br>PACAP-3<br>PVR3<br>VIP/PACAP2 | Helodermin<br>Ro 25–1553<br>Ro 25–1392 |  |  |  |

*Note*. Nomenclature for the PACAP-related receptor superfamily shown in comparison to their previous nomenclature and hormone affinity. Adapted from Harmar *et al.* (1998), with permission.

to inhibit IL-2 transcription in T cells through the reduction of c-Jun. In a recent study, VIP and PACAP were demonstrated to inhibit nuclear factor κB (NF-κB). VIP and PACAP inhibit p65 nuclear translocation and NF-κB DNA binding. In macrophages, VIP and PACAP have been shown to inhibit interferon-γ-induced activation of the Janus kinase 1 (JAK1)/JAK2/signal transactivators and activators of transcription/interferon regulatory signaling cascade.

## G. Tumor Biology

The pharmacological characterization of tumor cells expressing PACAP receptors was an integral part of understanding the biology and signal transduction of this hormone and its receptor. Therefore, the majority of the early work on their pharmacology and signal transduction relied on tumor cells such as the rat pancreatic cancer cell line AR-42J, the human neuroblastoma cell line NB-OK1, the human astrocytoma cell line, and the rat pheochromocytoma PC-12 cell line. It has been shown that PACAP stimulates the expression of c-*fos*,c-*myc*, and c-*jun* in a number of tumoral cell lines, indicating that it is a potent stimulator of cell proliferation. In human lung cancer cell lines, PACAP stimulates the growth of tumors injected into nude mice, an effect that can be antagonized by the partial PACAP antagonist PACAP 6–38 (see Table 3). In human tumors, radioligand-binding studies with either $^{125}$I-VIP or $^{125}$I-acetyl-PACAP-27 show expression of receptor in a large percentage of human tumors including breast, prostate, pancreas, lung, colon, stomach, and liver as well as lymphomas and meningiomas.

## V. CONCLUSIONS

PACAP is one of the most recently described neuropeptides. Its biological relevance is only beginning to be understood. Information thus far indicates that both the hormone and the PAC1 receptor are widely distributed in both the CNS and periphery and that this set of biological mediators is relatively important to normal physiology. With the recent study of genetic knockout mice, more information regarding the function of both the hormone and the receptor will be obtained.

## See Also the Following Articles

**Amino Acid and Nitric Oxide Control of the Anterior Pituitary • Cytokines and Anterior Pituitary Function • Glucagon-like Peptides: GLP-1 and GLP-2 • Monoaminergic and Cholinergic Control of the Anterior Pituitary • Neuropeptides and Control of the Anterior Pituitary • Secretin • Vasoactive Intestinal Peptide (VIP)**

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# Placental Development

Michael T. McMaster and Susan J. Fisher
*University of California, San Francisco*

I. INTRODUCTION
II. MORPHOLOGICAL ASPECTS OF NORMAL HUMAN PLACENTATION/CYTOTROPHOBLAST INVASION
III. MOLECULAR ASPECTS OF PLACENTATION/CYTOTROPHOBLAST INVASION
IV. REGULATION OF CYTOTROPHOBLAST ENDOVASCULAR INVASION

**Survival of the eutherian embryo/fetus depends on the formation of a transient but vital organ, the placenta. Therefore, key events early in placental development play a large role in determining the course and outcome of pregnancy.**

## I. INTRODUCTION

Placentation, the first test of the embryo's differentiative and organogenetic capacity, accomplishes two critical events: attaching the conceptus to the uterus and bringing the fetal and maternal circulations into close proximity to facilitate effective gas, nutrient, and waste exchange. These functions require that fetal placental cells (trophoblasts) acquire an invasive phenotype. In mammals that form a hemochorial placenta (e.g., humans and mice), fetal trophoblasts come in direct contact with maternal blood. Thus, placentation also entails the unique requirement for close cooperation and direct cellular contact between two immunologically distinct organisms.

During implantation and subsequent placental development, fetal trophoblasts and the uterine cells that they encounter intricately regulate the expression of several classes of molecules. These include adhesion receptors and their ligands, proteinases and their inhibitors, growth factors/cytokines and their receptors, immunomodulators, and transcription factors. This article focuses on the profound changes in trophoblast phenotype that occur during placental development, with emphasis on uterine invasion by cytotrophoblasts (CTBs). Data point to the central importance of the intimate relationship that develops between fetal trophoblasts and the maternal vasculature, as well as to strategies for uncovering the regulatory factors that control the formation of these highly unusual connections.

## II. MORPHOLOGICAL ASPECTS OF NORMAL HUMAN PLACENTATION/CYTOTROPHOBLAST INVASION

For many reasons, the human placenta has been difficult to study. With its 9-month life span, developmental processes that take months to years to complete in other organs are compressed into a narrow window of time. Within this short period, additional constraints exist: for example, the requirement that a functioning placenta be in place before substantial fetal growth occurs. As a result, the basic elements of placental development occur during the first half of pregnancy, after which additional growth elaborates on these themes. At term, this organ has reached the end of its life span. As a result of an autocrine program of planned obsolescence, pregnancies that continue beyond this point are endangered by complications that are related to a rapid decline in placental function. Accordingly, the concept that embryonic and fetal development occurs along a smooth continuum during the prenatal period applies only to intraembryonic processes. Differentiation of the extraembryonic lineages is by necessity an explosive event that occurs early in pregnancy. As a result, interpreting any picture of placental development depends entirely on when, during its 9-month life span, that snapshot was taken.

The complex anatomy of the placenta has also made this organ difficult to study. Commonly the placenta is depicted as a simple, pancake-shaped sponge connecting the embryo/fetus to the uterus. This portion of the placenta, which is expelled from the uterus during delivery, is easy to obtain and, consequently, frequently studied. But one of the most interesting parts of the placenta is rarely seen. This portion, which lies buried within the uterine wall, separates from the rest of the placenta during pregnancy termination or delivery. As a result, the only way to obtain this tissue is by uterine biopsy of the site where the placenta attached. Thus, special procedures that are similar to the methods used to obtain any other surgical specimen are required to collect this portion of the placenta.

A full understanding of the placenta can be obtained only by studying both its parts. Figure 1 diagrams these two parts and joins them into a single unit to show how they function, together with modified uterine structures, during human pregnancy. The placenta is made up of individual units termed chorionic villi. Each villus has a connective tissue core that contains fetal blood vessels and numerous macrophages, termed Hofbauer cells. The macrophages often lie adjacent to a thick basement membrane, which underlies a layer of cytotrophoblast stem cells that are the progenitors of all the trophoblast lineages.

The differentiation pathway that cytotrophoblast stem cells take depends on their location. In floating villi, the CTBs fuse to form a multinucleate syncytium, the syncytiotrophoblast, that covers the villus surface. These villi are attached at only one end to the tree-like fetal portion of the placenta. The rest of the villus floats in a stream of maternal blood, which optimizes exchange, across the syncytium, of substances between the mother and the fetus. In anchoring villi, cytotrophoblast stem cells detach from the basement membrane and form a column of nonpolarized mononuclear cells that invade the uterus. As a result, these villi are attached at one end to the fetal portion of the placenta and at the other end to the uterus. This arrangement anchors the villus to the uterine wall. Invasive CTBs rapidly traverse most of the uterine parenchyma (interstitial invasion). They also breach the uterine veins and arteries that they encounter (endovascular invasion). Their interactions with veins are confined to the portions of the vessels that lie near the inner surface of the uterus, but CTBs migrate in a retrograde direction along much of the intrauterine course of the arterioles. Eventually, these fetal cells completely replace the maternal endothelial lining and partially replace the muscular wall of these vessels. This unusual process diverts uterine blood flow to the floating villi.

## III. MOLECULAR ASPECTS OF PLACENTATION/CYTOTROPHOBLAST INVASION

Work from the authors' laboratory has focused on molecular aspects of the cytotrophoblast differentiation pathway that leads to uterine invasion. Given the unusual way in which these cells colonize the uterine blood vessels and channel maternal blood to the floating villi, it was hypothesized that they might replicate portions of standard vasculogenesis and/or angiogenesis programs. As a first test of this theory, the cells' expression of adhesion molecules during interstitial and endovascular invasion was examined. The onset of cytotrophoblast differentiation/invasion was found to be characterized by reduced staining for receptors characteristic of polarized cytotrophoblast epithelial stem cells—integrin $\alpha6\beta4$ and E-cadherin—and the onset of expression of adhesion

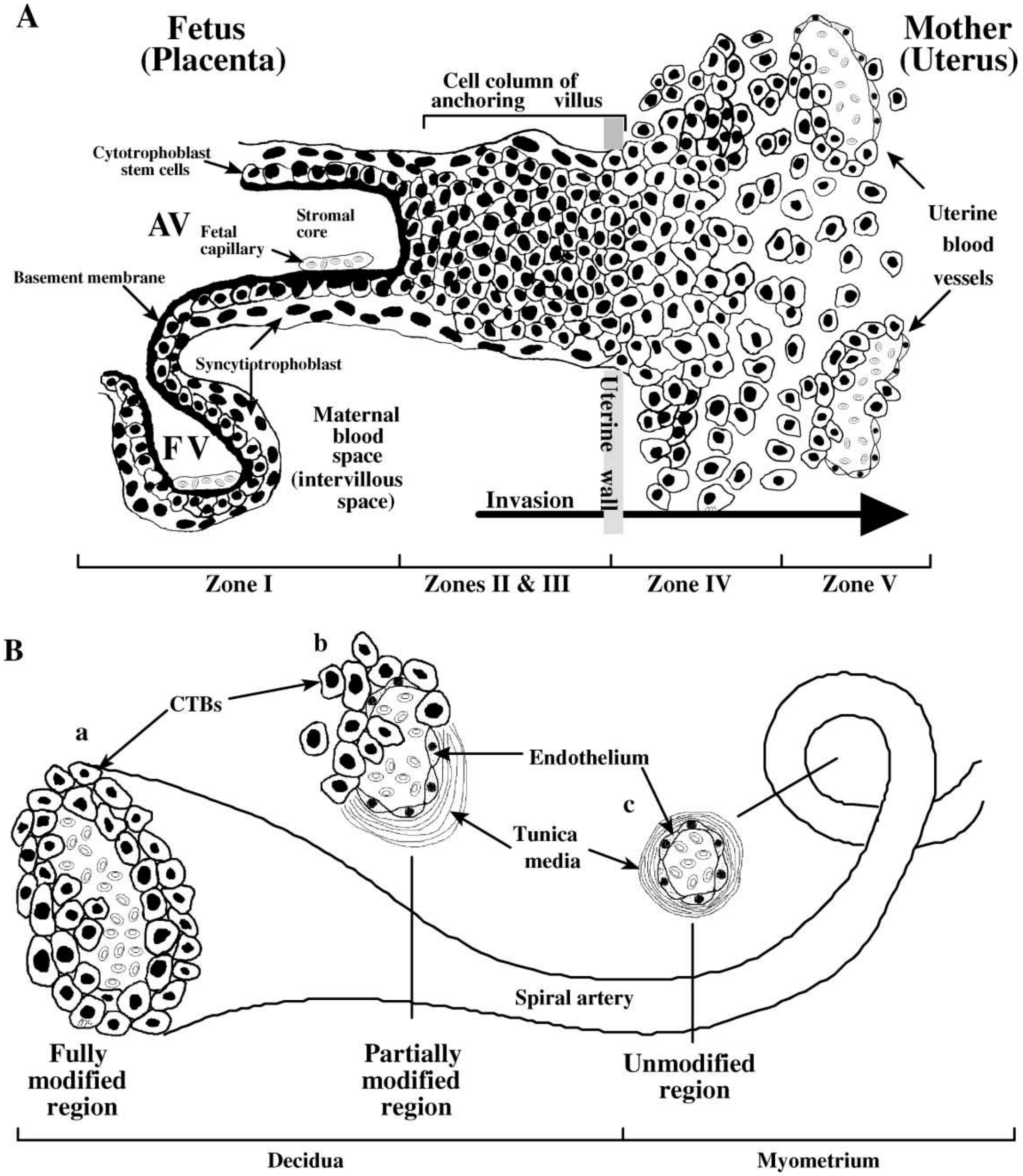
A
Fetus (Placenta)
Mother (Uterus)
Cell column of anchoring villus
Cytotrophoblast stem cells
AV
Fetal capillary
Stromal core
Basement membrane
Syncytiotrophoblast
FV
Maternal blood space (intervillous space)
Uterine wall
Invasion
Uterine blood vessels
Zone I
Zones II & III
Zone IV
Zone V
B
CTBs
a
b
c
Endothelium
Tunica media
Spiral artery
Fully modified region
Partially modified region
Unmodified region
Decidua
Myometrium

FIGURE 1 (A) Diagram of a longitudinal section of an anchoring chorionic villus (AV) at the fetal–maternal interface at approximately 10 weeks of gestational age. AV functions as a bridge between the fetal and the maternal compartments, whereas floating villi (FV) are suspended in the intervillous space and are bathed by maternal blood. CTBs (cytotrophoblasts) in the AV (Zone I) form cell columns (Zones II and III). CTBs then invade the uterine interstitium (decidua and first third of the myometrium: Zone IV) and maternal vasculature (Zone V), thereby anchoring the fetus to the mother and accessing the maternal circulation. Zone designations mark areas in which CTBs have distinct patterns of adhesion molecule expression. (B) Diagram of a uterine (spiral) artery in which endovascular invasion is in progress (10–18 weeks of gestation). Endometrial and then myometrial segments of spiral arteries are modified progressively. In fully modified regions (a), the vessel diameter is large. CTBs are present in the lumen and occupy the entire surface of the vessel wall. A discrete muscular layer (tunica media) is not evident. (b) Partially modified vessel segments. CTBs and maternal endothelium occupy discrete regions of the vessel wall. In areas of intersection, CTBs appear to lie deep in the endothelium and in contact with the vessel wall. (c) Unmodified vessel segments in the myometrium. Vessel segments in the superficial third of the myometrium will become modified when endovascular invasion reaches its fullest extent (approximately midgestation), whereas deeper segments of the same artery will retain their normal structure. Modified from Zhou *et al.* (1997), with permission.

receptors characteristic of endothelium—VE-cadherin, IgG family members vascular cellular adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) and integrins αVβ3 and α1β1. Accordingly, this phenomenon was termed pseudovasculogenesis. Other investigators showed that additional members of the cadherin family are also up-regulated on invasive CTBs and on decidualizing endometrial stroma. All CTBs in this pathway, regardless of location, stain for Mel-CAM, a melanoma-associated IgG family receptor also expressed on endothelium. Finally, CTBs within the maternal blood vessels turn on the neural cell adhesion molecule (CD56), an adhesion receptor that is also expressed by maternal natural killer cells that home to the pregnant uterus. Thus, as CTBs from anchoring villi invade and remodel the wall of the uterus, these epithelial cells of ectodermal origin acquire an adhesion receptor repertoire characteristic of endothelial cells. It is theorized that this switch permits the heterotypic adhesive interactions that allow fetal and maternal cells to cohabit the uterine vasculature during normal pregnancy.

Changes in the cytotrophoblast adhesion molecule repertoire take place in the context of the cells' equally dramatic modulation of their proteinase and proteinase inhibitor expression. Some aspects of this phenotypic transformation are undoubtedly linked to cytotrophoblast acquisition of an invasive phenotype. For example, expression and activation of matrix metalloproteinase-9 were found to be required for invasion *in vitro*. This observation fits well with recent observations, in other systems, that show that this same proteinase is a key regulator of angiogenesis. It is speculated that CTBs up-regulate the expression of other proteinases/inhibitors in order to present a nonthrombogenic surface to maternal blood. The urokinase-type plasminogen activator and plasminogen activator inhibitor-1, as well as the proteolytically activated thrombin receptor, might function in this manner.

The upstream regulatory factors that control these changes in proteinase and adhesion molecule phenotype include a variety of growth factors. In this regard, vascular endothelial growth factor (VEGF) family members, which are expressed at the maternal–fetal interface, are attractive candidates for regulating pseudovasculogenesis. Other factors, such as hepatocyte growth factor/scatter factor, can promote invasion by interacting with c-Met expressed on invading CTBs.

Finally, invading CTBs up-regulate the expression of other molecules that likely enable them to escape maternal immune rejection. Proteins expressed by trophoblasts that play a role in immunotolerance include human leukocyte antigen G (HLA-G), a unique major histocompatibility class Ib antigen with limited polymorphism, and interleukin-10, a potent immunosuppressive cytokine. HLA-G is expressed by invading CTBs that are in intimate contact with the decidua (which includes abundant maternal immune cells), suggesting a key juxtacrine regulatory role for this molecule. As shown in Fig. 2, HLA-G expression is a very sensitive marker of CTBs that have differentiated toward an invasive phenotype. Interstitial and endovascular CTBs express HLA-G, but syncytiotrophoblast and cytotrophoblast stem cells attached to the villus basement membranes do not. The regulated expression of these molecules by CTBs provides insights into how the placenta, a semiallograft, is able to develop in the context of a fully functional maternal immune system. However, the precise mechanisms whereby the placenta mediates maternal immune tolerance remain poorly understood.

## IV. REGULATION OF CYTOTROPHOBLAST ENDOVASCULAR INVASION

How can the knowledge of cytotrophoblast invasion be used to advance the understanding of the regulatory factors that govern placental development? One avenue that is being explored is the extent to which physiological parameters that are known to play important roles in the vasculature operate in the placenta. The first major effort in this direction led to the examination of the role of oxygen tension, one of the major factors at play in the vasculature. In this regard, it is interesting to consider that the placenta is the first organ to function during development. This hierarchy imposes novel requirements on cytotrophoblast growth and differentiation. For example, the critical early stages of placental development occur before the conceptus accesses a supply of maternal blood ($\leq 10$ weeks of gestation). In accord with this constraint, previous work showed that CTBs proliferate *in vitro* under hypoxic conditions that are comparable to those found during early pregnancy in the uterine lumen and the superficial decidua. As trophoblast invasion of the uterus proceeds, the placental cells encounter increasingly higher oxygen levels, which trigger their exit from the cell cycle and subsequent differentiation.

The discovery that CTBs acquire characteristics of vascular cells during endovascular invasion highlights

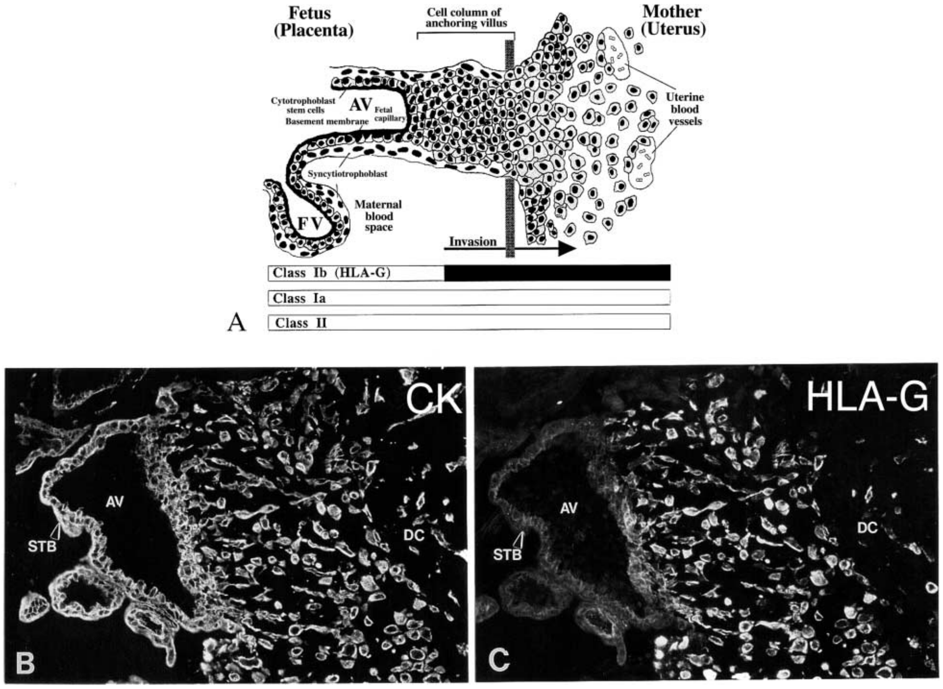

**FIGURE 2** Immunostaining of a section of the placental bed shows that HLA-G protein is produced by only invasive CTBs *in vivo*. (A) Summary diagram of the trophoblast populations in anchoring villi and their expression of major histocompatibility class I and II molecules. (B and C) Section of a 14-week placental bed biopsy double-labeled with rat monoclonal antibody 7D3 (anti-cytokeratin) and mouse monoclonal antibody 4H84 (anti-HLA-G). B shows cytokeratin staining, which labels all the trophoblast cells in the section. C shows HLA-G staining detected with fluorescein-conjugated antibodies. The section contains an anchoring villus (AV) with an associated cytotrophoblast cell column and decidua (DC). Note that the invasive CTBs stain strongly for HLA-G, whereas the syncytiotrophoblasts and cytotrophoblast stems cells do not. STB, syncytiotrophoblast.

the potential importance of factors that govern conventional vasculogenesis/angiogenesis in the regulation of placental development. Undoubtedly, this complex differentiation program is controlled by a hierarchy of regulatory factors. At a physiological level, it is already known that oxygen tension, which has profound effects on blood vessels, controls the switch between cytotrophoblast proliferation and differentiation. In these experiments, control anchoring villus explants (6–8 weeks of gestation) were maintained in either a 20 or an 8% $O_2$ atmosphere, mimicking standard culture conditions and the environment within the uterine interstitium (Fig. 1A, Zone IV), respectively. Other villi were cultured in 2% $O_2$, mimicking the hypoxic conditions in the fetal compartment near the uterine lumen at this time (Fig. 1A, Zone I; intervillous space). In 20 and 8% $O_2$, CTBs exit the cell cycle, up-regulate $\alpha1\beta1$, and become highly invasive. In hypoxia, CTBs continue to proliferate, but they fail to express $\alpha1\beta1$ integrin and do not invade. These observations suggest the following model. Before CTBs access the maternal blood supply (e.g., at 10 weeks of gestation), the hypoxic environment near the uterine lumen in which early placental development occurs favors cytotrophoblast proliferation, a phenomenon observed *in situ*. As interstitial invasion proceeds, invasive CTBs encounter a positive oxygen gradient that favors differentiation/invasion. Interestingly, hypoxia *in vitro* mimics some of the effects seen in preeclampsia, suggesting the possible consequences of failed endovascular invasion *in vivo*.

It can be envisioned that oxygen acts through a variety of downstream effectors that include molecular families known to play an important role in vasculogenesis/angiogenesis. In this regard, VEGF family members and their receptors are attractive candidates. There are several reports that CTBs, as well as fetal and maternal macrophages, express VEGF. CTBs also express placental growth factor (PlGF), a unique placental form of VEGF. Unlike VEGF, PlGF is not responsive to hypoxia; furthermore, its homodimers are not angiogenic and bind VEGF receptor-1 (Flt-1, Fms-like tyrosine kinase-1) but not VEGF receptor-2 (KDR, kinase insert domain-containing receptor). PlGF also forms heterodimers with VEGF that bind both receptors. VEGF up-regulates the expression of $\alpha 1\beta 1$ and $\alpha V\beta 3$ integrins in endothelial cells. These are the same proinvasive integrins that are up-regulated during cytotrophoblast differentiation, tempting speculation that VEGF family factors play analogous roles in invasive CTBs and angiogenic endothelial cells, a hypothesis that was recently proved. CTBs also express Flt-1 and KDR. Other factors and their cognate receptors that regulate vasculogenesis/angiogenesis, including fibroblast growth factors/receptors, TIEs/angiopoietins, and thrombospondins, are also expressed in the placenta.

Given this complexity, it is likely that the regulated balance of pro- and anti-angiogenic mechanisms is critical to coordinating the development of the hybrid vascular structures, composed of CTBs and maternal cells, that control blood flow to the fetus. Placental development actually requires the coordination of three different vasculogenesis/angiogenesis-like programs. First, fetal blood vessels develop in the stroma of floating chorionic villi (see Fig. 1A, fetal capillary). This process probably employs a vasculogenesis/angiogenesis program similar to that taking place within the embryo proper. Second, differentiating/invading CTBs undergo pseudovasculogenesis, the differentiation program that allows them to take on an endothelial adhesion phenotype (Fig. 1A, Zones II–V). Third, endovascular invasion by CTBs occurs without triggering significant angiogenesis in the maternal vessels, which suggests that CTBs also have novel paracrine mechanisms for rendering quiescent the resident uterine vasculature that they remodel (Fig. 1A, Zone V, uterine blood vessels). How are these different processes coordinated within the same microenvironment? The novel requirements of cytotrophoblast endovascular invasion suggest that known regulatory molecules may have novel actions; alternatively, novel regulatory molecules may control the behavior of these unusual cells.

The importance of trophoblast differentiation to embryonic/fetal development is also revealed by the numerous types of genetically engineered mice with placental defects. In the context of normal and abnormal human cytotrophoblast differentiation/invasion, some of the most interesting molecules implicated in knockout mouse experiments include hepatocyte growth factor/scatter factor and its c-Met receptor, VCAM-1 and the integrin $\alpha 4$ subunit, and the hypoxia-inducible transcription factor HIF-$1\beta$ (ARNT). Although the discovery that these and other molecules, e.g., Mash-2, Ets-2, Wnt2, and interleukin-11 receptor, play important roles in placentation has for the most part been unexpected, the trophoblast phenotypes of genetically engineered mice, which like humans form a hemochorial placenta, will continue to be extremely informative regarding mechanisms of human placental development and cytotrophoblast pseudovasculogenesis.

In summary, recent advances have uncovered the unexpected finding that specialized fetal cells of the human placenta—CTBs—undergo a novel pseudovasculogenesis differentiation program that enables them to masquerade as the endothelial and smooth muscle components of maternal uterine vessels. Discovery of the regulatory mechanisms that govern this unusual transformation will offer fascinating insights into how the placenta forms. Will the results of these studies be applicable to other normal and abnormal processes that require similar sorts of plasticity? One interesting possibility is that tumor cells, which like CTBs are marauders in search of a blood supply, might co-opt portions of the differentiation program described here. Whether lessons learned from placental development could be used to develop additional anti-angiogenesis cancer therapies, analogous to those described by Folkman and co-workers, remains to be investigated.

## Glossary

**cytotrophoblast stem cells** Mononuclear trophoblast precursors to invasive cytotrophoblasts and syncytiotrophoblasts.

**decidua** Stromal compartment of the endometrium during pregnancy.

**invasive cytotrophoblasts** Mononuclear trophoblasts that migrate into the uterine interstitium and vasculature.

**syncytiotrophoblasts** Multinucleate trophoblasts that line the surface of floating villi.

**trophoblast** Type of epithelial cell that forms the maternal–fetal interface.

## See Also the Following Articles

**Angiogenesis • Brain-Derived Neurotropic Factor • Decidualization • Insulin-like Growth Factor (Igf) Signaling • Oxytocin • Placental Gene Expression • Placental Immunology • Uterine Contractility**

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# Placental Gene Expression

AMRITA KAMAT AND CAROLE R. MENDELSON
*University of Texas Southwestern Medical Center*

I. INTRODUCTION
II. AROMATASE
III. HUMAN PLACENTAL LACTOGEN
IV. CONCLUSIONS

**The human placenta is composed of a core of proliferating mononuclear cytotrophoblasts; these trophoblast cells differentiate to form the syncytiotrophoblast layer, which covers the placental villi and has numerous secretory and transport/processing functions. It is this differentiation of cytotrophoblasts to syncytiotrophoblast that generates a cascade of regulatory signals that result in the production of various polypeptide hormones, growth factors, steroid hormones, and steroid-metabolizing enzymes. Cell culture techniques and transgenic technology are used to elucidate molecular events that promote and maintain syncytiotrophoblast differentiation and culminate in expression of different placenta-specific proteins factors.**

## I. INTRODUCTION

Maintenance of pregnancy and normal growth and development of the fetus are critically dependent on a biomolecular fetal–maternal communication, which is accomplished by the placenta. The placenta is, therefore, a critical organ, not only as the means of exchange of gases, nutrients, and waste products between the fetus and the mother, but also as an important source of hormones, growth factors, and other molecules that maintain uterine quiescence, stimulate fetal growth and development, and contribute to immune privilege. Placental molecules and growth factors include pregnancy-associated hormones (corticotropin-releasing hormone, progesterone, estrogen, and members of the growth hormone/prolactin gene family), enzymes (e.g., aromatase and side chain cleavage enzyme), leptin, interferons, angiogenic growth factors, cell adhesion molecules, and extracellular matrix metalloproteinases. These different molecules play varied but important roles in pregnancy; thus alterations in their expression underlie early pregnancy loss and complications.

In this article, we review work that has elucidated transcriptional regulation of two important human placental molecules, placental lactogen (chorionic somatomammotropin) and the enzyme aromatase, which catalyzes conversion of $C_{19}$ steroids to estrogen.

## II. AROMATASE

As normal pregnancy progresses, there is a gradual increase in plasma levels of estrogens and progesterone, which are synthesized primarily by the placenta. Near term in humans, there are extraordinarily high circulating levels of estrogen and progesterone, which decline abruptly with the delivery of the fetus and placenta. Thus, the human placenta, like the placentas of a number of ungulates, including cows, pigs, and horses, is able to synthesize estrogens, a property that is not shared by the placentas of rodent species, such as rats and mice. The physiological significance of the high levels of estrogen production by the human placenta is unclear at this time. However, it is likely that the human placenta has acquired the capacity to synthesize estrogens, in part, to metabolize the high levels of circulating androgens derived from the fetal adrenals, thus preventing their virilizing effects.

The human placenta lacks the enzyme 17α-hydroxylase, thus progesterone cannot be used as a substrate for estrogen biosynthesis. Instead, the high levels of estrogen produced in the human placenta are derived primarily from the $C_{19}$ steroid, 16α-hydroxyandrostenedione, which is secreted by the fetal adrenal. Aromatase P450 is the enzyme responsible for catalyzing the biosynthesis of $C_{18}$ estrogens (17β-estradiol, estrone, and estriol) from $C_{19}$ steroids. Aromatase P450 is expressed exclusively in estrogen-producing cells and is a product of the *CYP19* gene. Homozygous mutations of the single-copy human gene (h*CYP19*) result in virilization of the female fetus *in utero*, conferring subsequent primary amenorrhea.

The h*CYP19* gene spans ~130 kb in the human genome (Fig. 1). It is postulated that expression of h*CYP19* in various estrogen-producing tissues, including the gonads, brain, adipose tissue, and placenta, is driven by tissue-specific promoters that lie upstream of unique first exons, although the aromatase protein synthesized in each of these tissues is identical. Thus, the start site of transcription in placenta (in exon I.1) lies ~100,000 bp upstream of the start site of translation in exon II, whereas the ovary-specific first exon lies immediately upstream of exon II.

Primary cultures of human trophoblast cells transfected with various gene constructs have been used to create functional maps of sequences required for placenta-specific h*CYP19* gene expression. Mononuclear cytotrophoblast cells, which do not express aromatase, are isolated from the midterm placenta. When these cells are placed in culture, they fuse and differentiate to form a multinuclear syncytiotrophoblast. These morphological changes are associated with a marked induction of h*CYP19* gene expression. This primary cell culture system thus provides a physiologically relevant model to study molecular mechanisms in the regulation of h*CYP19* gene expression. Studies using this culture system suggest that 501 bp of exon I.1 5′ flanking DNA is sufficient for trophoblast-specific expression of h*CYP19* gene and that sequences between −501 and −246 bp contain silencer elements that may bind inhibitory transcription factors in nonplacental cells. Findings from deletion mapping analysis, site-directed mutagenesis, and electrophoretic mobility-shift assays also indicate that two overlapping hexameric sequences (AGGTCA, −183 to −191 bp), which may bind members of the nuclear receptor superfamily, and a G/C-rich sequence (−233 bp), which binds Sp1 and other as yet unidentified transcription factors, may contribute to the high levels h*CYP19I.1* promoter activity during syncytiotrophoblast differentiation.

In addition to primary cultures of placental cells, choriocarcinoma cell lines such as BeWo and JEG-3 have been used to study regulation of h*CYP19* gene expression. Using transfected JEG-3 choriocarcinoma cells, it has been observed that the proximal 301 bp upstream of exon I.1 is capable of conferring placental cell-specific h*CYP19* gene expression. Within this region, an element has been identified that is capable of binding a “glial cells missing” motif protein, the expression of which is restricted to the placenta. Other studies using BeWo choriocarcinoma cells have also localized a cell-type specific enhancer element between −242 and −166 bp relative to the transcriptional start site in exon I.1, which may play an important role in sustaining high levels of h*CYP19* expression.

In addition to studies in primary cultures of placental cells and choriocarcinoma cell lines, transgenic mice have been used to map h*CYP19* promoter regions required for appropriate tissue-specific and developmental regulation of gene expression. Initially, it was uncertain whether the mouse would serve as an appropriate model, because its placenta

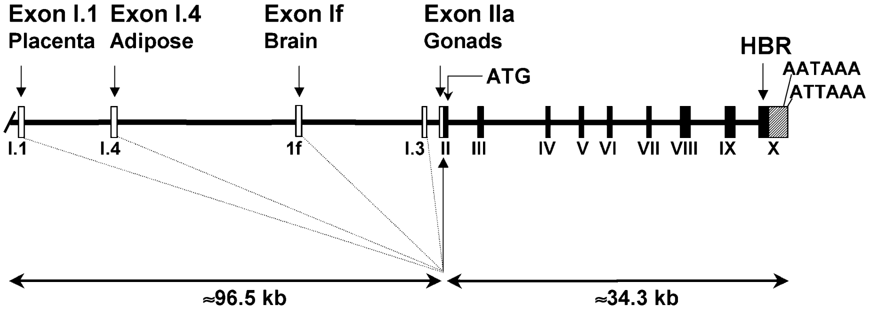

FIGURE 1 Schematic representation of the human *CYP19* gene and its alternative first exons. The protein coding sequences of the human *CYP19* gene, exons II–X (solid bars), and their introns comprise a region of ~34 kb. The heme-binding region (HBR) is in exon X, as are two alternative polyadenylation signals. The untranslated exons IIa, I.3/I.4, If, and I.1 (open bars) encoding the 5′ ends of the aromatase P450 mRNAs in the gonads, adipose, brain, and placenta, respectively, encompass a region of ~100 kb. These tissue-specific first exons are alternatively spliced onto a common site (shown by the septum) just upstream of the ATG codon in exon II. Based on the Celera Human Genome database (hcg 39857 CYP19GA_x2HTBL5L7WT), the human *CYP19* gene lies on chromosome 15, spanning q14–q15. Reproduced from Kamat *et al.* (2002), with permission from Elsevier Science.

does not express the endogenous *cyp19* gene. It was not known if the lack of estrogen synthesis in the mouse placenta was due to an absence of essential gene regulatory elements or of critical transcription factors. However, expression of h$CYP19I.1_{-2400}$:*hGH* (human growth hormone) or h$CYP19I.1_{-501}$:*hGH* fusion genes in transgenic mice was placenta-specific and developmentally regulated. Thus, just 500 bp of DNA, ~100 kb upstream of the coding region of the human *CYP19* gene, is sufficient to mediate placenta-specific expression in transgenic mice. These results clearly indicate that the transcription factors required to activate the human *CYP19* placenta-specific promoter are conserved between human and mouse genes, but that the mouse lacks the critical cis-acting elements required for expression of its endogenous *cyp19* gene.

To localize h$CYP19I.1_{-501}$:*hGH* fusion gene expression within the mouse placenta, *in situ* hybridization was performed using an antisense hGH probe. The main fetal components of the mouse placenta include the trophoblast giant cells, the spongiotrophoblast, and the labyrinthine trophoblast layer. Many of the placental polypeptide hormones and steroidogenic enzymes are expressed in the spongiotrophoblast and the trophoblast giant cells; however, the transgene is not expressed in these cells. On the other hand, the transgene is expressed as early as embryonic day 10.5 (E10.5) specifically in the mouse labyrinthine trophoblast layer (Fig. 2). This trilaminar layer is highly vascularized; its outer cellular layer, which covers two inner layers of syncytial cells, is bathed in maternal blood and thus plays an important role in nutrient and gas exchange. Therefore, the labyrinthine layer of the mouse placenta appears to be analogous to the human syncytiotrophoblast, which expresses aromatase and also is bathed in maternal blood. Thus, studies using trophoblast cells in culture and transgenic mice indicate that the proximal 501 bp flanking the 5′ end of exon I.1 of the h*CYP19* gene is sufficient for placenta-specific expression.

## III. HUMAN PLACENTAL LACTOGEN

Placental lactogens (PLs), or chorionic somatomammotropins, play an important role in the regulation of maternal and fetal metabolism and in the growth and development of the fetus. Human *PL* belongs to the growth hormone (*GH*) gene family, which includes the growth hormone gene (*GH-N*) and four growth hormone gene variants (*GH-V*, *PL-A*, *PL-B*, and *PL-L*). This five-gene family cluster shares greater than 90% sequence identity and has evolved by gene duplication. However, although *GH-N* is predominantly expressed in the pituitary somatotrophs, the other four genes are expressed selectively in the syncytiotrophoblast layer of the placenta. In addition to the major transcripts encoding the proteins, multiple alternatively spliced products for *GH-V*, *PL-A*, *PL-B,* and *PL-L* genes have been observed in the placenta.

Although members of the h*GH*/*PL* gene family share nearly 91–99% sequence identities throughout their coding regions and within a 500-bp 5′ flanking region just proximal to the transcription start sites, studies conducted over the years indicate that the critical trans-acting factors and the cis-acting

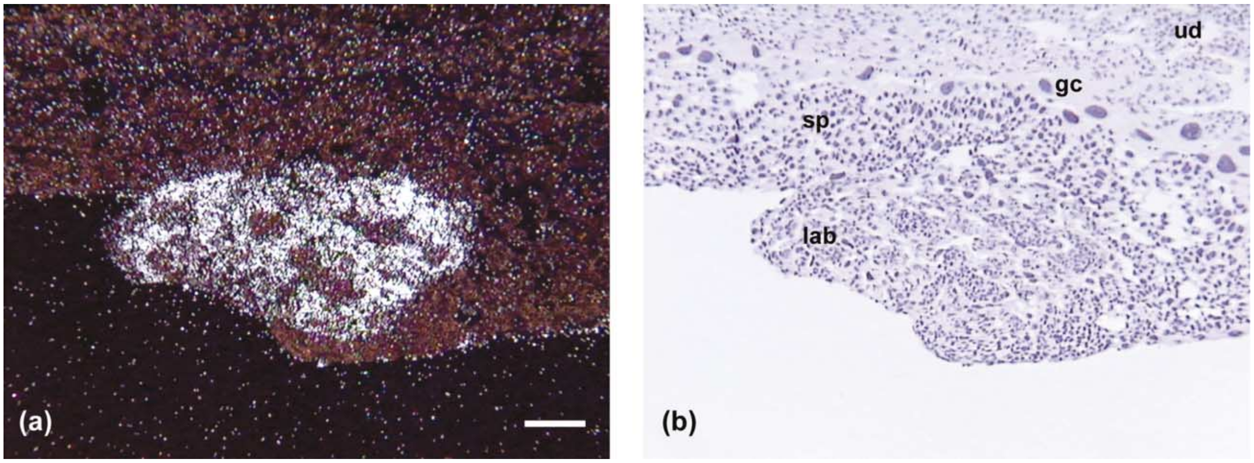

**FIGURE 2** Labyrinthine-specific expression of a *CYP19I.1*$_{-501}$:*hGH* fusion gene in transgenic mice. Placental tissue obtained from an E10.5 fetal transgenic mouse carrying the *CYP19I.1*$_{-501}$:*hGH* fusion gene was processed for *in situ* hybridization using a $^{35}$S-labeled antisense hGH cRNA probe and then exposed to photographic emulsion for 1–2 weeks. (a) Darkfield and (b) brightfield micrographs reveal trophoblast giant cells (gc), spongiotrophoblast (sp), labyrinthine trophoblast (lab), and the uterine decidua (ud). Reproduced from Kamat, A., Graves, K. H., Smith, M. E., Richardson, J. A., and Mendelson, C. R. (1999), A 500-bp region, approximately 40 kb upstream of the human CYP19 (aromatase) gene, mediates placenta-specific expression in transgenic mice, *Proc. Natl. Acad. Sci. USA* **96**, 4575–4580, with permission from the National Academy of Science, USA.

elements necessary for placenta-specific expression of *PL* are distinct from those that regulate the expression of h*GH* in the pituitary. Work done mainly in choriocarcinoma cell lines indicates that a ubiquitous Sp1 site, two activator protein-2 (AP-2) sites, and a trophoblast-specific initiator element (InrE) located within the 5′ flanking region are important for the regulation of *PL-A* gene expression. The Sp1 site, although found to be necessary, is not sufficient for maximal basal and enhancer-mediated transcription. However, a 70-kDa InrE-binding protein expressed in human choriocarcinoma cell lines BeWo and JEG-3 is observed to be required for maximal enhancer function and cell-specific h*PL* gene expression. Results also suggest that the InrE elements are required for accurate transcription initiation from the PL promoter in these cells. Expression of h*PL* in the placenta is also regulated by PL enhancers, CSEn1, CSEn2, and CSEn5, located downstream, or 3′, of the *PL-A*, *PL-B*, and *PL-L* genes, respectively. These enhancers are composed of multiple enhansons that work cooperatively to mediate maximal enhancer activity. Work done to characterize the enhancer CSEn2 indicates that several DNA–protein interactions occur within this region. Chorionic somatomammotropin gene enhancer factor-1 (CSEF-1), a 30-kDa protein found in BeWo cells, has been suggested to act through CSEn2 to enhance transcription. Transcriptional enhancer factor-1 (TEF-1) also binds to this element with the same affinity as CSEF-1 but reduces activation of the h*PL* promoter by inhibiting preinitiation complex formation. On the other hand, another member of the TEF family, TEF-5, binds to a unique element in CSEn2 and transactivates the enhancer. Further studies to characterize placenta-specific expression of *PL* genes have also indicated that there are mechanisms to suppress h*PL* gene expression in the pituitary. It has been demonstrated that CSEn2 in conjunction with either CSEn1 or CSEn5 forms a composite silencer in pituitary cells. Additionally, two orientation-dependent repressor elements (PSF-A and PSA-B) that exist in the 5′ flanking region of each of the *PL* and *GH-V* genes, but not in that of the pituitary *GH-N* gene, have been reported to bind pituitary nuclear factors that inhibit transactivation of the placental members of the growth hormone family in the pituitary. Thus, these various *in vitro* studies using both cultured placental cells and choriocarcinoma cell lines have indicated that placenta-specific transcriptional control of the h*PL* gene involves both positive and negative regulation.

Studies have also been conducted to ascertain if the critical cis-acting elements, as defined in cell transfection assays, are able to direct appropriate placenta-specific expression in transgenic mice. In initial studies, a 15-kb transgene fragment containing the *PL-A* gene with 5.4 kb of 5′ flanking sequence and

7.2 kb of 3′ flanking sequence was found either not to be expressed or to be expressed at low and variable levels in the mouse placenta. An 87-kb transgene was then created; it contained the majority of the h*GH* gene cluster linked to the entire locus control region, located − 15 to − 32 kb upstream of the hGH cluster, and was used to determine the expression pattern of the human genes as they exist in the native configuration of their family cluster. In the five transgenic mouse lines that were created, all of the h*GH* cluster genes were appropriately expressed, with h*GH-N* being specifically expressed at relatively high levels in the pituitary and the h*PL* genes being expressed specifically in the placenta. In contrast to the human genome, the mouse genome contains a single pituitary-specific *GH* gene and lacks any *GH*-related *PL* genes. Nonetheless, it is apparent that the mouse placenta has the transcription factors necessary to mediate placenta-specific expression of the human *PL*-containing transgene. Further *in situ* studies have indicated that expression of the h*PL-A*-containing transgene is localized to the labyrinth of the mouse placenta, which, as previously mentioned, is functionally analogous to the syncytiotrophoblast.

## IV. CONCLUSIONS

Aromatase and the placental lactogens participate in important placental functions in humans. Whereas aromatase is necessary for the conversion of $C_{19}$ steroids to estrogens, and lack of aromatase expression results in virilization of the fetus, the placental lactogens alter maternal carbohydrate and lipid metabolism to provide for fetal nutrient requirements. However, the biological functions of the products of both of these genes are not fully understood, and individuals with mutations in placental genes for *CYP19* or *PL* genes have had normal pregnancies. Studies on regulation of the h*CYP19* or h*PL* gene have thus far indicated that both negative and positive tissue-specific transcription factors are involved in regulation of the placenta-specific gene expression. Additionally, although the mouse placenta does not express aromatase or PLs, the h*CYP19* and h*PL* transgenes are specifically expressed in the labyrinthine layer of the mouse placenta, which is functionally analogous to the syncytiotrophoblast of the human placenta, where these genes are normally expressed. This suggests that specific cells within the labyrinthine layer of mouse placenta express the appropriate transcription factors required to activate the promoters of the h*CYP19* and h*PL* genes. Future studies in placental cells and in transgenic animals will likely contribute to our understanding of the positive and negative regulatory elements involved in tissue-specific regulation of aromatase and PL, and to the role that these two important molecules play in human placenta.

## Glossary

**enhancers** DNA sequences that increase the rate of gene transcription from a distance, irrespective of their orientation relative to the transcription start site in the gene.

**enhansons** Subunits of enhancers; some enhancers are composed of separate 15- to 20-bp elements that cooperate with one another to enhance transcription. These elements are made up of enhansons, which can be duplicated or interchanged to create new enhancer elements. Unlike enhancers, enhansons are sensitive to changes in spacing.

**labyrinthine zone** Murine placental complex of trophoblast, mesoderm, and vascular derivatives; functionally analogous to the floating chorionic villi in humans.

**spongiotrophoblast** Intermediate layer in the murine placenta that, like the trophoblast giant cells, arises from the ectoplacental cone; with the giant cell layer, separates the labyrinthine zone from the maternal decidua.

**trans-acting factors** Transcription factors (specific proteins) that bind to relatively short DNA sequence motifs, or cis-acting elements, to regulate gene transcription. The cis-acting elements can occur in various locations and at different distances and directions relative to the transcriptional start and stop sites within the gene.

**trophoblast cells** Progenitor cells, important in the formation of the placenta and responsible for various functions, including nutrition of the differentiating embryo and the growing fetus and secretion of hormones indispensable for the maintenance of pregnancy.

**trophoblast giant cells** Polyploid mouse trophoblast cells that surround the conceptus and lie in direct contact with the maternal decidua.

## See Also the Following Articles

**Aromatase and Estrogen Insufficiency • Knockout of Gonadotropins and Their Receptor Genes • Oocyte Development and Maturation • Ovulation • Oxytocin • Placental Development • Placental Immunology • Prolactin and Growth Hormone Receptors**

## Further Reading

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# Placental Immunology

Joan S. Hunt and Margaret G. Petroff
*University of Kansas Medical Center*

I. INTRODUCTION
II. PLACENTAL REGULATION OF HLA
III. INHIBITORY MOLECULES ON TROPHOBLASTS
IV. UTERINE LEUKOCYTE MODIFICATIONS
V. SUMMARY

**Placental immunology may be defined as the study of structural and functional features of the placenta that permit semiallogeneic pregnancy to proceed despite genetic differences between the mother and the fetus. Ordinarily, the presence of "foreign" tissue would stimulate a graft rejection response as is the case in transplantation of organs. In this article, the unique aspects of immune protection at the maternal–fetal interface are presented.**

## I. INTRODUCTION

In the middle of the past century, immunologists were just beginning to understand the design of the immune system. One general principle emerged; in healthy adults, the immune system is immensely efficient in repelling the invasion of foreign DNA and RNA.

A major enigma is presented by the state of human pregnancy, in which genetically different tissues reside side by side in apparent harmony. In 1953, P. Medawar was the first to offer ideas for mechanisms that could account for the surprising ability of the fetal semiallograft to survive in a potentially hostile environment. His suggestions included (1) anatomic separation of the mother and fetus; (2) antigenic immaturity of the fetus; and (3) tolerance in the mother. Although somewhat different from Medawar's original ideas, all these mechanisms have now been identified. The blood circulations of the mother and fetus are entirely separate, the major fetal cell surface molecules that stimulate graft rejection appear late, and mothers develop multiple tolerogenic mechanisms.

One of the most impressive features of immunological protection, which may be termed "immune privilege," is the wide range of devices used in maternal endometrium and fetal placenta to ensure tolerance. In humans, these may include (1) tight control over the expression of human leukocyte antigen (HLA) expression by cells in human placentas; (2) production of immunosuppressive substances such as prostaglandin E2, progesterone, and T-helper 2 ($T_H2$)-type cytokines at the maternal–fetal interface; (3) placental cell expression of the apoptosis-inducing cytokines, Fas ligand (CD95 ligand) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Apo-2 ligand), as well as proteins that inhibit the complement cascade and those that modulate leukocyte proliferation and cytokine secretion (B7-H1); and (4) restriction of migration of antigen-specific T and B lymphocytes into the decidua. Each of these mechanisms is of great interest and will be discussed briefly in this article.

## II. PLACENTAL REGULATION OF HLA

### A. HLA Class I Antigens

Both beneficial and potentially detrimental effects are exerted by the functionally unique HLA molecules. These cell surface structures are required for host protection against infectious agents but when they are perceived as foreign by the host, as is likely to happen with paternally derived antigens in human pregnancy, they may stimulate graft rejection.

The major histocompatibility complex (MHC) located on the short arm of chromosome 6 encodes the HLA class I antigens. The proteins comprise three ~100 amino acid "domains" with disulfide bonds looping the domains. HLA class I antigens usually have one transmembrane heavy chain (37 to 45 kDa) associated noncovalently with a light chain (β2-microglobulin, ~12 kDa). HLA class I glycoproteins

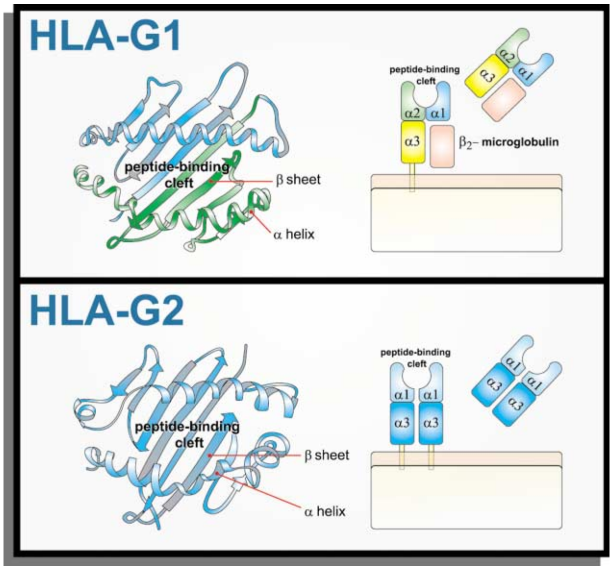

**FIGURE 1** Schematic representations of (left) the peptide-binding cleft in HLA-G1 and HLA-G2, as viewed from the top down, and (right) the likely structures of membrane-bound and soluble HLA-G1 and HLA-G2. Note that HLA-G1 is composed of a heavy chain and a light chain (β2-microglobulin), whereas HLA-G2 heavy chains form homodimers and do not bind β2-microglobulin.

carrying foreign peptides within a region called the peptide-binding groove (Fig. 1) are recognized by the T-cell receptor (TCR) on CD8$^+$ precursor cytotoxic T lymphocytes (CTL). The HLA-A, -B, and -C class Ia genes are highly polymorphic and their proteins are co-dominantly expressed on the cell membranes of essentially all eukaryotic cells, the exceptions being germ cells (oocytes, sperm) and placental trophoblast cells. Differences between class I antigens derived from each allele create a major barrier associated with transplantation of donor organs because immune cells recognize nonself alleles as foreign and mount a graft rejection response that includes cytotoxic cells and cytotoxic antibodies.

By contrast, the HLA-E, -F, and -G class Ib genes have few alleles and their proteins are restricted to certain types of cells. Figure 1 shows the probable antigen-binding clefts and stylized isoform structures of soluble HLA-G1 and soluble HLA-G2, two isoforms of HLA-G which are discussed below.

## B. HLA Class II (HLA-D) Antigens

The class II antigens have two transmembrane heavy chains, are also highly polymorphic, and also constitute barriers to transplantation. They are expressed primarily on antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes, but may be expressed on other cells during certain diseases. The class II antigens form homodimers rather than heterodimers. HLA class II (HLA-D) glycoproteins carrying foreign peptides in the homodimeric heavy chain cleft are recognized by TCRs on CD4$^+$ T-helper lymphocytes.

## C. HLA on Trophoblast Cells

The surprising success of semiallogeneic pregnancy may be attributed in large part to controlled expression of the HLA molecules in trophoblast cells. These cell surface structures are the main mediators of graft rejection, but HLA is so firmly regulated that there are no known instances of inappropriate expression in fetal tissues as a cause of pregnancy failure where maternal immune cells attack fetal tissues. Trophoblast cells may be subdivided into several subpopulations depending on their stage of differentiation and their anatomic location; regulation of their HLA proteins differs according to need.

## D. Trophoblast Subpopulations

Trophoblast cells are derived from the trophectoderm layer of the blastocyst. These cells are not uniform; they differentiate in a stage-specific manner into several subpopulations. The cytotrophoblast is the precursor cell and is driven into one of two pathways of differentiation. In one instance, cytotrophoblast cells coalesce into a multinucleated syncytium. This is an uninterrupted cell layer that, at term, would cover 9 m$^2$ if spread. The syncytiotrophoblast cell layer modulates bidirectional transport of nutrients and wastes, is responsible for most placental hormone production, and protects the inner cell mass-derived elements (the amnion and chorion membranes, the umbilical cord, and the embryo) from blood-borne maternal immune cells.

A second set of cytotrophoblast cells proliferates and emerges from the floating placental villi to form columns that ultimately contact the maternal decidua (Fig. 2). The extravillous trophoblastic cells invade the spiral arteries and replace the endothelial cells in the vessels. As pregnancy progresses, the extravillous cells cease migrating and regress to form the chorion membrane.

Unlike embryonic cells, trophoblast cells never express normal adult levels or patterns of HLA. As described below, specific HLA class I genes are

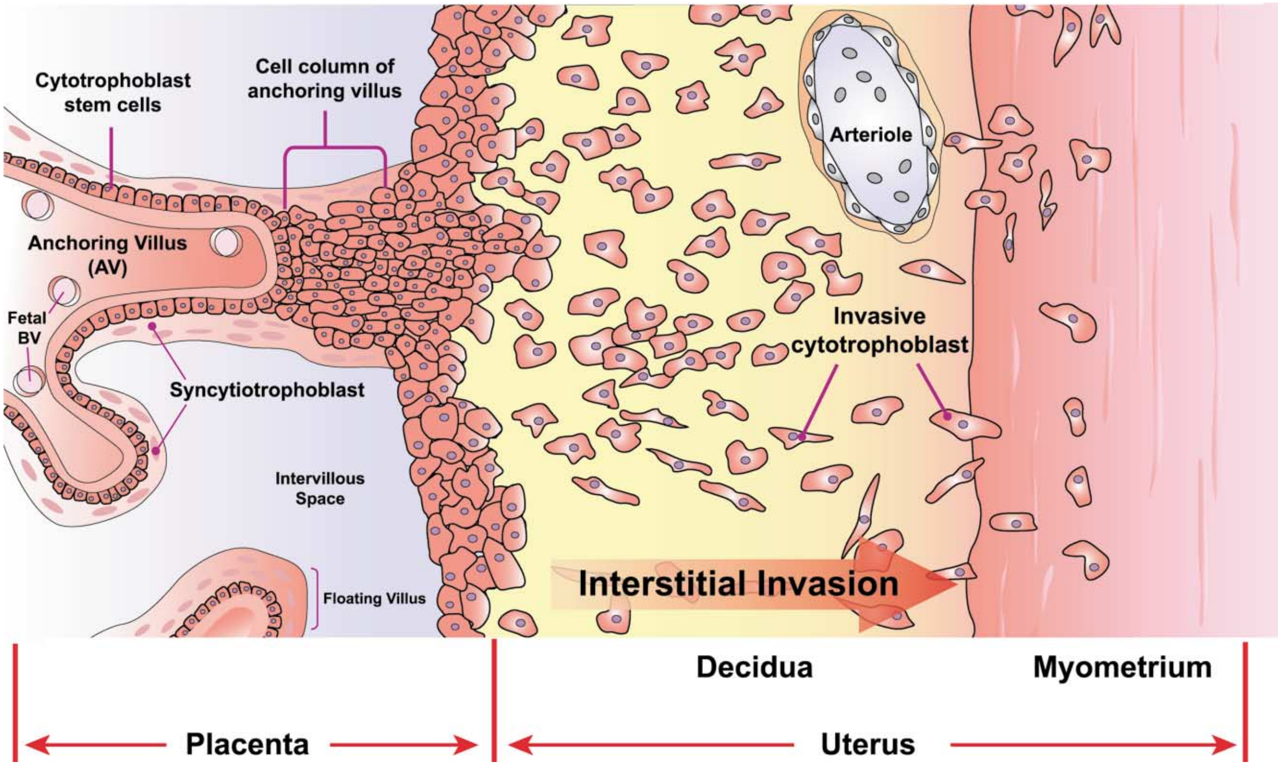

FIGURE 2 A representational drawing of the maternal–fetal interface at the point of contact. Some placental villi form cell columns. Cytotrophoblast cells invade the maternal uterine interstitium and may be found in the myometrium as well as lining maternal spiral arteries. Other villi float in a sea of maternal blood filling the intervillous space.

selected for expression from among the many members of this multigene family, whereas HLA class II genes are essentially unexpressed in trophoblasts. The absence of class II may be due to repressor elements on promoter and other elements in the class II gene sequences. More studies need to be performed to elucidate the nature of the restriction, which is effective even in the presence of class II inducers such as interferon-γ (IFN-γ) and type 1 interferons.

## E. HLA Class I in Trophoblasts

The exciting story of how placental expression of the HLA genes and the glycoproteins derived from these genes was revealed has been told frequently. In short, stage of gestation, state of differentiation, and anatomical location dictate expression in this unique cell lineage.

HLA class I message may be present but membrane-bound proteins are difficult to detect in early gestation syncytiotrophoblast and villous cytotrophoblast cells. In late gestation, neither subpopulation contains substantial class I mRNA or membrane-bound HLA class I protein. Although cytotrophoblast cells in early placentas that are proliferating in preparation for column formation are negative for both message and protein, the cytotrophoblast cells distal to the villus in the cytotrophoblastic shell are HLA class I positive. Class I positivity is retained in the chorion membrane cytotrophoblast cells, which are derived from the invasive extravillous cytotrophoblasts.

## F. HLA Class I Gene Selection

The ability to select specific members of the HLA class I multigene family for expression is a unique property of trophectoderm-derived cells. Three members of the HLA class I gene family are present in HLA class I positive trophoblasts; one is a class Ia gene, HLA-C, and two are class Ib genes, HLA-E and HLA-G. The highly polymorphic, widely distributed HLA-A and -B antigens are absent.

The function(s) of HLA-C remains unknown. Although the gene is polymorphic and it is clear that proteins derived from this gene are present on trophoblast cells, whether it has any influence on immune cells or the immunobiology of pregnancy is not known. Unlike other class Ia genes, expression is transient and at a comparatively low level. HLA-E is an interesting molecule; it uses the leader sequences of other class I proteins to gain access to the cell surface. Considerable experimentation has led to the idea that HLA-E interacts with natural killer (NK) cell inhibitory receptors and drives these cells into an anergic condition. The decidua in early and mid stages of pregnancy is packed with NK cells that would attack $HLA^{-}$ negative cells, so expression of a class I molecule is very important. HLA-G, which was the first HLA to be discovered on trophoblast cells, has been extensively studied. Much has been revealed about this unusual gene, as described in detail below, although the major functions of the spectrum of protein isoforms derived from this gene remain unclear.

To date, nothing is known of how these three genes are selected for expression in trophoblasts from among the members of the HLA class I multigene family. Their appearance appears to be synchronous, suggesting that whatever mechanism constitutes the "ON" signal influences the expression of all three genes.

## G. Immunogenetics of HLA-G

HLA-G is a gene exhibiting several unique features. Its cytoplasmic tail is shortened and may or may not transduce signals. Its promoter region contains a large deletion in the enhancer A/IFN consensus region, which results in crippling of IFN-induced transcription, and the γ-activated site has a single nucleotide substitution that prevents activation. As a consequence and unlike other HLA class I genes, HLA-G is only weakly enhanced by exposure to interferons.

Also in contrast to other HLA class I genes, alternative splicing is a feature of the HLA-G message. Mechanisms underlying the production of multiple transcripts from this gene are unknown. Importantly, some transcripts encode cell surface proteins and others encode soluble proteins. The latter is a consequence of a stop codon in intron 4, which precludes translation into the transmembrane region. All the transcripts are readily identified in placentas and trophoblast cell lines. Because the antibodies to HLA-G are poorly characterized regarding binding site and specificity, expression in adult cells and tissues remains uncertain.

## H. Functions of HLA-G

Functions clearly attributable to HLA-G are poorly defined at present. Because expression of this gene, which has few allelic variants, is a prominent feature of the maternal–fetal interface, its substitution for HLA-A or -B is widely believed to be critical to the maintenance of semiallogeneic pregnancy. The gene does express a few alleles, identified as $^{*}$0101, $^{*}$0103, $^{*}$0104, and $^{*}$0106, that might be immunostimulatory in mismatched couples, but it is not yet known whether mothers recognize the products of the different alleles.

Evidence has been presented for the postulate that HLA-G interacts with macrophage immunoglobulin-like transcript 4 (ILT4) receptors and conveys inhibitory signals to this cell lineage just as HLA-E does to NK cells. If this occurs, it could prevent activation of the macrophages in the decidua and subsequent killing of migrating trophoblastic cells. Presumably, these interactions would be cell–cell involvements and would rely on membrane-bound HLA-G on trophoblasts, but this does not preclude the utilization of soluble HLA-G, which is receiving considerable attention. It is important to remember that other suppressive substances such as progesterone and prostaglandins could have similar effects.

Soluble HLA-G is present in placentas and more recently has been demonstrated in the sera of pregnant women. These proteins could have profound effects on maternal immune cells. Evidence from studies employing soluble HLA-G1 partially purified from trophoblastic tumor cells indicates that this substance stimulates the apoptosis of lectin-activated T lymphocytes through the Fas/FasL pathway, a process that has been identified in immune cells exposed to other soluble HLA class I molecules. An immunoregulatory role for soluble HLA-G is indicated in the finding that levels are higher in mothers who have successful pregnancies following assisted reproductive technology as well as in heart transplant recipients who have less rejection. Furthermore, HLA-G transfected into pig islets increases graft acceptance.

Although it is possible that each of the isoforms derived from alternatively spliced messages has a different function, it seems more likely that there is at least some redundancy; pregnancy proceeds in the absence of HLA-G. The placentas of babies with a homozygous deletion that prevents synthesis of HLA-G1 protein contain other HLA-G isoforms; whether these alternative isoforms are the major players in the spectrum of HLA-G proteins or whether they are simply able to substitute for HLA-G1 remains to be ascertained.

## III. INHIBITORY MOLECULES ON TROPHOBLASTS

### A. Membrane-Bound Molecules

Since trophoblast subpopulations form the anatomical boundary between maternal blood and tissues and inner cell mass-derived fetal mesenchyme, it is not surprising to find that trophoblast cells display surface-bound proteins that actively participate in defending the fetus from potentially harmful components of the maternal immune system. Toxic entities could include maternal leukocytes as well as cytotoxic antibodies and complement. A number of protective entities on trophoblast membranes have been identified.

#### 1. *Tumor Necrosis Factor Superfamily Proteins*

Members of the TNF superfamily are emerging as potentially critical mediators of placental immune privilege. Essentially all the ligands may be either membrane-bound or soluble and the same is true of many of the receptors. FasL, which kills activated leukocytes through its receptor, Fas, is expressed discontinuously in villous and extravillous trophoblast cells throughout gestation. Trophoblasts have been shown to mediate apoptosis of leukocytes through the Fas/FasL system *in vitro*. Experiments in mice have shown that the ligand is highly important in protection of the placenta. In FasL-deficient mice, activated maternal leukocytes inappropriately infiltrate the maternal–fetal interface, and viability of the embryos is greatly reduced.

TRAIL is another member of the TNF superfamily that is likely to protect the fetus through expression on trophoblasts. This powerful inducer of apoptosis is incapable of causing death in trophoblasts, most likely because trophoblast cells express high levels of a TRAIL decoy receptor, DcR1. It is generally believed that in a similar fashion, protection against apoptosis by other toxic molecules is effected by receptor blocking. For example, soluble TNF receptor which compete with membrane-bound receptors, are abundant in placentas. FasL, TRAIL, and TNFα are not the only apoptosis-inducing superfamily members in placentas. Messenger RNA encoding other death-inducing ligands in first-trimester placentas include lymphotoxin-β, TNF-like weak inducer of apoptosis (TWEAK), and LIGHT (homologous to lymphotoxin), and term placentas contain these as well as lymphotoxin-α and 4-1B3 ligand (4-1BBL). The distribution of LIGHT protein has now been reported in term placentas, but proteins from most of the other newly identified members have yet to be evaluated systematically.

Importantly, the cytokines that were first identified in the immune system may have other critical functions in placentas such as governing developmental pathways through programmed cell death and perhaps also through effects on gene expression that do not lead to apoptosis. Nearly all the family members that have been examined to date stimulate a protective nuclear factor κB pathway in some cells under specific conditions.

#### 2. *Complement Regulatory Proteins*

Activation of the complement cascade leads to deposition of complement components on cellular surfaces and formation of lytic pore-forming units termed the membrane attack complexes. The complement system is intended to protect the host against bacteria, parasites, and virus-infected cells. However, damage to host cells can result if they are not protected by a series of complement regulatory proteins, which includes CD59, membrane co-factor protein (MCP, CD46), and decay accelerating factor (DAF, CD55). These complement regulatory proteins are broadly distributed in adult tissues. All three of these membrane-bound proteins are also highly expressed at the apical surface of the syncytiotrophoblast facing the maternal blood space as well by villous (CD59 and MCP) and extravillous (CD59 and DAF) cytotrophoblasts. The presence of these complement regulatory proteins is believed to confer resistance of trophoblasts to antibody-mediated cytotoxicity.

Murine Crry is a transmembrane glycoprotein that functionally resembles DAF and MCP. Crry is absolutely essential to pregnancy in mice. *Crry*$^{-/-}$ embryos fail to survive in normal mothers due to the spontaneous deposition of complement factor C3 in the developing placenta. Furthermore, *Crry*$^{-/-}$ embryos remain viable in mothers lacking C3. Activation of the complement system also leads to fetal loss in a murine model of anti-phospholipid syndrome, suggesting that a similar mechanism may occur in women suffering this disorder.

#### 3. *B7 Family Proteins*

Members of the B7 family of membrane glycoproteins are ligands that interact with receptors belonging to the CD28 family. Of the six known members of the B7 family, five are highly expressed in the human placenta. Expression of one of these, B7-H1 (also known as PD-L1), is relatively restricted, occurring mainly in macrophages, trophoblast cells, and cells constituting some organs. Consistent with the need for immunological protection of the allogeneic fetus, the B7-H1 receptor, PD-1, plays a major role in the maintenance of peripheral immunological tolerance. Mice lacking PD-1 develop severe autoimmune disorders that afflict organs whose cells express B7-H1 and its sister ligand, B7-DC (PD-L2). B7-H1 has been shown to inhibit CD3-activated T lymphocytes stimulate allograft survival, and protect tumor cells from T cell-mediated lysis. B7-H1 also alters their cytokine secretion patterns, possibly driving the T cells into the $T_H2$-type cytokine production profile that is thought to be important to pregnancy. In addition, B7-H1 induces apoptosis in activated, antigen-specific leukocytes, most likely through a receptor distinct from PD-1. The functional significance of placental B7 family members is not yet known, but their distribution within specific cellular elements of the human placenta indicates that this family of molecules plays an important role in the establishment and maintenance of maternal tolerance to the fetus.

### B. Soluble Molecules

Pregnancy is known to require adequate levels of progesterone for both implantation and maintenance. Early on, this hormone is synthesized in the corpus luteum of the ovary and subsequently in the placenta. Progesterone has many functions, one of which may be to discourage the proliferation and cytotoxicity of immune cells. As described below, the maternal–fetal interface is characterized by cells supplying the host (mother) with innate immunity rather than lymphocyte-specific acquired immunity. Progesterone may drive the substitution process, which occurs early following implantation. Furthermore, when exposed to high levels of progesterone, innate immune cells such as macrophages are programmed into a suppressive profile characterized by the absence of $T_H1$-type inflammatory cytokines (TNFα, IFN-γ) and production of $T_H2$-type anti-inflammatory cytokines [interleukin-10, transforming growth-β (TGF-β)] as well as the negative regulator of immune cells, prostaglandin E2.

Taken together, these elements probably account in large part for the well-described immunosuppressive environment of the pregnant uterus. However, researchers continue to identify additional protectors of the placenta; one recent entry is indolamine oxidase, an inhibitor of tryptophan metabolism.

## IV. UTERINE LEUKOCYTE MODIFICATIONS

Among the alterations that occur at the maternal–fetal interface, reassortment of leukocytes is one of the most profound. Although the cycling endometrium has lymphoid aggregates composed of T and B lymphocytes, immature NK cells, and randomly distributed macrophages, the antigen-specific T and B cells nearly disappear with the onset of implantation. Thus, the pregnant uterus is populated almost exclusively by cells supplying innate or natural immunity, the NK cells and macrophages. In the human decidua, the NK cells disappear in the second trimester, whereas the macrophages remain throughout pregnancy, providing a measure of host defense against infection.

It is clear that molecules present on the surfaces of migrating trophoblast cells and soluble products of the placenta target decidual immune cells, and there is evidence as well for reciprocal activity, with decidual leukocytes targeting placental cells. For the former, membrane-bound HLA-G and soluble HLA-G represent an excellent example. Decidual macrophages express inhibitory receptors for HLA-G, the ILT proteins termed ILT2 and ILT4. Although as yet untested in decidual macrophages, in other cells, ligand–ILT receptor interactions transduce inhibitory signals that reduce cellular cytotoxicity. Thus, membrane and soluble HLA-G could have a major impact on how leukocytes in the decidua are programmed for the benefit of pregnancy.

For the latter, experiments in gene-deleted mice strongly suggest that TNFα derived from decidual cells influences placental organization. Further, there is considerable information in mice on the interaction between uterine colony-stimulating factor-1 (CSF-1) and its receptor on trophoblast cells. CSF-1-stimulated trophoblast cells become phagocytic and then act as a component of the innate immune system at the maternal–fetal interface. Collectively, these types of studies have contributed to a better understanding of the unique utilization of immune system molecules to promote successful pregnancy.

## V. SUMMARY

More than four decades of research have brought reproductive immunologists to the understanding that the placental bed is a dynamic, ever-changing network of maternal–fetal interactions facilitated by a wide range of soluble and cell-surface molecules. Figure 3 illustrates some features of the bidirectional communication that facilitates successful pregnancy. Trophoblast cells secrete powerful soluble molecules such as prostaglandins and progesterone, soluble HLA-G, and immunomodulatory cytokines that include interleukins, members of the TNF supergene family, and members of the TGF-β gene family. Leukocytes are sources of both cytokines and prostaglandins. Membrane-bound molecules on trophoblast cells and leukocytes participate significantly in interactions in the decidua and may also modulate the actions of leukocytes circulating in the mother's blood. These include ILT, c-type lectin, and NK cell immunoglobulin-like receptors for HLA molecules, members of the B7 family of modulators, ligand and receptor proteins for the TNF and TGF-β gene families, and the critically protective complement regulatory proteins.

Identification of the many features of the maternal–fetal interface that circumvent the immune system designed for the destruction of cells carrying foreign DNA and/or RNA has provided the critical framework needed to understand pregnancy failures. Although experiments in women cannot be performed, new approaches to pinpointing the central players in the networks are being conducted in gene-modified mice as well as in nonhuman primates. Both rodent and primate experimental animal systems have attractive features and offer hope for demonstrating proofs of principle that will ultimately lead to new clinical therapies for problem pregnancies.

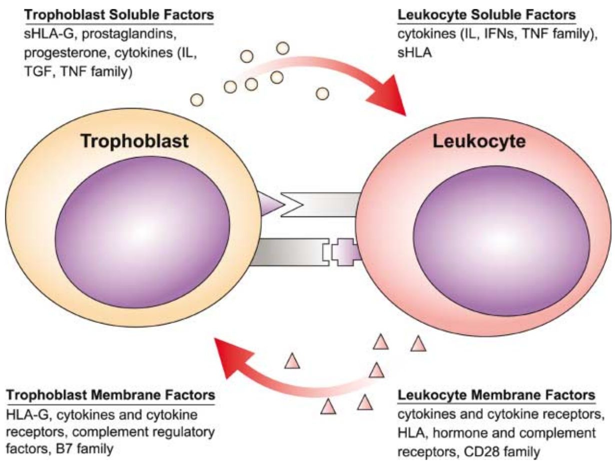
Trophoblast Soluble Factors
sHLA-G, prostaglandins, progesterone, cytokines (IL, TGF, TNF family)
Leukocyte Soluble Factors
cytokines (IL, IFNs, TNF family), sHLA
Trophoblast
Leukocyte
Trophoblast Membrane Factors
HLA-G, cytokines and cytokine receptors, complement regulatory factors, B7 family
Leukocyte Membrane Factors
cytokines and cytokine receptors, HLA, hormone and complement receptors, CD28 family

**FIGURE 3** Bidirectional interactions between trophoblast cells and leukocytes. Interactions include targeting of soluble molecules supplied by both types of cells as well as direct cell–cell contact and interaction via matching ligands and receptors. IFNs, interferons; IL, interleukins; sHLA-G, soluble HLA-G; TGF, transforming growth factor; TNF, tumor necrosis factor.

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## Glossary

| | |
|---|---|
| **B7 family molecules** | Cell surface molecules involved in both stimulation and inhibition of immune cells. |
| **complement** | The collective term for serum proteins that may cause lysis of cells. |
| **cytokines** | Molecules of 15 to 30 kDa that mediate cellular interactions. They may be either bound to the cell membrane or soluble and are often subdivided by their usual cell of origin, i.e., T-helper 1 ($T_H1$, pro-inflammatory) and $T_H2$ (anti-inflammatory) lymphocytes. |
| **cytotoxic T lymphocyte** | A type of cell that attacks foreign and infected cells. |
| **human leukocyte antigens** | Glycoproteins encoded by genes within the major histocompatibility complex on human chromosome 6. They are important self-recognition molecules in host defense but, when foreign, constitute targets for cytotoxic T lymphocytes and antibodies. |
| **leukocytes** | Bone marrow-derived cells involved in innate and immune host defense. |
| **macrophages** | Leukocytes that reside in tissues and have innate and immune host defense functions. |
| **major histocompatibility complex** | Genes that encode the human leukocyte antigen class I and class II proteins. |
| **natural killer cells** | Non-antigen-specific leukocytes that thickly populate the early to mid gestation pregnant uterus. |

## See Also the Following Articles

**Apoptosis • Decidualization • Implantation • Placental Development • Placental Gene Expression • Tumor Necrosis Factor (TNF)**

## Further Reading

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# Platelet-Derived Growth Factor (PDGF)

CARL-HENRIK HELDIN
*Ludwig Institute for Cancer Research, Uppsala, Sweden*

I. INTRODUCTION
II. PDGF ISOFORMS
III. PDGF RECEPTORS
IV. FUNCTION OF PDGF *IN VIVO*
V. PDGF IN DISEASE
VI. SUMMARY

**Platelet-derived growth factor (PDGF) is of particular importance for the growth, survival, and migration of mesenchymal cell types. It stimulates the differentiation of various cell types of the connective tissue during embryonic development and is important for the formation of blood vessels. In the adult, PDGF regulates the interstitial fluid pressure of the connective tissue and also stimulates the regeneration of connective tissue during wound healing. Overactivity of PDGF is connected with several disorders involving excess cell proliferation, including malignancies, atherosclerosis, and fibrotic conditions.**

## I. INTRODUCTION

Platelet-derived growth factor (PDGF) constitutes a family of dimeric isoforms, acting on connective tissue cells and certain other cell types. PDGF was originally discovered as a constituent of platelets, which are released into serum in conjunction with blood coagulation. Although the α-granules of platelets are a major storage site for PDGF, PDGF is also produced by many other cell types.

PDGF stimulates the growth of its target cells, but also affects chemotaxis, i.e., directed cell movement, and cell shape through reorganization of the actin filament system. PDGF also affects the differentiation of specific cell types and promotes cell survival. Through these effects, PDGF has important functions in certain organs during embryonic development, as well as in the adult in the stimulation of wound healing and in the maintenance of connective tissue homeostasis.

Overactivity of PDGF has been linked to certain diseases, such as malignancies in which PDGF production may promote tumor growth via autocrine or paracrine stimulation. PDGF is also implicated in other disorders that involve an excess of cell proliferation, e.g., atherosclerosis and fibrotic conditions.

## II. PDGF ISOFORMS

The PDGF family contains four different gene products, which are assembled into five different dimeric molecules (Fig. 1). The classical PDGF, purified from human platelets, consists of homo- and heterodimers of structurally related A and B polypeptide chains. More recently, two additional members of the family were discovered, i.e., PDGF C chain and D chain, which appear as homodimeric molecules. The PDGF isoforms are homologous to members of the vascular endothelial growth factor (VEGF) family of angiogenic factors.

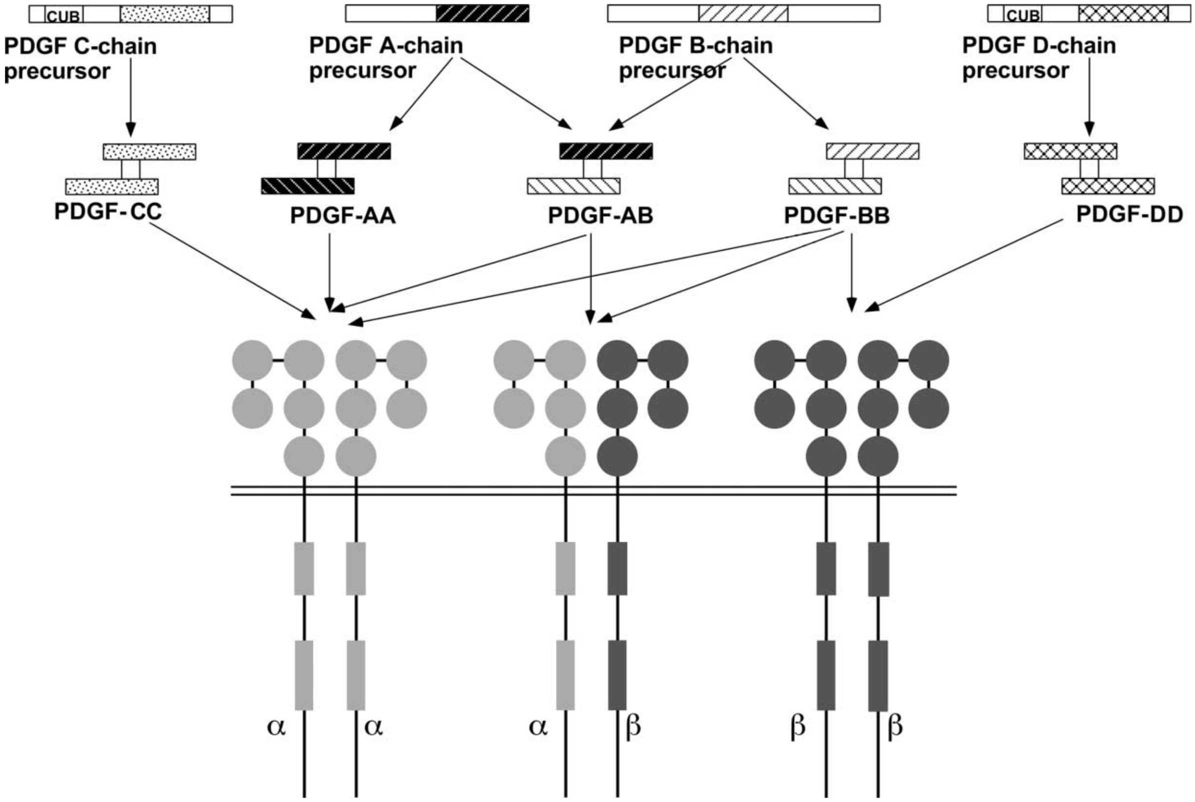

FIGURE 1 Processing and receptor-binding specificities of PDGF isoforms. The A and B chains of PDGF are synthesized as precursors that undergo processing during secretion from the producer cells. The C and D chains are also synthesized as precursors with CUB domains in their N-terminals; these isoforms are processed and activated after secretion from the producer cells. The PDGF isoforms bind to and dimerize α- and β-receptors with different specificities. Each of the receptors has five extracellular Ig-like domains and an intracellular kinase domain that is split into two parts by an intervening sequence.

## A. Synthesis of PDGFs

All PDGF isoforms are synthesized as precursor molecules, which undergo processing after synthesis. In the case of the A and B chains, the processing occurs during secretion from the producer cell, resulting in the export of active molecules. In contrast, the C and D chains are secreted as inactive precursors that need to undergo additional cleavage in the extracellular space in order to release the C-terminally located growth factor domain. The cleavages can be performed by plasmin, but the enzymes involved in normal tissues are not known. The N-terminals of the C and D chains contain CUB domains, which are so named because they also are present in complement factor, urchin epidermal growth factor-like protein, and bone morphogenetic protein 1. The CUB domains in PDGF-C and -D keep the growth factor domain inactive.

## B. Structure of PDGFs

The crystal structure of PDGF-BB is known; due to sequence conservation, it is likely that the other isoforms are folded similarly. PDGF-BB has a cysteine knot; i.e., one of its internal disulfide bonds passes through the hole formed by two other disulfides and intervening peptide sequences. Cysteine knots, which give a compact structure to the molecules, are also present in the homologous VEGFs, as well as in transforming growth factor-β and nerve growth factor, despite the fact that the latter molecules do not show any sequence similarity to PDGF. In addition to the six cysteine residues involved in the cysteine knot, another two cysteine residues are conserved in the growth factor domain; these residues form interchain disulfide bonds. The C and D chains contain four and two additional cysteine residues, respectively; how they are involved in disulfide bonds is not known.

From the compact cysteine knot of PDGF-BB, two loops (loops I and III) point in one direction and another loop (loop II) points in the other direction. Since the dimer is arranged in an anti-parallel manner, loops I and III from one subunit will be close to loop II of the other subunit; thus, two receptor-binding epitopes occur in the dimeric molecule, each one containing epitopes from loops I and III of one subunit and from loop II of the other subunit.

### C. Splice Forms of PDGFs

Certain PDGF isoforms are stored in the extracellular compartment of tissues. The A-chain gene occurs as two splice forms, one with and one without a C-terminal basic sequence, which mediate interactions with components of the extracellular matrix. It is not known whether the splicing of the A-chain gene is regulated, e.g., in response to external signals. A similar basic sequence is also present in the B chain. Whether the CUB domains have effects in addition to keeping the growth factors latent, such as mediating interactions with components of the extracellular matrix and thus providing a mechanism for storage of the PDGF isoforms in the tissues, remains to be elucidated.

Thus, the PDGF family of ligands contains five structurally related members that differ in their processing and other properties. In addition, they differ in their specificity of binding to different receptors, as discussed below.

## III. PDGF RECEPTORS

PDGF isoforms exert their cellular effects by binding, with different affinities, to two structurally related protein tyrosine kinase receptors, denoted α- and β-receptors. Each receptor contains five extracellular Ig-like domains and an intracellular kinase domain that is split into two parts by an intervening sequence. The two PDGF receptors form a subfamily among the tyrosine kinase receptors together with the stem cell factor receptor, the colony-stimulating factor 1 receptor, and Flk-1.

### A. Receptor Dimerization

Since PDGF isoforms are dimeric and contain two receptor-binding epitopes, they bring together receptors in dimeric complexes on binding. The A and C chains of PDGF bind to α-receptors, the D-chain preferentially to β-receptors, whereas the B chain binds to both α- and β-receptors with high affinity. Therefore, PDGF-AA and -CC cause the formation of αα receptor homodimers, PDGF-DD causes the formation of ββ receptor homodimers, PDGF-AB produces αβ receptor heterodimers as well as αα receptor homodimers, and PDGF-BB produces all three possible combinations of receptor dimers (Fig. 1).

### B. Receptor Autophosphorylation

Dimerization is a crucial event in receptor activation, since it brings the intracellular parts of the receptors close to each other so that autophosphorylation *in trans* can occur. The autophosphorylation serves two important functions; i.e., it activates the kinase activity and it creates docking sites for downstream SH2-domain-containing signaling molecules. Activation of the kinase involves autophosphorylation of a tyrosine residue in the activation loop of the kinase, Tyr-849 and Tyr-857 in the α- and β-receptors, respectively. The crystal structures of the PDGF receptor kinases are not known, but solutions of the structures of other kinases have elucidated mechanisms for control of the kinase domain. In the resting state, the activation loop of the kinase is folded over the active site and thereby prevents access to the substrate; phosphorylation causes the activation loop to swing out and thereby allows phosphorylation of the substrates. It is possible that other parts of the receptor, like the juxtamembrane region and the C-terminal tail, also interact with the kinase domain in a negative modulatory manner. Autophosphorylation at tyrosine residues located mainly outside the kinase domain may activate such inhibitory interactions and, in addition, allow binding of downstream signal transduction molecules, initiating a number of signaling pathways. The α- and β-receptors contain at least 9 and 11 such phosphorylation sites that bind, in a specific manner, to different SH2 domain proteins.

### C. Docking of SH2 Domain Proteins and Downstream Signaling

Some of the SH2 domain proteins that bind to the PDGF receptors contain intrinsic enzymatic activities, such as the protein tyrosine kinase Src, the protein tyrosine phosphatase SHP-2, phospholipase Cγ, GTPase-activating protein for Ras (RasGAP), and phosphatidylinositol 3′-kinase (PI3-kinase), which consists of a regulatory p85 subunit and a catalytic p110 subunit. Other SH2 domain proteins lack endogenous enzymatic activity and act like adapter molecules, i.e., Shc, Nck, Crk, and Grb2; Grb2 forms a stable complex with Sos1, a nucleotide exchange molecule for Ras.

Much effort has been put into the elucidation of which signaling pathways result in the various cellular effects of PDGF, i.e., cell proliferation, survival, chemotaxis, and actin reorganization. In general, PI3-kinase has been found to be important for the anti-apoptotic and motility responses of PDGF; Src and Ras, which activate the transcription factor Myc and the extracellular signal-related kinase mitogen-activated protein kinase cascade, respectively, are important for the growth-stimulating effect. However, the results depend on the cell type studied and the experimental conditions used for the studies. The difficulties in finding a clear and universal relationship between individual signaling pathways and specific effects can partly be explained by an extensive cross talk between the components in the various signaling pathways.

Both αα and ββ homodimeric receptor complexes transduce mitogenic signals; however, whereas the ββ homodimer stimulates chemotaxis, the αα homodimer inhibits chemotaxis. The molecular mechanism for the difference between the two homodimeric receptor complexes is not known. Moreover, there is evidence that the αβ heterodimeric complex has unique properties compared to the two homodimers; e.g., it appears to be the complex that causes the most potent mitogenic signal. A possible mechanism for this difference has been elucidated, i.e., the autophosphorylation sites in the PDGF β-receptor differ in a ββ homodimer, compared to an αβ heterodimer. For example, Tyr-771, which binds RasGAP, is phosphorylated efficiently in the ββ homodimer, but not in the αβ heterodimer. Thus, the αβ heterodimer cannot bind RasGAP. Since RasGAP converts active RasGTP to inactive RasGDP, the activation of Ras is therefore more efficient in a heterodimeric receptor than in a ββ homodimeric receptor, which may contribute to its higher mitogenic potency.

## D. Internalization of PDGF Receptors

After ligand-induced dimerization, the PDGF receptors are internalized in endosomes. Over time, the pH of the interior of the endosomes becomes acidified, leading to dissociation of the ligand from the receptor. The receptor is then degraded after fusion of the endosomes with lysosomes. An additional degradative route has recently been elucidated, i.e., proteasomal degradation in the cytoplasm, triggered by ubiquitination of the receptor. Alternatively, the receptor is recycled to the plasma membrane after dissociation from the ligand. In the situations that have been studied, the majority of PDGF receptors are degraded after internalization.

# IV. FUNCTION OF PDGF *IN VIVO*

PDGF has important functions at specific phases of embryonic development, as well as in wound healing and in control of the homeostasis of the connective tissue compartment in the adult.

## A. Embryonic Development

The important function of PDGF during embryogenesis is illustrated by the finding that mice with the A- or B-chain genes or α- or β-receptor genes inactivated die during embryogenesis or perinatally. Data on the phenotypes of mice with the C- or D-chain genes knocked out are not yet available.

A striking effect of knocking out the B-chain or β-receptor genes is that the mesangial cells of the kidney do not develop, causing defective glomeruli with poor filtrating capacity. Moreover, there is a defect in the development of blood vessels in the knockout animals with a dilated aorta. The actual cause of death of the animals is bleeding at the time of birth. The reason for the improper development of the vessels is deficient development of the smooth muscle cells, as well as the inability of the newly formed vessels to attract pericytes.

The major phenotype of animals with the A chain knocked out is emphysema of the lungs, leading to death at approximately 3 weeks of age. The reason for the emphysema is defective distal spreading of alveolar smooth muscle cell progenitors during development.

Since the α-receptor responds to all isoforms containing A, B, and C chains, it is not surprising that knockout of the α-receptor gene gives a more severe phenotype than knockout of the A-chain gene only. The α-receptor knockout animals die during embryogenesis with cranial malformations and deficiency of myotome formation.

Characterization of the expression patterns of PDGF isoforms and their ligands during embryonic development has revealed examples of the expression of a PDGF isoform and the corresponding receptor in the same cell, suggesting autocrine stimulation, as well as in adjacent cell layers, suggesting paracrine stimulation. PDGF receptors are often expressed in mesenchymal cells and PDGF isoforms in adjacent epithelial layers. In this manner, epithelial cells control the development of surrounding mesenchymal structures through the secretion of PDGF isoforms. Although PDGF is of particular importance for the development of connective tissue cells, PDGF receptors are also expressed in cells of nonmesenchymal origin. For instance, the α-receptor is expressed in the ectodermally derived neural crest and the β-receptor in mammary epithelial cells.

### B. Central Nervous System

PDGF isoforms and PDGF receptors are expressed in different types of neurons, as well as in the glial cells of the central nervous system (CNS). The importance of PDGF for glial cell differentiation has been particularly well characterized. The α-receptor is expressed on bipotential oligodendroglial–astroglial cell precursors from the rat optic nerve, spinal cord, and other parts of the CNS. PDGF controls the timing of the differentiation of these cells to astrocytes.

PDGF receptors are also expressed on certain postnatal neurons, and PDGF has been shown to have a neurotropic effect on cultured rat dopaminergic neurons.

### C. Vascular System

Capillary endothelial cells have been shown to express PDGF β-receptors, and PDGF has been shown to have an angiogenic effect. However, the angiogenic effect appears to be weaker than that of VEGFs or fibroblast growth factors. PDGF appears not to be important for the initial formation of novel vessels, since no apparent vascular abnormality is observed during early embryogenesis in mice with the genes for PDGF or PDGF receptors inactivated. It appears that PDGF isoforms have more important roles in the development of the smooth muscle layer and recruitment of pericytes to the vessels, which is required at later stages of vasculogenesis to give strength to the vessel wall.

PDGF also affects platelet aggregation. On platelet release and aggregation, induced, e.g., by thrombin or collagen, the released PDGF activates PDGF α-receptors present on the platelets, leading to a decreased platelet aggregation. PDGF, which is present in large amounts in platelets, may thus have an autocrine feedback role in the control of platelet aggregation.

### D. Tissue Homeostasis

The interstitial fluid pressure in tissues, which is normally slightly negative, is carefully controlled to allow an exchange of fluid and macromolecules between the extracellular compartment and the vascular system. PDGF exerts an important control on the interstitial fluid pressure, probably because of its ability to stimulate the formation of stress fibers in myofibroblasts of the connective tissue and to promote interactions between these cells and molecules of the extracellular matrix.

### E. Wound Healing

PDGF stimulates wound healing through actions on several cell types involved in the healing process. It stimulates chemotaxis of neutrophils and macrophages and both chemotaxis and proliferation of fibroblasts and smooth muscle cells. PDGF also stimulates macrophages to secrete other growth factors that are important for various phases of the healing process. Moreover, PDGF acts on connective tissue cells to stimulate the synthesis of matrix molecules, such as fibronectin, collagen, proteoglycans, and hyaluronic acid. PDGF may also be important during later stages of wound healing; for instance, it stimulates the contraction of collagen gels *in vitro*, suggesting that it may stimulate wound contraction *in vivo*.

Topical application of PDGF to large wounds in patients has been shown to increase their rate of healing. It is likely that PDGF also has a role in normal wound healing since it is present in wound fluid from soft tissue. There are several possible sources of PDGF in healing wounds; it has been shown to be released from platelets, activated macrophages, thrombin-stimulated endothelial cells, smooth muscle cells of damaged vessels, activated fibroblasts, and epidermal keratinocytes.

Whereas PDGF has a significant effect on healing of soft tissue wounds, its effect on fracture healing is less clear; there are reports that application of PDGF increases fracture healing, but other reports indicate that PDGF instead induces a soft tissue repair phenotype and response.

## V. PDGF IN DISEASE

There is evidence that PDGF is involved in autocrine as well as paracrine stimulation in malignancies. In addition, PDGF overactivity has been linked to other disorders involving an excess cell proliferation, such as atherosclerosis and fibrotic conditions.

### A. Malignancies

The finding that the retroviral oncogene v-*sis* is derived from the PDGF B-chain gene and that transformation by v-*sis* involves autocrine stimulation by a PDGF-like growth factor prompted investigations of whether overactivity of PDGF also occurs in human malignancies. Work in recent years has revealed several examples in which overactivity of the PDGF pathway drives the proliferation of tumor cells. There are examples of the classical autocrine situation, in which a PDGF receptor carrying cells starts the production of PDGF. For example, the relatively rare skin tumor dermatofibrosarcoma protuberance (DFSP) is associated with a fusion of the collagen 1A1 gene with the PDGF B-chain gene, causing the production of an excess of a collagen–PDGF fusion protein. The fusion protein is processed to a PDGF-like growth factor that stimulates the producer cell in an autocrine fashion. Other types of sarcomas and glioblastomas are also characterized by overexpression of PDGF, although the mechanisms behind the overexpression are not known.

There are also examples of perturbations of the PDGF signaling pathway intracellularly. Thus, in chronic myelomonocytic leukemia, the gene for the Ets-like transcription factor Tel is fused to the part of the PDGF β-receptor gene coding for the kinase domain. The result is the production of a fusion protein consisting of part of Tel and the PDGF β-receptor kinase. Since Tel forms a dimer, the fusion protein dimerizes, thereby causing a constitutive activation of the kinase. Constitutive PDGF β-receptor dimers can also be formed by interaction of the E5 oncoprotein of bovine papilloma virus with the transmembrane part of the receptor.

In addition to driving the proliferation of the tumor cells themselves, PDGF may be involved in paracrine stimulation of normal cells in tumors. Several types of tumor cells synthesize PDGF. In cases where the tumor cells do not express PDGF receptors, no autocrine stimulation will occur. However, the secreted PDGF may affect other cell types in the tumor. Thus, PDGF has been shown to have a weak angiogenic effect and to stimulate stromal cells. The paracrine effects of PDGF may be of importance for the balanced growth of tumors. Moreover, PDGF is important for the elevated interstitial fluid pressure that often characterizes solid tumors and that makes uptake of chemotherapeutic drugs less efficient.

### B. Atherosclerosis

Atherosclerosis is characterized by an inflammatory-fibroproliferative response, which includes an increased expression of PDGF. In experimental models, as well as in naturally occurring lesions, increased expression of PDGF and PDGF receptors is seen. PDGF may be released by platelets trapped in thrombi, by activated macrophages, by smooth muscle cells, or by endothelial cells, and it stimulates the migration of smooth muscle cells from the media of the vessel into the intima layer, where the cells also proliferate in response to PDGF. Such intimal thickening at sites of endothelial cell injury occurs at an early phase of the atherosclerotic process.

### C. Fibrotic Conditions

As discussed above, PDGF is important for the development of connective tissue compartments in several organs. In the adult, overactivity of PDGF causes fibrosis of the same organs. Thus, several types of pulmonary fibrosis have been shown to involve overexpression of PDGF. Moreover, intratracheal injection of PDGF-BB has been shown to cause transient proliferation of pulmonary mesenchymal and epithelial cells accompanied by collagen deposition. Overactivity of PDGF is also implicated in several types of glomerulonephritides, liver cirrhosis, and myelofibrosis.

### D. PDGF Antagonists

The fact that PDGF is involved in several serious disorders makes the development of clinically useful PDGF antagonists highly warranted. Several types of PDGF antagonists have been developed, including antibodies, DNA aptamers and soluble extracellular receptor domains that sequester PDGF, antibodies that bind to and block PDGF receptors, and low-molecular-weight inhibitors of the receptor kinase. Promising results of such antagonists have been obtained in animal models for malignancies, as well as atherosclerosis and fibrotic conditions; their usefulness in the treatment of patients is currently being evaluated.

## VI. SUMMARY

In the human genome, there are four genes encoding PDGF isoforms, PDGF-A, -B, -C, and -D. The four dimeric isoforms, PDGF-AA, -BB, -CC, and -DD, bind with different specificities to two related tyrosine kinase receptors, forming αα, αβ, or ββ receptor dimers.

Important aims of future research will be to elucidate the detailed *in vivo* function of the different PDGF isoforms, in particular, the novel isoforms PDGF-CC and PDGF-DD, and their potential involvement in disease. It will also be important to investigate whether additional receptors are involved in mediating signals from the novel PDGF isoforms. There is preliminary evidence that the different dimeric forms of PDGF α and β receptors transduce different intracellular signals. It will be important to elucidate the *in vivo* significance of such differences.

## Acknowledgments

Ingegärd Schiller is thanked for skillful secretarial help.

## Glossary

**autocrine, paracrine, or endocrine stimulation** The process whereby a cell produces a factor(s) that stimulates that cell itself, cells in the immediate environment, or distant cells, respectively.

**autophosphorylation** A process whereby a kinase phosphorylates itself or kinase-associated receptors in a dimeric complex phosphorylate one another.

**chemotaxis** Directed cell migration toward a gradient of a chemotactic factor, e.g., a growth factor.

**growth factor receptor** A transmembrane protein that consists of an extracellular ligand-binding domain and an intracellular effector domain that often is associated with a kinase activity that is activated on ligand binding.

**signal transduction pathway** A series of molecules activating one another through physical contacts and/or enzymatic modifications, leading to a specific effect, such as cell growth, survival, or migration.

## See Also the Following Articles

**Cancer Cells and Progrowth/Prosurvival Signaling • Epidermal Growth Factor (EGF) Family • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Nerve Growth Factor (NGF) • Vascular Endothelial Growth Factor**

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# Polycystic Ovary Syndrome

Stephen Franks
*Imperial College London*

I. INTRODUCTION
II. ENDOCRINE ABNORMALITIES IN PCOS
III. OVARIAN FOLLICULAR ABNORMALITIES IN PCOS
IV. GENETIC STUDIES OF PCOS
V. CLINICAL MANAGEMENT ISSUES
VI. SUMMARY

**Polycystic ovary syndrome (PCOS) is a very common, heterogeneous endocrine disorder that is a major cause of infertility, hirsutism, and metabolic disorders. Its most consistent biochemical feature is hypersecretion of ovarian androgens. PCOS is associated with an increased risk of type 2 diabetes in later life.**

## I. INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women, accounting for about three-quarters of all cases of anovulatory infertility and about 90% of the causes of hirsutism. Polycystic ovary syndrome is heterogeneous in its presentation and this has resulted in some discussion about how to define PCOS. The classic definition includes the association of anovulatory menses (or estrogen-replete amenorrhea) with clinical and/or

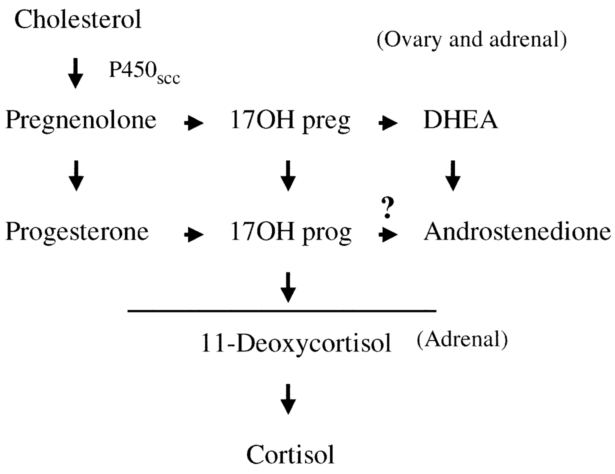

**FIGURE 1** The steroidogenic pathway in ovaries and adrenals. Note that cytochrome P450 side chain cleavage enzyme (P450scc) is the key enzyme in the production of pregnenolone, and hence all later stages of steroid production. There is a question concerning the conversion of 17-hydroxyprogesterone (17OH prog) to androstenedione: in the human ovary, it is more likely that androstenedione is derived mainly from conversion of dehydroepiandrosterone (DHEA) by the enzyme 3β-hydroxysteroid dehydrogenase.

biochemical evidence of excess androgen secretion. Using this definition, the estimated prevalence of PCOS is in excess of 5% of the female population of reproductive age. The range of clinical presentation of women with polycystic ovaries—as defined by pelvic ultrasonography—is, however, wide. It includes patients with anovulation who are nonhirsute and those with hirsutism who have regular menstrual cycles. Indeed, polycystic ovaries are found in over 80% of women who would otherwise have been labeled as having "idiopathic hirsutism." The results of ultrasound studies of "normal" populations suggest that polycystic ovaries are present in about 20% of women of reproductive age. The causes of polycystic ovaries (PCOs) and PCOS are not known for certain, but there is strong evidence for a major genetic contribution, as will be discussed in Section IV.

In addition to the reproductive consequences of the syndrome, PCOS is characterized by a metabolic disorder in which hyperinsulinemia and peripheral insulin resistance are the central features. This metabolic dysfunction may play a part in the etiology of anovulation but also has important implications for long-term health. Women with PCOS are two to three times more likely to develop type 2 diabetes mellitus in later life and may also be at increased risk of developing cardiovascular disease.

## II. ENDOCRINE ABNORMALITIES IN PCOS

The major endocrine abnormalities in women with PCOS are elevated serum concentrations of androgens and luteinizing hormone (LH) and, particularly in those with the classic definition of PCOS (i.e., including menstrual disturbances), hyperinsulinemia and insulin resistance.

### A. Hypersecretion of Androgens

The most common biochemical abnormality in women with polycystic ovaries is hypersecretion of androgens. The ovary appears to be the predominant source of excess androgen production, although many studies have pointed to evidence for an additional adrenal abnormality. Nevertheless, the ovary is clearly the more important contributor to hyperandrogenemia because suppression of LH in women with PCOS leads to a decrease in androgen concentrations to levels that are indistinguishable from those in menopausal or oophorectomized women. Cultured thecal cells from women with polycystic ovaries, regardless of presenting symptoms, produce some 20 times more androstenedione in primary culture than do cells from women with normal ovaries. Increased steroidogenic activity is, however, not confined to androgen production. All stages of the steroidogenic pathway—including progesterone production—appear to be amplified in PCO theca (Figs. 1 and 2). Importantly, these results

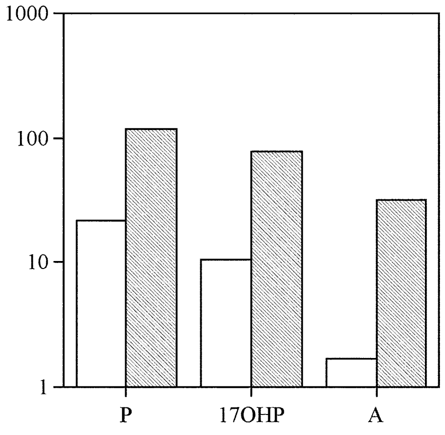

**FIGURE 2** Steroid production (in picomoles/1000 cells/48 h) by theca cells from normal (clear bars) and polycystic ovaries (PCO; shaded bars). P, Progesterone; 17OHP, 17-hydroxyprogesterone; A, androstenedione. Note the logarithmic scale. In each case, steroid production by theca cells from polycystic ovaries was significantly greater than that from normal theca (P, $p < 0.01$; 17OHP, $p < 0.01$; A, $p < 0.005$) Redrawn from Gilling-Smith *et al.* (1994).

have recently been confirmed using PCO and normal theca cells that have undergone several passages in culture. This suggests that this biochemical phenotype is an intrinsic feature of the polycystic ovary. Thus, it is unlikely that ovarian hyperandrogenism arises secondary to hypersecretion of LH in PCOS, particularly because this "typical" feature of PCOS occurs in little more than 50% of those with the classic syndrome and in the minority of those with hyperandrogenism and regular cycles.

### B. Metabolic Abnormalities in PCOS

In recent years, there has been a great deal of interest in the metabolic associations of PCOS. The classic syndrome is characterized by a distinctive form of insulin resistance. Women with PCOS have higher fasting and glucose-stimulated insulin concentrations and significantly reduced insulin sensitivity compared with weight-matched control subjects (Fig. 3). The cause of this abnormality is unclear, but clinical and laboratory-based studies in PCOS have variously pointed to abnormalities of insulin receptor binding, or, more plausibly, to postreceptor signaling as well as to evidence for a primary abnormality of insulin secretion. It has been demonstrated that weight reduction in obese women with PCOS results in normalization of insulin sensitivity, but "first-phase" insulin secretion in response to an intravenous glucose challenge remains abnormal. These data also illustrate an important principle in understanding the etiology of PCOS, which is that whatever the genetic basis for the syndrome, the phenotype can be influenced by environmental (in this case nutritional) factors.

## III. OVARIAN FOLLICULAR ABNORMALITIES IN PCOS

A further phenotypic feature of PCOS is the polycystic ovarian morphology. The polycystic ovary is characterized by the presence of an increased number not only of antral follicles but also of early-growing and preantral follicles. Because these earlier stages of follicular development are thought to be largely independent of gonadotropins, the implication is that local ovarian factors may have a role in genesis of the polycystic ovary. Many growth factors have been shown to have an influence on early folliculogenesis, including those of the transforming growth factor-β superfamily and growth factors signaling through tyrosine kinase coupled receptors such as insulin-like growth factors-I and -II and transforming growth factor α.

Anovulation in PCOS is characterized by arrested development of medium-sized antral follicles. Granulosa cells from these follicles display evidence of premature responsiveness to LH and increased steroidogenesis (indicative of advanced differentiation) compared with similarly sized follicles from normal ovaries. The reason for these

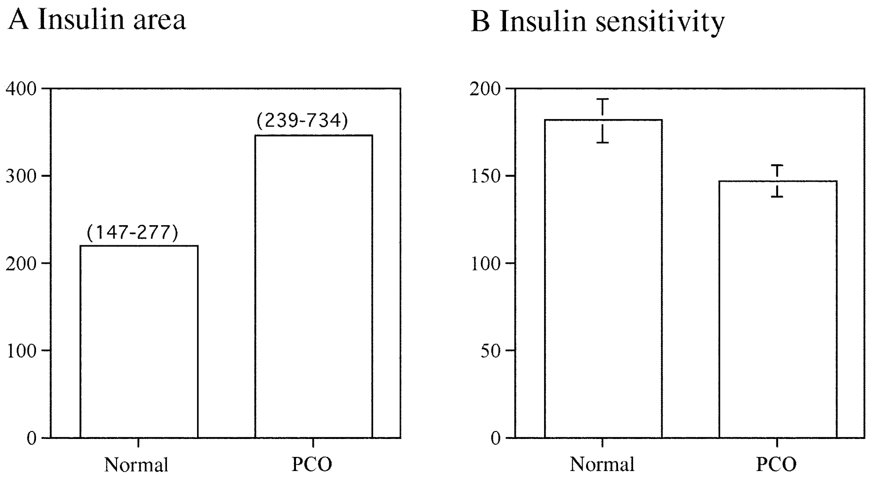

**FIGURE 3** (A) Insulin area (median, interquartile range; in milliunits/hour) following a 75-g oral glucose tolerance test dose and (B) insulin sensitivity (in micromoles/minute), measured by the short insulin tolerance test, in 29 women with polycystic ovary (PCO) syndrome and 24 weight-matched control subjects. Insulin area was significantly greater ($p < 0.01$) and insulin sensitivity significantly lower ($p < 0.01$) in women with polycystic ovary syndrome than in controls. Redrawn from Robinson *et al.* (1993).

differences are not yet entirely clear, but because insulin can greatly enhance the response of the granulosa cell to LH, it seems likely that hyperinsulinemia plays an important part in the mechanism of anovulation.

## IV. GENETIC STUDIES OF PCOS

The definition of abnormalities in the ovarian steroidogenic pathway, in the secretion and action of insulin, and in ovarian follicular development has paved the way for the use of a candidate gene approach in the identification of genetic susceptibility loci for PCOS.

### A. Genes Involved in the Biosynthesis and Metabolism of Androgens

Genes implicated in the pathway of androgen production and metabolism include those encoding the major endocrine regulator, LH, its receptor, and key P450 steroidogenic enzymes such as cholesterol side chain cleavage (P450scc) and 17α-hydroxylase/17,20 lyase (P450c17 α).

#### *1. CYP11a—Coding for P450 Cholesterol Side Chain Cleavage*

A polymorphic sequence [a pentanucleotide repeat—(tttta)$_n$] in the 5′ regulatory region of *CYP11a* has been identified, and both case-control association studies and nonparametric linkage analysis have been performed by Gharani and colleagues. Subjects were assigned to two groups according to genotype. The most common genotype, comprising four repeats, occurred with a frequency of 0.59, and was designated 216. Subjects were subdivided according to whether this allele was present (216 + ) or absent (216 − ). It was found that genotype was associated with serum testosterone concentrations, levels being significantly higher in women with the 216 − genotype (which consists of alleles of six repeats or longer) (Fig. 4). On more detailed analysis, this association held true only in those subjects with clinical evidence of hyperandrogenism. Supportive evidence for the association of PCOS with *CYP11a* comes from two recent European studies, the first of which has reported a relationship between the (tttta)$_n$ polymorphism and androgen levels in 88 hirsute women. This study found that the *CYP11a* genotype, together with endocrine markers, predicts the presence of PCO in hirsute women. Another group, in a case-control study, reported that the *CYP11a* genotype was associated with both PCO and total testosterone concentrations. Further support for this notion has been obtained from mutation screening of the *CYP11a* promoter in a 1.85-kb region 5′ to the start site of translation. Direct sequencing of fragments (amplified by polymerase chain reaction) of DNA samples from affected and unaffected family members has been carried out. Apart from the pentanucleotide repeat polymorphism at position −466 from the start site of transcription (and a previously identified dinucleotide repeat polymorphism at position −1314), no variation from the published sequence is found. Structure–function studies of the promoter region using expression systems need to be performed to explore the putative functional role of this element. An alternative explanation is that this polymorphic marker is in linkage disequilibrium with the disease locus, which may be located outside the promoter region.

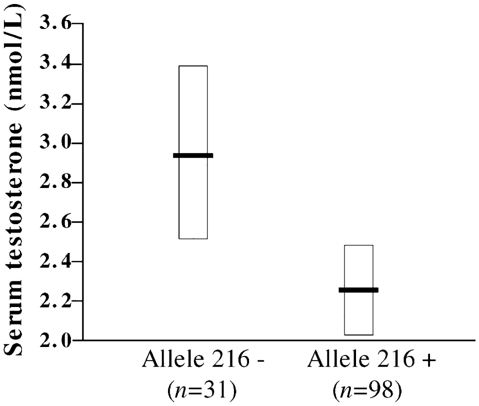

FIGURE 4 Association of *CYP11a* genotype with serum testosterone in a case-control data set of women with and without PCOS. Note significantly higher serum testosterone concentrations ($p = 0.0009$) in association with the 216 − genotype (more than six pentanucleotide repeats). Data shown are mean and 95% confidence intervals Redrawn from Gharani *et al.* (1997).

Segregation of *CYP11a* has been examined in 20 families. With the aid of a number of polymorphic markers [*D15S153*, *D15S125*, *CYP11a* (ac)$_n$, *D15S169*, and *D15S211*], in the region of *CYP11a*, nonparametric linkage analysis was performed using the GENEHUNTER (multipoint linkage) program. There is evidence for excess allele sharing, i.e., linkage, at the *CYP11a* locus [nonparametric logarithm of the odds (LOD) (NPL) score, 3.03; $p = 0.003$]. In a parallel parametric analysis, assuming the autosomal dominant model for inheritance, evidence of genetic heterogeneity between families is found, with about 60% of families showing linkage at the *CYP11a* locus. Thus, data from both association and linkage studies suggest that this is a major susceptibility locus for hyperandrogenism in PCOS.

#### 2. *CYP17—Coding for 17-Hydroxylase/17,20-Lyase*

Because of the reported abnormalities in regulation of 17α-hydroxylase/17,20 lyase in PCOS, initial studies have focused on the role of *CYP17* (coding for P450c17α). Results of a preliminary case-control study suggest that a variant form of *CYP17* is associated with PCOS, but there is no relationship between genotype and serum testosterone levels. Subsequent, larger, case-control studies have also been unable to confirm the putative association. Furthermore, linkage analysis excludes *CYP17* as a major susceptibility gene for PCOS within families.

### B. Genes Involved in the Secretion and Action of Insulin

#### 1. *The Insulin Gene Variable-Number Tandem Repeat*

There is evidence that the insulin gene (*INS*) variable-number tandem repeat (VNTR) is a major susceptibility locus for PCOS. The *INS* VNTR lies in the 5′ regulatory region of the gene; it has been shown to be involved in regulation of insulin gene expression and has been implicated in the etiology of type 2 diabetes. Class III alleles in the VNTR have been found to be associated with anovulatory PCOS in two independent populations and using two different methods of analysis [case-control studies and by the use of affected family-based controls (AFBAC)]. With the aid of a nonparametric linkage analysis program, it has also been established that there is excess allele sharing at the *INS* VNTR locus. The geometric mean of fasting serum insulin concentrations is significantly higher in families in which linkage is demonstrated than in those families without evidence of linkage. This suggests a functional role for the VNTR variant in the expression of hyperinsulinemia/insulin resistance in PCOS. It is also observed, using transmission disequilibrium (TDT) analysis, that there is a “parent of origin” effect in the transmission of alleles of the VNTR to affected subjects. Class III alleles are transmitted significantly more commonly from fathers than from mothers to the affected daughter. Interestingly, this finding has been mirrored recently in an analysis of families with type 2 diabetes for which PCOS is a known risk factor.

### C. Genes Involved in Folliculogenesis

#### 1. *The Follistatin Gene*

As part of a panel of candidate genes related to gonadotropin action, Urbanek and colleagues examined the follistatin locus on chromosome 5 and, somewhat unexpectedly, found the strongest evidence for linkage with PCOS of any of the 37 candidate genes they had chosen. In their affected sibling-pair analysis, 72% of sisters were concordant for the follistatin genotype, and this remained significant after correction for multiple testing. However, recent follow-up data from the same group suggest that this finding is no longer significant when further families are added to the database. This finding nevertheless remains intriguing, and the possibility arises that this and other genes implicated in folliculogenesis may have a causal role in this disorder, which is, after all, characterized by disordered follicle development.

## V. CLINICAL MANAGEMENT ISSUES

### A. Diagnosis of PCOS

The diagnosis of PCOS is reached primarily on a clinical basis. A patient presenting with irregular menses, oligomenorrhea, or amenorrhea and who has signs of hyperandrogenism is very likely to have PCOS. Even in the absence of hirsutism, PCOS is the most likely cause of these menstrual symptoms (it accounts for about 30% of cases of amenorrhea overall and about 90% of amenorrheic women with normal estrogen levels).

The majority of patients presenting with hirsutism have polycystic ovaries, irrespective of menstrual history. Much rarer but more serious causes of hirsutism and menstrual disturbances include Cushing’s disease, acromegaly, hyperprolactinemia, and tumors of the adrenal or ovary. In such cases, however, there are usually other clues, both clinical and biochemical, to the diagnosis, e.g., short history of increasing hirsutism and significantly elevated serum testosterone (>5 nmol/liter). For this reason, serum testosterone should be measured in all hirsute patients as a screening test to exclude more serious causes of hyperandrogenism. Late-onset (“nonclassical”) congenital adrenal hyperplasia due to 21-hydroxylase deficiency may be difficult to distinguish clinically from PCOS, but it is debatable whether this makes much practical difference to management of symptoms. Thus, in a typical situation in which the prevalence of nonclassical

21-hydroxylase deficiency is $<5\%$, for example, routine measurement of 17α-hydroxyprogesterone (the biochemical marker of 21-hydroxylase deficiency) is not necessary.

No single test is diagnostic of the syndrome and choice of investigations should be tailored to the clinical presentation. Serum LH levels are typically elevated in PCOS (follicle-stimulating hormone is normal), but up to 50% of women with all other clinical and biochemical features of the syndrome may have normal serum LH. Measurement of LH is therefore of limited diagnostic value; it is quite specific—elevated LH and normal follicle-stimulating hormone (FSH) essentially occur only in PCOS—but it is not very sensitive. Pelvic ultrasonography will define the polycystic ovarian morphology, but accurate assessment of the ovaries by ultrasound is a particular skill, and false negative results are not uncommon. Conversely, the presence of polycystic ovaries does not necessarily mean that the patient has polycystic ovary syndrome. Polycystic ovaries may be found coincidentally in women who have, for example, hypothalamic, estrogen-deficient amenorrhea.

In summary, pelvic ultrasonography and measurements of LH, FSH, and testosterone may be of some diagnostic value when set in the appropriate clinical context. By contrast, routine measurements of adrenal androgens are not indicated and measurement of sex hormone-binding globulin (which is primarily an index of body weight) is not at all helpful. Because of the increased risk of type 2 diabetes, it is recommended that obese women with PCOS should have a fasting glucose measurement at least once a year and, in view of the associated dyslipidemia, there may also be some merit in checking the lipid and lipoprotein profile at the same time.

## B. Management

Because the physiological basis of PCOS is unknown, treatment is largely to alleviate symptoms. Patients with anovulation may require induction of ovulation. The antiestrogen, clomiphene, is usually effective, but even this "simple" treatment should be monitored at a specialist center because of the risk of ovarian hyperstimulation and multiple pregnancy. For those not concerned about fertility, menstrual regulation by means of oral contraceptives or cyclical progestagens should be considered. Nonandrogenic progestagens (e.g., medroxyprogesterone acetate, desogestrel, and gestodene) are obviously preferable to norgestrel and norethisterone for women who may anyway have symptoms of androgen excess.

Symptoms of hyperandrogenism can be managed by antiandrogens such as cyproterone acetate. For women with acne and mild or moderate hirsutism, this can usually be given in the form of Dianette (cyproterone acetate, 2 mg, with ethinylestradiol, 35 mg). Cosmetic advice about removal of hair should not be forgotten, even with administration of antiandrogens.

Obese subjects with PCOS require particular attention. It has been clearly demonstrated that calorie restriction in obese women with PCOS improves insulin sensitivity and glucose tolerance. It also leads to resumption of spontaneous ovulatory cycles and normal fertility in many subjects. Significantly, such improvements in glucose–insulin homeostasis and reproductive function can be achieved with weight reduction of as little as 5% of the initial body weight. Insulin-sensitizing drugs may also have a role in reducing the risk of diabetes and improving ovarian function. The thiazolidinediones (TZDs) are a relatively new class of insulin-sensitizing drugs that have been introduced primarily for the control of type 2 diabetes. In a large randomized, multicenter study, troglitazone has been shown to improve insulin sensitivity and menstrual cyclicity in obese women with PCOS. Unfortunately, this drug has been withdrawn because of serious side effects, and although there are newer, apparently safer preparations available, there are concerns about the wisdom of prescribing TZDs in women of reproductive age. Metformin is a well-established medication in management of type 2 diabetes. Its mechanism of action is complex, but its effects include reduction of insulin receptors and insulin levels. Recent studies in women with PCOS have suggested that this may be a safe and effective means of improving the metabolic profile and reproductive function in both lean and obese women with PCOS. Results so far have been encouraging, but by no means conclusive. Randomized controlled trials have been few and have produced conflicting results.

## VI. SUMMARY

PCOS is clinically and biochemically heterogeneous. The major endocrine hallmark is hyperandrogenemia and although it is clear that hypersecretion of adrenal androgens may contribute to the hyperandrogenemia of women with polycystic ovary syndrome, the weight of evidence favors the ovary as the major source of excess androgen secretion.

The biochemical basis for the putative disorder of ovarian androgen biosynthesis remains controversial. There is evidence, from both clinical and *in vitro* studies of human ovarian theca cells, of dysregulation of the rate-limiting enzyme in androgen biosynthesis, cytochrome P450c17α, which catalyzes both 17α-hydroxylase and 17,20 lyase activities. Initial data have suggested that alleles of *CYP17*, the gene encoding P450c17α, are associated with PCOS but this has now been excluded as a candidate gene. However, *CYP11a*, which encodes cholesterol side chain cleavage enzyme, does appear to be a major susceptibility locus for hyperandrogenism in women with PCO. Nevertheless, the finding that the expression of other enzymes in the androgen biosynthetic pathway is also up-regulated suggests that this may not be the only focus for genetic abnormalities of steroidogenesis. The precise nature of the interaction of androgens and genetic loci affecting insulin secretion remains unclear.

From the viewpoint of clinical management, the major issues relate to correction of infertility, menstrual disturbance, and hirsutism. The metabolic abnormalities in PCOS have implications both for management of anovulatory infertility and for the increased risk of type 2 diabetes in later life. Dietary measures are very important in overweight subjects, but there is increasing evidence that insulin-sensitizing agents may have an important role to play in management both of anovulation and of metabolic consequences of PCOS.

## Glossary

***CYP11a*** Gene encoding the P450 side chain cleavage enzyme.

**insulin gene variable-number tandem repeat** Sequence in the regulatory region of the insulin gene.

**P450 cholesterol side chain cleavage enzyme** Key enzyme in ovarian steroidogenesis.

**polycystic ovaries** Characteristic ovarian multiple antral follicles, increased stroma, and androgen hypersecretion.

**polycystic ovary syndrome** Clinical and endocrine abnormalities, typically manifested as anovulation and hyperandrogenism; associated with polycystic ovaries.

## See Also the Following Articles

**Diabetes Type 2 • Follicle Stimulating Hormone (FSH) • Folliculogenesis • Inhibins, Activins, and Follistatins • Insulin Processing • Insulin Resistance in PCOS (Polycystic Ovary Syndrome) • Leptin Actions on the Reproductive Axis • Luteinizing Hormone (LH)**

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# Pregnancy

See ***Decidualization; Implantation; Placental Development***

**localized to a specialized region of steroid metabolism and action in the female reproductive tract. Steroid hormones synthesized by the adrenal glands (corticosteroids) and ovaries (progesterone) are natural anti-inflammatory agents that are intimately involved in these serial injury-repair processes. The immunoendocrine interplay between pro-inflammatory cytokines and anti-inflammatory steroids is therefore fundamental to reproduction.**

## I. INFLAMMATION AND REPRODUCTION

Ovulation, menstruation, implantation, and parturition share cardinal features of an acute inflammatory response: vasodilation, increased vascular permeability, and cellular infiltration. Each process involves a tightly controlled phase of local tissue breakdown and regeneration. During ovulation, the wall of the preovulatory follicle and overlying ovarian surface epithelium are breached to release an oocyte for fertilization. Implantation involves invasion of the uterine wall by the embryonic trophoblast to initiate pregnancy. Menstruation, at the conclusion of each nonconceptual ovarian cycle, entails shedding of spent uterine endometrial and vascular tissues. And if pregnancy occurs, parturition culminates in softening of the uterine cervix to permit delivery of the neonate.

In every case, the inflammatory reaction unfolds as a biochemical cascade involving the sequential production of cytokines, lipid mediators (steroids, prostanoids, and leukotrienes), vasoactive mediators, and matrix-degrading proteolytic enzymes. The central role of cytokines is to control the direction, amplitude, and duration of the inflammatory response, thereby localizing and limiting the site of tissue injury and repair.

## II. PRO-INFLAMMATORY CYTOKINES

Cytokines serve classic functions in host responses to injury and infection. They also function in the immunoendocrine system during ovulation, implantation, menstruation, and parturition, as paracrine signals that promote tissue remodeling. Cytokines are commonly classified as pro- or anti-inflammatory, as shown in Table 1.

TABLE 1 Common Pro- and Anti-inflammatory Cytokines

| Pro-inflammatory | Anti-inflammatory |
|---|---|
| Interleukin-1 (IL-1) | IL-4 |
| Tumor necrosis factor-α of GR | IL-10 |
| Interferon-γ | IL-13 |
| IL-6 | Interferon-α |
| IL-8 | Transforming growth factor-β |
| IL-12 | |
| IL-18 | |
| Granulocyte/macrophage colony Stimulating factor | |

This classification is undoubtedly overly simplistic, since individual cytokines can have multiple, overlapping, and sometimes opposing functions depending on their concentration, site of action, and the presence of other cytokines and mediators. The best characterized pro-inflammatory cytokines in the female reproductive tract are interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor α (TNFα).

Interleukin-1 and TNFα signal via membrane-associated receptors on target cells to increase the expression of genes with roles in inflammation. The type I IL-1 receptor is a member of the Toll-like receptor (TLR) superfamily involved in signal transduction during inflammation and host defense. The superfamily includes the *Drosophila melanogaster* protein Toll, the IL-18 receptor, and the Toll-like receptors TLR-2 and TLR-4. Ligand binding of the corresponding receptor activates postreceptor signaling networks that in turn activate stress-related transcription factors (SRTFs), such as nuclear factor-κB (NF-κB) and activated protein-1 (AP-1) (Jun/Fos or Jun/Jun dimers), as illustrated for IL-1 in Fig. 1. AP-1 and NF-κB are important in immune and inflammatory responses because they localize the expression of genes encoding chemoattractants, cytokines, cytokine receptors, cell adhesion molecules, and matrix-degrading metalloproteinases (MMPs). They are also molecular targets of anti-inflammatory steroid action. Genes activated by AP-1 and NF-κB include the inducible cyclooxygenase isozyme COX-2, otherwise known as prostaglandin-H synthase-2, which catalyzes the formation of prostanoids essential for ovulation ($PGE_2$), implantation ($PGI_2$), and parturition $PGF_{2\alpha}$.

## III. ANTI-INFLAMMATORY STEROIDS

The endocrine connection between pro-inflammatory cytokines and steroid hormones is provided by glucocorticoids, which are produced in increased amounts through cytokine action on the adrenal glands as a systemic response to infection. In addition

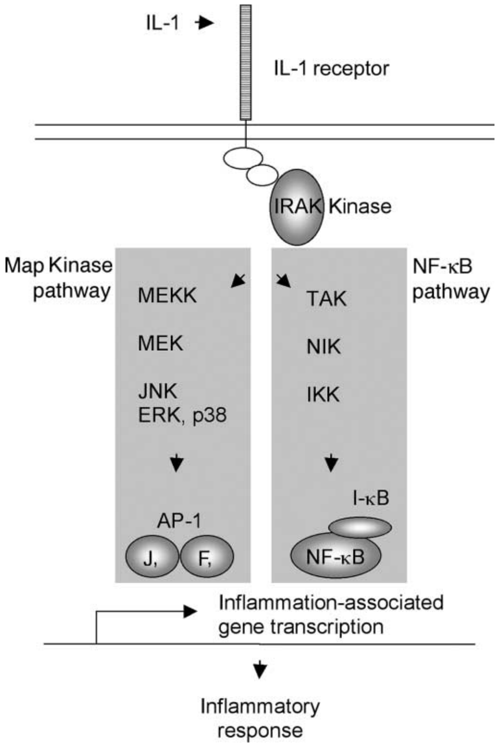

**FIGURE 1** Postreceptor mechanisms of inflammatory cytokine action. IL-1, the prototypical inflammatory cytokine, binds to the extracellular domain of the IL-1 receptor, which activates postreceptor signaling via IL-1 receptor-associated kinase (IRAK). IRAK kinases activate at least two major postreceptor signaling pathways, leading to activation of inflammation-associated gene transcription: mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways. ERK, Extracellular signal-regulated protein kinase; MAPK, p38; MEK, MAPK/ERK kinase; JNK, Jun N-terminal kinase; J, c-Jun; F, c-Fos; AP-1, activated protein-1; TAK, TGF-β-activated kinase; I-κB, inhibitory factor-κB; IKK, I-κB kinase; NIK, NF-κB-inducing kinase.

to classic roles in stress, injury, and nutrition, glucocorticoids exert anti-inflammatory effects via the ligand-activated glucocorticoid receptor (GR) signaling pathway. Mutually negative interactions between the GR and SRTFs underlie the immunosuppressive and anti-inflammatory actions of glucocorticoids.

Progesterone also has immunosuppressive and anti-inflammatory properties that can be explained by transcriptional interference between ligand-activated progesterone receptor (PR) and SRTFs.

## A. Glucocorticoid Activation

Reproductive tissues lack expression of key steroidogenic enzymes necessary for glucocorticoid synthesis. However, like most other tissues in the body, they express GR and are potential sites of glucocorticoid action. Glucocorticoids circulate in the blood in a binding equilibrium with corticosteroid-binding globulin, which sets the concentration of steroid available for interaction with the GR in target tissues. Glucocorticoid action is also influenced by metabolism through 11β-hydroxysteroid dehydrogenase (11β-HSD) activity in target tissues, which determines the relative availability of "active" cortisol or "inactive" cortisone for ligand activation of GR signaling (Fig. 2). Two 11β-HSD isoforms are known to exist in human tissues, both of which are microsomal enzymes belonging to the short-chain alcohol dehydrogenase/reductase superfamily. 11β-HSD type 1 is an $NADP^+$-dependent bidirectional enzyme with predominantly 11-oxoreductase activity, which therefore principally converts cortisone to cortisol. 11β-HSD type 2 (11β-HSD2) is an $NAD^+$-dependent enzyme with strong dehydrogenase activity, which inactivates cortisol to cortisone. Differential expression of 11β-HSD1 and 11β-HSD2 determines glucocorticoid tissue responsiveness. For example, the high level of 11β-HSD2 relative to 11β-HSD1 expressed in kidney promotes cortisol metabolism to cortisone and protects mineralocorticoid receptors from inappropriate occupation by cortisol. On the other hand, in liver, 11β-HSD1 converts cortisone to cortisol, ensuring that GRs are adequately exposed to cortisol.

Cells of tissues from throughout the body, including the reproductive tract, show increased activation of cortisone to cortisol when challenged with pro-inflammatory cytokines *in vitro*, suggesting a critical paracrine link between glucocorticoid activation and pro-inflammatory cytokine action.

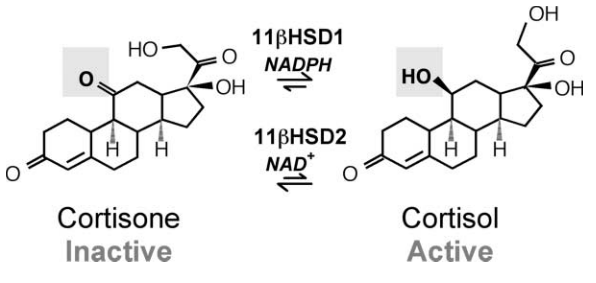

**FIGURE 2** Interconversion of cortisone and cortisol catalyzed by 11β-hydroxysteroid dehydrogenases (11β-HSDs). 11β-HSD1 is principally an 11-oxoreductase that reversibly converts "inactive" cortisone to "active" cortisol, which binds and activates glucocorticoid receptors. 11β-HSD2 is a strong 11-dehydrogenase, which inactivates cortisol to cortisone.

## IV. CYTOKINE–STEROID INTERPLAY

### A. Ovulation

Ovulation in women normally occurs halfway through each menstrual cycle, in response to stimulation of the preovulatory follicle(s) by gonadotropins. The midcycle luteinizing hormone (LH) surge initiates a cascade of biochemical changes in follicular cells and macrophages, leading to dissolution of the follicle wall and shedding of the oocyte. These changes include increased pro-inflammatory cytokine production, progesterone production, activation of COX-2, increased $PGE_2$ synthesis, histamine release, and increased proteolysis mediated by MMPs. Inhibition of progesterone biosynthesis or pharmacological blockade of the PR prevents ovulation. Mice with null PR mutations are unable to ovulate, demonstrating an absolute need for local progesterone. COX-2 inhibitors also prevent ovulation, and COX-2 and gene knockouts in mice confirm an absolute dependence on COX-2 activation during ovulation. Following follicular rupture, each bout of ovulation-associated damage to the ovary must be rapidly repaired. Inflammation—essential for wound healing—is contained and rapidly resolved. Locally produced progesterone and locally activated cortisol are believed to participate in this compensatory anti-inflammatory process.

Within the preovulatory follicle, a switch in the expression of 11β-HSD isoforms occurs during granulosa cell luteinization. Before induction of ovulation, nonluteinized human granulosa cells express mainly 11β-HSD2 mRNA and little or no 11β-HSD1 mRNA. After exposure to LH or human chorionic gonadotropin, 11β-HSD2 mRNA expression is suppressed and 11β-HSD1 mRNA expression is enhanced. This shift in potential for glucocorticoid metabolism from inactivation to reactivation is reflected in the ability of luteinizing human granulosa cells to undertake predominantly reductive (cortisone → cortisol) metabolism *in vitro* and substantially raised the concentrations of cortisol in follicular fluid. In experiments on cultured rat granulosa cells, treatment with IL-1β mimics the action of LH *in vitro*, up-regulating 11β-HSD1 and down-regulating 11β-HSD2. Treatment of cultured human ovarian epithelial cells with IL-1 also increases the gene expression of 11β-HSD1 and metabolism of cortisone to cortisol *in vitro*. Thus, cytokine-regulated 11β-HSD expression in the follicle wall and on the ovarian surface may determine the local availability of anti-inflammatory cortisol (Fig. 3).

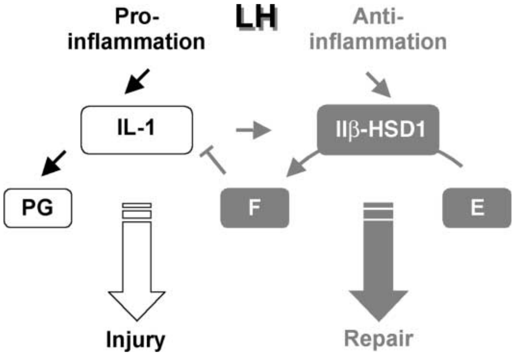

FIGURE 3 Proposed role of 11β-HSD1 in the resolution of LH-induced inflammation at the ovarian surface during ovulation. Ovulation is viewed as a natural injury-repair process. The ovulation-inducing LH surge induces an acute inflammatory response by increasing the local production of inflammatory cytokines (IL-1) and prostaglandins (PG), leading to proteolytic breakdown of the follicle wall and apoptosis of overlying ovarian surface epithelial cells. LH-induced IL-1 simultaneously up-regulates the expression of 11β-HSD1 in granulosa cells and OSE cells, serving to increase the metabolism of "inactive" cortisone (E) into anti-inflammatory cortisol (F). The increased local availability of cortisol is hypothesized to play a role in the resolution of this acute inflammatory event. Reprinted from Yong, P. Y. K., Harlow, C. R., Thong, K. J., Hillier, S. G. (2002). Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1. *Human Reproduction* **17** (**9**), 2300–2306. By permission of Oxford University Press.

### B. Menstruation

In the absence of pregnancy, progesterone withdrawal due to regression of the corpus luteum causes menstruation, which is associated with up-regulation of inflammatory mediators in the uterus (IL-8, monocyte chemoattractant peptide-1, and COX-2). Hypoxia due to arteriole vasoconstriction is coincident with progesterone withdrawal and in other body systems is a stimulus for IL-8 expression. IL-8 is not only a powerful leukocyte chemotaxin and secretagogue; it has also been shown to have important roles in neovascularization and mitogenesis and thus may be centrally involved in the local inflammatory and vascular events of menstruation.

The uterus expresses PR and the key components of the glucocorticoid response system, and there is evidence from experiments on rats that both activation of glucocorticoid through 11β-HSD enzyme activity and levels of GR are likely to have roles in modulating glucocorticoid action in the uterus. Although 11β-HSDs and GR are expressed in human endometrium, there are no data on regulation or expression in relation to hormonal status. High concentrations of glucocorticoids inhibit immunologic and inflammatory responses and also inhibit estrogen-stimulated epithelial proliferation. Glucocorticoids can down-regulate uterine estrogen receptors. In the rat, estradiol up-regulates both types of 11β-HSD. Furthermore, 11β-HSD2 mRNA has been localized to the stroma and stratum vasculare of the rat uterus. The physiological significance of the interaction between the GR and the 11β-HSD system in menstruation is as yet unexplored. It remains to be determined whether an altered availability of cortisol to bind to the GR (due to differential expression of 11β-HSD isoforms) is compensated for by activation of the PR by progesterone and vice versa.

### C. Implantation

Invasion of the uterine wall by trophoblastic tissue induces a natural inflammatory reaction, which must be contained to allow pregnancy to proceed. Maternal factors secreted into the lumen of the female reproductive tract as well as substances synthesized by the developing embryo itself help to regulate this process. Similar to ovulation, implantation involves increased local production of pro-inflammatory cytokines, dependence on progesterone, up-regulation of COX-2, and increased activity of MMPs that digest collagen as the uterus is penetrated.

During placentation, growth factors and cytokines produced by the placenta and decidual tissues accelerate the production of MMPs by trophoblasts. The release of IL-1β parallels the invasive potential of cytotrophoblasts, being produced in greater amounts by first-trimester cells than term cells. Inflammation-associated IL-1β production and MMP expression are decreased by glucocorticoid treatment *in vitro*. Thus normal trophoblast invasion may be regulated, in part, by the opposing actions of IL-1β and corticosteroids, since both are present in high concentrations at the maternal–fetal interface.

Human placental tissue is a rich source of 11β-HSD activity and a chronic suppressive action of glucocorticoid on cytokine production and nuclear binding of NF-κB and AP-1 proteins in human term placental cytotrophoblasts has been reported. This suggests a potential mechanism through which glucocorticoids may suppress inflammation at maternal–fetal interfaces across gestation.

### D. Parturition

Pro-inflammatory cytokines, prostaglandins, anti-inflammatory steroids, and matrix-degrading proteases directly participate in parturition. The pregnant uterus undergoes drastic remodeling during labor and the peripartum period, when the cervix becomes softened, effaced, and dilated to allow expulsion of the neonate. These changes to the cervix can be induced by mechanical trauma (as in "sweeping the membranes") or administration of prostaglandins, anti-progestins, or cytokines, such as IL-1 or IL-8. As in ovulation, menstruation, and implantation, MMPs, up-regulated by pro-inflammatory cytokines and prostaglandins, are instrumental to uterine and cervical tissue remodeling during parturition.

Anti-inflammatory steroids in turn participate in the cytokine- and prostaglandin-mediated mechanisms through which invading cells soften the cervix and initiate the natural onset of birth. Mifepristone, an anti-progestin that ripens the cervix when given during labor, is also a potent anti-glucocorticoid. Combined blockade of PR and GR by this substance presumably impedes ligand-activated anti-inflammatory signaling, thereby promoting the inflammation-associated process of cervical ripening.

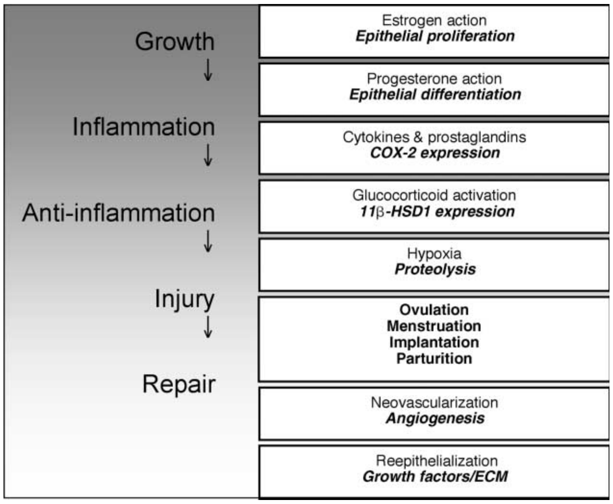

**FIGURE 4** Pro- and anti-inflammatory mechanisms common to ovulation, menstruation, implantation, and parturition. All four processes involve locally increased formation of inflammatory cytokines and prostaglandins. Systemic progesterone and glucocorticoids re-activated via local changes in 11β-HSD activity are believed to provide a compensatory anti-inflammatory response. Proteolytic tissue injury is thereby localized, minimized, and promptly healed.

The role of 11β-HSDs in regulating the availability of cortisol to participate in this process remains undetermined but 11β-HSD1 is richly expressed in amniotic membranes, where prostaglandins have been shown to increase the net conversion of cortisone to cortisol *in vitro*.

## V. SUMMARY

Locally produced pro-inflammatory cytokines promote inflammation-associated tissue injury and repair during ovulation, menstruation, implantation, and parturition. Each of these critical steps in reproduction depends absolutely on progesterone, which has anti-inflammatory properties. Furthermore, they all occur in regions of the reproductive tract where locally produced pro-inflammatory cytokines and prostaglandins have the potential to alter 11β-HSD enzyme expression, thereby influencing the local availability of cortisol (Fig. 4). Progesterone and cortisol activate anti-inflammatory signaling pathways through binding and activating the PR and GR, respectively. Both types of steroid may serve anti-inflammatory functions in the female reproductive tract, interacting with pro-inflammatory cytokines to localize and contain the natural inflammatory processes on which reproduction depends.

## Glossary

**corticosteroids** Steroid hormones secreted by the adrenal cortex that play crucial roles in nutrition, stress, and tissue responses to injury. In humans, cortisone and its synthetic analogues, such as prednisone and dexamethasone, are used therapeutically to control rheumatism and other inflammatory ailments.

**cytokines** Polypeptide messenger molecules that mediate cell-to-cell communication through binding to specific receptors on target cells and triggering postreceptor signaling pathways that alter cellular behavior. Cytokines are involved in reproduction, growth and development, injury repair, and the immunoendocrine system. Usually classified as interleukins, interferons, colony-stimulating factors, tumor necrosis factors, or growth factors, their classification is evolving. Interleukins are cytokines produced by leukocytes as part of the immune and inflammatory responses.

**inflammation** In higher organisms, a defense mechanism that protects the organism from infection and injury by localizing and limiting tissue damage, so that healing can begin. An inflammatory response lasting only a few days is called acute inflammation, whereas a response of longer duration is referred to as chronic inflammation.

**prostaglandins** Cyclic, unsaturated fatty acids derived from arachidonic acid, a phospholipid that is an integral component of the cell membrane. Inflammatory stimuli induce the rapid release of arachidonic acid, which is converted to prostaglandins, prostacyclin, and thromboxanes by cyclooxygenase (COX) enzyme activity. The inducible form of COX, COX-2, is mainly responsible for the local formation of the prostaglandins necessary for inflammation. Leukotrienes, produced from arachidonic acid due to lipoxygenase enzyme activity, are also important mediators of the inflammatory process, whereas thromboxanes and prostacyclin play roles in blood coagulation.

## See Also the Following Articles

**Anti-Inflammatory Actions of Glucocorticoids • Implantation • Interleukin-1 (IL-1) • Interleukin-18 • Ovulation • Tumor Necrosis Factor (TNF)**

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# Prolactin (PRL)

Nira Ben-Jonathan
*University of Cincinnati*

I. THE PROLACTIN GENE AND PROTEIN
II. PITUITARY AND EXTRAPITUITARY PRL
III. REGULATION OF PRL SYNTHESIS AND RELEASE
IV. PRL RECEPTORS AND SIGNAL TRANSDUCTION
V. BIOLOGICAL FUNCTIONS

**Prolactin (PRL) is a 23 kDa protein hormone that is produced and secreted by pituitary lactotrophs. Pituitary PRL synthesis and release are subjected to tonic inhibition by hypothalamic dopamine and are stimulated by many neuropeptides, steroid hormones, and growth factors. PRL binds to a single-span membrane receptor and exerts its action via several interacting signaling pathways. PRL is a multifunctional hormone that affects reproductive, developmental, osmoregulatory, and behavioral functions. In addition to the pituitary, PRL is produced by many tissues throughout the body and is concentrated from the blood into several cell types and fluid compartments. The heterogeneous nature of the PRL-producing cells together with the expression of PRL receptors by almost every tissue in the body supports the role of PRL as both a hormone and a cytokine and underlies its pleiotropic functions.**

## I. THE PROLACTIN GENE AND PROTEIN

The prolactin (PRL) gene is present as a single copy on human chromosome 6. PRL shares 40% homology with growth hormone (GH) and placental lactogen (PL), and the three hormones were derived by gene duplication from a common ancestral gene some 400 million years ago. Several proteins with similar structural features, generally named PRL-related or PRL-like proteins, are now included as members of the PRL/GH/PL gene family. The rat PRL gene is 10 kb in size and is composed of five exons. The mature mRNA is approximately 1 kb in length and encodes a 227-residue protein that includes a 28-residue signal peptide that is cleaved on entering the endoplasmic reticulum. A 2 to 2.5 kb sequence at the 5′-flanking region of the rat PRL gene (the proximal promoter) controls tissue-specific and hormone-regulated gene expression (Fig. 1). It is made of two distinct domains, a proximal region and a more distal enhancer, both of which are required for pituitary-specific expression. Eight *cis*-acting elements throughout the proximal promoter bind Pit-1, a homeobox transcription factor. Although Pit-1 is expressed only in the pituitary gland, it is not restricted to lactotrophs and requires interactions with other factors to confer the lactotroph phenotype.

The human PRL gene differs from the rat gene in several respects. It is composed of six, rather than five, exons and is more than 15 kb long. The extra noncoding exon, exon 1a, has a transcriptional start site 5.8 kb upstream of the pituitary start site (Fig. 1). In extrapituitary sites such as decidua, myometrium, and lymphoid cells, exon 1a is spliced to exon 1b, generating an mRNA transcript that is approximately 150 bp larger than the pituitary counterpart in the 5′-untranslated region. A superdistal promoter upstream of exon 1a regulates PRL gene expression in extrapituitary sites and is silenced in the pituitary gland. This region does not contain Pit-1-binding sites and its regulation differs from that of the proximal promoter.

The 23 kDa PRL protein is composed of a single chain of 199 residues. It has three intramolecular disulfide bridges between residues 4 and 11, 58 and 174, and 191 and 199, N- or O-linked glycosylation sites, and three phosphorylation sites. A three-dimensional model of PRL predicts that it is arranged in four anti-parallel α-helices organized in an "up-up-down-down" fashion. This bundle motif is shared with hematopoietic factors such as interferon and many interleukins. The 60 to 100% sequence homology between PRL molecules from different species reflects their phylogenetic relationship. Approximately 30 residues, clustered in four distinct regions, are highly conserved and may be important for binding to the receptor.

spatiotemporally in female reproductive tissues. However, the ratios of the individual isoforms vary in reproductive tissues as a consequence of developmental and hormonal status and during carcinogenesis.

Progesterone receptors have a modular protein structure consisting of distinct functional domains capable of binding steroidal ligand, dimerization of liganded receptors, interaction with hormone-responsive DNA elements, and interaction with co-regulator proteins required for bridging receptors to the transcriptional apparatus. Binding of progestin agonists to the hormone-binding domain induces conformational changes in receptor structure that promote the interaction of co-activator proteins with distinct activation function domains (AFs) located within both the amino- and the carboxy-terminal regions of the receptor. Such co-activators promote chromatin remodeling and bridging with general transcription factors, resulting in the formation of productive transcription initiation complexes at the receptor-responsive promoter. In contrast, binding of receptor antagonist compounds induces receptor conformational changes that render AFs nonpermissive to co-activator binding and instead promote interaction with co-repressor proteins that inhibit the transcriptional activity of the receptor. The ability of progesterone receptors to interact with a variety of co-activator and co-repressor proteins, together with the differing spatiotemporal expression of co-regulators, illustrates a key role of these proteins in mediating different tissue-specific responses of progesterone receptors to steroidal ligand. Importantly, receptors for progesterone can also be activated in the absence of steroidal ligand by phosphorylation pathways that modulate their interactions with co-regulator proteins.

The PR-A and PR-B isoforms differ in that the PR-B protein contains an additional sequence of amino acids at its amino-terminus that is not contained in PR-A. This PR-B-specific domain encodes a third transactivation function region (AF-3) that is absent from PR-A. Recent evidence has demonstrated that the presence of AF-3 allows binding of a subset of co-activators to PR-B that are not efficiently recruited by progestin-bound PR-A. Thus, when expressed individually in cultured cells, PR-A and PR-B display different transactivation properties that are specific to both cell type and target gene promoter context and are associated with the differential ability of PR-A and PR-B to recruit specific co-regulator proteins. Agonist-bound PR-B functions as a strong activator of transcription of several PR-dependent promoters and in a variety of cell types in which PR-A is inactive. Furthermore, when both isoforms are co-expressed in cultured cells, in cell and promoter contexts in which agonist-bound PR-A is inactive, the PR-A can repress the activity of PR-B. This repressor capability of PR-A also extends to other steroid receptors including estrogen receptor-α (ER-α). Finally, the PR-A and PR-B proteins also respond differently to P antagonists. Whereas antagonist-bound PR-A is inactive, antagonist-bound PR-B can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways. Although the sequences of the ligand-binding domains of PR-A and PR-B are identical, the ability of different ligands to induce different conformational changes in PR, together with the synergistic activity of the amino- and carboxy-terminal activation domains, predicts that PR-A or PR-B selective transcriptional regulation can be achieved by manipulating ligand interactions with the carboxy-terminal.

## III. PHYSIOLOGICAL ROLE OF PRs

Null mutation of the PR gene encoding both isoforms has provided evidence of an essential role of PRs in a variety of female reproductive and nonreproductive activities. Female mice lacking both PRs exhibit impaired sexual behavior, neuroendocrine gonadotropin regulation, anovulation, uterine dysfunction, impaired ductal branching morphogenesis, and lobuloalveolar differentiation of the mammary gland. PRs also play an essential role in the regulation of thymic involution during pregnancy and in the cardiovascular system through regulation of endothelial and vascular smooth muscle cell proliferation and response to vascular injury. Receptors for progesterone have also been identified in the central nervous system and bone, where progesterone has been implicated in both cognitive function and bone maintenance. However, the essential role of PRs in these regions has not yet been confirmed.

The more recent generation of novel mutant mouse strains in which either the PR-A (PRAKO) or the PR-B (PRBKO) isoform is selectively ablated has facilitated physiological analysis of the individual contributions of these proteins to the reproductive activities of progesterone.

## IV. PRs AND OVARIAN FUNCTION

Evidence that ovary-derived progesterone may participate in autocrine regulation of ovarian function first emerged with the demonstration that luteinizing hormone (LH), the primary signal for rupture of preovulatory ovarian follicles leading to ovulation, can stimulate the transient expression of PR mRNA and protein in granulosa cells isolated from preovulatory follicles and that the anti-progestin, RU486, can inhibit ovulation. Definitive proof that PRs are essential mediators of ovulation has been provided by analysis of the ovarian phenotype of the PRKO mouse. Despite exposure to superovulatory levels of gonadotropins, PRKO mice fail to ovulate. Analysis of the histology of these mice has revealed normal development of intraovarian follicles to the tertiary follicular stage. The follicles contain a mature oocyte that is fully functional when isolated and fertilized *in vitro*. However, follicular rupture is effectively eliminated. Despite the ovulatory block, the preovulatory granulosa cells within these follicles can still differentiate into a luteal phenotype and express the luteal marker, P450 side chain cleavage enzyme. Thus, PR is required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells to form a corpus luteum (luteinization). Follicular rupture requires induction of a prostaglandin-mediated inflammatory response to LH as well as tissue degradation at the apex of the preovulatory follicle, an event that is mediated by matrix-digesting proteinases. Recent investigations to examine the molecular events associated with ovulation that are mediated by PRs have shown that PRs are induced specifically in the mural granulosa cells of the mature tertiary follicle and are absent from the cumulus granulosa cells that surround the oocyte. Analysis of the expression of potential mediators of ovulation in PRKO mice has demonstrated that LH-induced regulation of COX-2, an enzyme that catalyzes the production of prostaglandins, is unaffected. COX-2 is required for ovulation and is expressed by cumulus granulosa cells. In contrast, the expression of two metalloproteinases, ADAMTS-1 (a desintegrin and metalloproteinase with thrombospondin motifs) and cathepsin-L (a lysosomal cysteine protease), is inhibited in granulosa cells of the mature follicle in PRKO mice. One of these proteases, ADAMTS-1, plays an essential role in ovulation and may represent a critical mediator of the progesterone-induced ovulatory event.

Both the PR-A and the PR-B proteins are induced in preovulatory follicles in response to LH stimulation. Stimulation of immature PRAKO mice with gonadotropins has shown that superovulation is severely impaired in these mice relative to their wild-type counterparts but, in contrast to the findings with PRKO mice, is not completely absent. In contrast, superovulation was unaffected in PRBKO mice expressing only the PR-A protein. Thus, PR-A expression is both necessary and sufficient to mediate the ovulatory response to progesterone.

Histological analysis of the ovaries of PRAKO mice showed numerous mature anovulatory follicles that contained an intact oocyte and were arrested at a stage similar to that previously observed in PRKO mice. Most surprisingly, however, in contrast to PRKO mice, the spatiotemporal regulation of ADAMTS-1 and cathepsin-L by progesterone was unaffected in these mice. Thus, despite its inability to mediate follicular rupture, the PR-B protein is functional in the ovary and capable of regulating a subset of progesterone-responsive target genes.

## V. PR ISOFORMS AND UTERINE IMPLANTATION

Female infertility in PRKO mice is also associated with defective uterine implantation and a lack of decidualization of uterine stromal cells in response to progesterone. Consistent with these findings, wild-type embryos failed to implant into the uterus when transferred into uteri of pseudo-pregnant PRKO females. Similarly, mating attempts between superovulated PRAKO females and wild-type males failed to result in successful pregnancies despite the release of small numbers of oocytes from PRAKO females. To determine whether the PRA protein is required for uterine decidualization, ovariectomized PRAKO mice were treated with progesterone and estrogen followed by mechanical stimulation of the left uterine horn of each animal in order to induce decidualization of stromal cells. Decidualization is associated with a marked increase in uterine weight and characteristic histological appearance associated with the differentiation of stromal cells into decidual cells. Both responses were inhibited in PRAKO mice, indicating that expression of the PRA protein in the uterus is required to mediate the decidualization response to progesterone.

The decidualization defect in PRAKO mice was also associated with aberrant regulation of progesterone-responsive target genes associated with implantation. Analysis of the regulation of three genes, calcitonin (CT), histidine decarboxylase (HDC), and amphiregulin (AR), whose expression is increased in the uterine epithelium in response to P in association with uterine receptivity and is abolished in PRKO mice showed that ablation of PR-A resulted in the loss of expression of CT and AR but the regulation of HDC was fully retained. These findings indicated that defective implantation in PRAKO uteri is associated with the loss of P-regulated expression of a subset of genes associated with uterine epithelial receptivity. Importantly, this differential target gene regulation by PR-B was not due to differences in spatiotemporal expression of PR-B relative to PR-A. The expression of PR-B in PRAKO mice showed the same pattern of intrauterine expression and regulation by estrogen as that observed in wild-type mice. Thus, the uterine defects observed in these mice are due to differences in the transcription factor activity of PR-B rather than to differences in the spatiotemporal expression of the protein relative to PR-A.

## VI. OPPOSING FUNCTIONS OF PR-A AND PR-B IN THE REGULATION OF UTERINE EPITHELIAL PROLIFERATION

Estrogen is the primary proliferative stimulus for uterine epithelium and its effects are inhibited by progesterone. Ablation of both the PR-A and the PR-B isoforms in PRKO mice results in marked hyperplasia of the lumenal and glandular epithelial tissue due to the unopposed action of estrogen. Selective ablation of PR-A, however, revealed an unexpected capacity of the PR-B protein to contribute to, rather than inhibit, epithelial cell proliferation. Treatment of PRAKO mice with estrogen alone induced epithelial hyperplasia in a manner similar to that observed in PRKO and wild-type mice. However, the addition of progesterone together with estrogen resulted in a marked increase in proliferation over that observed with estrogen alone, a response that was not observed in PRKO mice. These findings indicate that expression of the PR-B protein alone in the uterus results in a gain of proliferative activity. This acquisition of a proliferative activity of progesterone represents a PR-B-dependent gain of function not previously observed in the uterus, indicating that uterine expression of the PR-A isoform is required to oppose not only estrogen-induced proliferation but also that induced by progesterone acting through the PR-B protein.

The discovery that PR-B can contribute to, rather than inhibit, uterine epithelial cell proliferation is likely to have important clinical implications with regard to hormonal management of uterine endometrial dysplasias. Clearly, the relative expression of PR isoforms under these conditions will be an important determinant with regard to the effectiveness of progestin therapy. The results predict that progestin agonists selective for the PR-A protein should improve the effectiveness of progestin therapy for these conditions.

## VII. PRs AND MAMMARY GLAND DEVELOPMENT

Estrogen and progesterone are essential for the maintenance of postnatal developmental plasticity of the mammary gland and both hormones play a key role in mammary tumorigenesis. Null mutation of both PR isoforms in PRKO mice has demonstrated that PRs are specifically required for pregnancy-associated ductal proliferation and lobuloalveolar differentiation of the mammary epithelium. The mammary glands of PRKO mice failed to develop the pregnancy-associated side-branching of the ductal epithelium with attendant lobular alveolar differentiation despite normal postpubertal mammary gland morphogenesis of the virgin mice. Ablation of PR expression in these mice also resulted in a significantly reduced incidence of mammary tumor growth in response to carcinogen challenge. These observations underscore a specific role of PRs (as distinct from ERs) as obligate mediators of the intracellular signaling pathways that are essential for the initiation of murine mammary tumors induced by carcinogens.

The use of PRKO mice in combination with mammary gland transplantation techniques has provided important insights into the mechanisms underlying progesterone-dependent mammary gland morphogenesis. Throughout postpubertal mammary gland development, PRs are expressed exclusively in the epithelium. Consistent with these observations, tissue transplantation approaches using wild-type and PRKO mouse tissue to produce mammary gland recombinants that were devoid of PR in either the stromal or the epithelial compartments have provided strong support for the functional involvement of epithelial, rather than stromal, PRs in mediating mammary gland morphogenic responses to progesterone. The expression of PRs is localized to a scattered subset of epithelial cells throughout the ductal epithelium, the majority of which appear to be segregated from proliferating epithelial cells. The hierarchical organization of these receptors and their segregation from proliferating cells are conserved features in rodent and human mammary tissue. Such an expression pattern predicted that regulation of epithelial cell proliferation by progesterone may occur through a paracrine mechanism whereby PRs residing in nonproliferating cells induce the expression of a proliferative signal that promotes the proliferation of neighboring receptor-negative cells. Although PRKO mammary epithelium cannot undergo side-branching, mixing experiments with PRKO and wild-type epithelial cells demonstrate that the branching and differentiation defects can be overcome when PRKO cells are placed in close contact with $PR^+$ cells. Thus, although lacking $PR^+$ cells, the PRKO mammary epithelium still retains those $PR^-$ cells that are responsive to PR-mediated paracrine signaling. Recent attempts to uncover downstream mediators of the progesterone response have identified the secreted glycoprotein Wnt-4 as a potential PR target that is co-expressed in $PR^+$ cells, is regulated by P, and is essential for regulating ductal branching via paracrine regulation of proliferation.

Both isoforms of PR are expressed in the mammary gland of the virgin mouse and during pregnancy, although the levels of PR-A protein exceed those of the PR-B isoform by at least a 2:1 ratio in both cases. To examine the selective contributions of each isoform to the morphogenic responses of the mammary epithelium to progesterone, the morphology of mammary glands of ovariectomized wild-type, PRAKO, and PRBKO mice was compared after exposure to estrogen and progesterone. Ablation of PR-A in PRAKO mice did not affect the ability of PR-B to elicit normal progesterone responsiveness in the mammary gland. The morphological changes in ductal side-branching and lobular alveolar development in these glands were similar to those observed in wild-type mice. Thus, the PRB isoform is sufficient to elicit normal proliferation and differentiation of the mammary epithelium in response to progesterone and neither process appears to require functional expression of the A protein. In contrast, more recent analysis of the mammary glands of PRBKO mice under similar conditions has shown markedly reduced ductal side-branching, whereas lobular alveolar differentiation appeared to be unaffected. Thus, PR-B is the primary mediator of the proliferative response to progesterone, but both the PR-A and the PR-B proteins can provide the differentiative signals associated with alveologenesis.

## VIII. SUMMARY

Molecular dissection of progesterone signaling mechanisms using *in vitro* systems has demonstrated that the PR-A and PR-B proteins can respond to the same steroid ligand to induce both overlapping and distinct transcriptional responses that are promoter- and cell context-dependent. The use of genetically manipulated mouse models in which one or both of the PR isoforms are ablated has been pivotal in defining the physiological spectrum of progesterone receptor action as well as the contribution of the individual protein isoforms to the plieotropic activities of the hormone. These approaches have provided compelling evidence that the differences in the transactivation properties of the PR isoforms observed *in vitro* are reflected in a differential capacity to regulate the tissue-selective reproductive activities of progesterone.

## Glossary

**PRAKO** Progesterone receptor A isoform knockout.
**PRBKO** Progesterone receptor B isoform knockout.
**PRKO** Progesterone receptor knock-out.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen and Progesterone Receptors in Breast Cancer • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Implantation • Luteinizing Hormone (LH) • Ovulation • Oxytocin • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways**

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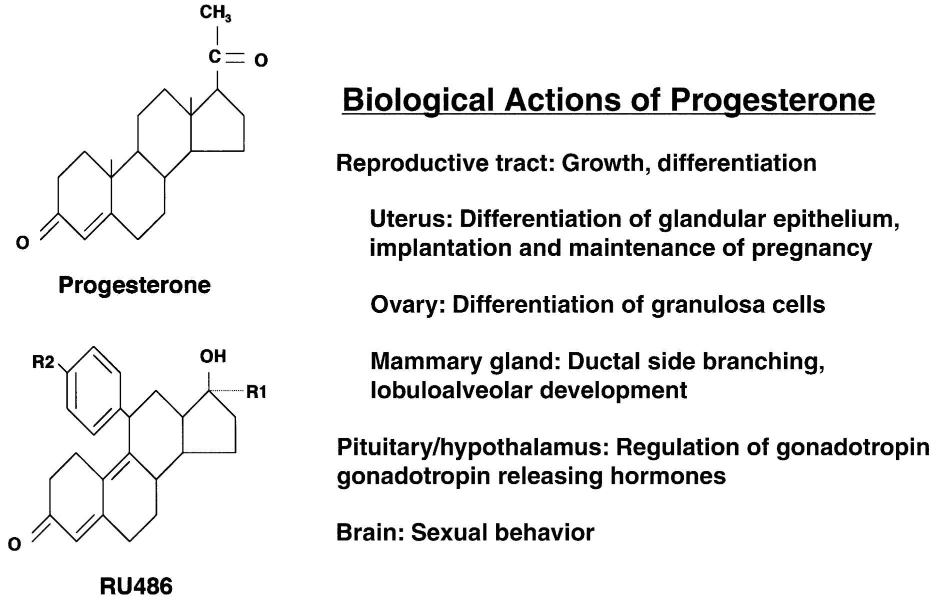
CH3
C = O
O
Progesterone
R2
OH
R1
O
RU486
Biological Actions of Progesterone
Reproductive tract: Growth, differentiation
Uterus: Differentiation of glandular epithelium, implantation and maintenance of pregnancy
Ovary: Differentiation of granulosa cells
Mammary gland: Ductal side branching, lobuloalveolar development
Pituitary/hypothalamus: Regulation of gonadotropin gonadotropin releasing hormones
Brain: Sexual behavior

FIGURE 1 Chemical structures of progesterone and the progesterone antagonist RU486 (Mifapristone) and the major biological actions of progesterone.

hormone receptor superfamily, including retinoid and thyroid hormone receptors, can actively silence gene transcription through recruitment of corepressors that recruit proteins with histone deacetylase (HDAC) enzyme activity. HDACs mediate effects opposite to those of HATs by promoting a condensation of nucleosome structure and impairing access of the general transcription machinery to the promoter.

## II. STEROID HORMONE RECEPTORS: GENERAL PROPERTIES

Members of the nuclear receptor superfamily share a similar domain organization consisting of a highly conserved DNA-binding domain (DBD) located in the central part of the molecule, a carboxyl-terminal ligand-binding domain (LBD), and an N-terminal domain that is the most variable region among

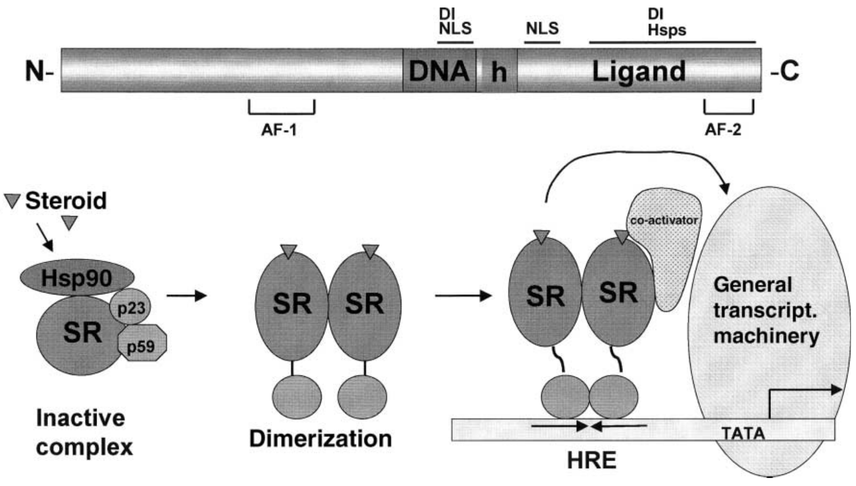
DI
NLS
NLS
DI
Hsps
N-
DNA
h
Ligand
-C
AF-1
AF-2
Steroid
Hsp90
p23
SR
p59
Inactive complex
SR
SR
Dimerization
co-activator
SR
SR
General transcript. machinery
TATA
HRE

FIGURE 2 General structural organization of steroid receptors as members of the nuclear receptor superfamily of ligand-dependent transcription factors, and activation mechanisms. DI, Dimerization domain; NLS, nuclear localization sequence; h, hinge; Hsps, heat-shock proteins; AF-1, AF-2, transcriptional activation domains; SR, steroid receptor; HRE, hormone response element.

superfamily members (Fig. 2). Three-dimensional atomic structures of isolated DBDs and LBDs have revealed common motifs for these regions. The core DBD contains two asymmetric zinc fingers, each with a zinc ion coordinated by four conserved cysteine residues. An α-helix extends between the two zinc fingers, which makes base-specific contacts in the major groove of HRE DNA. The LBD consists of 10–12 α-helices that fold into a three-layer α-helical sandwich containing a central core positioned between helix bundles on either side. This structure creates a hydrophobic wedge-shaped cavity in which the steroid hormone (ligand) is buried. By comparison, little is known about the structure of the N-terminal domain. Biophysical and biochemical data indicate that the N-terminal domain is in a non-globular extended conformation with little secondary structure. This is the least conserved region among superfamily members with respect to both length and amino acid sequence. The N-terminal domain is functionally important because it is required for full transcriptional activity of steroid hormone receptors and for many cell-specific and target gene-specific responses.

Other functional and structural determinants have been identified within these broader three domains. In addition to binding steroid hormone, the LBD contains determinants for dimerization (DI) in the absence of DNA, for binding of heat-shock proteins (Hsps), and for nuclear localization sequences (NLSs). The DBD contains a second NLS and a dimerization domain that is dependent on DNA binding. DNA-dependent dimerization stabilizes the receptor–DNA complex and facilitates orientation of the receptor dimer with the correct spacing of the HRE. Steroid receptors contain at least two transcription activation function (AF) domains. These are autonomous transferable domains required for the DNA-bound receptor to transmit a transcriptional activation response, and they function as specific binding sites for co-activators. AF-2, located in the LBD, is hormone dependent and becomes activated as a result of the steroid hormone inducing a repositioning of the C-terminal-most α-helix-12 in such a way as to create a specific hydrophobic binding pocket for members of the p160 family of steroid receptor co-activators (SRCs). Little is known about AF-1 in the N-terminus. It can function independently of AF-2 in a constitutive manner or can synergize with AF-2 in a ligand-dependent manner. The co-activators that bind to and mediate the activity of AF-1 are yet not well defined.

## III. PROGESTERONE RECEPTOR A AND B ISOFORMS

As a member of the nuclear receptor superfamily, the progesterone receptor (PR) shares the general structural domains, but has several unique features. In most species, PR is expressed as two isoforms, PR-A and PR-B. The exception is rabbits, which express only PR-B. In human tissues, PR-A is a truncated protein lacking the first 164 amino acids from the N-terminal domain; otherwise the two PRs have an identical amino acid sequence throughout the remainder of the protein, including the DBD and LBD (Fig. 3). PR-A and PR-B arise from a single gene by alternate transcription from two promoters. The two forms of PR have similar steroid hormone and DNA-binding activities, but they have distinct transcriptional activities due to differences in the N-terminal region. PR-A and PR-B are capable of forming heterodimers, and in the cell PR can exist in three molecular states of AA, AB, and BB dimers. This ability to produce three molecular forms of PR from a single gene expands the functional range of activities of the receptor without the need for a separate receptor subtype gene. The relative expression of PR-A and PR-B is regulated in a tissue-specific manner and by physiological conditions. The ratio of PR-A to PR-B varies significantly in a regular pattern in the uterus during the menstrual cycle; in some breast tumors, very high PR-A:PR-B ratios have been detected. In normal breast tissue, the ratios are close to 1:1, whereas PR-A appears to be the predominant form of receptor in endometriosis. Thus, a difference in relative expression of PR-A and PR-B is one way for a tissue to regulate response to progesterone. The two promoters responsible for expression of the PR isoforms are estrogen responsive. However, the gene region for these promoters is complex and has potential regulatory sites for multiple other factors, and is not likely to be regulated by estrogen alone.

The transcriptional activities of the two PR isoforms vary, depending on the cell type and the context of the target gene promoter. In general, on classical progesterone response element (PRE) targets, PR-B is a much stronger activator compared to PR-A. However, PR-A can be a strong activator under specific cell and target gene contexts. The stronger activation potential of PR-B is due in part to the existence of a third activation domain (AF-3) within the first N-terminal 164 amino acids that is unique to PR-B (Fig. 3). However, AF-3 is not an autonomous activation domain capable of activating transcription when linked to a heterologous DBD. AF-3 functions

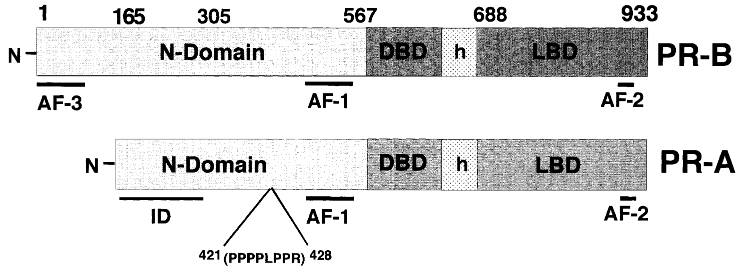

FIGURE 3 Domain organization of human progesterone receptor A and B isoforms (PR-A and PR-B). AF-1, AF-2, AF-3, Transcriptional activation domains; DBD, DNA-binding domain; h, hinge region; LBD, ligand-binding domain; ID, inhibitory domain.

only when linked to the PR DBD, and co-activators that interact with AF-3 have not yet been identified. Thus, AF-3 is thought to facilitate activity of AF-1 and AF-2 through intramolecular domain interactions. In support of this idea, the N- and C-terminal domains of PR (both A and B isoforms) are capable of directly associating with each other in a hormone agonist-dependent manner, but these interactions are more efficient for PR-B than for PR-A.

Under certain cell and target promoter contexts, PR-A is inactive as a transcription factor and can function as a ligand-dependent transdominant repressor of other steroid receptors, including PR-B, estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). PR-A can act in this repressor mode in response to binding either progestin agonists or antagonists. An inhibitory domain (ID) responsible for this transrepressor function has been mapped to the first 140 N-terminal (165–305) amino acids of PR-A (Fig. 3). The ID is functional and transferable to other steroid receptors, such as chicken PR and human ER, which do not exhibit this trans-repressor activity. Truncation of the ID from PR-A increases its transcriptional activity to the level of PR-B. The sequence within the ID is present in both PR isoforms but is active only in the context of PR-B, suggesting that a role of the PR-B-specific N-terminal segment is to suppress the activity of the ID. This is thought to occur through the PR-B N-terminal segment exerting a long-distance effect on the conformation of the PR-A N-terminus.

How PR-A can repress transcriptional activity of other steroid receptors remains unclear. This property does not involve the phenomenon of transcriptional squelching of a common limiting cofactor for PR-B and other steroid receptors. Instead, PR-A and PR-B exhibit a different ability to interact with coregulatory proteins. PR-A interacts more efficiently with the silencing mediator of retinoid/thyroid (SMRT) receptor corepressor than does PR-B, and this difference requires the inhibitory domain of PR-A. Conversely, PR-B interacts more efficiently with members of the SRC family of co-activators than does PR-A. Thus, the A isoform of PR may recruit a distinct coregulatory protein complex to promoters that contains corepressors and is functionally inhibitory to other DNA-bound complexes.

Studies with PR isoform-specific gene knockout mice and transgenic mice that overexpress either PR-A or PR-B have provided evidence that the two forms of PRs have distinct physiological roles *in vivo*. Selective knockout of PR-A in mice has a strong phenotype in the uterus but not in the mammary gland, suggesting that the PR isoforms have tissue-specific roles. Overexpression of PR-A or PR-B results in abnormal mammary gland development, but the phenotypes of the two transgenic mouse lines are not the same.

Transcription factors that harbor both activation and repression domains, or are expressed as truncated forms capable of functioning as dominant transrepressors, have been identified in several different families of transcription factors. Such factors include the Id protein of the MyoD transcription factor family, the jun dimerization proteins (JDP-1 and JDP-2) of the AP-1 (fos/jun) family, and isoforms of the basic-region leucine zipper (bZIP) containing activating transcription factor-2 (ATF-2) and CCAATT/ enhancer-binding protein (C/EBP) transcription factors. These naturally occurring transrepressors have important physiological roles in shutting off activation responses at specific times during development and differentiation or under specific physiological conditions. Among the steroid hormone receptors, PR-A has been suggested to have a similar role that may be particularly relevant in the uterus, in which progesterone is known to antagonize the growth-stimulatory activity of estrogen.

## IV. PHOSPHORYLATION OF PR

As with other steroid hormone receptors, PR in human tissues is phosphorylated on multiple serine/threonine residues in a highly specific and hormone-regulated manner. Specific sites of phosphorylation on the PR *in situ* (PR expressed in mammalian cells) that have been confirmed by peptide sequencing are shown in Fig. 4. Most of the sites are located throughout the N-terminal domain; five are unique to PR-B whereas the others are within the N-terminal domain in common with PR-A and PR-B. The exception is phosphorylation of Ser-676 within the hinge region between the DBD and LBD. The PR phosphorylation sites are classified as basal and hormone dependent. Three hormone-dependent sites (Ser-102, Ser-294, and Ser-345) are unphosphorylated in the absence of ligand and become fully phosphorylated in response to hormone *in situ*. Some of the remaining sites are basally phosphorylated without hormone and increase phosphorylation rapidly within 5–10 min after hormone treatment of cells. The hormone dependence of the other sites has not been determined. Most of the phosphorylation sites reside within Ser-Pro motifs and there are at least three kinases capable of phosphorylating human PR, including the cyclin-dependent kinase CDK-2/cyclin A (Ser-130, Ser-162, Ser-190, Ser-213, Ser-400, and Ser-676), mitogen-activated protein kinase (MAPK; Ser-294), and casein kinase II (Ser-81). The kinases that phosphorylate the hormone-dependent sites at Ser-102 and Ser-345 and basal sites at Thr-430, Ser-554 have not yet been identified. The fact that subsets of sites are substrates for different kinases suggests that these groups of phosphorylation sites have distinct roles in PR structure/function and are regulated by different signaling pathways.

The function of PR phosphorylation has not been well defined. Analysis of various phosphorylation site mutants (serine to alanine substitutions) reveal no effect of phosphorylation on PR–DNA binding or steroid-binding activities. However, up to a 50% decrease in hormone-dependent transcriptional activity is observed with two mutants, Ser to Ala-190 and Ser to Ala-676. Other mutations have little to no effect on transcriptional activity of the PR. The Ser-294 phosphorylation site has been reported to be a signal for hormone-dependent down-regulation of the PR that targets the receptor for degradation by proteasomes. The PR from chicken oviduct (cPR) is also phosphorylated on multiple serine/threonine residues in the N-terminal domain and on a single site in the hinge. Phosphorylation site mutations in the N-terminal domain of cPR result in as much as a 75% reduction in transcriptional activity *in situ*, whereas mutation of the hinge region site reduces the hormone sensitivity of cPR-mediated transcription without altering steroid binding affinity. The magnitude of the effect of these phosphorylation site mutations on PR activity varies and is dependent on cell and target gene promoter context, suggesting that phosphorylation has a role in modulating PR interactions with other proteins for which expression may be cell type or target gene specific. Thus, phosphorylation does not appear to be a regulatory on/off switch but seems to have a more subtle role in

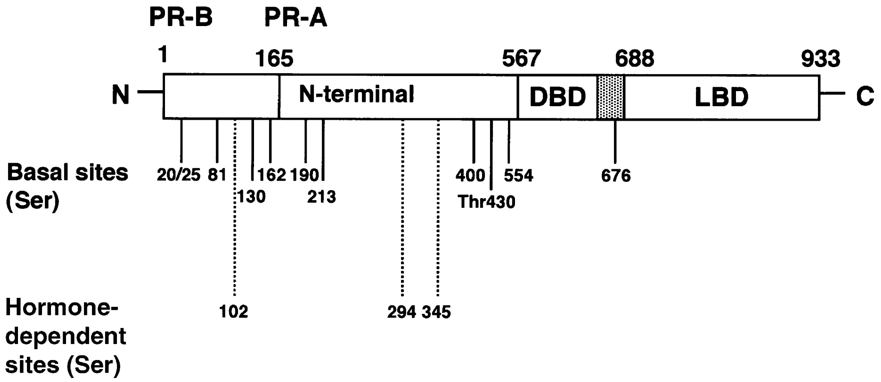

FIGURE 4 Phosphorylation sites of the human progesterone receptor. All sites are on serine residues, with the exception of position 430 (threonine). PR-A, PR-B, Progesterone receptor isoforms A and B; DBD, DNA-binding domain; LBD, ligand-binding domain.

modulating different functional activities of the PR. Additionally, phosphorylation could have a structural role in stabilizing the folded state of the receptor, in particular the N-terminal domain, which is fairly devoid of secondary structure. A physical mapping of the surface structure of the N-terminal domain of the PR by limited proteolysis shows that protease-accessible sites are limited to phosphorylation sites, suggesting that these sites are surface exposed and may stabilize domain interactions or folding within the N-domain.

## V. PR CROSSTALK WITH OTHER TRANSCRIPTION FACTORS

Steroid receptors can regulate transcription of genes that lack HREs as a primary response through protein-protein interactions with other DNA-bound transcription factors (Fig. 5). Although this mode of regulation can be either positive or negative, it is more commonly a pathway for negative gene regulation by steroid receptors, and in some cases a mutual repression between steroid receptors and the other transcription factor has been observed. Genes that contain composite response elements consisting of a less than optimal DNA-binding site for the steroid receptor (often a HRE half-site) that overlaps, or is adjacent to, a good binding site for another sequence-specific transcription factor are a variation of this mode of regulation. Regulation of composite elements often involves both receptor–DNA and receptor–protein interactions with other transcription factors. The ability to enhance the activity of other transcription factors arises through recruitment of receptor transcription activation domains; in essence, the receptor acts as a co-activator. Negative regulation can occur via the steroid receptor interfering with activation domains of other transcription factors or competing for DNA-binding sites. Examples of negative PR crosstalk with other transcription factors are repression of nuclear factor κB (NF-κB) activity (through interaction with RelA/p65 subunit), interference with the dioxin arylhydrocarbon receptor (AhR) signaling, inhibition of prolactin-induced Stat5-mediated activation of the β-casein gene, and repression of AP-1 (fos/jun) activity. Not all crosstalk with the PR results in repression; unliganded PR can enhance AP-1 in human endometrial carcinoma cells, although addition of progesterone reverses this enhancement. This positive crosstalk between PR and AP-1 appears to be cell type specific suggesting that other cellular factors are required. Because NF-κB is activated by various cytokines, crosstalk with the PR is thought to be involved in the immunosuppressive effects of progesterone during pregnancy. Progesterone receptor crosstalk with AP-1 and Stat5 is thought to be involved in proliferative and differentiation functions of progesterone, respectively, in the mammary gland and uterus.

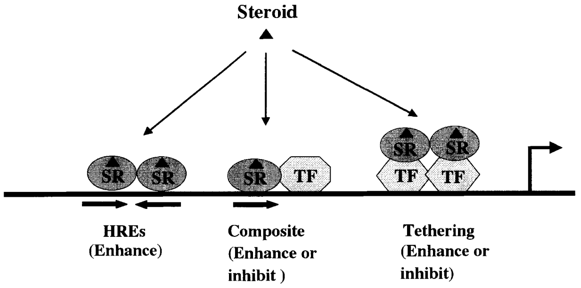

FIGURE 5 Different mechanisms by which steroid receptors activate or inhibit gene transcription as a primary gene regulation response. Left: Direct binding of the steroid receptor (SR) to hormone response elements (HREs) within promoters of steroid-responsive genes. Middle: Composite element consisting of a weak HRE half-site and a neighboring optimal site for another sequence specific transcription factor (TF). Steroid receptors can either enhance or inhibit transactivation mediated by the other transcription factor. Right: Tethering response element. Steroid receptor interacts with another DNA-bound transcription factor through a protein–protein interaction to either enhance or inhibit transactivation.

## VI. PR CROSSTALK WITH CELL SIGNALING PATHWAYS

Steroid receptors as ligand-dependent transcription factors and cell membrane/cytoplasmic signal transduction pathways have traditionally been viewed as completely separate pathways for regulating gene expression in response to external signals. However, it was discovered in the early 1990s that steroid receptors are nuclear targets of certain signal transduction pathways, suggesting a convergence of these two major pathways. Modulation of protein kinases or phosphatases in mammalian cells can either activate steroid receptors in the absence of ligand or potentiate ligand-dependent activity of receptors. Crosstalk with other signal transduction pathways was first reported with cPR and subsequently with human PR and all classes of steroid receptors. Agents that elevate intracellular cAMP (8-bromo-cAMP) to activate protein kinase A (PKA), inhibitors of protein phosphatases 1 and 2A (okadaic acid), and natural peptides that activate membrane receptor-linked signal transduction pathways, including the neurotransmitter dopamine and epidermal growth factor (EGF), can all activate cPR in the absence of progesterone. Although the human PR is not activated in the absence of ligand, 8-bromo-cAMP, okadaic acid, EGF, and activators of protein kinase C, all stimulate hormone-dependent PR transactivation in different cell lines, including breast cancer cells. Activation of cPR in the absence of progesterone by these agents and potentiation of human PR activity in the presence of ligand are not accompanied by changes in receptor phosphorylation. The lack of effect on direct PR phosphorylation suggests that receptor-interacting co-activators are the targets of phosphorylation by these different protein kinase signaling pathways. As evidence for this idea, SRC-1 becomes phosphorylated on two specific MAPK sites (Ser-1179 and Ser-1185) in response to activation of PKA pathways. This phosphorylation does not facilitate direct SRC-1 interaction with the PR; rather, its role is to modulate functional cooperation between SRC-1 and CREB-binding protein (CBP), which is required for activity of a larger multiprotein SRC co-activator complex (Fig. 6).

A reverse crosstalk was recently discovered between human PR and cell membrane/cytoplasmic signaling pathways. The N-terminal region common to PR-A and PR-B contains a short, contiguous polyproline-rich sequence (amino acids 421–428, written as PPPPPLPR using the single-letter code, where P is proline, L is leucine, and R is arginine) that conforms to a consensus type II motif for binding the Src homology domains (SH3) of cell membrane/cytoplasmic signaling molecules. These polyproline sequences form a left-handed helix conformation that interacts with a binding pocket of SH3 domains. PR interacts *in vitro* and in cells with SH3 domains of various signaling molecules. including c-Src tyrosine kinases. This interaction is stimulated by progestins and is mediated directly by the proline-rich motif in the N-terminus of PR. The consequence of this interaction is a rapid progesterone-dependent stimulation of Src kinase enzyme activity through PR-mediated displacement of an intramolecular SH3 domain interaction that maintains Src kinases in an inactive state. Point mutations in the proline-rich motif that abolish progestin-induced activation of Src do not affect the transcriptional activity of PR. Conversely, point mutations in the DBD or AF-2 that cripple PR as a transcription factor have no effect on the ability of PR to mediate progestin activation of Src kinase. This PR–SH3 domain interaction is of biological consequence. Progestins can rapidly and transiently (10 min) activate the entire Src/ras/MAPK pathway in mammalian cells in a manner that is dependent on the integrity of the proline-rich–SH3 domain interaction motif within the PR. Also, PR–SH3 domain interactions contribute to two known

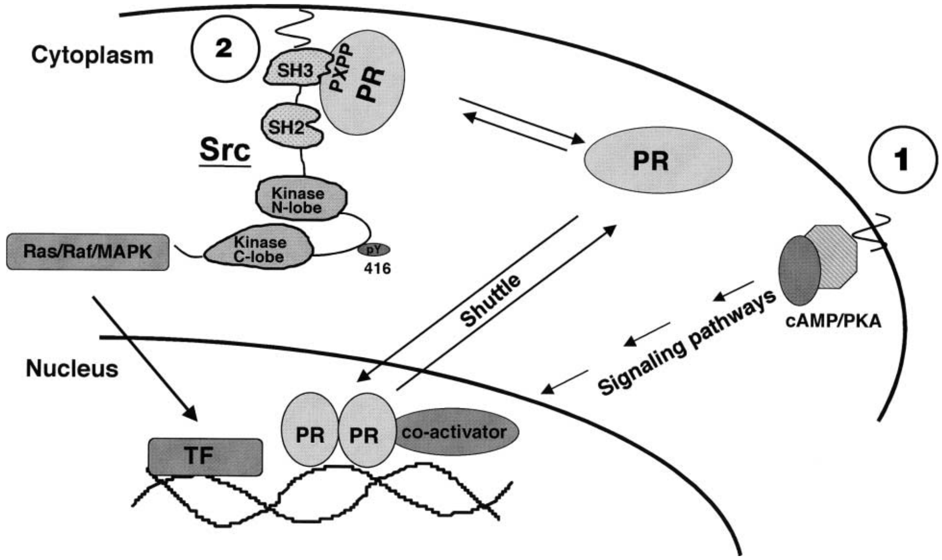

**FIGURE 6** Two-way crosstalk between the progesterone receptor (PR) and cell signal transduction pathways. Cell signal transduction pathways can potentiate the transcription activity of nuclear PRs through phosphorylation of receptor-interacting co-activators. (1) Phosphorylation enhances the transcription activity of the receptor–co-activator complex. (2) The PR can modulate other cell-signaling pathways through a direct interaction in the cytoplasm with regulatory SH3 domains of signaling molecules, including Src kinases. This extranuclear function of the PR is rapid (5–10 min) and is not dependent on transcription.

biological responses of progesterone, including inhibition of proliferation of normal breast epithelial cells and induction of meiosis in *Xenopus* oocytes. These data suggest that the PR is a dual-function protein capable of directly interacting with target DNA in the nucleus in its well-established role as a transcription factor, and also interacting with SH3 domains to modulate cytoplasmic signaling pathways (Fig. 6). Although the function of the PR is traditionally thought to take place in the nucleus, it rapidly shuttles between the cytoplasm and nucleus by active nuclear import and export mechanisms. Thus, the PR has the opportunity to encounter signaling molecules in the cytoplasm and to have extranuclear functions.

## VII. PROGESTERONE ANTAGONISTS

Several steroid analogues of progesterone that have been developed function as potent PR antagonists. The most important antagonist clinically is RU486 (Mifapristone), which is used as an antifertility agent and in experimental treatment of diseases such as brain meningiomas, endometriosis, uterine fibroids, and breast cancer. The structural features of RU486 that confer antagonist activity are the aromatic ring at the 11β-carbon position and the side chain at the carbon-17 position of the steroid ring structure of progesterone (see Fig. 1). RU486 competes with progesterone for binding to the PR and has a higher affinity for the receptor than does the natural hormone. RU486 binding effectively inactivates the PR by a complex mechanism. The receptor activation steps of dissociation from the sequestering Hsp complex, dimerization, and binding to PREs are not impaired when the PR is occupied by RU486. PR interaction with DNA, however, is nonproductive as a result of RU486 inducing a conformation in the carboxyl-terminal tail of the PR that is distinct from that induced by hormone agonist. This alternate conformation inactivates AF-2 and does not permit interaction with co-activators. However, RU486 is a more potent antagonist than is predicted by a simple inactivation of AF-2 of those PR molecules occupied by RU486. First, PR bound to RU486 is capable of heterodimerization with PR bound to hormone agonist and of inhibiting the activity of the agonist-bound PR *in trans*. Thus, effective inhibition of PR bound to agonist requires less than stoichiometric amounts of RU486. Additionally, the alternate conformation of PR induced by RU486 results in a substantial enhancement of corepressor binding to PR. Thus, in the presence of RU486, PR is capable of actively repressing gene transcription.

As with most steroid antagonists, RU486 is not a pure antagonist and exhibits partial agonist/antagonist activity, dependent on physiological conditions. PR crosstalk with cAMP and protein kinase A signaling pathways results in a dramatic potentiation of the agonist activity of RU486. This antagonist-to-agonist switch appears to be fairly specific to crosstalk with cAMP/PKA signaling pathways. Activation of protein kinase C and growth factor pathways does not cause this functional switch. Only the B receptor responds to cAMP in this manner, suggesting a requirement for both AF-3 and AF-1 together for RU486 to act as an agonist. The mechanism by which the cAMP/PKA pathway potentiates the agonist activity of RU486 involves a decreased association of corepressors (NcoR and SMRT) with PR. However, dissociation of corepressor alone is not sufficient for PR activation. Because AF-2 becomes inactivated by RU486, recruitment of an N-terminal domain co-activator would also be required, but such a co-activator has not yet been identified. Thus, the relative agonist/antagonist activity of RU486 is thought to be a reflection of the balance in the cell between expression and availability of specific N-terminal co-activators and corepressors. This concept is of potential relevance to the clinical use of RU486 and other steroid antagonists and could explain the variable efficacy of steroid antagonists in clinical settings, especially in the treatment of breast cancer.

## VIII. SUMMARY

Many biological responses to progesterone are mediated through the PR interacting directly with target DNA, thus acting as a transcription factor, or through the PR interacting with other DNA-bound proteins, thus acting as a transcription cofactor. Transcription activities of PR can be further modulated by variable expression of the A and B isoforms and through crosstalk with other signal transduction pathways. PR-A may also function as a naturally occurring repressor of other members of the steroid hormone group of nuclear receptors. The progesterone receptor also has rapid nontranscription functions through its ability to interact directly with SH3 domains and to activate cytoplasmic signal transduction pathways.

## Glossary

**DNA-binding domain** Region of steroid receptors that makes specific contact with DNA; has a conserved structural motif among members of the nuclear receptor family and can function autonomously.

**hormone response elements** Specific DNA sequences in the promoter region of steroid-responsive genes; bind and mediate transcriptional responses to steroid receptors.

**ligand** In the context of the nuclear receptor superfamily, a small lipophilic molecule, in some cases a steroid hormone, that binds to the ligand-binding domain of a receptor.

**ligand-binding domain** Region of receptor in the C-terminus that binds steroid hormone; has a conserved structural motif and can function autonomously.

**progesterone response elements** Hormone response elements that are specific for the progesterone receptors.

**SH3 domain** Src tyrosine kinase homology domain 3; a conserved regulatory region of many signaling molecules that interacts with other proteins.

**steroid receptor co-activators** Family of proteins of 160,000 molecular weight (p160); interact with transcriptional activation domains of nuclear hormone receptors and act as bridging factors between the DNA-bound receptor and the general transcriptional machinery.

**transcriptional activation domain** Region of steroid receptors that binds co-activators and mediates transcriptional enhancement activity.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Crosstalk of Nuclear Receptors with STAT Factors • Estrogen and Progesterone Receptors in Breast Cancer • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • Progesterone Action in the Female Reproductive Tract • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Pro-inflammatory Cytokines and Steroids

Stephen G. Hillier
*University of Edinburgh*

I. INFLAMMATION AND REPRODUCTION
II. PRO-INFLAMMATORY CYTOKINES
III. ANTI-INFLAMMATORY STEROIDS
IV. CYTOKINE–STEROID INTERPLAY
V. SUMMARY

**Pro-inflammatory cytokines produced by somatic cells and activated macrophages are mediators and modulators of ovulation, menstruation, implantation, and parturition. Each of these hormonally regulated processes is**

investigations showed that GHR and PRLR must be dimerized to properly transmit signals, which allowed the design of potent PRL and GH antagonists that interfered with the efficient dimerization of these receptors. Until the beginning of the 1990s, however, the downstream mechanisms involved in signal transmission by PRL and GH receptors were only poorly elucidated, and not until the discovery of the Janus tyrosine kinase (JAK) and signal transducers and activators of transcription (STAT) factor families was the JAK/STAT pathway identified as the first major signaling cascade responsible for the effects of PRL and GH. Within the past 6 years, there have been numerous reports describing molecules involved in or interacting with known pathways or even identifying new pathways/molecules.

This article is divided into four sections aimed at elucidating the molecular basis of signaling properties of these receptors: (1) the structure–function relationships of naturally occurring receptor isoforms; (2) the mechanism of ligand-induced receptor activation; (3) the major signaling pathways; and finally (4) the ways in which animal models (knockout, transgenics) can correlate with and highlight molecular studies of PRLR/GHR signaling.

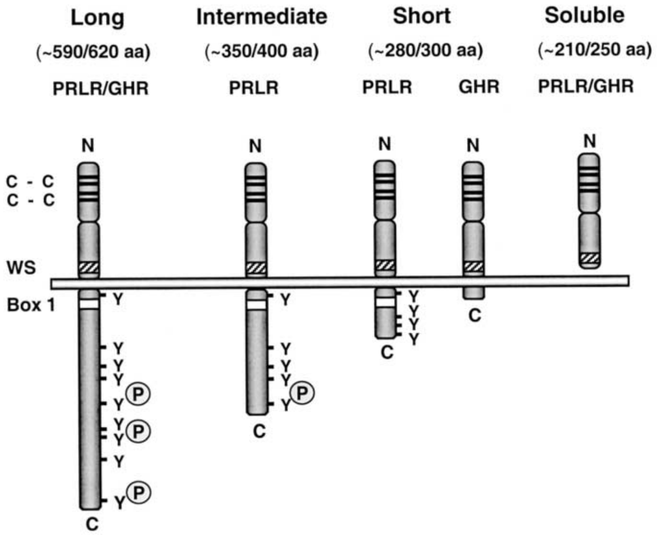
## II. PRLR AND GHR ISOFORMS HAVE DIFFERENT SIGNALING CAPABILITIES

PRLR and GHR are single-pass transmembrane receptors with the N-terminal outside the cell (the ligand-binding domain) and the C-terminal inside the cell (the signaling domain). The three structural features conserved among hematopoietic cytokine receptors are found in both the PRLR and the GHR: two pairs of disulfide-linked cysteines in the N-terminal part of the extracellular domain (C12–C22 and C51–C62 in hPRLR), the typical "WS motif" (a double W-S repeat, conservatively mutated into Y-G-E-F-S in the GHR) in the membrane-proximal region of this domain, and finally, the proline-rich region (called Box 1) in the juxtamembrane region of the cytoplasmic domain (Fig. 1). The first two features are required for correct folding and therefore for the functioning of the extracellular domain (including ligand binding and receptor trafficking to the cell surface), whereas Box 1 is required for triggering (all) signaling cascades (see below). These conserved features are thus essential for the PRLR and GHR to elicit their activities.

One of the characteristics of cytokine receptors is that alternative splicing of the primary transcript (a single gene exists for each receptor) leads to the occurrence of multiple protein isoforms, which in most cases are not correlated with pathological states. These isoforms differ mainly in their cytoplasmic tail, which affects their signaling properties. The classical isoform, called the "long" form, contains ~600 amino acids (591 for hPRLR and 620 for hGHR) and is considered to elicit all the actions attributed to the ligand. The "short" forms are truncated at their C-terminal and lack part of, or almost all of, the cytoplasmic tail. In rat, the short PRLR contains 291 amino acids, and in human cells, a 288-residue PRLR isoform has been recently described. All PRLR isoforms identified thus far from natural sources contain Box 1. Regarding the hGHR, two short isoforms containing 277 or 279 residues and lacking Box 1 have been identified. These short GHRs are thus devoid of signaling properties and act as dominant negatives of the full-length receptor by trapping the ligand and/or by forming inactive heterodimers with long receptor isoforms (see below). Intermediate forms have also been described, such as in rat Nb2 lymphoma cells (393 amino acids, due to a deletion in the PRLR gene) or in human cells (376 amino acids, due to alternative splicing). At least for the Nb2 receptor, none of the major signaling properties displayed by the full-length receptor are affected by this deletion. Finally, soluble receptors corresponding to the extracellular domain of membrane receptors have been identified; they originate either from alternative splicing or from limited proteolysis of full-length receptors (Fig. 1). As deduced from cloned cDNAs encoding these binding proteins (BPs), their overall length is 246 amino acids for the hGHBP and 206 for the PRLBP. Soluble receptors are intrinsically devoid of intrinsic signaling activity, although they may be indirectly involved in PRL/GH functions by controlling the levels of ligand available (BPs increase the hormone's half-life).

## III. LIGAND-INDUCED RECEPTOR DIMERIZATION: THE FIRST STEP OF SIGNALING

The extracellular domain is the ligand-interacting region of these receptors. The three-dimensional structure of genetically engineered hPRLR and hGHR extracellular domains has been determined by crystallographic analysis (Fig. 2A). Not only are the GHR and PRLR extracellular domains structurally related (they fold in two antiparallel β-sheets),



| Signaling properties | Long | Intermediate | Short *Box 1* | *No Box 1* | Soluble |
|---|---|---|---|---|---|
| -Independent of receptor phosphorylation | Active | Active | Active | Inactive, dominant negative | Inactive |
| -Dependent on receptor phosphorylation | Active | Active | Inactive, dominant negative | Inactive, dominant negative | Inactive |

**FIGURE 1** Schematic representation of the PRLR and GHR isoforms. The PRLR and GHR exist in various isoforms that differ only in their cytoplasmic tail regions. The four types of receptor isoforms, referred to as long, intermediate, short, or soluble with respect to their overall length, are illustrated. The conserved features among cytokine receptors are indicated: C–C, internal disulfide bonds; WS, for the Trp–Ser repeat, hatched box; and Box 1, for the proline-rich region, white box. "Y" represents tyrosines (number and position of tyrosines are random) of the cytoplasmic domain, some of which are phosphorylated (denoted by "P" in gray circle) by JAK2; short isoforms are not tyrosine-phosphorylated. Signaling properties of each isoform are summarized at the bottom (see text for more details).

their ligands also share a high level of structural similarity since both PRL and GH adopt the antiparallel four-α-helix-bundle fold characteristic of hematopoietic cytokines (Fig. 2B). Thus, it is not surprising that the ligand-induced activation of the PRLR and that of the GHR share very similar mechanisms. PRL and GH interact with two molecules of their receptors via two distinct regions, binding sites 1 and 2 (Fig. 2B). The active hormone–receptor complex is thus a trimer, comprising one molecule of ligand and two (identical) molecules of receptor (homodimer). Thus far, no specific receptor cDNA has been identified for placental lactogen (PL, another member of the PRL/GH hormone family). Interestingly, PRLR/GHR heterodimers have been proposed to constitute such a specific receptor complex, but the physiological relevance of this observation remains to be demonstrated. Since receptor homodimerization is required and is presumably sufficient to trigger downstream signaling cascades, receptor antagonists that interfere with this process have been designed. Impairing binding site 2 of PRL or GH by introducing sterically hindering mutations within this region leads to ligands that are unable to induce functional dimerization of the receptor and, therefore, act as inhibitors of the natural hormones by competing for receptor-binding sites. In many biological contexts, the ability of PRL or GH antagonists to inhibit PRL- or GH-induced signaling cascades has been clearly demonstrated.

## IV. MAJOR SIGNALING PATHWAYS

With the exception of constitutively active N-terminal truncated receptors experimentally engineered for research purposes, all actions mediated by the PRLR and GHR result from their interaction with

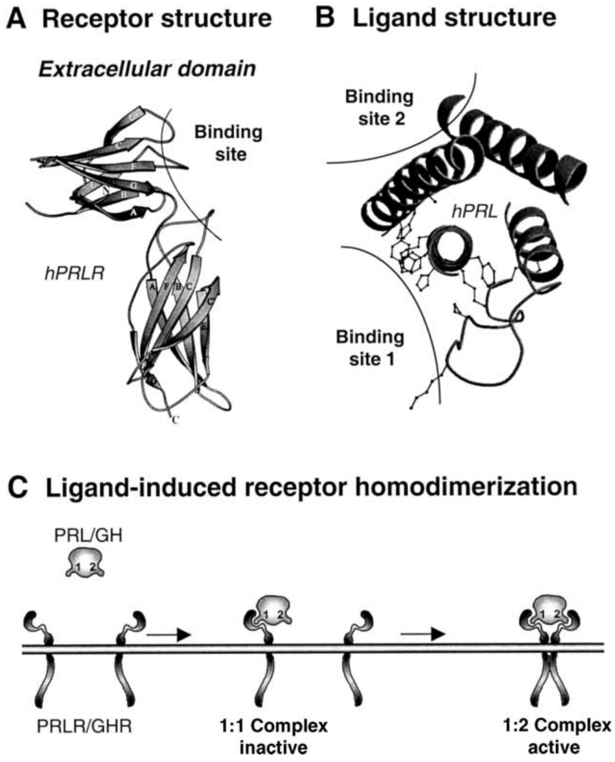


**FIGURE 2** Mechanism of receptor activation. PRLR and GHR extracellular domains adopt the seven-β-strand sandwich fold typical of cytokine receptors (A), and hormones adopt the four-α-helix-bundle fold typical of hematopoietic cytokines (B). Regions involved in hormone–receptor interactions are indicated. Following ligand binding, receptors are homodimerized in a sequential manner: the first receptor molecule binds to binding site 1 of its ligand, and then the second receptor molecule binds to binding site 2 to form the active trimeric complex (C).

any of their natural ligands, which leads to receptor dimerization and the activation of cascades in the intracellular space. Thus far, in contrast to the majority of cytokine receptors, there is no evidence that any "accessory" membrane protein is required or that the type of ligand (e.g., hPRL, hPL, or hGH for the hPRLR) affects the nature of signals that are transmitted inside the cell.

## A. JAK/STAT Pathway

### 1. *Activation of the JAK/STAT Pathway*

The PRLR and GHR are devoid of any intrinsic enzymatic activity. In 1993–1994, JAK2 was identified as the Janus tyrosine kinase associated with these receptors. Although the involvement of JAK1 and JAK3 was also proposed later for both receptors, their role is clearly less relevant. In contrast to the GHR, which recruits JAK2 to the receptor complex on ligand binding, the kinase is constitutively associated with the PRLR; i.e., its recruitment is not induced by ligand binding. For the GHR, SH2-Bβ has been recently identified as a cytoplasmic protein tightly binding to and potentiating the activity of phosphorylated JAK2.

The receptor–JAK2 interaction requires integrity of Box 1, which does not preclude the involvement of additional C-terminal regions of the receptor. Thus, with the exception of short GHR isoforms lacking Box 1 (Fig. 1), all PRL/GH receptor isoforms are able to interact with and activate JAK2 and, therefore, to exhibit some signaling properties. Mutational analysis of Box 1 has assigned a critical role to proline residues in the interaction with JAK2, suggesting an SH3 domain-mediated interaction. However, due to the absence of a typical SH3 domain in the JAK2 sequence, the question of whether the interaction between kinase and the receptors is direct or involves an intermediate protein (adapter) remains unresolved.

JAK2 activation is a prerequisite for triggering many, if not all, downstream signaling cascades (Fig. 3). Accordingly, receptor mutants unable to associate with JAK2, such as engineered Box 1-deleted PRLR or short GHR isoforms (Fig. 1), are unable to trigger tyrosine phosphorylation cascades and downstream activation of target genes. Interestingly, heterodimerization of the short and intermediate PRLR cytoplasmic tails results in complexes that are unable to stimulate JAK2 autophosphorylation, whereas both can associate with and activate the kinase in the context of their respective wild-type receptor. This observation suggests that only "perfect" homodimers can signal properly, which might be important in a physiological context since many tissues express different receptor isoforms, potentially forming heterodimers. Accordingly, short PRL receptors have been assigned a dominant negative role *in vitro* by inhibiting the transcriptional activation of milk protein genes induced by the full-length receptor; the physiological relevance of this observation remains to be demonstrated. Short GHR isoforms have been cloned from patients displaying a novel form of GH insensitivity syndrome, clearly indicating a dominant negative role of this short receptor isoform *in vivo*.

Once it is tyrosine-phosphorylated (i.e., activated), JAK2 phosphorylates the receptor on several tyrosines. No correlation between tyrosines that are preferentially phosphorylated by JAK kinases and their surrounding amino acids has been established

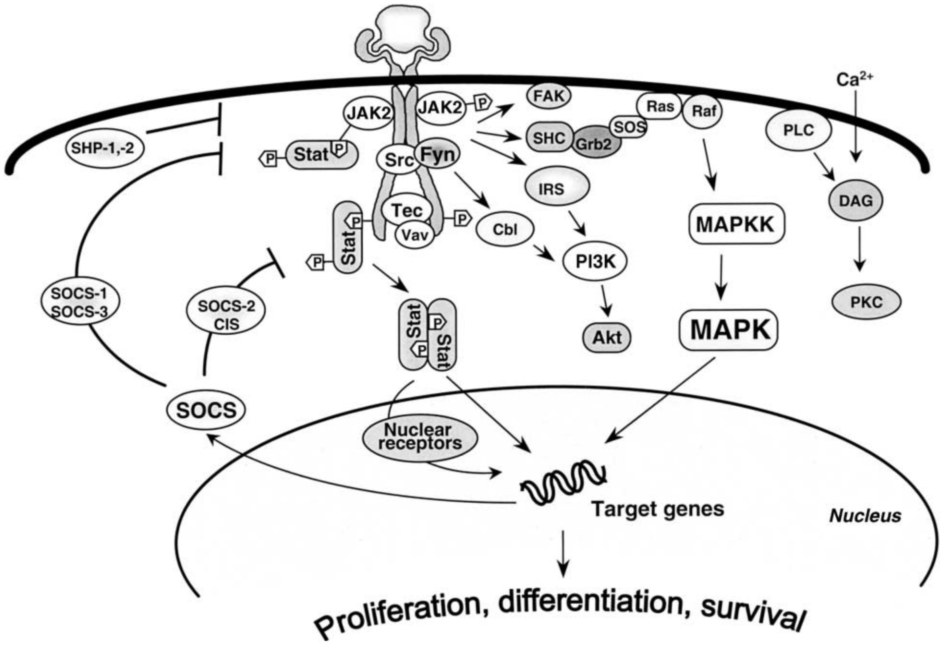


**FIGURE 3** Signaling pathways. Major signaling pathways activated by the PRLR and GHR are represented (see text for differences). The JAK/STAT pathway and the MAPK pathway are among the best characterized cascades. In addition to these pathways, several other kinases/adapters have been shown to be involved in PRLR signaling, although their downstream effectors may be less well defined.

(no consensus sequence). Therefore, the reason that the short PRLR isoform does not undergo tyrosine phosphorylation despite its ability to activate JAK2 and the presence of tyrosines in its cytoplasmic domain remains unknown. The phosphotyrosines of receptors, as well as of JAK2, become sites of interaction with signaling molecules containing phosphotyrosine-binding motifs (SH2, PTB). Among these, recruitment of STAT factors to the receptor complex has been extensively studied. Both the PRLR and the GHR activate three of the seven known STATs: Stat1, Stat3, and mainly Stat5 (A and B isoforms). Stat1 and Stat3 bind to phosphotyrosines of the receptors and/or of JAK2; in agreement with these findings, short isoforms containing Box 1 (short PRLR or engineered C-terminal-truncated GHR) activate these STATs although the receptor is not phosphorylated. In contrast, Stat5 interacts only with the phosphotyrosine(s) of full-length and intermediate receptors. Although redundancy between some of these tyrosines has been reported, C-terminal tyrosines play a critical role, at least in the PRLR. Thus, one of the major differences between short and intermediate/long isoforms lies in the ability to activate Stat5-dependent downstream targets, which is directly correlated with the phosphorylation status of the receptor (Fig. 1).

Once recruited to the receptor complex, STATs are phosphorylated by JAK2 on their C-terminal tyrosine, dissociate from the JAK–receptor complex, and translocate as dimers into the nucleus, where they specifically activate transcription by interacting with consensus DNA sequences within the promoters of PRL or GH target genes. The transcriptional specificity exhibited by identical STAT factors activated by distinct cytokines remains poorly understood. The answer may partly reside in the arsenal of other transcription factors that are also activated and can interact with STATs, modulating their transactivation activity (cross talk). Among these are nuclear receptors (estrogen, progesterone, glucocorticoid, etc.), Sp1 (specificity protein 1), CREB-binding protein/p300, peroxisome proliferator-activated receptor α (PPARα), nuclear factor κB, members of the interferon regulator factor (IRF) family, c-jun, MCM5 (mini-chromosome maintenance protein 5), and BRCA1 (breast cancer 1 gene). Different STATs can also interact with one another; e.g., Stat1 and Stat5 have been reported to exert opposing actions on IRF-I gene transcription.

#### 2. *Down-regulation of the JAK/STAT Pathway*

The recent discovery of a family of proteins down-regulating the activation of the JAK/STAT pathway has greatly helped in understanding how these activated (phosphorylated) proteins return to their steady state after hormone stimulation. These proteins are denoted SOCS (suppressor of cytokine signaling) or CIS (cytokine-inducible SH2 proteins) and down-regulate the JAK/STAT pathway by interfering with either JAK2 enzymatic activity (SOCS-1, -3) or STAT recruitment to the receptor complex by competing for phosphotyrosine binding (SOCS-2, CIS). Importantly, the SOCS/CIS genes are targets of the cytokine-induced JAK/STAT pathway, meaning that they encode proteins functioning as regulators of this pathway in a feedback manner. Internalization and proteasome-dependent degradation of a GHR/JAK2/CIS complex have been proposed to be important steps in the time-dependent CIS inhibition mechanism. However, not all SOCS proteins inhibit receptor signaling; SOCS-2, which binds directly to the PRLR, potentiates receptor signaling.

In addition to SOCS, SH2-containing tyrosine phosphatases (SHP-1 and SHP-2) are important in signaling down-regulation by dephosphorylating JAK and/or receptors. SIRP (signal-regulated protein), a transmembrane protein interacting with JAK2 on GHR stimulation, recruits signaling molecules to the receptor complex, including the phosphatase SHP-2, which leads to JAK2 dephosphorylation; SIRP is thus currently regarded as a negative regulator of GH signaling. The role of phosphatases remains poorly understood, however, since SHP-2 also appears to be necessary for initiating PRLR and GHR signaling.

### B. Other Pathways

The JAK/STAT pathway is undoubtedly one of the major cascades triggered by these receptors. However, many other signaling proteins have been shown to be activated by the PRLR and GHR. This section is aimed at providing an overview, albeit certainly not an exhaustive one, of these additional signaling cascades.

#### 1. *Mitogen-Activated Protein Kinase*

The well-known mitogen-activated protein kinase (MAPK) pathway involves the Shc (Src homology and containing protein)/SOS (Son of Sevenless)/Grb2 (growth factor receptor-bound protein 2)/Ras/Raf/MAPK cascade. This pathway has been demonstrated to be activated by both the PRLR and the GHR, including the short PRLR isoform, in agreement with the observation that the membrane proximal Box 1 region is sufficient to activate the MAPK pathway. Shc directly interacts with the receptor complex and becomes tyrosine-phosphorylated, presumably by JAK2. Whether activation of the MAPK cascade requires kinases other than JAK2, e.g., Src kinases, is yet to be elucidated. Also, indirect activation of the MAPK cascade via JAK2-induced phosphorylation of the EGF receptor and subsequent recruitment of Grb2 by phosphorylated EGF receptor have been reported in the case of GH stimulation. More recently, activation of c-jun N-terminal kinase and p38 by PRL has also been described.

#### 2. *Src Kinases*

At least two members of the Src tyrosine kinase family, namely, Src and Fyn, are activated by the PRLR and this activation seems to occur independent of JAK2. Fyn has been shown to be constitutively bound to the PRLR. Src tyrosine kinases play an important role in PRLR signaling, especially in PRL-induced cell proliferation. There are as yet no reports demonstrating the involvement of Src kinases in GHR signaling.

#### 3. *Insulin Receptor Substrate and Phosphatidyl Inositol 3-Kinase*

Insulin receptor substrate members (IRS-1, -2, -3) also interact with phosphorylated receptors and themselves become tyrosine-phosphorylated (by JAK2), which creates docking sites for SH2-containing proteins, including the p85 subunit of phosphatidyl inositol 3-kinase (PI3K). One downstream effector of PI3K has been shown to be the serine/threonine kinase Akt, the stimulation of which is involved in the anti-apoptotic effect of GH.

#### 4. *Other Molecules*

The above-mentioned pathways do not exhaustively summarize the current knowledge in the field of PRLR/GHR signaling, but rather represent a broad overview. Other tyrosine kinases (ZAP-70, Tec), serine/threonine kinases (protein kinase C), phospholipase C$\gamma$ and the downstream pathway modulating intracellular calcium concentration, adapters (Cbl, a substrate of Src kinases), guanine nucleotide exchange factors (Vav, complexed to Tec), proteins of the cytoskeleton (FAK), the 17$\beta$-hydroxysteroid dehydrogenase/17-ketosteroid reductase (known as PRAP, for PRLR-associated protein), and proteins linked to the apoptotic pathway (including Bax, Bcl-2, and Bag-1) have also been identified as molecules that are associated with and/or involved in signaling by the PRLR and/or GHR.

## V. HOW ANIMAL MODELS CAN HIGHLIGHT *IN VITRO* SIGNALING STUDIES

The analysis of genetically modified animal models (knockout, transgenic) is extremely informative in evaluating the involvement of a particular signaling protein in the physiological development and function of a given organ. For example, the conditional knockout of Stat3 has clearly demonstrated a dramatic delay in the involution process of the mammary gland after weaning, establishing this STAT as a key factor in signaling the initiation of physiological apoptosis *in vivo* in this organ. However, it may prove difficult to correlate this kind of information with the identification of signals involved in upstream activation of signaling proteins in a normal context [e.g., which cytokine(s) or growth factor(s) regulates Stat3 activation in the mammary gland to control involution]. Examples of knockout models that have clearly highlighted the role of signaling proteins involved in PRLR- and GHR-mediated functions are provided below.

Stat5A and Stat5B are two closely related STAT proteins that, although they are encoded by distinct genes, have been often considered to be redundant in most molecular (*in vitro*) studies involving Stat5 analysis. It is only when Stat5A- and Stat5B-deficient mice were shown to exhibit distinct phenotypes that their nonredundant roles began to be understood. Stat5A-deficient mice display alterations of mammary gland development, including defective proliferation of the lobulo-alveolar ductal epithelium during pregnancy and lactation failure. All these phenotypes are very similar to those observed in PRL or PRLR knockout mice, suggesting that Stat5A, originally designated mammary gland factor, is tightly linked to PRLR-mediated signaling. In contrast, Stat5B-deficient mice exhibit a loss of normal sexually dimorphic growth, a phenotype reminiscent of Laron dwarfism in humans, which suggests that Stat5B is presumably more closely linked to GHR- than PRLR-mediated signaling.

Another very interesting knockout is that of SOCS-2, which leads to a gigantism phenotype very similar to that observed in GH transgenic mice. At 6 weeks of age, SOCS-2-deficient mice are 40% heavier than wild-type mice and their long bones are significantly longer, which is strong evidence that SOCS-2 is a natural negative regulator of GH and/or insulin-like growth factor-I (the main second messenger of GH) signaling.

Finally, a negative regulator of PRL signaling, SOCS-1, has recently been proposed as a factor capable of preventing lactation prior to parturition. In fact, mice deficient for SOCS-1 that were rescued from neonatal death by concomitant deletion of the interferon-γ gene were shown to have accelerated mammary lobulo-alveolar development. Interestingly, when a single allele of the SOCS-1 gene was deleted and these animals were crossed with PRLR$^{+/-}$ mice, the lactational defect normally seen in heterozygous mice was rescued. The functional pathways involved in SOCS-1 inhibition of the mammary gland remain to be identified.

## VI. CONCLUSIONS

As for all cytokine receptors, the JAK/STAT pathway appears to play a central role in PRLR/GHR signaling, and receptor isoforms unable to activate JAK2 (naturally occurring or experimentally engineered) have been shown to be devoid of signaling properties. However, the variety and multiplicity of signaling pathways activated by PRL and GH receptors are presumably correlated with the unusually extended range of biological functions displayed by these hormones, whose receptors are widespread in the organism. Interconnection (cross talk) of these cascades now appears to be one of the key components to achieving some degree of specificity using a limited set of signaling molecules (JAKs, STATs, MAPKs, etc.) activated by distinct cytokines, hormones, and growth factors. Understanding how these activated protein pathways are organized in space as well as in time is a major challenge for future signaling studies.

## Acknowledgments

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## Glossary

**binding protein** In many species, prolactin- and growth hormone-binding proteins are generated and result either from alternative splicing of transcripts encoding full-length receptors or from limited proteolysis of membrane-bound receptors. Soluble binding proteins bind circulating ligands and prolong their half-life. They are devoid of intrinsic signaling properties, but may interfere with membrane-receptor signaling by forming inactive heterodimers (one soluble receptor and one membrane-bound receptor).

**cytokine receptor** A family of single-pass transmembrane receptors identified in the late 1980s based on sequence comparison of receptors for prolactin, growth hormone, erythropoietin, interleukin-2 (IL-2), and IL-6. Additional members of the class I hematopoietic cytokine receptor superfamily include receptors for leptin, thrombopoietin, and many cytokines regulating the immune system, such as most interleukins. The main conserved features of class I cytokine receptors are two pairs of cysteines and a Trp-Ser repeat (WS motif) in the extracellular domain and a proline-rich region (called Box 1) in the intracellular domain, which is essential for signaling properties. Cytokine receptors are devoid of intrinsic enzymatic activity and signal through associated kinases, the most classical of which are Janus tyrosine kinases (which bind to the Box 1 region of the receptors). Receptors for prolactin and growth hormone are very similar with respect to overall structure and signaling properties.

**growth hormone (GH)** A polypeptide hormone (191 amino acids in humans) that adopts the α-helix-bundle fold typical of hematopoietic cytokines. GH is secreted mainly by the pituitary gland, although it is also produced by other cell types, such as lymphoid cells. Its actions are related mainly to growth (soft tissues, long bones, etc.) and metabolism. It belongs to a family of hormones that includes prolactin and placental lactogens, as well as other placental factors.

**homodimerization** Cytokine receptors are activated by clustering of two or more receptor subunits, identical or not. Prolactin and growth hormone receptors are both activated by ligand-induced homodimerization of two identical receptor chains. In fact, a single molecule of ligand (prolactin or growth hormone) contains two binding sites (binding sites 1 and 2), each interacting with one receptor molecule. Based on this homodimerization model for receptor activation, hormone antagonists have been designed by impairing binding site 2 in prolactin or growth hormone, leading to ligands that are able to bind the receptor via their site 1 but unable to induce efficient receptor dimerization.

**Janus kinase/signal transducers and activators of transcription (JAK/STAT)** The most typical signaling pathway activated by cytokine receptors. This pathway involves a family of four tyrosine kinases designated “Janus” or “JAK” kinases (members are JAK1, JAK2, JAK3, and Tyk2). JAK2 is the main JAK involved in growth hormone and prolactin receptor signaling. Substrates of JAK tyrosine kinases include cytokine receptors and STAT factors. The eight members of the STAT protein family must interact with tyrosine-phosphorylated cytokine receptor complexes to be activated by tyrosine phosphorylation (by JAKs); they then migrate into the nucleus and transactivate cytokine target genes. With respect to growth hormone and prolactin signaling, Stat5 and, to a lesser extent, Stat1 and Stat3 are activated.

**mitogen-activated protein kinase (MAPK) pathway** One of the major pathways activated by membrane receptors. It involves a cascade of serine/threonine and dual-specificity (Tyr/Ser/Thr) kinases, leading to the activation of several target genes. The MAPK pathway has been historically linked to cell proliferation, but recent data have shown that it is involved in many cell responses and crosstalk with other pathways, including the Janus kinase/signal transducers and activators of transcription pathway. Activation of the MAPK cascade by prolactin and growth hormone receptors involves Box 1 but not the phosphotyrosines of the receptor.

**prolactin (PRL)** A polypeptide hormone (199 amino acids in humans) that adopts a four-α-helix-bundle fold typical of hematopoietic cytokines. PRL is secreted mainly by the pituitary gland, although it is also produced by other cell types and tissues, such as mammary gland, endometrium, lymphoid cells, and prostate. Its actions are essentially related to reproduction and lactation, but its involvement in an extremely wide spectrum of biological responses has been reported (osmoregulation, immunoregulation, behavior, growth, metabolism, etc.). Prolactin belongs to a family of hormones that includes growth hormone and placental lactogens as well as other placental factors.

**receptor isoform** Prolactin and growth hormone receptors are encoded by single genes. However, alternative splicing of primary transcripts leads to the existence of many receptor isoforms, which differ in the length of their cytoplasmic domains and are thus referred to as short, intermediate, or long receptors. Short isoforms have a truncated C-terminal tail and are not tyrosine-phosphorylated, which correlates with their inability to exhibit all signaling properties of long (full-length) or intermediate isoforms. In some instances, short isoforms have been suggested to act as dominant negative receptors, presumably because heterodimerization of short and long/intermediate receptors achieves inactive complexes.

**suppressor of cytokine signaling (SOCS)** A recently identified family of proteins that play an important role in regulating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. They are currently viewed as negative regulators of this pathway, either by interfering with Janus kinase activity or by competing with STATs for binding to phosphorylated tyrosines of the receptor complex. Since SOCS genes are themselves targets of the JAK/STAT pathway triggered by activated cytokine receptors, they function by negative feedback regulation.

**tyrosine phosphorylation** Many stages of signaling cascades triggered by cytokine receptors involve tyrosine phosphorylation of various proteins. With respect to the receptor complex, several tyrosine residues within the cytoplasmic domains of prolactin and growth hormone receptors, or on Janus kinase 2 (JAK2) itself, are phosphorylated (by JAK2) and serve as docking sites for downstream effectors containing phosphotyrosine-binding domains (e.g., SH2 domains); candidates are signal transducers and activators of transcription proteins, suppressor of cytokine signaling proteins, phosphatases, or adapters (e.g., Grb2). The short receptor isoforms are not tyrosine-phosphorylated, which prevents some of these interactions from occurring.

## See Also the Following Articles

**Crosstalk of Nuclear Receptors with STAT Factors • Growth Hormone (GH) • Growth Hormone-Releasing Hormone (GHRH) and the GHRH Receptor • Placental Gene Expression • Prolactin (PRL)**

## Further Reading

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# Protein Kinases

Alain Enjalbert and
Caroline Le Pechon-Vallee
*ICNE, Institut Jean-Roche, Université de la Méditerranée, Marseille, France*

I. INTRODUCTION
II. DEFINITION OF PROTEIN KINASES
III. STRUCTURE, CLASSIFICATION, AND SUBSTRATE RECOGNITION
IV. REGULATION OF PROTEIN KINASE ACTIVITY
V. TRANSDUCTION MECHANISMS
VI. MAJOR PHYSIOLOGICAL ROLES OF PROTEIN KINASES
VII. PROTEIN KINASES IN HUMAN PATHOLOGIES

**Protein kinases are enzymes that catalyze the phosphorylation of proteins. Phosphorylation is an important intracellular signal-generating protein modification used in signal transduction. Protein kinases phosphorylate many proteins and are involved in major cellular processes such as differentiation, proliferation, and cell death.**

## I. INTRODUCTION

The great importance of the protein kinases within the cell can be understood through review of their function and major physiological roles. This article focuses on the mechanisms that ensure the specificity and tight regulation of protein kinases and their involvement in pathology and disease.

## II. DEFINITION OF PROTEIN KINASES

### A. The Enzymatic Reaction Catalyzed by Protein Kinases

Protein kinases, members of the family of phosphotransferases, catalyze the transfer of the γ-phosphate of adenosine triphosphate (ATP) to a hydroxyl residue of a protein substrate (see Fig. 1). This covalent

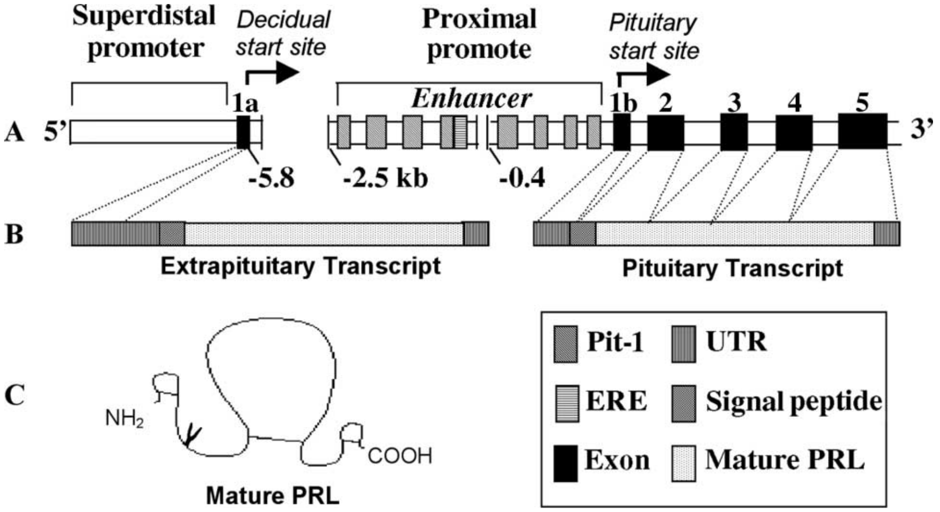

FIGURE 1 Diagram of the human PRL gene (A), extrapituitary and pituitary transcripts (B), and the mature protein (C). Note the use of exon 1a as the transcription start site for extrapituitary PRL and the longer 5′-untranslated region (UTR). The site of N-glycosylation (Y, $Asn^{31}$) is also shown. ERE, estrogen-response element.

Variants of PRL are formed by transcriptional or translational mechanisms and increase the biological specificity as well as the diversity of PRL. Most variants are generated by posttranslational modifications and differ in size or functional groups. Larger variants are formed by dimerization or aggregation and smaller variants are formed by proteolysis, both at the sites of synthesis and at some target tissues. Some variants retain PRL-like activities and others have unique properties or no known functions. Two variants, a 22 kDa isoform and a 16 kDa isoform, are of interest. The 22 kDa form, $PRL_{1-173}$, may be important in female reproduction and is generated by kallikrein, a trypsin-like serine protease. The 16 kDa PRL possesses anti-angiogenic activity and is generated by cathepsin D, an acid protease. The angiostatic activity of 16 kDa PRL may occur via a unique receptor since it has low binding affinity to the classical PRL receptor.

Human PRL is N-glycosylated on $Asp^{31}$, adding 2–3 kDa to its molecular mass. Glycosylated PRL is detected in the pituitary and in several body fluids, ranging from 15 to 20% of total PRL in both the pituitary and the plasma to over 50% in the amniotic fluid and milk. The carbohydrate moieties vary among species and tissues and their heterogeneity accounts for differences in bioactivity, immunoreactivity, receptor binding, and metabolic clearance rate of PRL. Although glycosylation often decreases the bioactivity of PRL, unique physiological functions of glycosylated PRL have not been yet identified. PRL can also be mono- or diphosphorylated on serine and/or threonine residues, a modification that results in charge variability. The ratio of phosphorylated/nonphosphorylated forms is altered during various physiological states and could be involved in determining the balance between the mitogenic and the anti-mitogenic effects of PRL.

## II. PITUITARY AND EXTRAPITUITARY PRL

PRL is the secretory product of the lactotrophs, acidophilic-staining cells of the pituitary. Lactotrophs are the last anterior pituitary cells to differentiate during fetal development and are preceded by GH- and dual GH/PRL-producing cells, the somatolactotrophs. Pit-1 is required for the development of somatotrophs, lactotrophs, and thyrotrophs and its inactivation results in the virtual absence of these cell types. The factors that induce terminal differentiation of lactotrophs are unknown, but several growth factors, including nerve growth factor, epidermal growth factor (EGF), and basic fibroblast growth factor, have been implicated in this process.

Lactotrophs constitute 20–30% of total anterior pituitary cells and are heterogeneous in terms of morphology, basal hormone release, electrical activity, and responsiveness to secretagogues. The dual-secreting cells can be interconverted to somatotrophs or lactotrophs, depending on the nature of the stimulus. This process facilitates the rapid recruitment of PRL-producing cells while bypassing metabolically costly cell division. Unlike most pituitary cells, lactotrophs retain a robust proliferative capacity during adulthood and their number increases during pregnancy and lactation. This proliferative potential also accounts for the higher incidence of lactotroph tumors (prolactinomas) compared to other types of pituitary tumors. Some rat strains are especially sensitive to induction of prolactinomas by estrogens, but a similar role for estrogens in the etiology of human prolactinomas has not been demonstrated.

Although PRL was initially regarded as an exclusive pituitary hormone, many nonpituitary tissues were later found to contain immunoreactive PRL. The widespread distribution of tissues capable of PRL synthesis as well as those that contain PRL is illustrated in Fig. 2. The most established extrapituitary sites that produce PRL are the decidua, immune system, brain, and myometrium, with emerging evidence for PRL synthesis by the skin and exocrine glands, including mammary glands, sweat glands, and lacrimal glands. PRL is produced by a wide variety of cells of different embryonic origins, morphology, and physiological functions. Some, e.g., lymphocytes and epithelia, are less differentiated and have a high proliferative capacity, whereas others, e.g., neurons, are postmitotic and terminally differentiated. Immunoreactive PRL is also present in tissues that do not produce PRL but are capable of taking up and concentrating PRL from the blood.

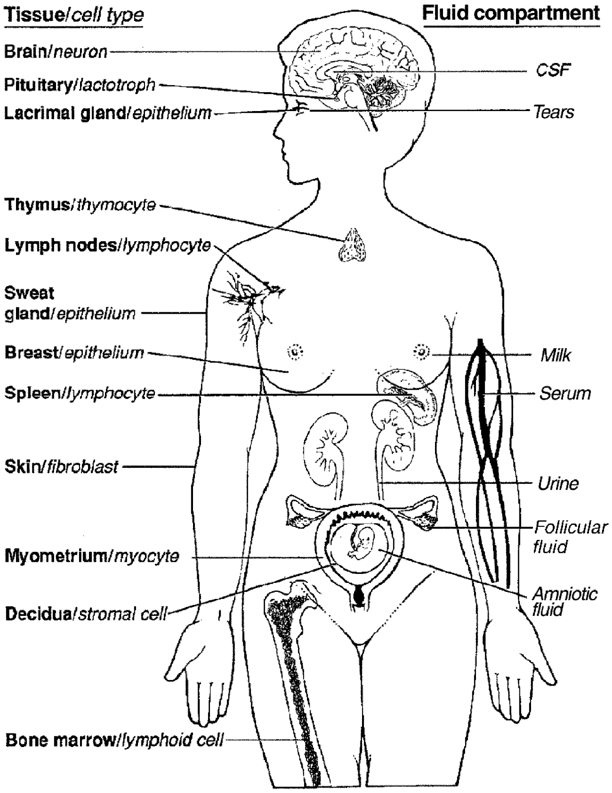

FIGURE 2 Distribution of PRL throughout the human body. Tissues and cells that produce PRL are shown on the left and fluid compartments that contain PRL are shown on the right. Note the heterogeneity of PRL-producing cells (italics) in terms of embryonic origin, morphology, and function.

Another remarkable feature of PRL is its presence in most body fluid compartments. Although all hormones are present in serum and most are excreted into urine, PRL is also found in cerebrospinal fluid (CSF) and the amniotic fluid and is secreted into milk, tears, and follicular fluid. Whereas PRL in the amniotic fluid originates from a local source (decidua), that in milk and CSF is derived from both locally produced and circulating PRL. Significant cellular resources must be spent in transporting PRL into these compartments and yet little is currently known about the functions subserved by PRL in these sites.

## III. REGULATION OF PRL SYNTHESIS AND RELEASE

Consistent with its function as an adaptive rather than an indispensable hormone, the profile of PRL release varies greatly under many physiological conditions and is dissimilar among species. In humans, serum PRL progressively rises throughout gestation in both the maternal and the fetal circulation and is episodically released in response to suckling during lactation. Adult women have higher serum PRL levels than men but do not exhibit marked changes in the secretory profile of PRL throughout the menstrual cycle. This is in contrast to rodents, in which a clear preovulatory surge of PRL is evident. Stress conditions, including anesthesia, surgery, electric shock, strenuous exercise, and insulin-induced hypoglycemia, also stimulate PRL release in both men and women. PRL is also secreted in an episodic fashion throughout the day with some evidence for sleep-related increases in its circulating levels.

The synthesis and release of PRL by the pituitary lactotrophs are subjected to multiple regulators that can be classified into four broad categories: endocrine, paracrine, juxtacrine, and autocrine (Fig. 3). Endocrine agents originate from the hypothalamus and gonads and reach the lactotrophs via a humoral

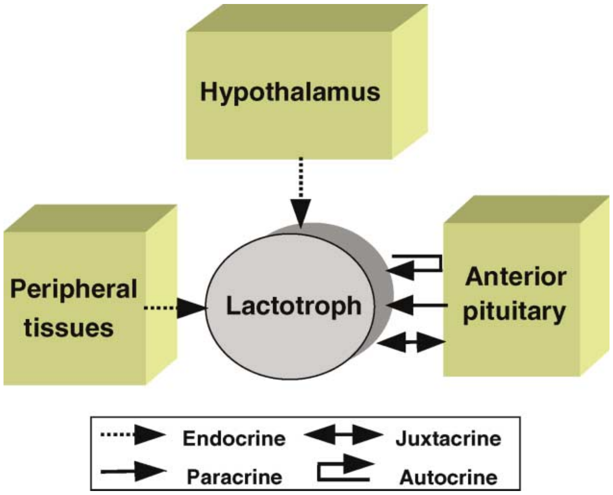

**FIGURE 3** Diagram of the four types of regulation of pituitary lactotrophs.

route. Paracrine factors are produced by other pituitary cell types and reach the lactotrophs by diffusion. Juxtacrine interactions emanate from the extracellular matrix and cells adjacent to the lactotrophs. Autocrine agents are synthesized by the lactotrophs themselves. Consequently, the overall secretory activity of the lactotrophs reflects a balance between local and distant releasing and inhibiting factors. In nonhuman species, there is also evidence for PRL-regulating factors that originate from the posterior pituitary.

The pituitary lactotroph is unique in its capacity for constitutive synthesis and secretion of PRL. Unlike pituitary tropic hormones such as luteinizing hormone or adrenocorticotropin hormone, whereby the hypothalamus provides a positive stimulus and peripheral target glands supply negative feedback inhibition, PRL does not have a singular target organ. Hence, its main regulation is provided in the form of tonic inhibition by dopamine, the physiological PRL release-inhibiting factor. Acting via a type 2 dopamine receptor, dopamine exerts multiple actions on the lactotrophs, including suppression of intracellular calcium and cyclic AMP levels, inhibition of PRL gene expression and release, and suppression of cell proliferation. Interruption of dopaminergic input to the lactotrophs caused by physiological inhibition of dopamine release, damage to the pituitary stalk, or dopamine receptor antagonists results in hyperprolactinemia. PRL itself, acting via a short-loop negative feedback mechanism, is the primary regulator of the dopaminergic system. Despite intensive searching, a singular releasing factor for PRL has not been identified, but several neuropeptides, including thyrotropin-releasing hormone (TRH) and vasoactive intestinal peptide (VIP), are capable of stimulating PRL release under some physiological conditions. In contrast to the pituitary, little is known about the regulation of PRL production/release in extrapituitary sites except that they do not respond to dopamine, neuropeptides, or estrogens but appear to be regulated by local autocrine/paracrine factors.

Pituitary PRL gene expression is affected by many hormones, neurotransmitters, and growth factors. Compounds that bind to G-protein-linked receptors, e.g., TRH, VIP, and dopamine, activate protein kinase A, protein kinase C, and/or calcium/calmodulin-dependent pathways. These are mediated via a variety of transcription factors that bind to consensus sequences within the PRL promoter. Estrogens, on the other hand, freely diffuse into the nucleus, where they bind to either estrogen receptor-α or -β. The activated receptor dimerizes and acts as a transcription factor and binds to a unique DNA sequence called the estrogen-response element that is located in the enhancer region next to a Pit-1-binding site (see Fig. 1). Among the growth factors, insulin and EGF stimulate the PRL gene, whereas transforming growth factor-β suppresses the PRL gene. Many of the growth factors bind to transmembrane receptors with intrinsic tyrosine kinase activity and exert pleiotropic actions such as stimulation of PRL gene transcription, increases in hormone storage, and alterations of lactotroph morphology. Again, details about the control of PRL gene expression in extrapituitary sites are largely unknown.

## IV. PRL RECEPTORS AND SIGNAL TRANSDUCTION

PRL exerts its actions by binding to specific, high-affinity plasma membrane receptors. The gene encoding the human PRL receptor is located on chromosome 5 and contains at least 10 exons encompassing over 100 kb. The PRL receptor belongs to the cytokine/GH/PRL receptor superfamily that is characterized by a single-pass transmembrane stretch that divides the receptor into an extracellular ligand-binding domain and an intracellular domain. The extracellular domain contains two disulfide bonds and a WS motif (Trp-Ser-X-Trp-Ser) that is required for correct folding of the receptor and may participate in the formation of a ligand-binding pocket. The cytoplasmic domain has a proline-rich motif (Box 1) near the plasma membrane that couples to intracellular signaling molecules. Another motif (Box 2′) is less conserved.

Humans express primarily one "long" form of the receptor, whereas rats have three: long, intermediate, and short. An intermediate isoform that is uniquely expressed by the rat Nb2 lymphocyte cell line renders these cells dependent on PRL for growth and survival. Given this property, proliferation of Nb2 cells in response to PRL is widely used as a sensitive bioassay for PRL. An intermediate receptor isoform with a deleted segment within the intracellular domain, differentially spliced short receptors, and a soluble receptor form that contains only the extracellular domain have been detected in human tissues, but their exact functions remain to be determined. The PRL receptor is expressed by most tissues, with the highest level of expression in the liver and mammary gland. The PRL receptor concentrations fluctuate under many physiological conditions, especially in response to changes in circulating PRL and steroid hormones. Both PRL and its receptor are also internalized within several cell types but neither the exact intracellular localization nor the function of the internalized receptor or PRL is well understood.

Binding of PRL to its receptor induces sequential receptor dimerization. Two binding sites on the PRL molecule, site 1, comprising helices 1 and 4, and site 2, comprising helices 1 and 3, are required for the induction of receptor homodimerization and formation of an active trimeric complex. The receptor is devoid of intrinsic tyrosine kinase activity, utilizing instead the Janus kinase (JAK)2–signal transducers and activators of transcription (STAT) pathway as its main signaling cascade (Fig. 4). JAK2 (Janus kinase 2), which is constitutively associated with Box 1 on the PRL receptor, is rapidly phosphorylated on receptor dimerization. In turn, the activated JAK2 induces phosphorylation of the receptor, other associated kinases, and Stat proteins. Of the seven known Stat proteins, Stat1, Stat3, and Stat5 are activated by PRL. As revealed by studies with knockout mice, Stat5a and Stat 5b are especially important for mammary gland development and function. The activated (phosphorylated) Stat proteins dimerize and are rapidly translocated into the nucleus, where they bind to specific sequences on target genes. The Ras/Raf/mitogen-activated protein kinase cascade and fyn, a member of the Src kinase family that phosphorylates phosphatidyl inositol 3-kinase, are also activated by PRL in a cell-specific manner but appear to be of lesser importance in mediating PRL action than the JAK2–STAT pathway. Notably, all the above-mentioned pathways have been characterized under acute conditions, whereas the mechanism mediating the long-term effects of PRL are not well understood. In addition, human GH and placental lactogens bind to the PRL receptor with high affinity and mimic some of the actions of PRL.

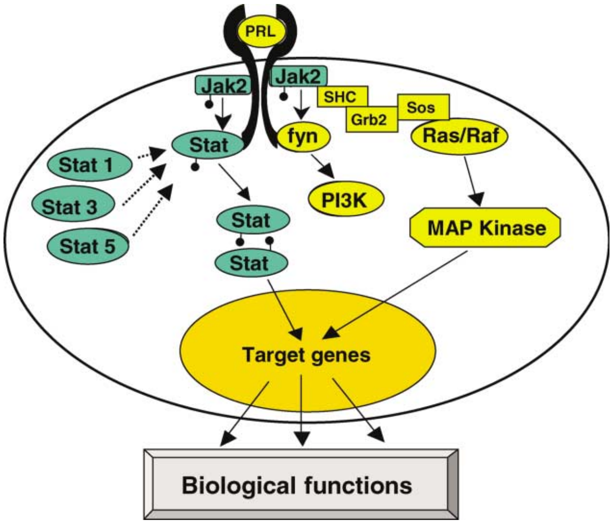

FIGURE 4 Schematic presentation of the PRL receptor and the intracellular signaling pathways that mediate PRL actions.

## V. BIOLOGICAL FUNCTIONS

PRL is one of the most versatile hormones, with more than 100 functions ascribed to it, far exceeding the number of all known actions of other pituitary hormones combined. These functions are broadly associated with reproduction, growth and development, osmoregulation, metabolism, immunoregulation, brain function, and behavior. Several caveats should be considered, however, when evaluating these functions. First, although both males and females produce PRL and express the PRL receptor, PRL functions have been most extensively studied in females. Second, some PRL effects in lower vertebrates may have been lost during evolution and are recapitulated in higher animals only during certain developmental stages. Third, PRL often exerts species-specific actions among mammals (e.g., luteotropic activity in rodents only), complicating the interpretation of studies with transgenic animals. Fourth, fetal exposure to very high PRL levels via the blood and the amniotic fluid is unique to humans and cannot be carefully examined due to lack of suitable animal models. Fifth, the uneven activity of various PRL isoforms in some functional assays leads to incomplete assessment of the spectrum of actions of PRL. Finally, the ability of related hormones, i.e., GH and placental lactogens, to bind to the PRL receptor and mimic some of the actions of PRL can lead to erroneous interpretations of experimental manipulations.

The effects of PRL on reproduction encompass multiple systems and tissues that differ in their importance in a species-specific manner. The principal target for PRL is the mammary gland. Together with several other hormones, PRL promotes growth and differentiation of the mammary epithelium and is essential for the initiation and maintenance of lactation by increasing the synthesis of all major milk components: the milk proteins α-lactalbumin and casein, lactose, and lipids. In some species, however, continuous lactogenesis is supported by GH rather than by PRL. In rodents, PRL has both luteotropic and luteolytic actions on the ovary and supports progesterone production. Therefore, in these species PRL plays a major role in modulating the reproductive cycle and is crucial for pregnancy and lactation. PRL has well-established mitogenic, secretory, and morphogenic effects on the prostate, but its actions on the testes are not well defined. Under normal conditions, PRL has permissive effects on human reproduction. However, overproduction of PRL (hyperprolactinemia) is one of the major causes of neuroendocrine-related anovulation and infertility in women and impotence in men. The inhibitory effects of excess PRL on these reproductive processes occur at both central (hypothalamic–pituitary) and peripheral (gonadal) sites. Recent evidence indicates that PRL may also play a role as a mitogen/anti-apoptotic factor in breast and prostatic cancer.

PRL plays a major role in regulating water and electrolyte balance in fish and amphibians, with lesser osmoregulatory actions in birds and mammals. Whereas the control of development and body growth is normally ascribed to GH, there are some functional overlaps between GH and PRL, especially in lower vertebrates. Both the metabolic and the immunoregulatory actions of PRL have been matters of controversy. PRL stimulates the proliferation of the pancreatic islets and increases insulin secretion primarily during pregnancy. In the immune system, PRL induces the proliferation and differentiation of functional activity of various lymphoid cells, but transgenic animals lacking PRL or its receptor have little if any immune disturbances. Within the brain, PRL affects the production and release of several hypothalamic releasing/inhibiting hormones, e.g., dopamine and gonadotropin-releasing hormone, and has significant effects on maternal behavior.

## Glossary

**extrapituitary prolactin sites** Several nonpituitary tissues, e.g., decidua, myometrium, brain, and breast, that are capable of *de novo* synthesis of prolactin (PRL). The regulation of PRL synthesis and release from extrapituitary sites differs markedly from that in the pituitary.

**Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway** Intracellular signaling cascade that mediates the action of many cytokines and some growth factors. JAK2 and STAT5 constitute the main pathway that is rapidly activated by prolactin.

**lactotrophs** A class of anterior pituitary cells that produce prolactin. They constitute over one-third of all pituitary hormone-secreting cells. Lactotrophs are heterogeneous in structure and function and are normally subjected to inhibition by dopamine.

**posttranslational modifications** Changes in the structure of a protein hormone that include glycosylation, phosphorylation, and cleavage. These modifications affect hormone binding to the receptor or clearance from the circulation and thus alter its biological properties.

**prolactin promoter** A DNA sequence that is used to regulate the transcription of the prolactin gene. The pituitary proximal promoter is located immediately 5′ upstream of the transcription initiation site, whereas the extrapituitary superdistal promoter is located 5.8 kb further upstream.

**prolactinomas** Nonmalignant tumors of the anterior pituitary that are composed of lactotrophs and result in hyperprolactinemia or abnormally high serum prolactin levels. They usually develop from a single cell (monoclonal) but their etiology is unclear.

## See Also the Following Articles

**Cytokines and Anterior Pituitary Function • Endocrine Rhythms: Generation, Regulation, and Integration • Growth Hormone (GH) • Neuropeptides and Control of the Anterior Pituitary • Prolactin and Growth Hormone Receptors**

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# Prolactin and Growth Hormone Receptors

Vincent Goffin and Paul A. Kelly
*Faculty of Medicine Necker, Paris*

I. INTRODUCTION
II. PRLR AND GHR ISOFORMS HAVE DIFFERENT SIGNALING CAPABILITIES
III. LIGAND-INDUCED RECEPTOR DIMERIZATION: THE FIRST STEP OF SIGNALING
IV. MAJOR SIGNALING PATHWAYS
V. HOW ANIMAL MODELS CAN HIGHLIGHT *IN VITRO* SIGNALING STUDIES
VI. CONCLUSIONS

**The prolactin receptor (PRLR) and growth hormone receptor (GHR) are among the class I hematopoietic cytokine receptors. PRLR is expressed is almost all tissues and cell types, although the mammary gland and gonads are the major targets for prolactin (PRL). In primates, both PRL and growth hormone (GH) can activate the PRLR. The GHR bears a strong resemblance to the PRLR but is involved only with the actions of GH, which promotes the growth of several tissue types and has a variety of metabolic functions. Although the Janus kinase/signal transducers and activators of transcription pathway plays a central role in PRLR/GHR signaling, a number of other signaling pathways are also activated by PRLR and GHR < likely relating to the wide spectrum of biological functions carried out by these hormones.**

## I. INTRODUCTION

It was not until the early 1970s that prolactin (PRL) and growth hormone (GH) were revealed to be two distinct hormones in humans (h). The reason is that in primates, but not in lower species, both PRL and GH are able to activate the PRL receptor (PRLR). The actions mediated by the PRLR (also referred to as the lactogen receptor) are historically linked, but certainly not restricted to milk production and control of reproductive functions; mammary gland and gonads are thus major PRL targets, although the nearly ubiquitous expression of the PRLR renders almost all tissue and cell types potential targets of lactogenic hormones. The GH receptor (GHR) closely resembles the PRLR, but mediates only GH actions (PRL does not bind to the GHR in any species). As suggested by its name, GH exerts growth-promoting activity on several tissues (e.g., soft tissues and long bones) and shows a wide range of metabolic functions.

These two receptors were cloned at almost the same time (in the late 1980s) and are among the five membrane receptors whose sequence analysis led to the identification of a new receptor family, the class I hematopoietic cytokine receptors, which includes receptors for cytokines (e.g., interleukins), erythropoietin, and leptin. Within 5 years after the receptor cDNAs were cloned, biochemical and structural

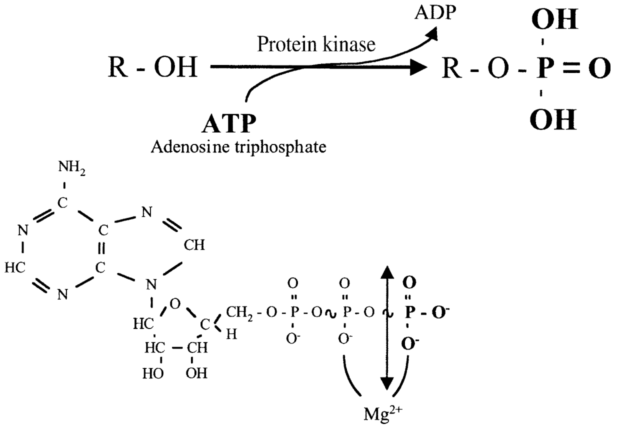

FIGURE 1 The biochemical reaction catalyzed by the protein kinases, showing the structure of ATP and the γ-phosphate that is transferred to the substrate hydroxyl group.

reaction takes place on the alcoholic aliphatic side chain of serine and threonine residues in the case of serine/threonine kinases. It can also take place on the phenolic group of tyrosine residues with protein–tyrosine kinases and on both serine/threonine and tyrosine residues in the case of dual-specificity kinases (see Fig. 2).

Histidyl kinases transfer the phosphate group onto an aspartate residue in their targets. The phosphate group comes from a histidyl residue that has been previously autophosphorylated within the kinase. These protein kinases have been discovered as a two-component histidine–aspartate phosphorelay in prokaryotes and plants. They are quite distinct from the conventional serine/threonine or tyrosine kinases.

The reverse reaction, or dephosphorylation, catalyzed by protein phosphatases, determines the ratio of phosphorylated and unphosphorylated forms. A vast number of protein phosphatases (PPs) exist, including serine/threonine phosphatases, such as the PP1s, PP2A, and PP2B, and a magnesium-dependent protein phosphatase, PP2C (also called PPM) for. The serine/threonine phosphatases are essentially cytosolic or nuclear. They localize within the cell through their binding to subcellular structures such as the endoplasmic reticulum and the cytoskeleton. Another class of phosphatases is the protein tyrosine phosphatases (PTPs). Some of the PTPs have membrane-spanning domains. They dephosphorylate tyrosine residues but some of them dephosphorylate both tyrosine and serine/threonine residues and are dual-specificity phosphatases. Some protein phosphatases have a very narrow specificity for their substrate, whereas others dephosphorylate a large number of proteins.

## B. The Protein Kinase Superfamily

Protein kinases are part of a huge superfamily. There exist many kinases and isoforms. According to the human genome, there should be more protein kinases than those discovered to date and some of the new kinases should be quite different from the ones already known. Phosphorylation is a primary means of signal transduction. Signal transduction is based on two main principles. First is the posttranslational modification of pre-existing proteins, such as phosphorylation. But isoprenylation, lipidation, methylation, glycosylation, or partial proteolysis also occurs. Second is production of new proteins or second messengers. Nevertheless, phosphorylation is the most common protein modification used in signal transmission, maybe because it is reversible and because protein kinases use a ubiquitous cofactor, ATP.

# III. STRUCTURE, CLASSIFICATION, AND SUBSTRATE RECOGNITION

## A. Structure

Protein kinase A (PKA), a cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase,

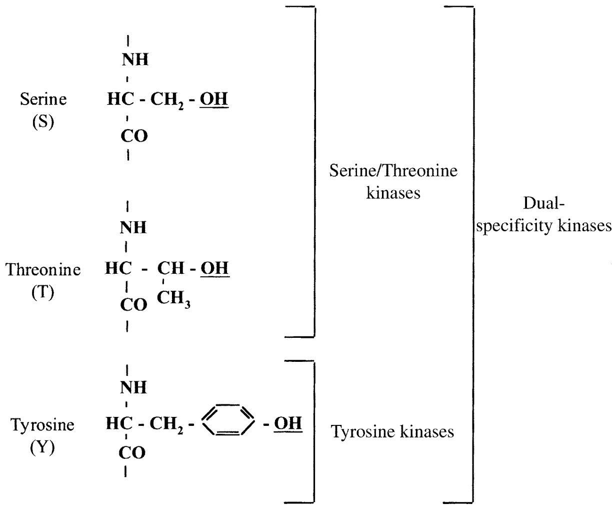

FIGURE 2 Phospho-acceptor amino acids. Protein kinases transfer a phosphate group onto the hydroxyl residue of an amino acid according to the specificity of the kinase. Serine/threonine kinases use either serine or threonine amino acids, tyrosine kinases use tyrosine, and dual-specificity kinases use serine, threonine, and tyrosine residues.

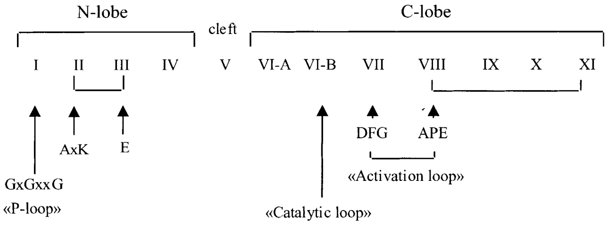

**FIGURE 3** Subdomains of the catalytic domain of protein kinase A, showing some of the major residues involved in the structure or catalytic activity of the kinase (see text for discussion).

was one of the first kinases studied and its structure is well known. In the absence of cAMP, PKA enzymes are tetramers composed of two regulatory (R) subunits and two catalytic (C) subunits. Two classes of R subunits, RI and RII, and several isoforms for each class have been identified. The catalytic domain of the PKAs can be divided into 12 subdomains according to the localization of highly conserved regions (see Fig. 3).

The structure of the kinase domain of the PKAs has long served as a model. It folds into a bilobal structure separated by a deep cleft. The smallest N-lobe consists of subdomains I–IV. It is almost a single β-sheet and is involved in proper ATP orientation and binding. The cleft consists of subdomain V and interferes in substrate recognition and catalytic activity promotion. The C-lobe consists of subdomainsVI-A, VI-B, and VII–XI and is almost entirely composed of α-helices. Structure and regulatory processes are unique to each kinase but almost every subdomain contains positions in which structurally similar residues are conserved throughout the entire protein kinase superfamily (Fig. 3).

The most important domains for the structure and the function of the protein kinases are subdomains I, II, VI-B, VII, and VIII. Subdomain I (amino acids 43–64; numbering is derived from the PKA sequence) contains the phosphate-anchoring loop, or the P-loop, which is a glycine-rich β-ribbon ATP-binding consensus sequence. This sequence is GxGxxG, in which G represents glycine (in the single-letter amino acid code) and x denotes positions where any residues would be tolerated. Subdomain II (amino acids 65–83) contains the AxK motif, in which the lysine (K) at position 72 is necessary for maximum activity because it is engaged in a salt bridge with the glutamine (E) in position 91 in subdomain III.

Subdomain VI-B (amino acids 161–177) contains the catalytic loop and is directly involved in catalysis. It is a sequence with at least three conserved residues: an aspartate, a leucine, and an asparagine (DLN). The aspartate residue (D166 in PKA) within the sequence is known as the "catalytic base" because it removes the proton from the hydroxyl group of the phospho-acceptor, producing an anionic oxygen that is involved in the nucleophilic attack of the γ-phosphate of the ATP. In serine/threonine kinases, a lysine (K) is also present (determining a DLKxxN motif) and is thought to be important to neutralize the negative charge of the γ-phosphate and to stabilize the intermediate state during the chemical reaction. In the case of tyrosine kinases, the lysine is replaced by an arginine (R) residue in a DLRAAN (A, alanine) sequence in the case of nonreceptor tyrosine kinases and in a DLAARN sequence in the case of receptor tyrosine kinases. Such conserved features, which characterize subgroups of protein kinases, exist also in other subdomains.

Subdomain VII (amino acids 178–193), also called the "metal-binding loop," referring to the metal ions bound to ATP, contains a DFG triplet (F, phenylalanine). The mutation of the aspartate (D) in this triplet results in an inactive kinase. Subdomain VIII (amino acids 194–210) contains another very important motif for the kinase activity: the APE motif (P, proline). The glutamate (E) is engaged with an arginine residue from subdomain XI in an interaction that contributes to the stability of the C-lobe. The region lying between the DFG and the APE motifs is the activation loop. It is a highly variable sequence but it always contains one or several residues for which phosphorylation induces the conformational rearrangement necessary for kinase activation.

## B. Classification

From catalytic domain alignment, Hanks and Quinn, in 1991, provided a general classification of protein kinases. This classification, which is still used, delineates five kinase family groups: (1) AGC, (2) CaMK, (3) GMCC, (4) PTK, and (5) OPK.

1. *AGC group.* This group is so called because it includes protein kinases A, G, and C. The PKA and the PKG families include protein kinases activated by cyclic nucleotides cAMP and cGMP, respectively. The protein kinase C family includes several isoforms, distributed in three subclasses according to their allosteric regulators. The cPKCs (α, βι, βιι, and γ) are the conventional PKCs regulated by calcium, diacylglycerol (DAG), and

phosphatidylserines. The nPKCs (δ, ϵ, η, and θ) are the novel, or nonclassical, calcium-independent PKCs. The aPKCs (ζ and ι/λ) are the atypical calcium- and DAG-independent PKCs. The AGC group also includes the G-protein receptor kinases (GRKs), which phosphorylate G-protein-coupled receptors. These kinases, such as the β-adrenergic receptor kinase (βARK) and the rhodopsin kinase (RK), are involved in desensitization and scaffold construction on the activated receptors (see below).

2. *CaMK group*. The second group of the Hanks and Quinn classification corresponds to the kinases regulated by calcium/calmodulin (CaM). CaMKs are involved in many cellular processes in response to changes of the intracellular calcium concentration. Within the cells, calcium is generally captured by high-affinity calcium-binding proteins such as calmodulin or troponin, which is found in muscles. In the CaMK family of protein kinases (CaM group I, CaM group II, and CaM "other"), the most well-known calcium/CaM-regulated kinases are the CaMKI, CaMKII, and CaMKIV. CaMKI and CaMKIV are closely related and distinct from CaMKII. CaMKII is also called the multiprotein kinase, because it actually comprises a family of closely related enzymes that phosphorylate numerous substrates. Each CaMKII is made of several catalytic subunits. Autophosphorylation may lead to calmodulin trapping, making the kinase calcium independent until it is dephosphorylated by protein phosphorylases or is phosphorylated on additional residues. In the absence of autophosphorylation, the binding of $Ca^{2+}$/CaM is necessary to promote the transition from an inactive state to a nonactivated state of the CaMK, which has to be phosphorylated to be fully activated.
3. *GMCC group*. The GMCC group is so called because it includes, among others, glycogen synthase kinases (GSKs); mitogen-activated protein kinases (MAPKs), which include the extracellular signal-regulated kinases; cyclin-dependent kinases (Cdks), which are involved in cell cycle progress; and the carboxy-terminal domain (CTD) kinases, which phosphorylate the long carboxy-terminal domain on the largest subunit of RNA polymerase II.
4. *PTK group*. The fourth group is composed of the conventional protein tyrosine kinases (PTKs). It includes nonmembrane (such as Src, Csk, and Fak families) and membrane-spanning protein tyrosine kinases (such as the large growth factor receptor families).
5. *OPK group*. The last group includes various "other protein kinases," i.e., the Raf family from the extracellular signal-related kinase (ERK) cascade and the MAPK/ERK kinase (MEK) family. It includes also other MEK kinases that are intermediates of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK pathways.

Beyond the distinction based on the catalytic domain amino acid sequence proposed by Hanks and Quinn, protein kinases can be distinguished by the nature of the residues they prefer to phosphorylate or by their subcellular localization. This would lead us either to consider AGC, CaMK, and GMCC kinases plus the other serine/threonine kinases from the OPK group as a unique serine/-threonine kinase family, or to view the superfamily of protein kinases as two groups: the cytosolic group and the membrane-spanning protein kinase group. The cytosolic kinase group would include serine/-threonine kinases (such as PKAs and PKCs) and tyrosine kinases (such as Src). On the other hand, the group of membrane-spanning protein kinases would include some serine/threonine protein kinases (such as the transforming growth factor-β receptor) and the large group of tyrosine kinase receptors. In addition to membrane or cytosolic localization, subcellular targeting is also important and is different from one kinase to another.

Compartmentalization of protein kinases plays a crucial role in their activity and is frequently determined by specific anchoring proteins. Several of these have already been identified. Several A-kinase-anchoring proteins (AKAPs) target the PKAs to subcellular loci (plasma membrane and mitochondrial surface). AKAPs are characterized by their ability to bind RI or RII regulatory subunits of the PKAs. This binding has two consequences. First, it is supposed to change the catalytic activity of the PKAs. Second, it specifically brings the PKAs into close contact with upstream effectors or downstream substrates via protein–protein or protein–lipid interactions mediated by the AKAPs. In addition, AKAPs can bind several PKAs at once and protein phosphatases as well. Such is the case of AKAP79, which anchors PKA, PKC, and PP2B on the plasma membrane. This mechanism may be useful for the fine and coordinated regulation of signal transduction. Several proteins also regulate PKC anchoring and translocation to particulate fractions, including myristoylated alanine-rich protein kinase C (MAPCK) substrate proteins and the receptors for activated C kinase (RACK) proteins.

### C. Substrate Recognition

Protein kinases use proteins as substrates. The phosphorylated residue is identified by the kinase thanks to specificity determinants surrounding it in the substrate sequence. In the case of the PKAs, which are basotropic kinases, the consensus sequence is R-(R/K)-x-S/T-B (where B stands for a hydrophobic residue). Such a small sequence cannot determine by the high specificity needed to select a substrate within the cell. This suggests that additional mechanisms exist, such as the targeting of the substrate and/or the kinase, the accessibility of the substrates, and the existence of a "docking site" on the substrate surface. A docking site corresponds to a larger sequence of amino acids seated astride the phosphorylated residue and an acute three-dimensional folding of the substrate that allows high-affinity interaction with the catalytic core of the kinase and increases the catalytic efficiency. For the moment, only a few docking sites are known. Some kinases are very specific for a protein substrate whereas others phosphorylate a large number of substrates.

## IV. REGULATION OF PROTEIN KINASE ACTIVITY

Frequently, the presence of a sequence homologous to the substrate recognition motif in the sequence of the protein kinase forces a refolding of the kinase, which prevents substrate and ATP binding. In the PKA inactive conformation, interaction between regulatory and catalytic subunits is responsible for the autoinhibition of the kinase. In the case of the PKCs, a pseudo-substrate site, located inside the N-terminal conserved "C1" domain of the kinase, interacts with the C-terminal conserved "C4" domain, and the kinase bends back into a closed, inactive conformation. As for the CaMKs, extension of the regulatory domain of the enzyme across the catalytic core leads to multiple inhibitory interactions. In the case of the cytoplasmic tyrosine kinase Src, access to the active site is blocked because of Src homology domain 2 (SH2) and Src homology domain 3 (SH3) intramolecular interactions. Src recruitment via its SH2 domain and dephosphorylation remove the inhibitory constraint from the kinase. In the tyrosine kinase receptor inactive state, the activation loop obtrudes the substrate and the ATP binding site and leads also to autoinhibition. Thus, almost every protein kinase needs an ultimate rearrangement to achieve the activated state. Several events induce separately or concomitantly this reorganization.

### A. Second-Messenger-Mediated Activation

PKA is a prime example of protein kinases regulated by a second messenger. The specific binding of two cAMP molecules onto each PKA regulatory subunit leads to the release of the two catalytic subunits and to the relief of the autoinhibition process. Many of the kinases of the AGC group are activated following production of second messengers. For instance, calcium and diacylglycerol participate in the activation of some PKCs. Others kinases are regulated by the local calcium concentration within the cells. Such is the case of the CaMKs. Indeed, the binding of $Ca^{2+}$/CaM on the CaMKs releases the constraint of the autoinhibitory domain.

### B. Ligand-Mediated Activation of Receptor Protein Kinases

The signal transduction initiated by the ligand binding to tyrosine kinase receptors starts with homo- or heterodimerization. The dimerization results in a conformational change and an auto- or interphosphorylation of several tyrosine residues on the cytoplasmic tail of the transmembrane receptor. Current research suggests that tyrosine kinase receptors can also undergo intracellular transactivation. An example of this is the extracellular growth factor (EGF) receptor transactivation downstream of the G-protein-coupled receptors, but some researchers think that this implies a pseudo-ligand binding. This underlines the importance of the ligand-mediated release of the autoinhibition of protein kinase receptors.

### C. Regulation by Phosphorylation

Because protein kinases are proteins, they can be used as a substrate and can then be phosphorylated and activated by other protein kinases. Thus, protein kinase activation in series can take place within cells, as illustrated by activation of the ERK MAPK cascade (Fig. 4). The mitogen-activated protein kinase superfamily includes the extracellular signal-regulated kinases, the c-Jun N-terminal kinases/stress-activated protein kinases, and the p38MAPKs. The MAPKs are

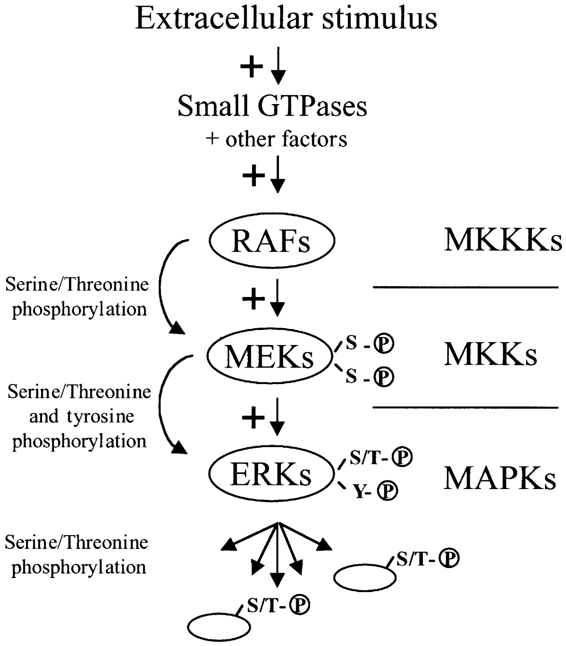
Extracellular stimulus
+
Small GTPases
+ other factors
+
RAFs
MKKKs
Serine/Threonine phosphorylation
+
MEKs
S-P
S-P
MKKs
Serine/Threonine and tyrosine phosphorylation
+
ERKs
S/T-P
Y-P
MAPKs
Serine/Threonine phosphorylation
S/T-P
S/T-P

FIGURE 4 The extracellular-regulated kinase cascade is a prime example of phosphorylations occurring in series within the cell. This cascade is initiated by Raf protein activation (MKKKs). Activated Rafs then phosphorylate MEK proteins (MKKs) on serine (S) residues. MEKs, in turn, phosphorylate extracellular signal-related kinase (ERK) proteins or mitogen-activated protein kinases (MAPKs) on serine/threonine (S/T) and tyrosine (Y) residues. This cascade ends with the phosphorylation on serine/threonine of many substrates by the ERKs.

activated at the very last step of a three-kinase cascade. MAPKs are phosphorylated and activated by the MAP/ERK kinases, or MEKs, also called MAPK kinases (or MKKs), as indicated Fig. 4. The MEKs are phosphorylated and activated by the MEK kinases, also called MAPK kinase kinases (or MKKKs). When considering the ERK MAPK cascade, which ends with the activation of ERK1 or ERK2, MEK kinases are Raf proteins; MEKs are either MEK1 or MEK2. Raf proteins phosphorylate MEKs on serine residues. MEKs, which are dual-specificity kinases, phosphorylate ERKs on serine/threonine and tyrosine residues, and ERKs phosphorylate a large number of substrates on serine and threonine residues.

## D. Multifactorial Activation

Raf-1 is one of the MEK kinases. Raf-1 regulation is complex and may illustrate what should be the integrated regulation of most protein kinases. Raf-1 is activated downstream of the small GTPase Ras, but Ras is not sufficient to activate Raf-1. Raf-1 activation requires protein–protein interactions, binding to phospholipids, and phosphorylations on serine, threonine, and tyrosine residues, as well as appropriate relocalization within the cell. Several protein kinases have been reported to phosphorylate Raf-1, including Src, PKCs, p21PAK, and Akt. The subcellular localization of Raf-1 is dictated by its binding to Ras but also to several other proteins (such as heat-shock protein 90, p50, and 14-3-3 proteins). According to the combination of these regulations, Raf-1 may exhibit graded activity states.

An additional degree of complexity is achieved because of the multiplicity of isoenzymes acting at each level of the MAPK cascade. Ras and Rap-1 are able to promote Raf protein activation. The Raf protein family includes Raf-1, B-Raf, and A-Raf. The fact that several isoenzymes can act at the same step in the cascade could suggest a redundancy, but it is now agreed that it instead involves a precise regulation that results from the great specificity of regulation and function of each isoenzyme.

## V. TRANSDUCTION MECHANISMS

As already mentioned, protein kinases can act on the enzymatic activity of other protein kinases, initiating cascades of protein kinases within the cell, as illustrated with the MAPK pathway. In addition, protein kinases can activate or inhibit other enzymes that are not protein kinases. For instance, in hepatocytes (Fig. 5), PKA not only phosphorylates a protein kinase (the phosphorylase kinase) but also phosphorylates and inactivates glycogen synthase I (an enzyme that catalyzes glycogen synthesis).

Protein kinases also phosphorylate nonenzymatic proteins. For instance, ionic channel opening can be regulated by phosphorylation. In addition, cytoskeletal components or adhesion molecules are phosphorylated by protein kinases. Phosphorylation of some proteins blocks their proteolysis, but more generally serine/threonine phosphorylation promotes ubiquitinylation and subsequent degradation of the protein by the proteasome. Phosphorylation of G-protein-coupled receptors by PKAs, PKCs, or the highly specialized G-protein receptor kinases is the first step in G-protein receptor desensitization. Indeed, the receptor phosphorylation induces arrestin binding, G-protein uncoupling, and the receptor internalization. In addition, it is now believed that it may also initiate cross talk with other pathways and even with G-protein-independent pathways.

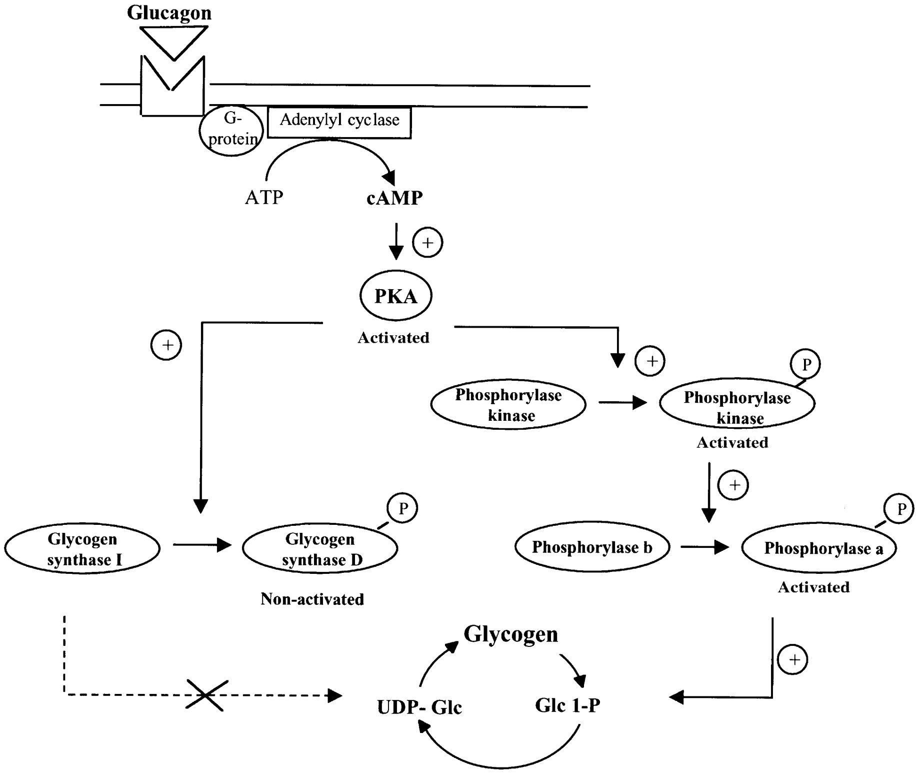

FIGURE 5 The protein kinase cascade regulating glycogen metabolism. In hyperglycemia, glucagon induces protein kinase A (PKA) activation in hepatocytes. PKA activation induces both the inhibition of the glycogen synthase I (a nonkinase enzyme) and the activation of the phosphorylase kinase (a protein kinase). Note that the multiplicity of substrates of PKA renders possible this concomitant effect and prevents a futile cycle from taking place in this metabolic pathway. UDP, Uridine diphosphate; Glc, glucose.

Phosphorylation can affect the activity of transcription factors by modulating their transcriptional activity, but also by regulating their nuclear translocation to the vicinity of DNA. In fact, bidirectional trafficking across the nuclear envelope is tightly regulated for many proteins. The nuclear localization sequence (NLS) targets proteins to the nucleus, whereas the nuclear export sequence (NES) targets proteins to the cytoplasm. The presence of the NLS is not sufficient, however, and a phosphorylation regulatory module for nuclear import (PrNLS), which includes sites in addition to the NLS, has been specified and suggests a significant role of phosphorylation in either promoting or preventing the nuclear localization.

Protein kinases are also involved in the construction of multiprotein scaffolds. Most often they are built on tyrosine kinase receptors through high-affinity phosphotyrosine docking sites. Less frequently, they are built on integrins and on G-protein-coupled receptors. Some investigators think that these scaffolds are crucial in channeling the signal to the right pathway in the intracellular network of signaling cascades and in determining the quantity of proteins to enter signal transduction by delineating a microenvironment. On the contrary, other workers consider that scaffolds generate a high degree of diversity, because the scaffolds increase considerably the number of outputs by multiplying the number of protein–protein interactions. Most agree that the scaffolds promote cross talk between the linear transduction pathways by recruiting proteins from parallel cascades. Cross talk may be the key for a selective, specific, and efficient signaling. The more signal transduction is studied, the more it is seen to proceed through combinatorial interactions of a limited repertoire of proteins and to depend on a few kinds of protein modifications, such as phosphorylation. Thus, the multiplicity of cross talk between canonical pathways is probably necessary to transmit diverse and specific signals.

## VI. MAJOR PHYSIOLOGICAL ROLES OF PROTEIN KINASES

In view of the multitude of substrates they phosphorylate and of the variety of transduction mechanisms they use, it is clear that protein kinases participate in many cellular functions. They are at least involved in shaping cell and adhesion regulation, in cellular metabolism, endocytosis, exocytosis, and cellular growth and differentiation. In particular, protein kinases are implied in the cell cycle and in regulation of apoptosis.

Specific cyclin-dependent kinases complexes regulate transitions between two subsequent phases of the cell cycle. The Cdks are serine/threonine kinases. The Cdk association with cyclins, of which the level of expression is regulated during the cycle, is the major means of regulation of Cdk activity, but phosphorylation also modulates activity. Thus, at least, two phosphorylations steps interfere in the cell cycle regulation. The upstream phosphorylation of the Cdks is followed by the serine/threonine phosphorylation of substrates by the activated Cdks.

Apoptosis is a physiologically and genetically programmed cell death that proceeds both through posttranslational modification of pre-existing factors and through regulation of a specific gene expression level. Thus, every intracellular signaling pathway involving phosphorylation can either inhibit or promote apoptosis at a distance. In addition, some proteins of the apoptotic program are also directly regulated by phosphorylation. Such is the case of caspase 9, for which proteolytic activity is reduced when it is phosphorylated. The unphosphorylated caspase 9 cleaves and activates caspase 3, which is one of the terminal caspases. These caspases are responsible for the cleavage of specific cellular proteins and for the DNA fragmentation seen in the cells committed to apoptosis.

Phosphorylation also takes part in almost every single step of the intracellular signaling pathway, from the membrane to the nucleus. This can be exemplified with the platelet-derived growth factor (PDGF) receptor. The phosphorylation of tyrosine residues on the cytoplasmic tail of such a tyrosine kinase receptor and the binding of PTB- or SH2-domain-containing proteins have already been discussed. Among these proteins, Grb2 is constitutively bound to Sos. Sos is an exchange factor that promotes small GTPase activation. Once the GTPases are activated, the MAPK cascades can continue. Finally, ERKs phosphorylate pre-existing transcription factors (such as Elk-1, CREB, and ATF2), which very quickly stimulate transcription of the so-called early gene products of the Fos/Jun family. Later, Fos and Jun transcription factors, associated in an activator protein 1 (AP-1) complex, the composition of which depends on the previous step, induce transcription of specific target genes.

## VII. PROTEIN KINASES IN HUMAN PATHOLOGIES

Because phosphorylation and dephosphorylation play an essential role in almost every regulatable cellular process, any abnormality in protein kinase activity is likely to be a feature in human pathology. For example, current research on diabetes and neurodegenerative and cardiovascular diseases suggests the potential for beneficial spin-offs from the study of protein kinase defects. In particular, the role of tyrosine kinases in cancer is well established. Cancers are characterized by unregulated cell growth, differentiation, and apoptosis, processes in which protein kinases are largely involved. Alteration of protein kinase activity, including tyrosine kinase, can be the result of several mechanisms. First is an abnormal protein kinase expression because of mutation, deletion, or chromosomal translocation. For instance, the activating mutations of Ras proteins, which are upstream protooncogene activators of the MAP/ERK cascade, are encountered in 30% of human neoplasms, including 90% of pancreatic adenomas and 30% of colon adenocarcinomas. In chronic myeloid leukemia (CML), the t(9,22) reciprocal translocation, which causes the Philadelphia chromosome, juxtaposes the sequences of the breakpoint cluster region (BCR) and the c-ABL tyrosine kinase. The resulting fusion protein exhibits an elevated tyrosine kinase activity compared to the normal c-ABL. Second, an overexpression of a normal protein kinase and/or of its endogenous activator is sufficient to induce an adverse autocrine loop. Such is the case in some lung cancers in which a sustained stimulation of the EGF-proliferating pathway has been reported. In addition, a positive correlation between an increased tumor neovascularization, an increased risk of tumor invasion, and a decreased survival is well established. Growth factors such as fibroblast growth factors (FGFs), platelet-derived growth factor β (PDGFβ), and vascular endothelial growth factor (VEGF) are stimulators of angiogenesis. This suggests that the tyrosine kinase pathways that they initiate may be involved in the further growth and invasion or in the switch from a dormant to an active state of the malignant cells.

Nevertheless, the use of protein kinase inhibitors as therapeutic agents is an unfulfilled goal. Most of the protein kinase inhibitors that have been developed are ATP-competitive inhibitors, but because all protein kinases use this nucleotide as a cofactor, it is difficult to get specificity. Adverse toxic effects may result from the inhibition of all of the intracellular phosphorylations and may limit any use of protein kinase inhibitors. It is necessary to target tumoral tissue and to preserve the normal protein kinase activity elsewhere. In addition, cross talk between transduction pathways is likely to render impossible or vain the inhibition of a unique kinase.

A few protein kinase inhibitors and angiosuppressive agents are being used in clinical trials, administered alone or in combination with other anticancer drugs. One example is the c-ABL-specific tyrosine kinase inhibitor, STI571, which is currently being tested for chronic myeloid leukemia. Another is the SU101 PDGF receptor inhibitor, which has already shown promising results in hormonorefractory prostate cancer clinical trials. Moreover, much effort has been made to develop farnesyltransferase inhibitors to prevent Ras protein binding to cell membranes and to block transduction of mitogenic signals.

Considering the crucial role of protein kinases in all major cellular processes, a better knowledge of protein kinase function is essential for a better understanding of cell physiology and pathology. There has been an enormous increase in literature on protein kinases since 1968, when E. G. Krebs first discovered the glycogen phosphorylase kinase and PKA. Ongoing research on protein kinases continues to be a promising field of study. Most efforts likely will continue to focus on discovery of new protein kinases and on gaining insight into the molecular mechanisms of regulation of the protein kinases already known. However, the importance of signal transduction compartmentalization in protein kinase function and the role of protein kinases in establishing cross talk between pathways are already under investigation.

## Glossary

**enzyme** A protein that specifically catalyzes a chemical reaction in order to accelerate the reaction speed.

**phosphorylation** Binding of a phosphate group onto a molecule.

**phosphotransferase** An enzyme that catalyzes the transfer of a phosphate group from one molecule to another.

**signal transduction** A set of events taking place in a cell in response to extracellular stimuli, including input reception, intracellular transmission of the information from the membrane to the nucleus, and integration of concomitant signals, resulting in production of a suitable biological response.

**substrate** The molecule transformed by the enzyme.

## See Also the Following Articles

**Angiogenesis • Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Membrane Receptor Signaling in Health and Disease • Signaling Pathways, Interaction of**

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# PTH

See *Parathyroid Hormone*

## II. METHODS TO STUDY PROTEIN–PROTEIN INTERACTIONS

### A. Functional Complementation Assays

One of the techniques used for the study of protein–protein interactions has been functional complementation. For this, mutant receptors that are nonfunctional or partially functional are generated either by site-directed mutagenesis or by exchanging individual domains between two distinct types of receptors (chimeras). If co-expression of these receptors results in increased functional activity as compared to individual receptors, it is taken as a measure of physical association between receptors. Functional complementation studies have documented the association between receptors of the same family as well as between receptors of distantly related families. Such functional complementation studies have been useful in predicting the domains involved in the interaction and have led to modeling studies examining the mechanisms of receptor–receptor interaction.

### B. Biochemical Techniques

In order to facilitate physical isolation of the interacting receptor–receptor complexes, differential epitope tagging and immunoisolation have been used. Typically, epitope tags (short peptide sequences from proteins that are not commonly expressed in eukaryotic tissues) are expressed at the N- or C-termini of GPCRs. cDNAs harboring distinct epitopes are co-expressed in heterologous cells. Selective immunoprecipitation of the epitope-tagged complex is achieved using antisera to one of the epitopes. The immunoprecipitate is subjected to size fractionation and the second receptor in the complex is visualized using antisera to the epitope on the second receptor. Depending on the conditions of solubilization and immunoprecipitation, there is a possibility that artifactual aggregation of proteins occurs (due to the inherent hydrophobic nature of GPCRs). This can be overcome by using stringent buffers for solubilization and immunoprecipitation. For example, the presence of a disulfide capping agent (such as iodoacetamide) in the buffers used in the receptor complex isolation process reduces chances of spurious formation of disulfide bonds. Treatment of cells with cross-linking agents followed by extraction with a combination of detergents with diverse physical properties is also helpful to disrupt nonspecific aggregation. An important control for these studies is to mix cells individually expressing receptors that are epitope tagged with distinct epitopes and to subject the mixture to solubilization and immunoprecipitation procedures identical to procedures using cells co-expressing these receptors. In the majority of cases, receptor–receptor interaction is seen only on co-expression of the two receptors. Another control is to co-express GPCRs that are known not to interact. Therefore, when the appropriate controls are used, immunoprecipitation is a valid technique for detecting GPCR dimers.

### C. Biophysical Techniques

Proximity-based energy transfer assays have been used to study receptor–receptor interactions in living cells. The bioluminescence resonance energy transfer (BRET) technique measures the transfer of energy between a luminescent donor (luciferase) and a fluorescent acceptor [a mutant form of green fluorescent protein (GFP)]. The catalytic degradation of the substrate coelenterazine, by luciferase, leads to the release of bioluminescent energy that can excite mutant GFP; the resulting fluorescence emission is taken as a measure of physical proximity between the two proteins. Because the Forster energy transfer occurs only when the distance between the donor and acceptor is less than 100 Å, this method is ideally suited to examine receptor–receptor interactions. For this, fusion proteins of GPCRs are generated by genetically fusing luciferase or the mutant GFP to their N- or C-termini.

If a BRET signal is detected on co-expression of these fusion proteins in heterologous cells in the absence of agonist treatment, it is taken as a measure of constitutive dimers in intact cells. The selectivity of the BRET signal is measured by expressing GPCR fusion proteins of different families. BRET signals can detect interactions that occur within the cells as well as on the cell surface. Agonist treatment can cause an increase in the number of receptors in the clathrin-coated pits and this can cause an increase in the BRET signal as a result of increased clustering of receptors. Conditions that block receptor clustering (such as 0.4 *M* sucrose treatment or a dominant-negative mutant of dynamin that is known to block agonist-mediated internalization of GPCRs) have been used to examine receptor–receptor interactions at the cell surface.

Fluorescence resonance energy transfer (FRET) measures the Forster resonance energy transfer between two fluorophores that emit fluorescence at nonoverlapping wavelengths. The efficiency of FRET depends on the overlap in the spectrum of the two fluorophores, their relative orientation, and the distance between them. Typically, fusion proteins of GPCRs (which are fused to two different forms of green fluorescent proteins) are used as donor and acceptor. An external light source is used to excite the donor and the light emitted by the donor excites the acceptor. This method has been used to demonstrate agonist-mediated changes in the level or conformation of receptor–receptor association. A related method, photobleaching FRET, has also been used for examining changes in interactions of receptors on agonist exposure. In this method, antisera conjugated to two different fluorophores (such as fluorescein and rhodamine) are used to bind the receptor. The decrease in the fluorescence intensity of the donor (fluorescein) as a result of photobleaching during prolonged exposure to excitation light is reduced by the acceptor (rhodamine). Because the close proximity of the acceptor slows down the photobleaching process, association between receptors leads to an increase in the photobleaching time constant. Photobleaching FRET is a useful technique to examine the interaction between receptors at the cell surface and has been used to measure changes in interaction mediated by the agonist with a variety of combinations of GPCRs of the rhodopsin family.

BRET and FRET can also be used to measure the strength of interactions between two receptors. For this, increasing amounts of the untagged receptor are co-expressed with a fixed amount of the luciferase-tagged and mutant GFP-tagged GPCR. The concentration of the competitor that decreases the signal by 50% is used to compare the strength of interactions.

## III. ROLE FOR RECEPTOR–RECEPTOR INTERACTIONS IN RECEPTOR ACTIVITY

Receptor–receptor interactions have varied effects on the activities of GPCRs. It appears that receptors that are closely related (i.e., members of the same subfamily) as well as distantly related (i.e., members of distinct subfamilies) interact with each other, and this interaction differentially modulates their function (a few examples are shown in Table 1). In some cases, receptor interactions are required for surface expression and generation of functional receptors. In other cases, interactions between two functional receptors lead to the generation of receptors with novel binding properties. Some of these receptors are refractory to selective ligands; activation of both receptors is required for efficient ligand binding and signaling. In some cases receptor–receptor association leads to alterations in the agonist affinity as well as the efficacy and in others only the agonist efficacy is altered. Receptors that couple to distinct G-proteins are able to interact with each other, and this interaction has been shown to differentially affect the signal transduction pathway and/or level of signaling. Receptor–receptor interactions can increase or decrease the level of receptor desensitization due to alterations in agonist-mediated endocytosis of the receptor. Finally, a role for receptor–receptor interaction in the development of pathology has been documented in the case of angiotensin–bradykinin receptor interactions.

TABLE 1 **Regulation of GPCR Function by Heteromeric Interactions**

| Receptor | Modulation of function |
|---|---|
| **Members of the same subfamily** | |
| $\mathrm{GABA_BR_{1a}}$–$\mathrm{GABA_BR_2}$ | Surface expression and signaling |
| κ–δ opioid | Signaling and trafficking |
| μ–δ opioid | Signaling |
| $\mathrm{SSTR_1}$–$\mathrm{SSTR_5}$ (somatostatin) | Signaling and trafficking |
| $\mathrm{SSTR_2}$–$\mathrm{SSTR_3}$ (somatostatin) | Signaling and trafficking |
| $\mathrm{M_2}$–$\mathrm{M_3}$ (muscarinic) | Signaling |
| **Members of the same family** | |
| $\alpha_{2c}$-adrenergic–$\mathrm{M_3}$ (muscarinic) | Signaling |
| $\mathrm{SSTR_5}$ (somatostatin)–$\mathrm{D_2}$ (dopamine) | Signaling |
| $\mathrm{AT_1}$(angiotensin)–$\mathrm{B_2}$ (bradykinin) | Signaling and trafficking |
| δ opioid–$\beta_2$-adrenergic | Trafficking |
| κ opioid–$\beta_2$-adrenergic | Trafficking |
| **Members of different families**[a] | |
| $\mathrm{A_1}$ (adenosine)–$\mathrm{mGluR_1}$ (glutamate) | Signaling |

[a] Families A and C.

## IV. FACTORS THAT MODULATE RECEPTOR–RECEPTOR INTERACTIONS

There is a growing body of evidence to support the proposal that GPCRs interact with each other in the absence of agonist. In a large number of cases, receptor–receptor interactions appear to occur in the biosynthetic compartments (i.e., in the endoplasmic reticulum) and to be required for efficient maturation of receptors. However, in some cases, receptors appear to exist as noninteracting units that undergo increased association mediated by the agonist, as evidenced by biochemical and biophysical studies.

In a subset of cases, GPCRs have been shown to interact with other proteins (such as growth factor receptors, ion channels, receptor activity-modifying proteins, and other intracellular signaling molecules), and these interactions result in efficient surface expression, change in the cellular localization, and/or alteration in their functional activity.

## V. DOMAINS OF RECEPTOR–RECEPTOR INTERACTIONS

An examination of the possible sites for receptor–receptor interactions has implicated the involvement of extracellular, transmembrane, and/or C-terminal regions in GPCR association. The evidence accumulated thus far points to transmembrane domain-mediated contacts that lead to receptor–receptor interactions. Hydrophobic interactions within the transmembrane domain are thought to provide the proper receptor conformation to facilitate additional interactions at other domains. Hydrophobic interaction via the coiled-coil domain in the C-tail has also been documented in the case of metabotropic $\gamma$-aminobutyric acid receptor. Although in the majority of the cases the interactions are mainly hydrophobic in nature, in some cases covalent interactions do occur. Crystallographic studies of the extracellular domain of metabotropic glutamate receptors have identified cysteine residues involved in the covalent homomeric interactions.

## VI. SUMMARY

In conclusion, the study of GPCR interactions is a nascent field and studies thus far support the notion that receptor–receptor associations lead to changes in receptor function by modulating their ligand affinity, signaling, and trafficking properties. These interactions could be useful to modulate receptor activation following the corelease of selective endogenous ligands *in vivo*. Alternatively, these interactions could lead to the generation of a hitherto uncharacterized receptor for a unique endogenous ligand. The number of endogenous peptide ligands far exceeds the number of cloned GPCRs. These endogenous peptides could bind and activate interacting receptors that exhibit novel pharmacology; this could explain GPCR subtypes in some cases. Direct physical interactions between GPCRs have enormous ramifications for our understanding of how their actions are regulated. Furthermore, these findings provide a new strategy for the development of novel therapies.

## Glossary

**BRET/FRET** Measures of the bioluminescence/fluorescence energy transfer from the light emitted by catalytic degradation of the substrate coelenterazine, by Renilla luciferase, to the energy acceptor green fluorescent protein. FRET is similar to BRET with the exception that the energy donor molecule, generally a mutant form of green fluorescent protein (cyan fluorescent protein) is excited by an external light source and the emitted energy is used to excite an acceptor, another mutant of green fluorescent protein (yellow or red fluorescent proteins).

**differential immunoprecipitation** Technique for isolation and visualization of interacting proteins using distinct antisera to individual proteins.

**dimers/oligomers** Two monomers/multiple monomers in association; a given G-protein-coupled receptor can exist as monomer, homo- or heterodimer, or homo- or heterooligomer.

**functional complementation** Technique in which coexpression of two receptors that are nonfunctional or partially functional leads to improved functional activity.

**G-protein-coupled receptor** Heptahelical or serpentine seven-transmembrane-domain receptor.

**G-protein-coupled receptor families** Three major receptor families: Family A is characterized by a relatively short N-terminal extracellular region, conserved residues in transmembrane helices, and a palmitoylated cysteine in the carboxy-terminal tail. Family B is characterized by the presence of a large N-terminal extracellular domain that contains several well-conserved cysteine residues. Family C is characterized by a very long N-terminal extracellular domain that is sufficient for ligand binding.

**homomers/heteromers** Physical association between identical proteins leads to homomers and association between nonidentical proteins leads to heteromers.

## See Also the Following Article

**GPCR (G-Protein-Coupled Receptor) Structure**

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# Reproductive Stress

See *Stress and Reproduction*

# Retinoid Receptors

Peter Ordentlich and Richard A. Heyman
*X-Ceptor Therapeutics, Inc., California*

I. INTRODUCTION
II. RECEPTOR IDENTIFICATION
III. RECEPTOR STRUCTURE
IV. MECHANISM OF ACTION
V. RECEPTOR PHYSIOLOGY
VI. RXR HETERODIMERIZATION
VII. RETINOID RECEPTORS AND CANCER
VIII. SUMMARY

**Retinoids are metabolic and synthetic derivatives of vitamin A that have been shown to play an essential role in regulating multiple processes including development, vision, growth, and physiological homeostasis.**

## I. INTRODUCTION

The majority of these effects are mediated through direct binding to a group of receptors that belong to the nuclear hormone receptor superfamily of proteins. These retinoid receptors fall into two families, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are three receptors in each group, RARα, RARβ, RARγ, and RXRα, RXRβ, RXRγ, respectively. The retinoid receptors function as transcriptional regulators in the presence or absence of ligand, through the recruitment of multisubunit regulatory protein complexes to promoter sites. Other factors that influence the action of retinoids include the enzymes and proteins that are required to transport and metabolize retinoids. This article will provide an overview of the RAR and RXR receptors with regard to their structure, mechanism of action, biological activities, and role in disease states.

## II. RECEPTOR IDENTIFICATION

Two independent groups identified the first retinoid receptor, RARα, in 1987 using similar approaches. In one case, low-stringency hybridization screening of a cDNA library, with a consensus oligonucleotide sequence based on a highly conserved region of the DNA-binding domain, resulted in successful cloning of a novel sequence with homology to other nuclear hormone receptors. The other group designed a similar screening strategy based upon the finding that in a human hepatocellular carcinoma, the hepatitis B virus genome inserted upstream of a sequence that showed strong homology to other nuclear hormone receptors. Subsequently, it was shown that this receptor, RARα, could bind and be activated by retinoic acid.

Based on sequence homology in the DNA-binding domain, RARβ and RARγ were later identified and also shown to bind and be activated by retinoic acid (Fig. 1). The three RARs are highly conserved in both cell (of B) at time $t$ as a concentration time average over a time delay $[l_1, l_2]$ is represented as

$$\fint_{t-l_1}^{t-l_2} Y(r)dr = \frac{1}{l_2 - l_1} \int_{t-l_1}^{t-l_2} Y(r)dr,$$

where $Y(r)$ is either a hormone concentration or its rate of change at time $r$. The effect of a feedback/feed-forward signal for a peptide is different from that for a steroid. If hormone A is a steroid, its molecules are able to diffuse through the target cell membrane, whereas if hormone A is a peptide, its molecules do not penetrate but rather attach to receptors on the target cell membrane. Either way, a cascade of biochemical reactions results.

These feedback interactions can be modeled via monotonic logistic dose–response functions

$$H(x_1) = \frac{C}{1 + \exp[-(A + B_1 x_1)]} + D,$$

of one input,

$$H(x_1, x_2) = \frac{C}{1 + \exp[-(A + B_1 x_1 + B_2 x_2)]} + D,$$

of two inputs,

or where the coefficients themselves are described by logistic functions

$$H(x_1, x_2) = \frac{C(x_2)}{1 + \exp[-(A + B_1 x_1 + B_2 x_2)]} + D.$$

If $B_i > 0$, the feedback is positive (i.e., feed-forward effect); if $B_i < 0$, the feedback is negative. For each hormone, its resulting nonbasal rate of synthesis $S(\cdot)$ will depend on time-delayed feedback from the system through some nonlinear dose–response function $H(\cdot)$, plus noise $\xi(\cdot)$. The $H(\cdot)$ function serves the role of an average rate (a "conditional expectation") for all the cells in the endocrine gland; the actual realized rate of synthesis $S(\cdot)$ varies about $H(\cdot)$. Response adaptation and desentization can then be formulated as allowable variation in the parameters of the interfaces.

Whereas dose–response relationships in the above three axes have been largely defined for individual nodes acting in isolation (e.g., GnRH's stimulation of LH secretion, LH's receptor-mediated stimulation of testosterone and estrogen secretion), the implicitly dynamic nature of this network arises from the time-lagged nonlinear feedback and feed-forward interactions among all three interconnected loci.

## II. THE HPT (GnRH–LH–Te) MODEL, AS REPRESENTATIVE OF RECEPTOR-MEDIATED SYSTEMS

Secretion of a hormone is usually viewed as occurring in two fractions. There is an approximately constant basal secretion as well as nonbasal secretion, the latter being highly variable and fluctuating. For steroid hormones (e.g., Te), nonbasal secretion occurs as a continuous release. For peptide hormones (e.g., GnRH and LH), there is variable mass accumulation within the endocrine gland, which is released in a pulsatile manner. In either case, upon release from a gland, hormones within the capillary bloodstream are immediately subjected to at least two primary dispersive forces: physical diffusion within the plasma space and advective effects of blood flow within the tubular capallaries. The process of irreversible (metabolic) removal of the hormone molecules can take place in the systemic circulation, since blood is delivered to metabolizing tissues, such as the liver, kidneys, and bone marrow macrophages.

Suppose that the systemic circulation can be described topologically as a circle ($S^1$) of length $L$. Let $X(x,t)$ be the concentration and $Z(x,t)$ be the rate of secretion at time $0 \leq t \leq T$ and at location $x \in S^1$. Also, let $\alpha_i$ be the elimination rate constant and $D_i$ and $A_i$ the diffusion and advection constants. It is assumed that the concentration dynamics, with initial conditions $X_i(x,0) = X_i^0(x)$, are described by

$$\frac{\partial X_i(x,t)}{\partial t} = D_i \frac{\partial^2 X_i(x,t)}{\partial t^2} + A_i \frac{\partial X_i(x,t)}{\partial x} - \alpha_i X_i(x,t) + Z_i(x,t)$$

where G denotes GnRH, L denotes LH, and Te denotes testosterone.

The difficulty with that implementation is that blood sampling is ordinarily performed at a single site $x_*$ and for practical reasons probably always will be. Hence, a representation of the above equation applicable to the sampling at a single location ($x_*$) is needed. The following approximation can be justified (for a given hormone), where the dependency on $x = x_*$ is left implicit; e.g., $X_G(x_*, t)$ and $Z_G(x_*, t)$ are represented as $X_G(t)$ and $Z_G(t)$, respectively, $i =$ G, L, Te,

$$X_i(t) \approx X_i(0)(a_i^{(1)} e^{-\alpha_i^{(1)} t} + a_i^{(2)} e^{-\alpha_i^{(2)} t}) + \int_0^t (a_i^{(1)} e^{-\alpha_i^{(1)}(t-s)} + a_i^{(2)} e^{-\alpha_i^{(2)}(t-r)}) Z_i(s) ds,$$

where $\alpha_i^{(1)}$ can be interpreted as a fast half-life of elimination (primarily diffusion and advection) and $\alpha_i^{(2)}$ as a slow half-life of elimination (irreversible clearance). The $a_i^{(1)}$ and $a_i^{(2)}$ are fractional amounts of secretion, with $a_i^{(1)}$ estimated as 0.63 for LH and 0.76 for Te. The variations among individuals and within a given individual appear to be quite stable, with 0.63 and 0.76 being reasonable representative values. At present, these values have not yet been determined for GnRH.

In order to apply the above model for concentration ($X_i(\cdot)$), models for the rates of secretion for GnRH, L, and Te are formulated in the following sections.

## A. GnRH Pulse Generator

The pulsatile nature of GnRH and LH secretion was first discovered in the rhesus monkey and then later in human and other species. There are on the order of 800–1200 GnRH-secreting neurons, each connected to its own individual network of glial cells; the resulting random pulsatile structure is partly modulated by feedback inhibition via testosterone receptors, as well as by a synchronization of network firing frequencies. It is assumed that GnRH signaling dictates the pulse times for LH after a finite time delay $\tau_L$, reflecting hypothalamo–pituitary portal blood transit, and a poststimulus refractory interval, $r_L$, when further GnRH inputs are ignored. Thus, there will be two corresponding sets of pulse times $T_G^0, T_G^1, T_G^2, \ldots$ and $T_L^0, T_L^1, T_L^2, \ldots$, where

$$T_L^k = Min_j\{T_G^j \mid T_G^j \geq T_L^{k-1} + r_L\} + \tau_L,$$
$$\text{with } T_G^0 = T_L^0 = 0.$$

Here the pulse times are viewed as a point process, with $N_i(\cdot)$, $i = G$, L being the associated counting processes (i.e., the number of pulses up to that time). The GnRH pulse times are then assumed to be given by a rate parameter process $\lambda(\cdot)$ (number of pulses/day), modulated by feedback:

$$\lambda(t) = H_{1,2}\left[\int_{(t-l_{1,2})}^{(t-l_{1,1})} X_{Te}(r)dr,\ \int_{(t-l_{2,2})}^{(t-l_{2,1})} X_G(r)dr\right]$$

and a parameter $\gamma$, which controls the regularity of interpulse interval lengths. The conditional probability densities for $T_G^k$ given $T_G^{k-1}$ are

$$p\left[s \mid T_G^{k-1}, \lambda(\cdot)\right] = \gamma \times \lambda(s)\left(\int_{T_G^{k-1}}^{s} \lambda(r)dr\right)^{\gamma-1} \times \exp^{-\left[\int_{T_G^{k-1}}^{s} \lambda(r)dr\right]^{\gamma}}.$$

## B. Synthesis

A pulsatile rate of secretion means that release is not continuous, but rather at certain times (the pulse times), the rate of secretion rapidly increases, followed by a less rapid decrease; this combined increase and decrease in the rate of secretion will be called a pulse. A pulse at time $t$, having started at pulse time $T^j$, is represented by a function $M^j \times \psi(t - T^j)$, where the pulse shape $\psi(\cdot)$ represents the instantaneous rate of secretion per unit mass per distributional volume and $M^j$ is the total mass available for release.

The mathematical effect of cascading target-tissue reactions to a GnRH signal input is (approximately) the multiplication of the initial feedback/feed-forward signal by a linear combination of exponential functions, denoted by $\Gamma_G(\cdot)$, which allows ongoing glandular responses after the signal is withdrawn. Accordingly, synthesis ($S$), accumulation ($C$), and fractional mass remaining for later secretion ($\Psi$) are given as

$$\psi_i(s) = \frac{\beta_i^{(3)}}{\Gamma(\beta_i^{(1)})(\beta_i^{(2)})^{(\beta_i^{(1)}\beta_i^{(3)})}} s^{(\beta_i^{(1)}\beta_i^{(3)})-1} \times e^{-(s/\beta_i^{(2)})^{\beta_i^{(3)}}}$$

(normalized rates of secretion for $i = \mathrm{G}, \mathrm{L}$) (a 3-parameter gamma function)

$$\Psi_i(T_i^{j-1}, T_i^j) = \int_{T_i^{j-1}}^{T_i^j} \psi_i(s - T_i^j)ds$$

(fraction of $(j-1)$ – st mass remaining at time $T_i^j, i = G, L$)

$$S_L(t) = H_{5,6}\left[\int_{T_L^{N_L(t)}-l_{5,2}}^{T_L^{N_L(t)}-l_{5,1}} X_G(s)ds \times \Gamma_G\left(t - T_L^{N_L(t)}\right),\ \int_{t-l_{6,2}}^{t-l_{6,1}} X_{Te}(s)ds + \xi_L(t)\right]$$

(LH synthesis) (expected LH synthesis rate + allowable variation ($\xi$))

$$S_G(t) = H_3\left(\fint_{t-l_{3,2}}^{t-l_{3,1}} X_{Te}(s)ds\right) + \xi_G(t)$$

(GnRH synthesis)

$$A_L^j = \int_{T_L^{j-1}}^{T_L^j} \xi_L(t)dt \text{ and}$$

$$A_G^j = \int_{T_G^{j-1}}^{T_G^j} \xi_G(t)dt$$

(allowable variation in LH and GnRH mass accumulation)

$$C_L^j = \int_{T_L^{j-1}}^{T_L^j} H_{5,6}\left(\fint_{T_L^{N_L(t)}-l_{5,2}}^{T_L^{N_L(t)}-l_{5,1}} X_G(s)ds \times \Gamma_G(t - T_L^{N_L(t)}), \fint_{t-l_{6,2}}^{t-l_{6,1}} X_{Te}(s)ds\right) dt + A_L^j$$

(the storage of newly synthesized LH granules)

$$\approx [\eta_L^{(0)} + \eta_L^{(1)} \times (T_L^j - T_L^{j-1})] + A_L^j$$

$$C_G^j = \int_{T_G^{j-1}}^{T_G^j} H_3(\fint_{t-l_{3,2}}^{t-l_{3,1}} X_{Te}(s)ds)dt + A_G^j$$

$$\approx [\eta_G^{(0)} + \eta_G^{(1)} \times (T_G^j - T_G^{j-1})] + A_G^j$$

$$M_i^j = \Psi_i(T_i^{j-1}, T_i^j)\ M_i^{j-1} + C_i^j$$

(the $j$-th pulse mass, for $i = L, G$)

$$\approx \Psi_i(T_i^{j-1}, T_i^j)\ M_i^{j-1} + [\eta_i^{(0)} + \eta_i^{(1)} \times (T_i^j - T_i^{j-1})] + A_i^j$$

$$F_L(t) = \fint_{t-l_{4,2}}^{t-l_{4,1}} X_L(s)\, ds$$

(LH feed-forward signal on Te synthesis)

$$S_{Te}(t) = H_4(F_L(t)) = H_4(\fint_{t-l_{4,2}}^{t-l_{4,1}} X_L(s)ds)$$

## C. Secretion

Based upon the above constructions, the corresponding interactively controlled rates of secretion for L, G, and Te are given as

$$Z_L(t) = \beta_L + \sum_{j:T_L^j \le t} [(\eta_L^{(0)} + \eta_L^{(1)} \times (T_L^j - T_L^{j-1}) + A_L^j]\psi_L\ (t - T_L^j)$$

$$Z_G(t) = \beta_G + \sum_{j:T_L^j \le t} [(\eta_G^{(0)} + \eta_G^{(1)} \times (T_G^j - T_G^{j-1}) + A_G^j]\psi_G\,(t - T_G^j)$$

$$Z_{Te}(t) = S_{Te}(t) = \eta_0 + \frac{\eta_1 + A_{Te}^j}{1 + e^{-(\eta_2 + \eta_3 \times F_L(t))}}, \quad T_L^{j-1} < t \le T_L^j$$

($A_{Te}^j$'s, allowable random variations in upper asymptote (efficacy))

Hence, the basic secretory model for LH and GnRH consists of two components: basal ($\beta$) and pulsatile (non-basal) secretion. The amount of mass that accumulates is assumed to be proportional to the previous interpulse interval, plus an allowable random variation (the $A_L^j$'s and $A_G^j$'s). If there were mass accumulation at a rate that randomly varied about a steady-state rate, then the result would be precisely this. Testosterone, also has two components (basal and non-basal), except that the non-basal secretion is assumed to be released continuously, without storage. Moreover, the non-basal component is assumed to be described by a logistic interface function of an LH feed-forward signal, a time-delayed averaging of LH concentration. Again, as in the modeling of the LH and GnRH secretion, flexibility in the structure needs to be allowed. Here, there is allowable pulse-by-pulse variation in the upper asymptote (i.e., efficacy) of the dose-response function, reflecting potential desensitization or adaptation. These models for GnRH, LH, and testosterone secretion reflect the essence of the title: receptor-mediated interlinkages.

## D. Concentrations

Incorporating the above secretion rates, the resulting concentration processes are then (approximately) given as: $i$ = G, L, Te.

$$X_i(t) = X_i(0)\Big[a_i^{(1)}e^{-\alpha_i^{(1)}t} + a_i^{(2)}e^{-\alpha_i^{(2)}t}\Big] + \int_0^t \Big[a_i^{(1)}e^{-\alpha_i^{(1)}(t-s)} + a_i^{(2)}e^{-\alpha_i^{(2)}(t-s)}\Big]Z_i(s)ds.$$

What one then observes is a discrete-time sampling of these processes, plus joint uncertainty due to blood withdrawal, sample processing, and hormone measurement errors, $\epsilon_i(k)$, $k = 1, \ldots, n$,

$$Y_i(t_k) = X_i(t_k) + \epsilon_i(k), \quad k = 1, \ldots, n, \quad i = G, L, Te.$$

Using the statistical methods developed, which implement the above models, statistical fits can be obtained for the GnRH, LH, and testosterone

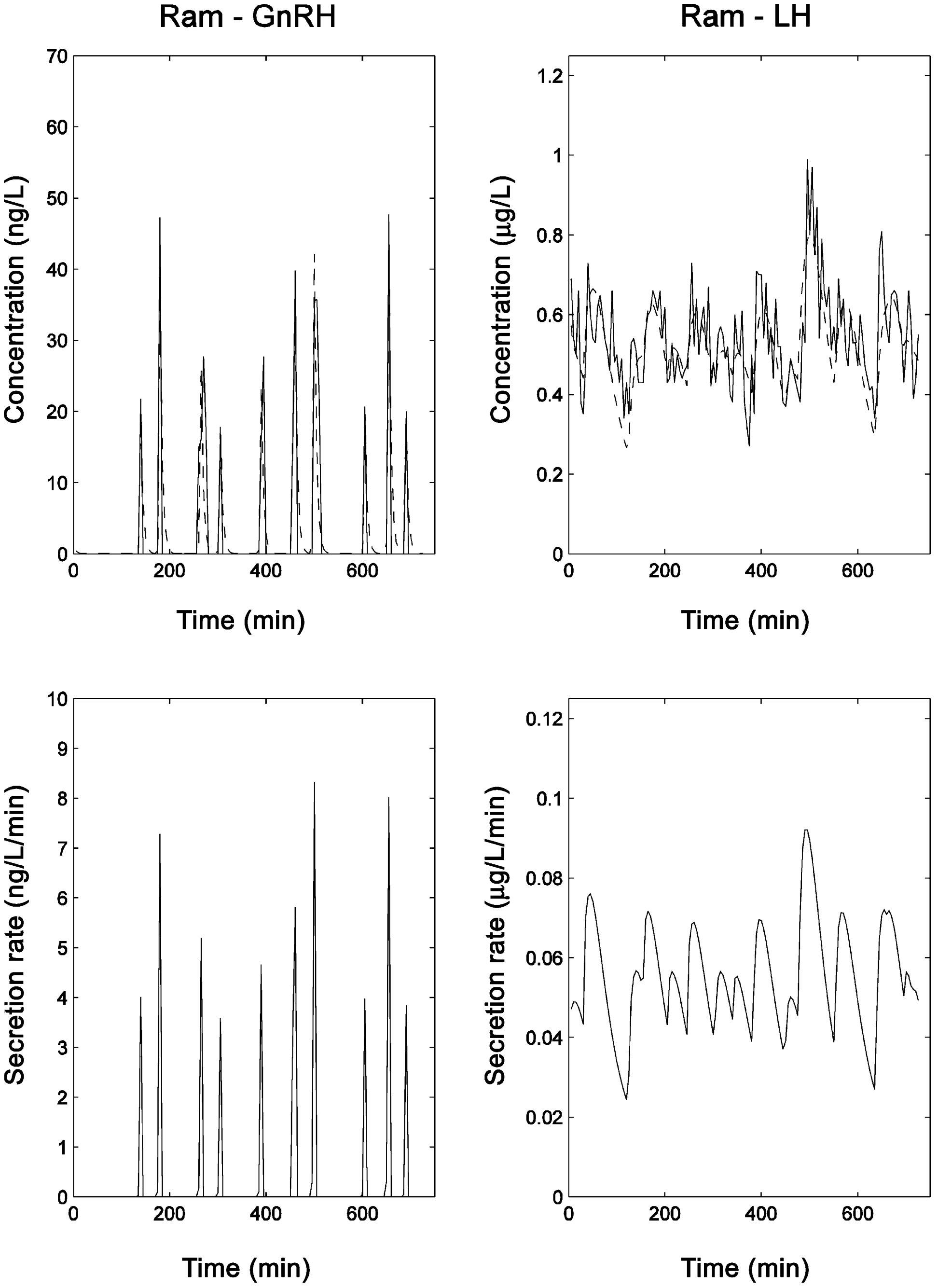

FIGURE 2 GnRH, LH and Te concentrations were obtained in a conscious ram, every 5 min for 12 hours. (Top) GnRH (left) and LH (right) concentrations (continuous line) and their model fits (dashed). (Bottom) Estimated GnRH (left) and LH (right) secretion rates.

concentrations, as well as the estimation of their unobserved secretion rates. The next section presents two applications of the methodology.

## E. Applications

For exposition of the above model, data obtained from a ram and a stallion is used. In each, all three of the hormones (GnRH, LH, and Te) were measured. In the ram, blood was sampled every 5 minutes for 12 hours. In the stallion, the sampling was over 6 hours, with GnRH and LH sampled every 5 minutes at the pituitary, and Te and LH sampled every 15 minutes at a jugular. The results are presented in Figs. 2 and 3 for the ram and in Figs. 4 and 5 for the horse. Figure 2 (top) displays the GnRH and LH

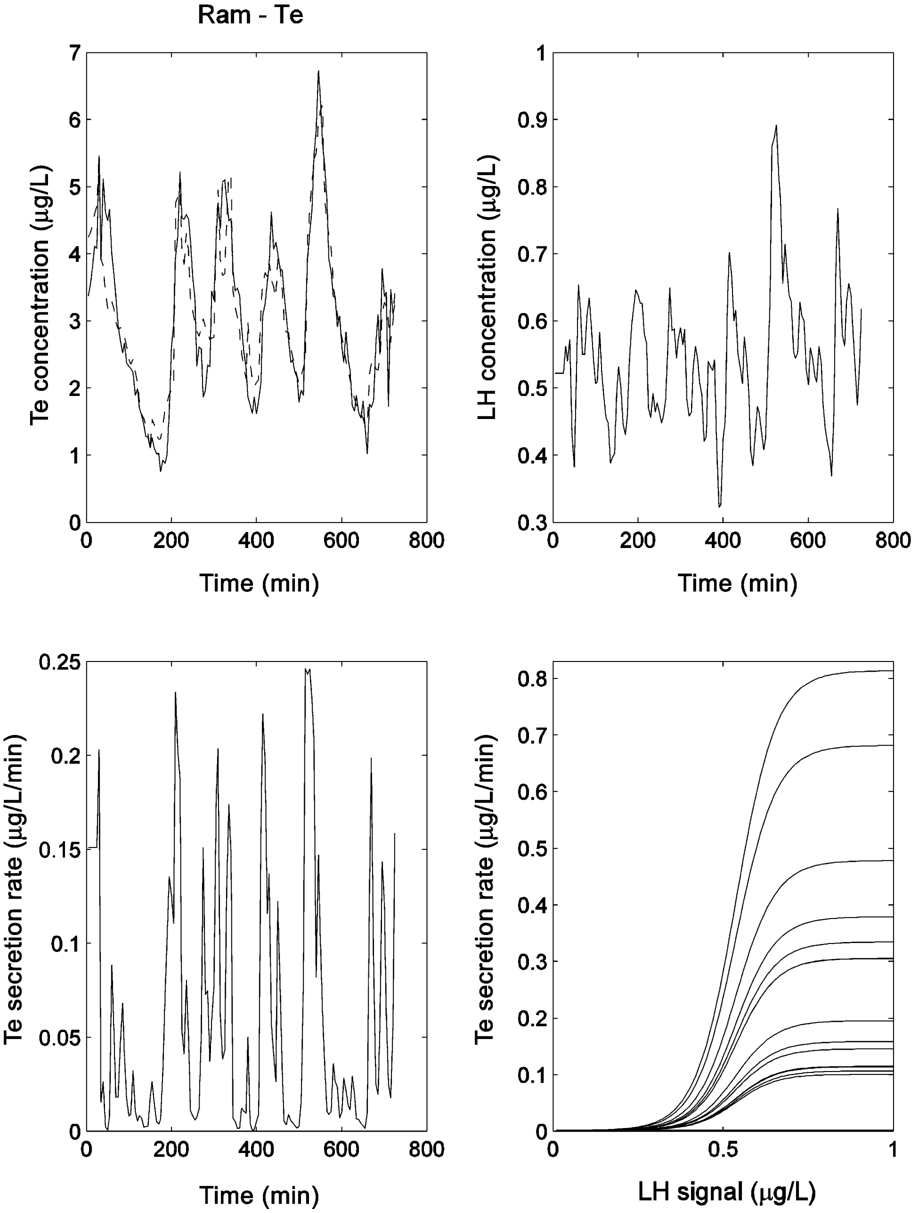

FIGURE 3 (Top left) Te concentration (continuous line) and the model fit (dashed), and (right) the LH feed-forward signal on Te. (Bottom left) The estimated Te secretion right, and (right) the dose-response function, with the allowable, random, pulse-by-pulse responsitivity (efficacy) shifts.

concentrations (continuous line) and their respective model fits (dashed line). The bottom half of Fig. 2 shows the corresponding estimated secretion rates for GnRH and LH. Figure 3, top left, shows the observed Te concentrations (continuous line) and the model fit (dashed line) and the LH feed-forward signal is shown in the top right. The bottom left of Fig. 3 shows the estimated Te secretion rate and in the bottom right is the dose-response function, with the allowable, random, pulse-by-pulse responsitivity (efficacy) shifts. Figures 4 and 5 contain the same plots for the horse, with the addition of LH being observed at both the pituitary and the jugular.

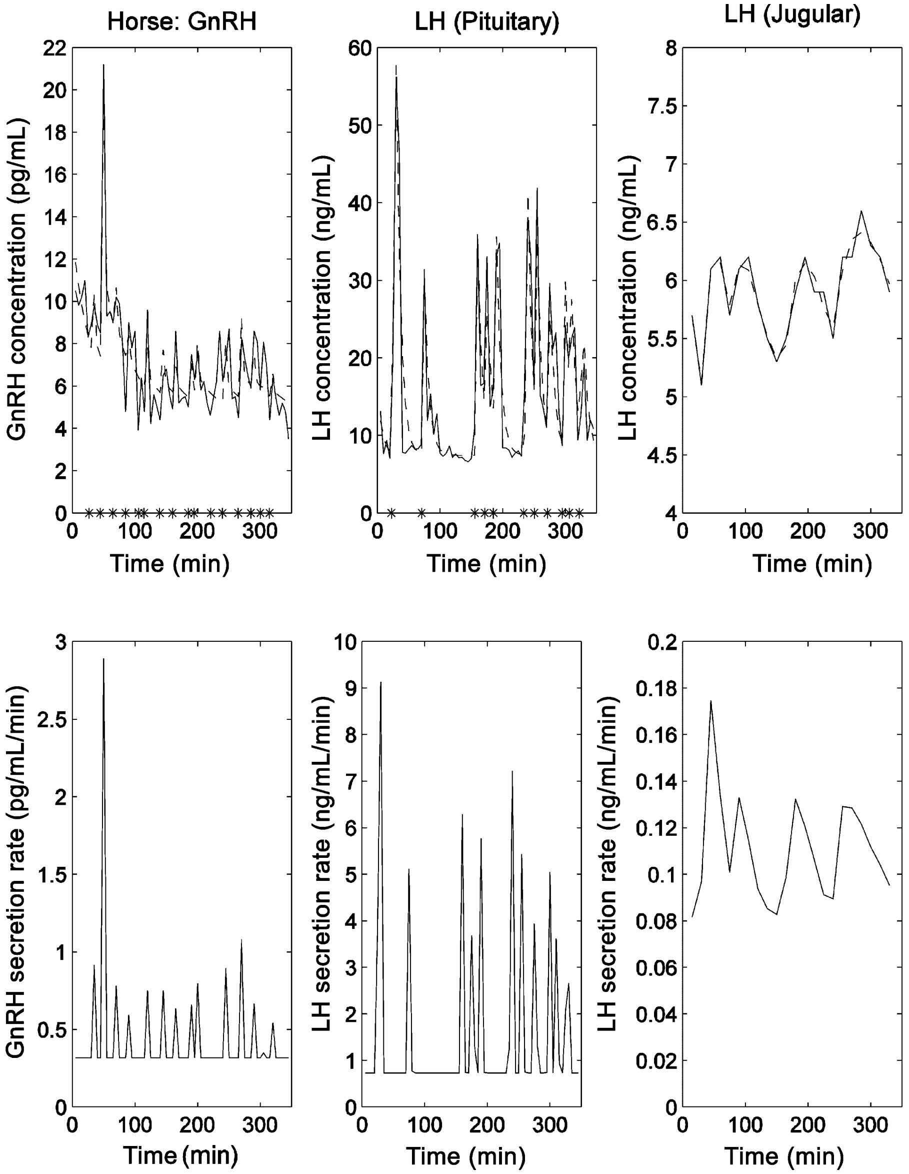

FIGURE 4 GnRH and LH concentration time series monitored every 5 min in pituitary blood, and Te and LH were monitored every 15 min in jugular blood, for 6 hours in an awake stallion. Figures 4 and 5 include the same information format as in Figs. 2 and 3 (with an additional panel for jugular LH). (Top) GnRH (left) and LH (right) concentrations (continuous line) and their model fits (dashed). (Bottom) Estimated GnRH (left) and LH (right) secretion rates.

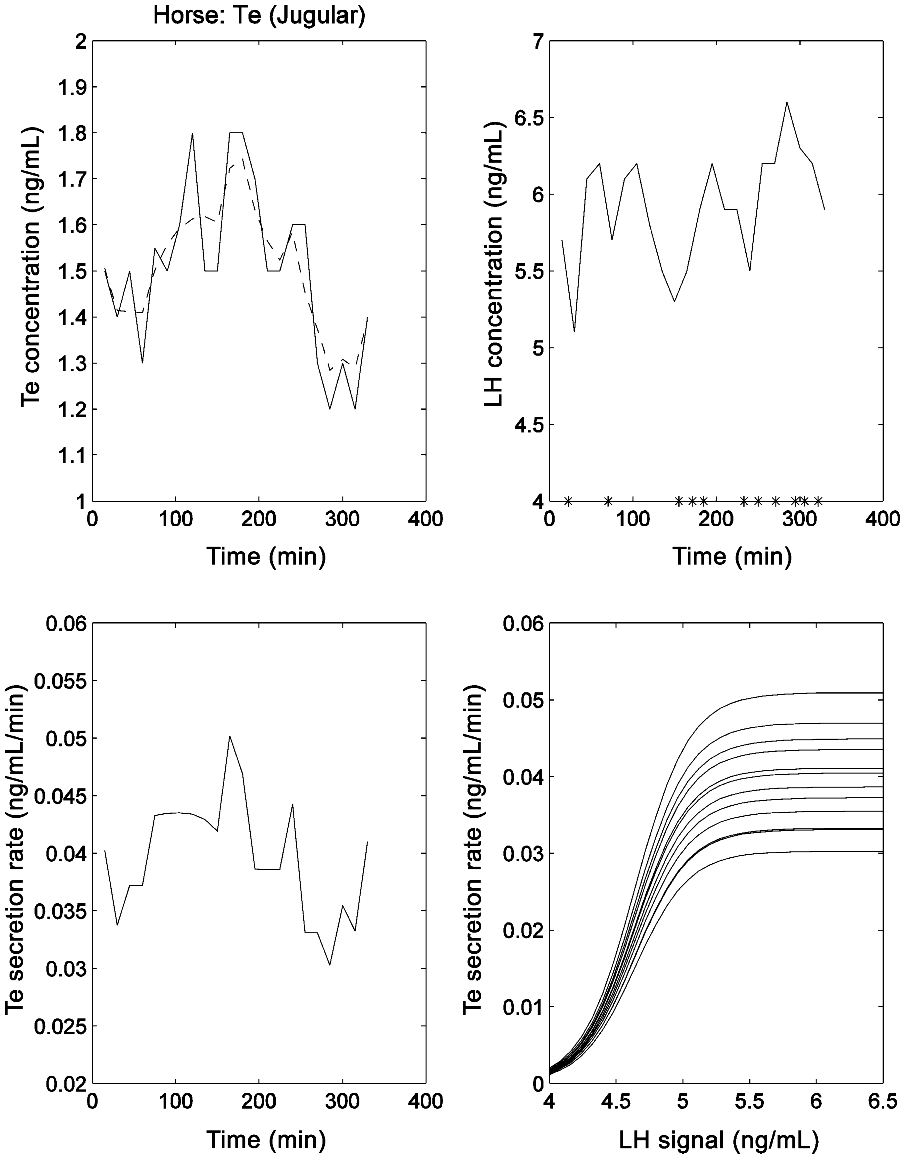

**FIGURE 5** (Top) GnRH (left) and LH (right) concentrations (continuous line) and their model fits (dashed). (Bottom) Estimated GnRH (left) and LH (right) secretion rates Figure 5 (Top left) Te concentration (continuous line) and the model fit (dashed), and (right) the LH feed-forward signal on Te. (Bottom left) The estimated Te secretion right, and (right) the dose-response function, with the allowable, random, pulse-by-pulse responsitivity (efficacy) shifts.

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## Glossary

**dose–response interface function** A function (e.g., a logistic function) whose response output is an instantaneous rate of hormone synthesis, mass accumulation, or release.

**fast and slow elimination** Secreted molecules undergo combined diffusion and transport in the bloodstream at very rapid rates (short half-life component, $\alpha^{(1)}$) and are removed more slowly but irreversibly (long half-life component, $\alpha^{(2)}$).

**feedback and/or feed-forward signal** A signal at time *t*, constructed as a time-delayed, time-averaging of a hormone concentration (or its rate of change) that serves as an input (driver) to the dose–response interface function.

**pulse generator** A process, possibly modulated by feedback, that governs the resulting, variable-pulse time-release pattern for a pulsatile secreting gland.

**receptor-mediated system** A physiological system (e.g., a hormonal axis) whose linkages are interconnected via receptor mechanisms (interface functions).

## See Also the Following Article

**Receptor–Receptor Interactions**

## Further Reading

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# Receptor–Receptor Interactions

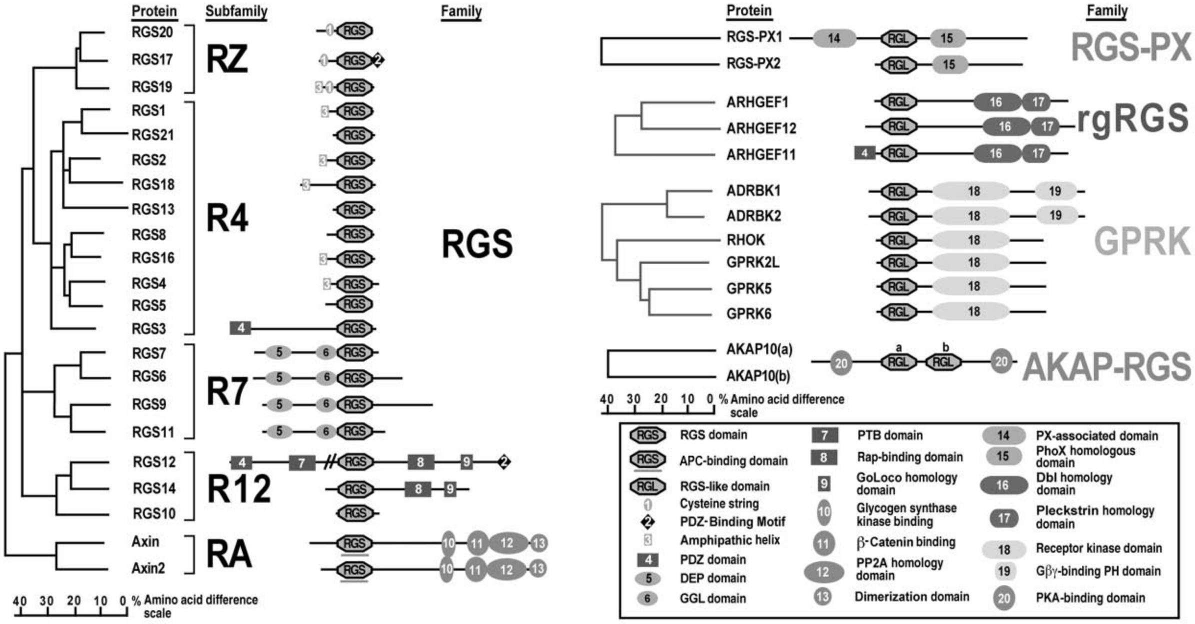
Lakshmi A. Devi
*Mount Sinai School of Medicine*

I. INTRODUCTION
II. METHODS TO STUDY PROTEIN–PROTEIN INTERACTIONS
III. ROLE FOR RECEPTOR–RECEPTOR INTERACTIONS IN RECEPTOR ACTIVITY
IV. FACTORS THAT MODULATE RECEPTOR–RECEPTOR INTERACTIONS
V. DOMAINS OF RECEPTOR–RECEPTOR INTERACTIONS
VI. SUMMARY

**The function of every cell in the body is regulated by plasma membrane receptors. The dimerization of receptors has been an extensively studied phenomenon. Receptor–receptor associations lead to changes in receptor function by modulating receptor ligand affinity, signaling properties, and trafficking properties. These interactions could be useful to modulate receptor activation and may provide strategies for therapeutic applications.**

## I. INTRODUCTION

The vast majority of plasma membrane receptors belong to the superfamily of G-protein-coupled receptors (GPCRs), which at current estimates account for ~1% of the genes present in a mammalian genome. Models describing the interaction of GPCRs with their G-protein targets have been based on the assumption that the receptors exist as monomers and couple to G-proteins in a 1:1 ratio. These classical models of receptor/G-protein coupling may be oversimplified, because a number of studies have reported the presence of dimeric and oligomeric arrays in the case of a number of GPCRs. Direct protein–protein interaction (GPCR oligomerization) has not been previously recognized despite a significant amount of indirect evidence derived from cross-linking experiments, target size analysis, and hydrodynamic studies. The availability of GPCR complementary DNAs (cDNAs) now allows direct examination of receptor–receptor association by biochemical and biophysical techniques.


Protein
Subfamily
Family
RGS20
RGS17
RGS19
RZ
RGS1
RGS21
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RGS4
RGS5
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rgRGS
ADRBK1
ADRBK2
RHOK
GPRK2L
GPRK5
GPRK6
GPRK
AKAP10(a)
AKAP10(b)
a
b
AKAP-RGS
RGS domain
APC-binding domain
RGS-like domain
1 Cysteine string
2 PDZ-Binding Motif
3 Amphipathic helix
4 PDZ domain
5 DEP domain
6 GGL domain
7 PTB domain
8 Rap-binding domain
9 GoLoco homology domain
10 Glycogen synthase kinase binding
11 β-Catenin binding
12 PP2A homology domain
13 Dimerization domain
14 PX-associated domain
15 PhoX homologous domain
16 Dbl homology domain
17 Pleckstrin homology domain
18 Receptor kinase domain
19 Gβγ-binding PH domain
20 PKA-binding domain

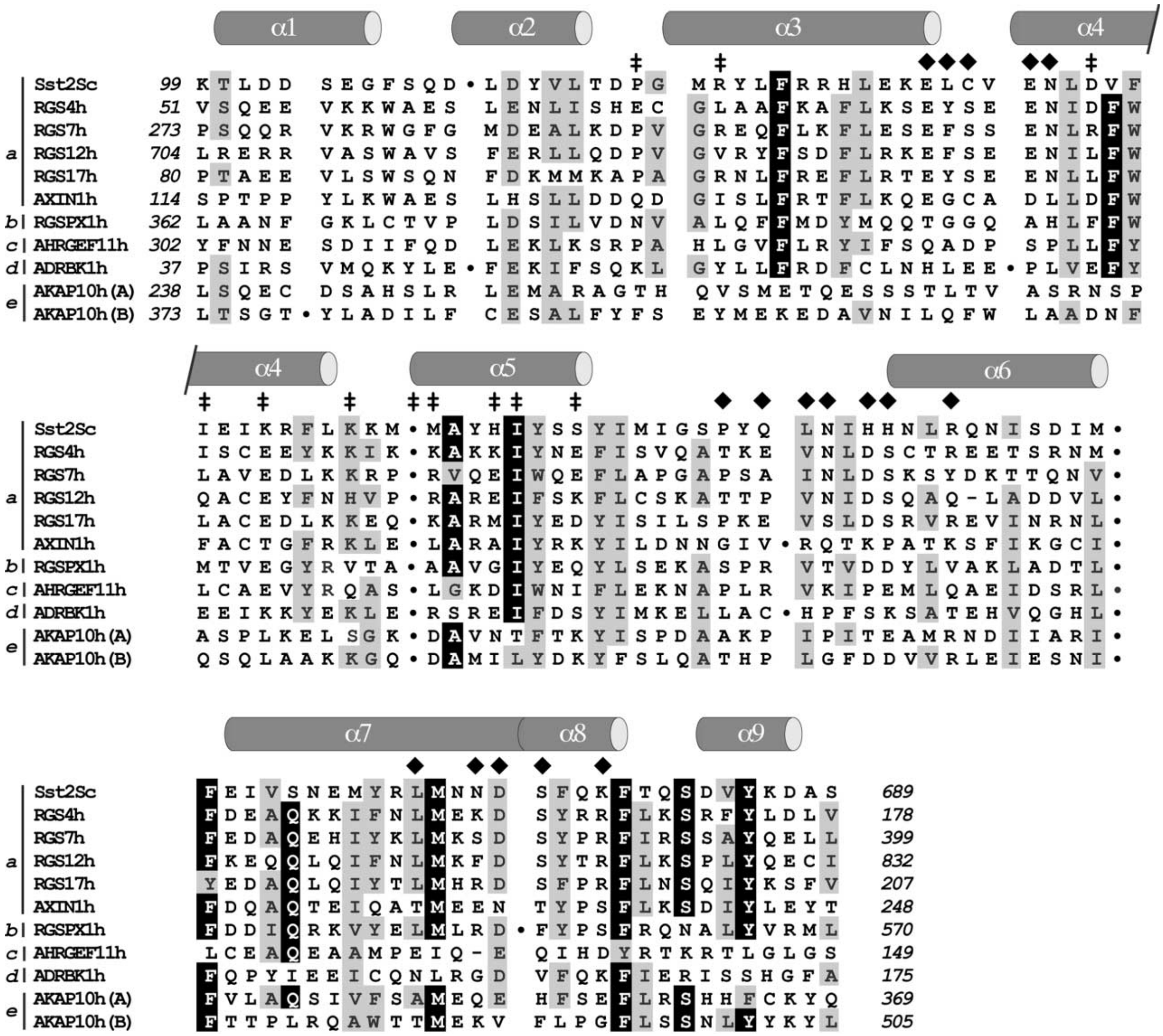
**FIGURE 1** The superfamily of RGS proteins. Amino acid identity comparisons within the RGS domain defined five families of RGS and RGS-like proteins: *RGS*, *RGS-PX*, *rgRGS*, *GPRK*, and *AKAP-RGS*. The RGS family is further subdivided into five subfamilies: *RZ*, *R4*, *R7*, *R12*, and *RA*. Human RGS domains contain between 127 and 130 amino acids. Branch junctions approximate the values calculated by DNAStar for sequence identity for each pair of sequences calculated as 100% minus the sum of the horizontal distance to and from the common branch point. Protein domains flanking the RGS domain are identified in the figure, and functions are summarized in Table 2. Common aliases for *RGS* genes are indicated in Table 1.

similarity within the RGS domain (Fig. 1). RGS proteins in the RZ, R4, R7, and R12 subfamilies all display GAP activity (Table 1). By contrast, the RA subfamily proteins lack GAP activity; they are scaffold proteins that help assemble components of the wnt signaling pathway during development and cancerous growth.

The structure of the RGS domain was solved by both X-ray crystallography and nuclear magnetic resonance. Interestingly, even though the entire RGS4 protein was crystallized (complexed with a $G_{\alpha i}$ subunit), only the RGS domain of RGS4 was resolved, whereas the N- and C-terminal residues were disordered. The correspondence of the ordered structure to the evolutionarily conserved RGS domain, which conveys GAP activity, indicates that the RGS domain is a discrete unit of folding and function. The RGS domain is a globular structure composed of two four-helical bundles. Residues within three loops on one surface are important for GAP activity and substrate specificity (Fig. 2). This surface has been termed the "A" or active site. However, RGS proteins do not have residues that directly contribute to catalysis, but rather appear to stabilize the transition state of GTP hydrolysis on the $G_{\alpha}$ subunit. RGS proteins with amino acid substitutions, deletions, or insertions within this region (from experimental mutagenesis or found naturally, as in the RA subfamily) lack GAP activity. A potential regulatory or "B" site occurs within an acidic domain in helices 4 and 5; in axin, this region binds a peptide fragment of adenomatous polyposis coli (APC), and R4 family RGS proteins are thought to alternatively bind phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) and $Ca^{2+}$/calmodulin to regulate GAP activity. Other regions of RGS proteins bind additional proteins and lipids (Table 2). Interestingly, the most highly conserved residues within the RGS domain face inward and maintain structure. Several outward-facing residues are conserved within subfamilies and, together with flanking domains, may interact with other proteins or lipids in the signaling complexes that convey specific regulatory functions.

## B. Superfamily of RGS Domain Proteins

Sequence homology between families of RGS-like proteins is low and difficult to detect. The different


α1
α2
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α8
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b
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e
Sst2Sc 99 K T L D D S E G F S Q D • L D Y V L T D P G M R Y L F R R H L E K E L C V E N L D V F
RGS4h 51 V S Q E E V K K W A E S L E N L I S H E C G L A A F K A F L K S E Y S E E N I D F W
RGS7h 273 P S Q Q R V K R W G F G M D E A L K D P V G R E Q F L K F L E S E F S S E N L R F W
RGS12h 704 L R E R R V A S W A V S F E R L L Q D P V G V R Y F S D F L R K E F S E E N I L F W
RGS17h 80 P T A E E V L S W S Q N F D K M M K A P A G R N L F R E F L R T E Y S E E N L L F W
AXIN1h 114 S P T P P Y L K W A E S L H S L L D D Q D G I S L F R T F L K Q E G C A D L L D F W
RGSPX1h 362 L A A N F G K L C T V P L D S I L V D N V A L Q F F M D Y M Q Q T G G Q A H L F F W
AHRGEF11h 302 Y F N N E S D I I F Q D L E K L K S R P A H L G V F L R Y I F S Q A D P S P L L F Y
ADRBK1h 37 P S I R S V M Q K Y L E • F E K I F S Q K L G Y L L F R D F C L N H L E E • P L V E F Y
AKAP10h (A) 238 L S Q E C D S A H S L R L E M A R A G T H Q V S M E T Q E S S S T L T V A S R N S P
AKAP10h (B) 373 L T S G T • Y L A D I L F C E S A L F Y F S E Y M E K E D A V N I L Q F W L A A D N F
Sst2Sc I E I K R F L K K M • M A Y H I Y S S Y I M I G S P Y Q L N I H H N L R Q N I S D I M •
RGS4h I S C E E Y K K I K • K A K K I Y N E F I S V Q A T K E V N L D S C T R E E T S R N M •
RGS7h L A V E D L K K R P • R V Q E I W Q E F L A P G A P S A I N L D S K S Y D K T T Q N V •
RGS12h Q A C E Y F N H V P • R A R E I F S K F L C S K A T T P V N I D S Q A Q - L A D D V L •
RGS17h L A C E D L K K E Q • K A R M I Y E D Y I S I L S P K E V S L D S R V R E V I N R N L •
AXIN1h F A C T G F R K L E • L A R A I Y R K Y I L D N N G I V • R Q T K P A T K S F I K G C I •
RGSPX1h M T V E G Y R V T A • A A V G I Y E Q Y L S E K A S P R V T V D D Y L V A K L A D T L •
AHRGEF11h L C A E V Y R Q A S • L G K D I W N I F L E K N A P L R V K I P E M L Q A E I D S R L •
ADRBK1h E E I K K Y E K L E • R S R E I F D S Y I M K E L L A C • H P F S K S A T E H V Q G H L •
AKAP10h (A) A S P L K E L S G K • D A V N T F T K Y I S P D A A K P I P I T E A M R N D I I A R I •
AKAP10h (B) Q S Q L A A K K G Q • D A M I L Y D K Y F S L Q A T H P L G F D D V V R L E I E S N I •
Sst2Sc F E I V S N E M Y R L M N N D S F Q K F T Q S D V Y K D A S 689
RGS4h F D E A Q K K I F N L M E K D S Y R R F L K S R F Y L D L V 178
RGS7h F E D A Q E H I Y K L M K S D S Y P R F I R S S A Y Q E L L 399
RGS12h F K E Q Q L Q I F N L M K F D S Y T R F L K S P L Y Q E C I 832
RGS17h Y E D A Q L Q I Y T L M H R D S F P R F L N S Q I Y K S F V 207
AXIN1h F D Q A Q T E I Q A T M E E N T Y P S F L K S D I Y L E Y T 248
RGSPX1h F D D I Q R K V Y E L M L R D • F Y P S F R Q N A L Y V R M L 570
AHRGEF11h L C E A Q E A A M P E I Q - E Q I H D Y R T K R T L G L G S 149
ADRBK1h F Q P Y I E E I C Q N L R G D V F Q K F I E R I S S H G F A 175
AKAP10h (A) F V L A Q S I V F S A M E Q E H F S E F L R S H H F C K Y Q 369
AKAP10h (B) F T T P L R Q A W T T M E K V F L P G F L S S N L Y Y K Y L 505

**FIGURE 2** Primary structure alignment of RGS superfamily proteins. Amino acid sequence alignment of the RGS domains from a representative member of each RGS subfamily, *R4*, *R7*, *R12*, *RZ*, and *RA*, and the yeast RGS protein Sst2 (*a*) and the four families of RGS-like domains (*b*, *RGS-PX*; *c*, *rgRGS*; *d*, *GPRK*; *e*, *AKAP-RGS*). The features noted in the RGS domain include the position of the α-helices in RGS4, AXIN, and ARHGEF11 (rods), $G_\alpha$ contact residues in RGS4 (diamonds), residues in AXIN that bind APC (double dagger), highly conserved, inward-facing amino acid identities (black), and amino acids of similar chemical function (gray). Dots signify amino acid insertions that were omitted from the alignment, spaces show where other proteins in the RGS superfamily have amino acid insertions, and dashes signify that an amino acid is not present in that RSG subfamily at that position within the alignment.

families are so divergent that only a few inward-facing residues important for structural integrity are conserved (Fig. 2). On the other hand, the crystal structures of RGS and rgRGS proteins are very similar, with the conserved residues identically placed within the RGS domain structure, supporting the notion that these RGS-like proteins are evolutionarily related. Presumably, members of the RGS-PX, GPRK, and AKAP-RGS families also have similar structures, but this is yet to be determined. As a cautionary note, the first protein demonstrated to have $G_\alpha$-GAP activity, the effector protein phospholipase C-β (PLC-β), is structurally unrelated to the RGS proteins. Thus, $G_\alpha$-GAP activity has evolved independently at least twice.

Within the superfamily of RGS domain proteins, GAP activity is observed in some but not necessarily all of the proteins of the RGS, rgRGS, RGS-PX, and

GRPK families and not in either RGL domain of AKAP10 (Table 1). Interestingly, the different RGS-like GAPs accelerate GTP hydrolysis on distinct types of $G_\alpha$ subunits. This substrate selectivity may help provide specificity in the regulation of G-protein signaling pathways.

Mammals, flies, and roundworms express four classes of $G_\alpha$ subunits, termed $G_i$, $G_q$, $G_{12}$, and $G_s$. The four classes are defined on the basis of similarity in sequence, gene structure, and regulation of effector proteins. The $G_q$ class activates phospholipase C-β, and thus regulates the production of the second messengers inositol 1,4,5-triphosphate ($IP_3$), diacylglycerol (DAG), and oscillations in the concentration of intracellular $Ca^{2+}$. The $G_i$ class regulates diverse effectors, including the inhibition of adenylyl cyclase, activation of cyclic GMP (cGMP) phosphodiesterase, potassium channel opening, and activation of phospholipase C-β. The $G_{12}$ class activates a guanine nucleotide exchange factor on the small GTP-binding

**TABLE 1** RGS Superfamily $G_\alpha$-GAP Activity

| Family | Subfamily | Locus (alias) | $G_\alpha$-GAP | LocusLink ID No. |
|---|---|---|---|---|
| RGS | R4 | RGS1 (BL34) | $G_{i/q}$ | 5996 |
| | | RGS2 (GOS8) | $G_q > G_i$ | 5997 |
| | | RGS3 (RGS15, PDZ-RGS) | $G_{i/q}$ | 5998 |
| | | RGS4 | $G_{i/q}$ | 5999 |
| | | RGS5 | $G_{i/q}$ | 8490 |
| | | RGS8 | $G_{i/q}$ | 85397 |
| | | RGS13 | $G_{i/q}$ | 6003 |
| | | RGS16 (RGS-r) | $G_{i/q}$ | 6004 |
| | | RGS18 | $G_{i/q}$ | 64407 |
| | | RGS21 | [a] | [b] |
| | R7 | RGS6 | $G_{i/q}$ | 9628 |
| | | RGS7 | $G_{i/q}$ | 6000 |
| | | RGS9 | $G_{i/q}$ | 8787 |
| | | RGS11 | $G_{i/q}$ | 8786 |
| | R12 | RGS10 | $G_{i/q}$ | 6001 |
| | | RGS12 | $G_{i/q}$ | 6002 |
| | | RGS14 | $G_{i/q}$ | 10636 |
| | RZ | RGS17 (RGSZ2) | $G_z > G_i$ | 26575 |
| | | RGS19 (GAIP) | $G_i$ | 10287 |
| | | RGS20 (RGSZ1, Ret-RGS1) | $G_z > G_i$ | 8601 |
| | RA | AXIN1 | — | 8312 |
| | | AXIN2 (AXIL, CONDUCTIN) | — | 8313 |
| RGS-PX | | RGS-PX1 (SNX13, KIAA0713) | $G_s$ | 23161 |
| | | RGS-PX2 (MSTP043) | [a] | 83891 |
| rgRGS | | ARHGEF1 (P115-RHOGEF, LSC) | $G_{12}$ | 9138 |
| | | ARHGEF11 (GTRAP48, KIAA0380, PDZ-RHOGEF) | $G_{12}$ | 9826 |
| | | ARHGEF12 (LARG, KIAA0382) | $G_{12}$ | 23365 |
| GPRK | | ADRBK1 (GRK2, BARK1) | $G_q$ | 156 |
| | | ADRBK2 (GRK3, BARK2) | $G_q$ | 157 |
| | | GPRK2L (GRK4, GPRK4) | ND | 2869 |
| | | GPRK6 (GRK6) | ND | 2870 |
| | | RHOK (RK, GRK1) | ND | 6011 |
| AKAP-RGS | | AKAP10 (PRKA10, D-AKAP2) | — | 11216 |

*Note*. LocusLink (http://ncbi.nlm.nih.gov).

[a] GAP activity untested.

[b] LocusLink number not assigned; ND, not done; —, no GAP activity.

TABLE 2 RGS Protein Domains and Functions

| Family | Subfamily | Domain number and name | | Function |
|---|---|---|---|---|
| RGS | RZ | RGS | RGS domain | $G_Z$-, $G_i$-GAP |
| | R4, R7, R12 | RGS | RGS domain | $G_i$-, $G_q$-GAP |
| | RZ | 1 | Cysteine string | Membrane localization |
| | RZ, R12 | 2 | PDZ-binding motif | Scaffold binding |
| | R4, RZ | 3 | Amphipathic helix | Membrane localization and receptor selectivity |
| | R4, R12 | 4 | PDZ domain | Scaffold/protein-binding motif |
| | R7 | 5 | DEP domain | Unknown function: possible membrane protein and phospholipid binding |
| | R7 | 6 | GGL domain | $G_{\beta5}$ binding |
| | R12 | 7 | PTB domain | Scaffold–phosphotyrosine binding |
| | R12 | 8 | Rap-binding domain | Binds small GTPase Rap |
| | R12 | 9 | GoLoco homology domain | Binds $G_{\alpha i}$-GDP, $G_{\alpha o}$-GDP |
| | RA | RGS | APC-binding domain | Scaffold function: binds adenomatous polyposis coli protein |
| | RA | 10 | GSK-binding domain | Scaffold function: binds glycogen synthase kinase |
| | RA | 11 | β-Catenin-binding domain | Scaffold function: binds β-catenin, prevents nuclear translocation |
| | RA | 12 | PP2A homology domain | Scaffold function: binds protein phosphotase PP2A |
| | RA | 13 | Dimerization domain | Homodimerization domain |
| RGS-PX | | RGL | RGS-like domain | $G_s$-GAP |
| | | 14 | PX-associated domain | Unknown |
| | | 15 | PhoX homologous domain | Vesicle binding |
| rgRGS | | RGL | RGS-like domain | $G_{12}$-GAP |
| | | 4 | PDZ domain | Scaffold function |
| | | 16 | Dbl homologous domain | Rho guanine nucleotide exchange factor (GEF) |
| | | 17 | Pleckstrin homology domain | Unknown function: possible membrane localization |
| GPRK | | RGL | RGS-like domain | $G_q$-GAP |
| | | 17 | Pleckstrin homology domain | Unknown function: possible membrane localization |
| | | 18 | Receptor kinase domain | Phosphorylates activated G-protein-coupled receptors |
| | | 19 | Gβγ-binding PH domain | Binds βγ-subunit of G-protein |
| AKAP-RGS | | RGL | Rgs-like domain | Unknown |
| | | 20 | PKA-binding domain | Binds PKA regulatory subunit |

protein Rho (RhoGEF). $G_\alpha$ subunits of the $G_s$ class activate the effector protein adenylyl cyclase, which stimulates the production of the second messenger cyclic AMP (cAMP). The activity of these four classes of G-proteins is opposed by the GAP activity of proteins in the RGS superfamily. $G_q$ and $G_i$ proteins are substrates for proteins in the RGS family, $G_{12}$ proteins are regulated by rgRGS proteins, and $G_s$ proteins are substrates for RGS-PX proteins (Table 1). An aspect of signaling specificity is that RGS proteins in the RGS, rgRGS, and RGS-PX families have no GAP activity on the G-protein substrates of the other two RGS families. $G_q$ proteins are exceptional because GTP hydrolysis on these α-subunits can be accelerated by RGS and GPRK proteins within the RGS superfamily and the unrelated effector protein PLC-β.

At least two other families of proteins that contain RGS-like domains are expressed in metazoans (Table 2). AKAP10 contains two RGS-like domains; although the function of this protein is not known, it is tempting to speculate that it regulates protein kinase A (PKA) activity in response to $G_s$-stimulated cAMP production. GPRK is the fifth family of RGS-like proteins, including at least two proteins, ADRBK1 and ADRBK2, that have weak $G_q$-GAP activity. It is not known whether ADRBK1 and ADRBK2 interact with RGS $G_q$-GAPs to regulate $G_q$ signaling complexes or whether GAP activity by GPRKs, RGS-PX, and/or RGS may influence receptor desensitization and internalization. Several of the GPRK genes have been deleted in mice, leading to a wide range of defects including light-dependent retinal degeneration, embryonic cardiomyocyte hypoplasia, and increased sensitivity and/or lack of desensitization to physiologically relevant doses of pharmacological agents.

## C. Multidomain RGS Proteins

Proteins in the RGS superfamily are defined by their RGS domains, but each has additional flanking domains with distinct functions. These modules mediate protein or lipid interactions that are unique to individual families and even subfamilies of RGS proteins (Fig. 1). A brief description of protein domains specific to different RGS protein families and their functions is presented in Tables 2 and 3. These groupings suggest that the functional domains contribute to subfamily-specific catalytic activities, subcellular localization, and/or regulation (summarized in Fig. 3). For example, the kinase domain in GPRK family proteins phosphorylates activated heptahelical receptors, leading to their internalization and down-regulation. A different family of RGS-like proteins, the rgRGS proteins, are distinguished by a dbl homology domain that binds and activates its effector protein Rho, thereby regulating cell shape changes and motility. The RGS-PX proteins have a PX domain, which is usually found in proteins called nexins, involved in vesicular trafficking within cells. Genetic and biochemical evidence from vertebrates and invertebrates indicates that RGS, GPRK, and rgRGS proteins regulate $G_q$, $G_i$, and $G_{12}$ signaling via G-protein-coupled receptors but it is unclear whether the $G_s$-GAPs of the RGS-PX family regulate hormonal signaling or vesicular transport.

Some RGS proteins serve as scaffolds that bring proteins into proximity to allow specific interactions. For example, the RA protein axin is a scaffold protein that negatively regulates the wnt signaling pathway. The RGS domain of axin has no GAP activity but binds another scaffold protein on the wnt pathway, APC. Flanking the axin RGS domain are additional domains that bind the serine/threonine kinase GSK, its phosphorylation target β-catenin, and its negative regulator, protein phosphatase 2A. Axin and APC together assemble a signaling complex that regulates the stability and activity of the transcription factor β-catenin, thereby influencing development and tumor progression.

Scaffolding functions can also serve to bring the RGS domain into a particular receptor complex where it can act on the $G_\alpha$ subunit and serve to

**TABLE 3** RGS Deficiencies in Eukaryotes

| Species | RGS family | Gene | Phenotype of genetic deficiency |
|---|---|---|---|
| *Saccharomyces cerevisiae* | RY[a] | *Sst2* | Supersensitive to pheromone, delayed recovery from cell cycle arrest |
| | | *Rgs2* | Enhanced glucose response |
| *Schizosaccharomyces pombe* | | *Rgs1* | Increased sensitivity to mating factor, mating defects |
| *A. nidulans* | | *FlbA* | No conidiophore formation or asexual reproduction |
| *Caenorhabditis elegans* | R7 | *Egl-10* | Hypokinesis and delayed egg-laying |
| | | *Eat-16* | Hyperkinesis and precocious egg-laying |
| | RC[b] | *Rgs1*<br>*Rgs2* | Rgs1;Rgs2 double-mutant delayed recovery from fasting |
| *Drosophila melanogaster* | R12 | *Loco* | Impaired glial cell function and dorsal–ventral axis formation |
| | rgRGS | *DRhoGEF* | Gastrulation defects |
| *Mus musculus* | R7 | *RGS9* | Delayed recovery to light in photoreceptor cells |
| | R4 | *RGS2* | Reduced male aggression, T-cell defects, hypertension |
| | RA | *axin* | Duplication of anterior structures during embryogenesis |
| | GPRK | *GRPK2* | Embryonic lethality from cardiomyocyte hypoplasia |
| | | *GRPK3* | Reduced olfactory desensitization, increased muscarinic-evoked airway response |

[a] RY is an RGS subfamily found in fungi.
[b] RC is an RGS subfamily found only in *C. elegans*.

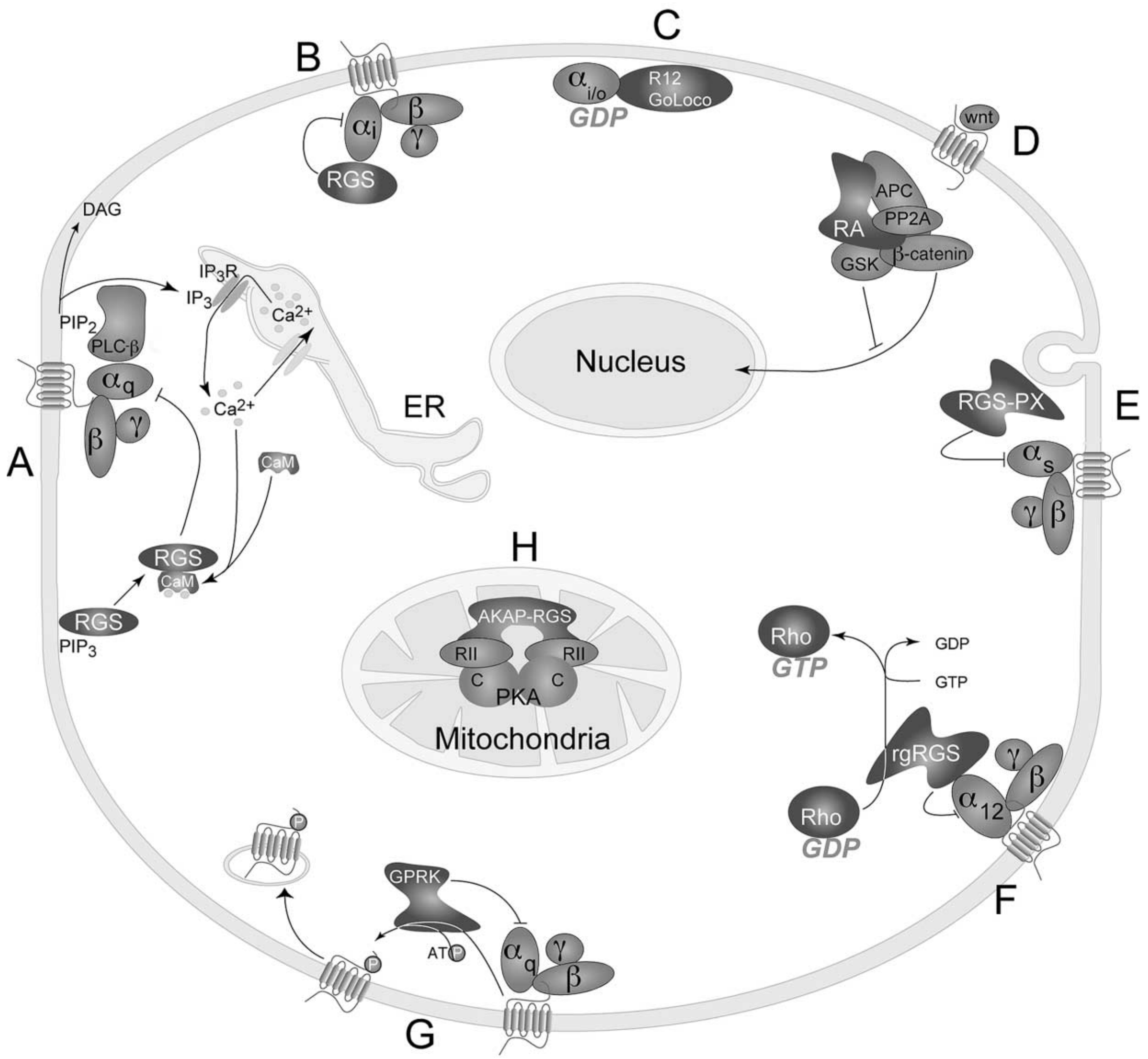
C
B
$\alpha_{i/o}$
R12
GoLoco
GDP
$\alpha_i$
β
γ
RGS
wnt
D
APC
PP2A
RA
β-catenin
GSK
DAG
$IP_3R$
$IP_3$
$Ca^{2+}$
$PIP_2$
PLC-β
$\alpha_q$
β
γ
$Ca^{2+}$
Nucleus
ER
RGS-PX
E
$\alpha_s$
γ
β
A
CaM
RGS
CaM
RGS
$PIP_3$
H
AKAP-RGS
RII
RII
C
C
PKA
Mitochondria
Rho
GTP
GDP
GTP
rgRGS
γ
β
$\alpha_{12}$
Rho
GDP
F
P
GPRK
ATP
P
$\alpha_q$
γ
β
G

**FIGURE 3** Actions of RGS superfamily proteins in cellular signaling complexes. (A) $G_q$ class G-proteins activate PLC-β to produce DAG and $IP_3$. $IP_3$ causes the release of the second messenger $Ca^{2+}$ from intracellular stores. $Ca^{2+}$/calmodulin (CaM) displaces $PIP_3$ on RGS proteins of the R4 subfamily, which acts as a GAP on the $G_\alpha$ subunit. (B) $G_i$ class G-proteins are negatively regulated by RGS proteins of the R4, R7, R12, and RZ subfamilies. (C) The GoLoco domain of R12 RGS proteins interacts with $G_{\alpha i/o}$-GDP. (D) RA RGS proteins function as structural components of the wnt signaling pathway, bringing β-catenin into proximity of its negative regulator, GSK kinase, which phosphorylates β-catenin, marking it for proteosome-mediated degradation. (E) RGS-PX proteins function as GAPs for $G_{\alpha s}$ and also bind to vesicles. (F) rgRGS serve as GAPs for $G_{12}$ class G-proteins and as guanine nucleotide exchange factors for small Rho G-proteins. (G) GPRKs can serve as GAPs for $G_q$ class G-proteins and also phosphorylate activated receptors, promoting their internalization and down-regulation. (H) AKAP10 contains two RGS-like domains of unknown function and two domains that bind the regulatory subunit of PKA, and it is highly enriched in mitochondria.

distinguish hormonal signals sent through different receptors that couple to the same G-protein. For example, in pancreatic acinar cells, RGS4 has been shown to preferentially inhibit signaling through m3-muscarinic receptors, relative to cholecystokinin receptors, despite the fact that they both couple to $G_{\alpha q}$. RGS4 contains an N-terminal domain shared by some other R4 subfamily members that is responsible for this receptor selectivity. This allows the cell to regulate signaling specificity through a combination of receptor-dependent activation by agonists and attenuation by RGS proteins.

## III. GAP ACTIVITY REGULATES SIGNALING

### A. G-Proteins Transduce Extracellular Signals to Regulate Intracellular Responses

All higher eukaryotes, including yeast, *Dictyostelium discoides*, plants, and animals, utilize G-protein signaling to mediate intracellular responses to extracellular stimuli. G-protein signaling systems have several components, each encoded by distinct multigene families. Extracellular signals received by heptahelical receptors are coupled by heterotrimeric G-proteins to the regulation of effector proteins that generate intracellular second messengers. Following agonist stimulation, cells must recover to maintain homeostasis. RGS proteins can rapidly attenuate G-protein signaling through feedback regulatory mechanisms. Thus, G-proteins act as signal transducers under the positive and negative regulation of receptors and RGS proteins, respectively. Activation and inactivation may occur within a G-protein signaling complex, composed of perhaps six distinct proteins, which acts like a molecular machine to convey extracellular signals and coordinate intracellular responses. All of the molecular components of G-protein signaling cascades, from ligands to effector proteins, are required to relay information from the outside of the cell to the inside.

### B. Regulation of GTP Binding and Hydrolysis

The essential regulatory feature of G-protein signaling is the cycle of GTP binding and hydrolysis on the $G_\alpha$ subunit. Many of the proteins that interact with the $G_\alpha$ subunit regulate its transit through this cycle. Signaling is initiated on receptor binding of extracellular agonists, such as nucleotides or small peptides, or the activation of a prebound chromophore by light. RGS proteins may help assemble a signaling complex that brings receptor, G-protein, and effector protein into proximity to stimulate rapid activation on ligand binding. The GAP activity of RGS proteins accelerates GTP hydrolysis and thereby can rapidly terminate signaling. The balance of activation by hormone binding and inactivation by RGS GAP activity controls the signaling flux through G-proteins.

The heterotrimeric $G_{\alpha\beta\gamma}$ protein complex is required for the receptor to stimulate intracellular signaling (Fig. 4). $G_\alpha$ is the largest subunit in the complex, ranging from 41 to 45 kDa, and binds the guanine nucleotide. $G_{\beta\gamma}$ forms a stable heterodimer of 35 and 7 kDa subunits, respectively. In the inactive state, $G_\alpha$ binds GDP to assume a conformation with high affinity for $G_{\beta\gamma}$. $G_\alpha$-GDP$\beta\gamma$ can interact with the intracellular loops and tail of the receptor. Agonist binding to receptor on the outside of the cell conveys conformational changes to the intracellular surfaces of the receptor that catalyze the dissociation of GDP from the $G_\alpha$ subunit. In the open state, the $G_\alpha$ subunit will bind GDP or GTP with equal affinity but the cytosolic concentration of GTP is 100-fold higher that GDP, which promotes GTP binding and progression of the activity cycle. GTP binding induces a conformational change in the switch regions of the $G_\alpha$ protein. The switch regions bind at least three different proteins ($G_{\beta\gamma}$, effector proteins, and RGS proteins) at different times during the cycle of GTP binding and hydrolysis. Conformational changes in the switch regions favor dissociation of the previous binding partner and association of the next partner in the cycle. GTP binding to the $G_\alpha$ subunit weakens its interaction with $G_{\beta\gamma}$ and receptor but induces a conformational change that favors interaction with effector proteins. Both $G_\alpha$-GTP and $G_{\beta\gamma}$ can regulate independent effector proteins. Thus, information is transduced from extracellular ligand binding to effector proteins that regulate the production of intracellular second messengers, such as cAMP and $Ca^{2+}$. $G_\alpha$ remains active until GTP is hydrolyzed. Conformational changes in $G_\alpha$-GDP drive the dissociation of the effector protein, thus terminating signaling and favoring reassociation of $G_{\beta\gamma}$. The cycle of GTP binding and hydrolysis can be repeated in the presence of persistent agonist.

RGS proteins are important regulators of G-protein activity because they are GAPs for $G_\alpha$ subunits, thereby inactivating or attenuating signaling. GAP activity is conveyed by the RGS domain. The RGS domain from several different RGS proteins can stimulate GTP hydrolysis up to 2000-fold above the basal activity of the $G_\alpha$ subunit, with all of the catalytic residues being supplied by the $G_\alpha$ subunit. Additionally, recent studies have shown that some RGS proteins also serve as scaffolds to help assemble active receptor complexes that are tightly coupled to effector proteins. Thus, RGS proteins regulate the kinetics of the G-protein activity cycle, and some apparently can accelerate both the activation and the inactivation of signaling. Current models of G-protein signaling suggest that signaling initiates within a multiprotein complex including receptor, G-protein, effector, RGS protein, and perhaps membrane lipids and other proteins (Figs. 3 and 4).

The duration and intensity of G-protein signaling can be modulated by posttranslational modifications of the receptors and RGS proteins that regulate the cycle of GTP binding and hydrolysis. Signaling is

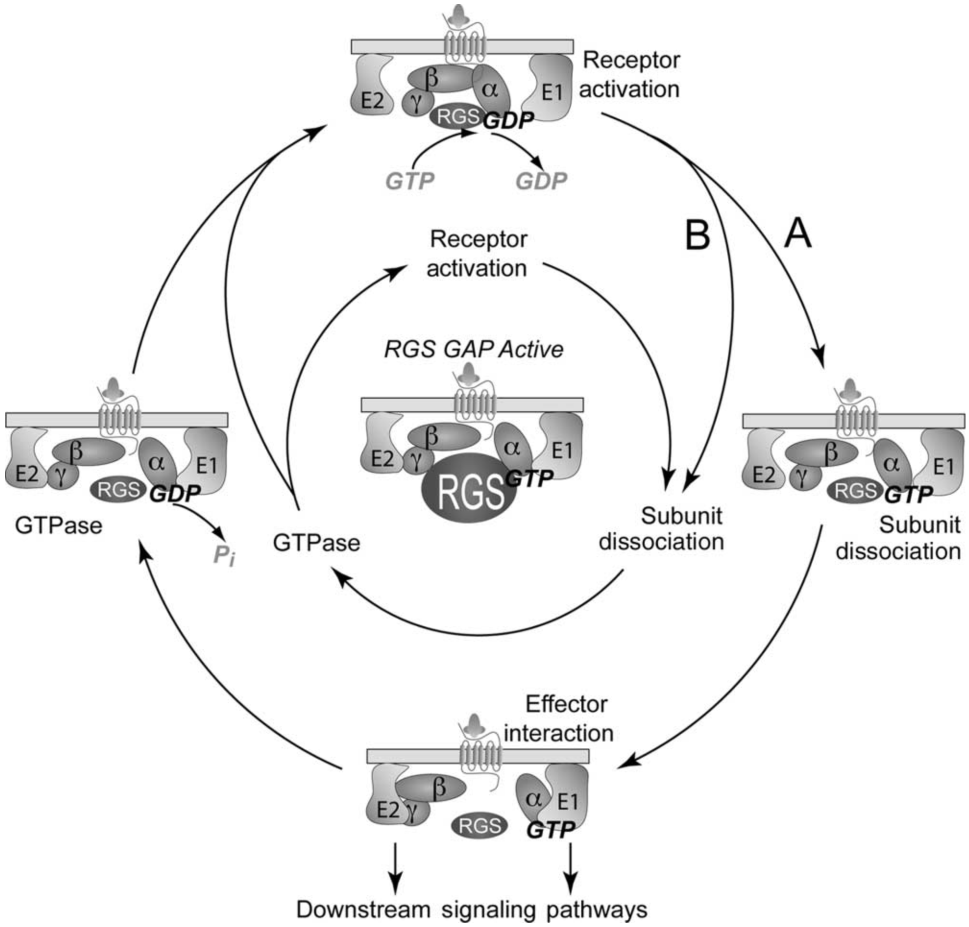

**FIGURE 4** Action of RGS within the cycle of guanine nucleotide binding and hydrolysis in heterotrimeric G-protein signaling complexes. In the presence of hormone agonist, receptor activation causes the exchange of GDP for GTP on the α-subunit. In the presence of low or rising second-messenger levels, RGS is inactive, promoting pathway A, where subunit dissociation is followed by effector interaction, and the activation of downstream signaling pathways, causing an increase in second messenger levels. Intrinsic GTPase activity of $G_\alpha$ cleaves the terminal phosphate from GTP, and the cycle is poised for another round in the continued presence of hormone agonist. When second-messenger levels are high, pathway B is promoted, where receptor activation is followed by subunit dissociation, and the GAP activity of RGS accelerates GTP hydrolysis before effector interaction can take place. In the continued presence of hormone agonist, multiple cycles of pathway B can uncouple hormone binding from effector protein activation, thereby terminating signaling. Repeating switches between pathways A and B can initiate oscillations in the production of second messengers, as observed in $Ca^{2+}$ oscillations evoked by $G_q$-coupled agonists.

activated by agonist (Fig. 4, pathway A) and can be terminated when agonist is depleted or overwhelmed by antagonist. Signaling can also be terminated by constitutively active RGS GAP activity, despite agonist stimulation of the G-protein. Thus, RGS proteins can uncouple effector protein activation even in the presence of persistent agonist (Fig. 4, pathway B). If RGS GAP activity is transiently activated and inactivated in response to the rise and fall of intracellular second messengers, then second-messenger production and downstream signaling can oscillate. RGS proteins have been proposed to play an important role in generating $Ca^{2+}$ oscillations in response to $G_q$-coupled agonists and thus regulate diverse biological processes such as the release of peptides from neuroendocrine cells and the proliferation and growth of cardiomyocytes during development and progression to heart disease.

## C. Effectors as GAPs

At least three G-protein-coupled effectors have GAP activity or stimulate an associated GAP. Although this accelerates the rate of return to the basal state, effector activation, and thereby signaling, is maintained by persistent agonist stimulation. However, signaling can rapidly terminate on agonist dissociation from the receptor. Thus, effector GAP activity allows a rapid response to both activating and inhibitory inputs.

The rgRGS proteins are both GAPs and effector proteins for $G_{12}$ class α-subunits. These proteins mediate cell shape changes during development and cell migration. Cell shape changes are regulated by the small GTPase Rho, which controls the assembly of actin stress fibers. Rho can be activated by a variety of guanine nucleotide exchange factors (GEFs). rgRGS proteins are RhoGEFs that are uniquely responsive to activation of $G_{12}$ class α-subunits. The rgRGS proteins have an RGS-like domain that binds and accelerates GTP hydrolysis on $G_{12}$ class α-subunits and a dbl-homology domain with RhoGEF activity. This signaling pathway, which couples heterotrimeric G-proteins to the small GTPase Rho, is conserved in vertebrates and invertebrates.

A second example of an effector with GAP activity was discovered in vertebrate photoreceptor cells. In this case, the G-protein transducin interacts with separate GAP and effector proteins, RGS9 and the γ-subunit of cGMP phosphodiesterase (PDE), respectively. RGS9 GAP activity on transducin is intrinsically weak but is augmented by both PDEγ and a recently discovered protein termed R9AP. Thus, a multiprotein complex of G-protein, RGS effector, and ancillary proteins serves the signaling requirements of vertebrate photoreceptor cells.

The regulation of $G_q$ class proteins is a third variation on this theme of multiprotein signaling complexes. As mentioned previously, like rgRGS, PLC-β is both a $G_\alpha$-GAP and an effector protein, but its sequence and structure are unrelated to those of RGS proteins. In addition to PLC-β, several RGS proteins also have $G_q$-GAP activity and appear to regulate PLC-β activity within a signaling complex composed of receptor, G-protein, RGS, and PLC-β. Currently under debate is why $G_q$ signaling is regulated by both PLC-β and RGS GAPs. One proposal is that GAP activity of PLC-β is constitutive, and this drives a rapid cycle of GTP binding and hydrolysis in the presence of persistent agonist. This is sufficient to maintain $G_\alpha$ and $G_{\beta\gamma}$ in proximity to the active receptor, acting like a kinetic scaffold to prevent G-protein dissociation from the receptor complex. Despite $G_q$-GAP activity, PLC-β still generates second messengers in the presence of persistent agonist, but only while the RGS GAP is inactive. By contrast, the GAP activity of many RGS proteins appears to be regulated by the rise and fall of second messengers inside the cell. Thus, GAP activity drives a repeating cycle of GTP binding and hydrolysis; PLC-β generates second messengers, whereas RGS proteins act as feedback inhibitors to uncouple G-protein activation from effector protein activation (Fig. 3). The combined action of these two GAPs may initiate pulses, or oscillations, of second messenger, as observed in many cell types during the initiation of $Ca^{2+}$ signaling by $G_q$-coupled agonists.

## IV. SUMMARY

GTP binding and hydrolysis on G-proteins drive cycles of protein–protein interactions that mediate signaling inside cells in response to extracellular signals. Heterotrimeric G-protein α- and/or βγ-subunits can independently regulate effector proteins, and thus, a single stimulus can generate a bifurcating signal. G-protein signaling via both $G_\alpha$ and $G_{\beta\gamma}$ is subject to feedback regulation through their attendant RGS proteins. The five families of RGS proteins in the RGS superfamily bind, and often accelerate GTP hydrolysis, to distinct types of G-proteins via interactions through their RGS domains. In addition to their catalytic properties, several RGS proteins function like scaffolds to organize multiprotein complexes that rapidly initiate and terminate signaling (summarized in Fig. 3). Each subfamily of RGS protein has protein domains, in addition to the RGS domain, that bind proteins and/or lipids that convey distinct regulatory properties. Thus, regulatory specificity is achieved by the combination of G-protein substrate specificity, cellular targeting, and accessory protein interactions with RGS proteins. Regulation of G-protein signaling by RGS proteins is found in fungi, *Dictyostelium*, and metazoan organisms at all stages of their life cycles and is fundamental to intercellular communication in higher eukaryotic organisms.

## Glossary

**effector protein** $G_\alpha$ and $G_{\beta\gamma}$ bind and regulate proteins that either increase or decrease the intracellular concentration of small molecules, termed second messengers, that stimulate cellular responses to extracellular hormones.

**G-protein** Proteins that are composed of three subunits; the α-subunit binds GTP and has GTPase activity, the β- and γ-subunits form stable heterodimers, and both $G_\alpha$ and $G_{\beta\gamma}$ can independently activate effector proteins in response to hormone stimulation.

**G-protein-coupled receptors** Heptahelical transmembrane proteins that bind hormones, chromophores, or other extracellular ligands to promote G-protein signaling inside the cell.

**GTPase accelerating proteins (GAPs)** Proteins that accelerate the hydrolysis of GTP on distinct types of G-protein α-subunits. GAPs occur in four of the five families of RGS-like proteins and the effector protein phospholipase C-β.

**regulators of G-protein signaling proteins** GTPase accelerating proteins for the α-subunit of heterotrimeric G-proteins.

**second messenger** Small molecules, such as cyclic AMP, inositol 1,4,5-triphosphate, diacylglycerol, or $Ca^{2+}$, that alter the activity of target enzymes and ion channels to influence cellular responses to extracellular hormones, neurotransmitters, and other stimuli.

## See Also the Following Articles

**Effectors • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Multiple G-Protein Coupling Systems • Receptor–Receptor Interactions**

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# Salicylic Acid

D'Maris Amick Dempsey and Daniel F. Klessig

*Boyce Thompson Institute for Plant Research, New York*

I. INTRODUCTION
II. SA-REGULATED PROCESSES IN PLANTS
III. SA METABOLISM
IV. MECHANISMS OF SA ACTION
V. SA-INDUCED GENE EXPRESSION
VI. GENETIC ANALYSIS OF THE SA SIGNALING PATHWAY
VII. SUMMARY

**Salicylic acid, a phenolic compound synthesized by plants, plays an important role in signaling mechanisms that regulate plant defenses against pathogens. Plant genetic studies show that salicylic acid regulates components of its own signaling pathway and is involved in cross talk with other pathways involved in mediating disease resistance and thermogenesis.**

## I. INTRODUCTION

Salicylic acid (SA) and its derivatives, collectively known as salicylates, are just some of the many phenolic compounds synthesized by plants.

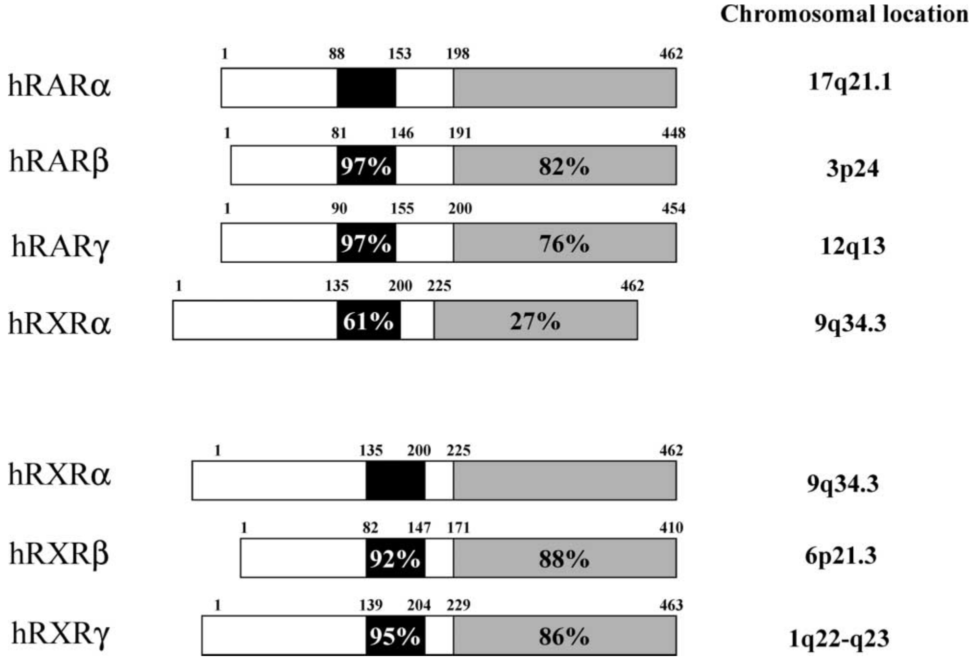


**FIGURE 1** Retinoid receptors and homologies A schematic representation of the members of the retinoid receptor family. The diagram provides the amino acid boundaries of the DNA-binding domains (black) and ligand-binding domains (gray) for the receptors. Included also is the amino acid homology of the ligand-binding domains within each class, i.e., RAR and RXR, and also the homology between RXRα and RARα. The DNA-binding domains are generally conserved (greater than 60%) among all the retinoid receptors, whereas the ligand-binding domains are conserved (greater than 75%) within each receptor family, but not between the RAR and RXR (less than 30%) groups. The chromosomal location of each receptor that has been identified in humans is shown as reference.

the DNA-binding domain (97%) and the ligand-binding domain (~75% or greater). The RARs exhibit a broad tissue distribution with the specific isoforms having both overlapping and distinct patterns of tissue and developmental expression. RARα expression is ubiquitous, whereas RARβ is more restricted to neural tissues, heart, lung, and spleen. RARγ is the primary isoform expressed in the skin. In addition to the α, β, and γ subtypes of RAR, differential promoter usage and alternative splicing generate multiple isoforms of each subtype to significantly increase the complexity of retinoid responses. Evolutionarily, the RAR receptors are well conserved and the finding of a retinoid-responsive RAR receptor in ascidians (sea squirts) suggests that retinoid receptor signaling existed early in vertebrate development as long as 500 million years ago.

In an attempt to identify other members of the retinoid receptor family, further screening of cDNA libraries resulted in the isolation of an RARα-related receptor, retinoid X receptor (RXRα). The other isoforms of RXR, β and γ, were subsequently isolated (see Fig. 1 for homologies and chromosomal locations). As with the RARs, multiple splice forms and transcripts expressed from different promoters add to the multiplicity of RXR isoforms. It is possible that the alternative splicing of retinoid receptor promoters and intron–exon boundaries may result in differential tissue expression, transactivation capabilities, and ligand responsiveness of the individual splice forms. RXRα is highly expressed in liver, kidney, lung, muscle, and spleen. RXRβ is generally found in most tissues and RXRγ is expressed predominantly in muscle and brain. Importantly, RXRs have been shown to be critical heterodimer partners for numerous other nuclear hormone receptors, as discussed in detail below. RXR is also well conserved throughout evolution and is considered one of the parental nuclear hormone receptors from which many of the others have evolved. Functional RXRs have been isolated from organisms as primitive as jellyfish, which indicates that they have existed for 600 million or more years. Unlike RAR, a homologue of RXR called ultraspiracle exists in the fruit fly, *Drosophila melanogaster*, although this receptor does not bind 9-*cis*-retinoic acid (9-*cis*-RA) and may not bind any ligand. It is unclear therefore when retinoid receptors developed the response to retinoids or whether, through evolution, some retinoid receptors lost the ability to respond.

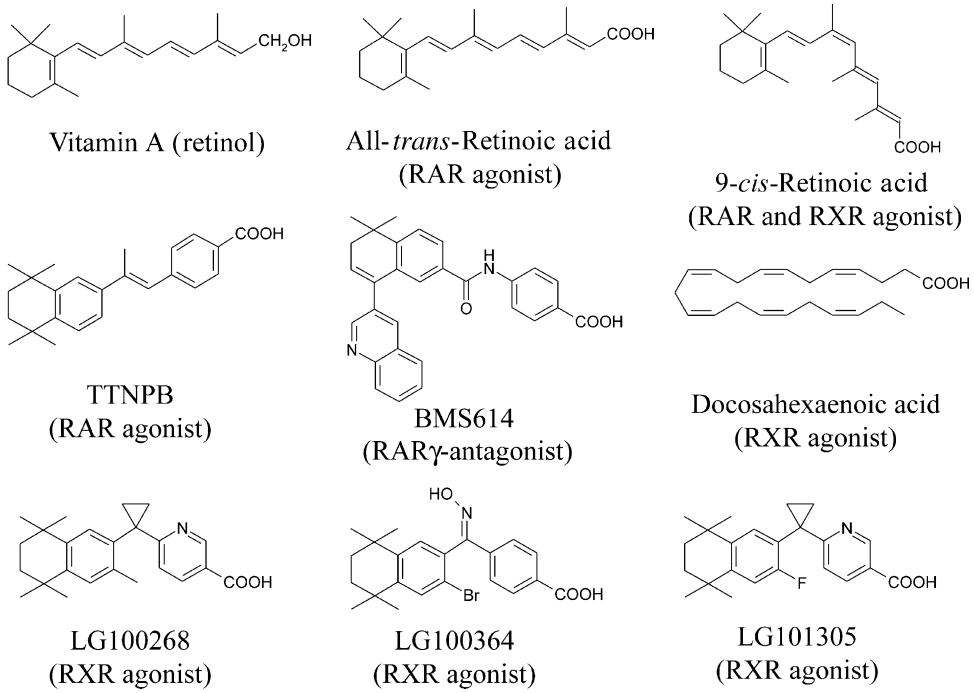
The first natural ligand to be identified for retinoid receptors was all-*trans*-retinoic acid (AtRA), the active metabolite of vitamin A. The AtRA was shown to bind with nanomolar affinity to all three isoforms of RAR. Although the homology of RXRs to RARs in the DNA-binding domain is relatively high (61%), the homology in the ligand-binding domain is less than 30%, suggesting that these receptors respond to different classes of ligands (Fig. 1). In experiments designed to identify RXR ligands, it was therefore surprising to find that AtRA could, at high levels, activate RXRα. Ligand-binding assays could not show binding of AtRA to RXR, and further testing of metabolites of retinoic acid (RA) resulted in the identification of 9-*cis*-RA as a high-affinity ligand for RXRs as well as RARs.

In addition to different ligand specificity, another important finding related to the mechanism of how retinoid receptors function was the identification of RXR as an obligatory heterodimeric partner for a number of nuclear hormone receptors including RARs, thyroid hormone receptor (TR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR), constitutive androstane receptor (CAR), and vitamin D receptor (VDR). Although the consequences of heterodimerization between RXR and these receptors are discussed in greater detail below, it is important to introduce this as a central concept in the identification of ligands that are specific for both RAR and RXR independently as well as those ligands that may regulate heterodimer function through the RXR. Compounds that specifically bind and activate RXR have been termed rexinoids. Supporting the pharmacological importance of rexinoids are findings demonstrating that RXR ligands working through specific heterodimers can recapitulate the activities observed with ligands specific to the heterodimeric partner. One example of this is the result that an RXR ligand, LG100268, can activate a PPARγ/RXR heterodimer and function as an effective insulin sensitizer in animal models of insulin resistance, similar to that observed with the PPARγ-regulating thiazolidinediones. Another example has been the finding that rexinoids such as LG100268, LG100364, and LG101305 can affect both cholesterol levels and atherosclerotic lesion size by virtue of working through another permissive heterodimer partner, LXR. The evidence that rexinoids can control multiple aspects of physiology through the regulation of RXR heterodimers demonstrates the importance of retinoid receptors as tools for pharmacological intervention in a number of diseases. As with RXR, there are also significant efforts to develop synthetic RAR compounds that are isotype specific to both reduce unwanted side effects and focus the pharmacological action of such compounds to specific tissues and disease states. See Fig. 2 for a selection of RAR and RXR ligands.

## III. RECEPTOR STRUCTURE

The retinoid receptors exhibit the general modular structure typical of nuclear hormone receptors in that they have five domains separable by sequence, structure, and function (Fig. 3). These domains are termed A/B, C, D, and E. In some receptors, there is also an F domain, for which a clear function has not yet been identified. The N-terminal part of the receptors contains the A/B domain, which harbors an autonomous transcriptional activation function domain termed AF-1. This domain has been shown to be important in regulating cell- and promoter-specific gene expression. The activity of this domain is ligand independent. AF-1 can be regulated by modifications such as phosphorylation in the case of PPARγ and estrogen receptor-β and these modifications result in the recruitment of transcriptional cofactors. The AF-1 can also affect the overall transcriptional activity of the receptor by modulating the activity of the other transcriptional regulator domain, AF-2, which is part of the C-terminal ligand-binding domain. Work by several groups using transgenic and knockout mice has established, among other things, that the primary mediator of many of the effects of RA during development is the RAR/RXRα heterodimer. Based on this observation, the contribution of the AF-1 region in the context of retinoid signaling was assessed by generating transgenic mice expressing wild-type and AF-1 deletion mutants of RXRα. The results indicate that the AF-1 domain is required for the transcriptional activity of the RAR/RXRα heterodimer, but that the ligand-dependent AF-2 domain plays a greater role in effecting most of the RAR/RXRα-mediated downstream events.

The C domain of nuclear receptors contains the DNA-binding domain (DBD) and a weak dimerization function. The approximately 66-amino-acid DNA-binding domain is the most conserved feature among the nuclear hormone receptor family. The DNA-binding domain consists of two zinc-fingers that have been well characterized as protein motifs utilized in many transcription factors for DNA



Vitamin A (retinol)

All-*trans*-Retinoic acid
(RAR agonist)

9-*cis*-Retinoic acid
(RAR and RXR agonist)

TTNPB
(RAR agonist)

BMS614
(RARγ-antagonist)

Docosahexaenoic acid
(RXR agonist)

LG100268
(RXR agonist)

LG100364
(RXR agonist)

LG101305
(RXR agonist)

**FIGURE 2** Selected retinoid receptor ligands. Several retinoid receptor ligands that represent both endogenous and synthetic small molecule regulators of RAR and RXR are depicted. The structures of vitamin A and the metabolites all-*trans*-retinoic acid and 9-*cis*-retinoic acid are provided as examples of high-affinity endogenous ligands. The other endogenous ligand shown is docosahexaenoic acid, an RXR agonist that may have effects on neural function. Examples of synthetic ligands that are referred to in the text include an RAR agonist, TTNPB, an RAR antagonist, BMS614, and a series of RXR agonists, LG100268, LG100364, and LG101305.

binding and protein–protein interactions. Within the first zinc-finger lies a highly conserved 13-amino-acid region termed the proximal or P-box (Fig. 3). The P-box determines the specificity of binding of the receptor to its half-site. In the second zinc-finger, there is a region, termed the distal (D)-box, that contributes to dimerization. A third functionally characterized region within the DNA-binding domain is an approximately 25-amino-acid carboxy-terminal extension (CTE). In the case of RAR/RXR heterodimer DNA binding, the CTE plays a role in promoting both receptor–DNA interactions and receptor–receptor contact. The DBDs of RARα, and RXRα have been crystallized as a heterodimer complex bound to a direct repeat of AGGTCA with spacing of 1 bp. The results of these studies indicate that both the DNA and the receptors induce conformational changes in each other, resulting in a more stable DNA-bound complex. The ability of nuclear receptors, specifically retinoid receptors, to bind and regulate through multiple DNA elements is therefore a function of both the flexibility of the receptor and the DNA site.

The region that connects the DBD and the ligand-binding domain (LBD) has been referred to as the hinge or D region. This is a highly variable domain both in sequence and in length. As the name implies, the main purpose of this part of the receptor is to connect the DNA-binding component to the ligand-binding domain and to allow a high degree of freedom of movement for these domains.

The most important domain for ligand-mediated activity is the LBD, also referred to as the E region. The LBD contains the ligand-binding site, a strong dimerization function, cofactor interaction sites, and a ligand-dependent activation domain (AF-2). The LBD consists of 12 α-helices that form the ligand-binding pocket. Helix 12, which is at the C-terminus of the LBD, contains AF-2. The ligand-binding domains of RARγ and RXRα have been crystallized in the presence of compounds and, in the case of RXRα, also in their absence. The RXRα LBD structure was solved in the presence of 9-*cis* retinoic acid, and the RARγ LBD structure was determined in the presence of both agonists such as all-*trans* retinoic acid and antagonists such as BMS614 (Fig. 2).

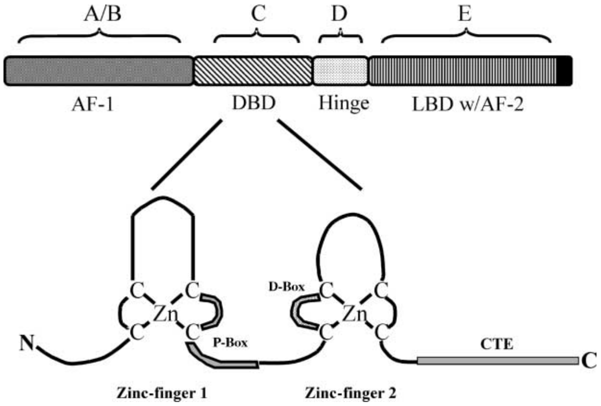

A/B
C
D
E
AF-1
DBD
Hinge
LBD w/AF-2
C
C
Zn
C
C
D-Box
C
C
Zn
C
C
N
P-Box
CTE
C
Zinc-finger 1
Zinc-finger 2

FIGURE 3 Nuclear hormone receptor functional domains. The diagram presents a general view of nuclear hormone receptor structure through delineation of the functional domains. The domains referred to in the text include the N-terminal A/B region that contains a ligand-independent transcriptional activation domain, the C or DNA-binding domain (DBD), the D or hinge region, and the E or ligand-binding domain (LBD). The LBD contains a ligand-dependent transcriptional activation function domain, AF-2 (in black). Shown in greater detail is the structure of the DNA-binding domain. There are two $C_2C_2$-type zinc-fingers that mediate protein–DNA and protein–protein interactions. Other regions highlighted include the P-box (DNA-binding specificity), the D-box (dimerization), and the carboxy-terminal extension (CTE; DNA and protein interactions).

These studies, along with the structures of other nuclear receptor ligand-binding domains, allowed several conclusions to be made. The 12 α-helices of the LBD form a novel fold termed an anti-parallel α-helical sandwich. In the apo state, helix 12 extends away from the ligand-binding pocket, whereas in the holo state, there is a significant rearrangement of H11, H12, and the region between H1 and H3. The shift in structure of these helices, e.g., H1–H3, H11, and H12, between the ligand-bound active state and the apo- or unbound inactive state has been referred to as a "mouse-trap" mechanism. As implied in this name, once a ligand enters the ligand-binding pocket, H12 is moved into a position in which it essentially traps the ligand in the pocket.

## IV. MECHANISM OF ACTION

### A. DNA Binding/Heterodimerization

Nuclear hormone receptors have been shown to bind to specific DNA-response element sequences as homodimers or heterodimers. The DNA binding is ligand independent. For RAR and RXR, the retinoid-responsive elements (RAREs and RXREs) that have been isolated in target gene promoters have the consensus half-site sequence AGGTCA organized in different conformations. One type consists of a direct repeat (DR) of the half-site with an intervening spacer of 1, 2, or 5 (DR1, DR2, or DR5, respectively) bp. The DR5 element is the most common RARE. Another element is a palindromic or inverted half-site repeat, and a third response element is an inverted palindrome or everted half-site repeat (Fig. 4). It is likely as well that sequences flanking the half-sites further contribute to the affinity and specificity of receptor binding to these response elements.

RAR binding to DNA requires formation of a heterodimer with RXR. In the RAR/RXR heterodimer, it is the RAR that determines the ligand-dependent activity of the receptors, so that an RXR ligand is unable to activate the heterodimer in the absence of an RAR ligand. In this situation, RAR is referred to as a nonpermissive partner for RXR activity. As mentioned previously, RXR has also been shown to heterodimerize with many other nuclear hormone receptors including TR, VDR, PPARs, FXR,

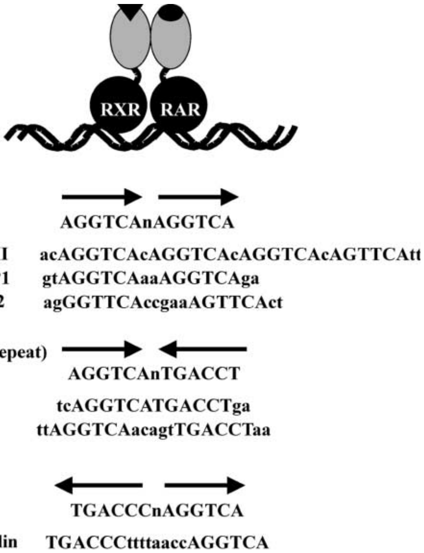

RXR
RAR
Direct repeat
Consensus
AGGTCAnAGGTCA
DR1: rCRBPII
acAGGTCAcAGGTCAcAGGTCAcAGTTCAtt
DR2: mCRBP1
gtAGGTCAaaAGGTCAga
DR5: hRARβ2
agGGTTCAccgaaAGTTCAct
Palindrome (inverted repeat)
Consensus
AGGTCAnTGACCT
IR0: TRE-pal
tcAGGTCATGACCTga
IR5: IR+5
ttAGGTCAacagtTGACCTaa
Everted repeat
Consensus
TGACCCnAGGTCA
ER8: γF-crystallin
TGACCCttttaaccAGGTCA

FIGURE 4 Retinoid receptor-response elements. There have been many retinoid receptor-response elements identified in the literature and examples of consensus and selected examples of these elements are shown in this figure. As indicated, the consensus half-site for retinoid receptors is AGGTCA, and the different elements are generated through variable spacing between these half-sites (DR1, DR2, and DR5) or orientation of the half-sites (IR0, IR5, and ER8). The DR1 is taken from the rat cellular retinal-binding protein II (rCRBPII); DR2 from mouse cellular retinal-binding protein I (mCRBPI); DR5 from human RARβ2 gene; IR0 and IR5 are synthetic elements; and ER8 is from the γF-crystallin promoter.

Nurr1, nur77/NGFI-B, and LXRs. Of these, TR and VDR are nonpermissive, and the others are referred to as permissive partners as they can be activated by RXR ligands or the appropriate dimer ligand. In addition to serving as a heterodimeric partner, RXR can bind as a homodimer to DR1 and certain palindrome elements. An important observation relating to how retinoid receptors respond to ligand is that the ternary structure of the receptor bound to DNA dictates the level of response to a given ligand. This is due to the specific RARE contributing to the determination of the affinity and specificity of a compound. This predicts that retinoids and/or small molecules can be synthesized to regulate specific target genes.

Similar to other steroid hormone receptors such as the glucocorticoid receptor, RAR and RXR can also directly or indirectly interact with other transcription factors to regulate target gene expression independent of binding to RAREs or RXREs. This has been demonstrated through inhibition of activator protein-1 (AP-1) and $Ca^{2+}$/cyclic AMP-response element-binding protein (CREB) activity by RAR and RXR ligands. In the case of RAR, the *trans*-repression can be observed in the absence of the induction of RAR transcriptional activity by certain compounds such as SR11203 and SR11238. It is likely that a combination of retinoid receptor-dependent events contribute to this repression, among which is competition for DNA-binding sites, sequestering of regulatory factors such as CREB-binding protein/p300 (CBP), and direct protein–protein interactions.

## B. Transcriptional Regulation

The retinoid receptors function to regulate gene expression through the ability to adapt conformations that induce transcriptional activity in the presence of ligand or repress transcriptional activity in its absence. The gradient of activity that can be achieved, from full repression to full activation, is dependent on the ability of the ligand-binding domain to undergo a series of conformational changes. Much of the work over the past several years has established that large multisubunit protein complexes are recruited to DNA through binding to the receptors. As described previously, in the presence of an agonist, the LBD adopts a conformation in which the AF-2 containing helix 12 moves almost 90° to stabilize the receptor structure and create a binding interface for a class of proteins termed transcription co-activators. Examples of these factors include CBP, p300/CBP-associated factor (pCAF), thyroid hormone receptor-associated proteins (TRAPs), receptor interacting protein 140 (RIP140), and the p160 proteins including steroid receptor co-activator-1 (SRC-1), SRC-2, and SRC-3. A common characteristic of many of these cofactors is that they have intrinsic histone acetyltransferase activity. Another shared feature is the presence of a protein interaction motif composed of the amino acids Leu-X-X-Leu-Leu (LXXLL). This short peptide sequence is sufficient to promote interaction of the cofactor with the receptor. Amino acids flanking the core sequence have been demonstrated to regulate specificity between cofactor and receptor. The co-activator complex, which is predicted to be approximately 2 MDa, contains an overlapping set of these factors and it is likely that they have regulator functions in addition to acetylating histones. The number, diversity, and expression level of co-activators in different tissues correspond to the tissue- and promoter-specific effects of a given ligand on its receptor. Histone acetylation by the co-activator complex recruited to the receptor in the presence of ligand leads to a loosened and more accessible chromatin structure (Fig. 5). This is expected to facilitate and promote expression of the target gene. The co-activator complex also has been demonstrated to contain kinase and methyltransferase activity.

In the absence of an agonist or in the presence of some antagonists, retinoid receptors, like some other nuclear hormone receptors, have been shown to repress the basal activity of a target gene promoter. This silencing ability is attributed to the presence of another large multifactorial complex. Two co-repressor proteins, nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT), have been identified as playing a major role in receptor controlled repression. These proteins share structural similarities, such as multiple repression and receptor interaction domains. Analogous to co-activator proteins, consensus SMRT and N-CoR receptor interaction motifs have been isolated with a minimal shared sequence of Leu/Ile-X-X-Leu/Ile-Leu/Ile. SMRT and N-CoR appear to function partly as platform proteins upon which the remainder of the co-repressor complex is assembled. This complex contains multiple proteins that have intrinsic histone deacetlyase (HDAC) activity. The HDAC activity is expected to maintain the chromatin structure in a tightly bound state that is incompatible with transcription. The ability of nuclear receptors, such as RAR and RXR, to interact in a ligand-dependent manner with at least two large protein complexes with opposing actions helps to explain the wide range of activities of these receptors

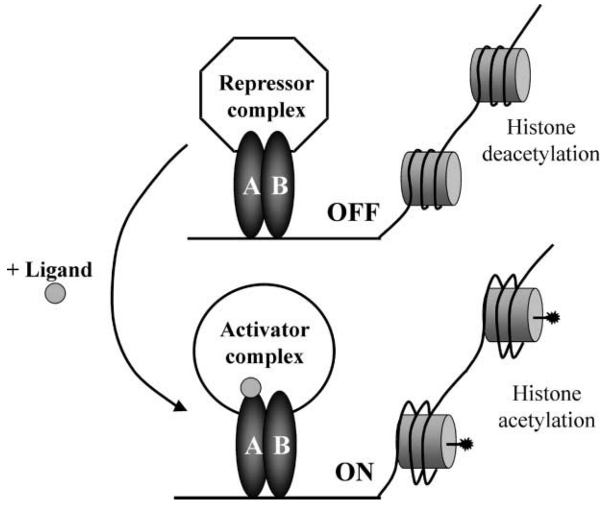


**FIGURE 5** Mechanism of transcription regulation by retinoid receptors The diagram illustrates the ligand-mediated activation of transcription by retinoid receptors. A and B refer to any receptor heterodimer or homodimer partner. In the absence of ligand, the receptor dimer is bound to DNA and a large multiprotein co-repressor complex. The co-repressor complex is recruited to the receptors through specific protein–protein interactions between the receptor LBD and short hydrophobic peptide motifs found in various co-repressor proteins, such as SMRT and N-CoR (see text). The repressor complex contains certain enzyme activities, including histone deacetylases, that function to configure the chromatin structure into a state that is inhibitory to transcriptional activation. Upon ligand binding, the repressor complex is displaced through recruitment of a multiprotein activator complex. Similar to co-repressors, the co-activator complex is recruited through protein–protein interactions between the receptors and well-defined peptide motifs in the co-activator proteins. The co-activator complex contains enzyme activities, such as histone acetylases, that alter the chromatin structure to a more transcriptionally permissive state.

and their attractiveness as targets for therapeutic intervention.

The importance of the interaction of RAR with the co-repressors SMRT and/or N-CoR can be seen in the different responses of patients with acute promyelocytic leukemia (APL) to all-*trans*-retinoic acid therapy. Those patients with a translocation of the promyelocytic leukemia (PML) gene with the RARα gene (PML–RAR) respond well to RA, whereas those with a translocation of the promyelocytic leukemia zinc-finger (PLZF) gene with RARα (PLZF–RAR) respond poorly. This can be attributed to the ability of both PLZF and RAR to interact with co-repressors as opposed to just RAR in the PML–RAR fusion. The PLZF–co-repressor interaction is ligand independent and thus treatment by RA has minimal effect. This finding is discussed below in greater detail.

## V. RECEPTOR PHYSIOLOGY

Vitamin A and retinoids have been shown to be essential for proper embryonic development and many physiological functions. Some of the characteristics of vitamin A deficiency include blindness, immune system dysfunction, infertility, congenital malformations, heart malformation, and epithelial hyperkeratinization. Vitamin A excess also can be detrimental and lead to birth defects. The effects of retinoids can be classified as receptor mediated and receptor independent. The use of retinoid receptor-specific ligands, cells from receptor null mice, overexpression of receptors, and overexpression of dominant-negative receptors have all contributed experimentally to linking retinoid effects with receptor-mediated effects. This next section provides a brief overview of a selected set of receptor-dependent activities that contribute to proper development and homeostasis, beginning with a review of the phenotypes of the various receptor null mice that have been made.

### A. Receptor Mutant Mice

For the RARs, mice lacking the major isotypes have been generated. These mice have also been crossed with one another to generate compound mutants. RARα − /− mice died early after birth and showed signs of degenerative testis. Additional defects include homeotic transformations and congenital defects. RARγ − /− mice also have these defects, leading to improper development of the skull, vertebrae, and ribs. RARγ − /− mice also have decreased viability. RARβ − /− mice display some minor changes including some ocular and long-term memory defects. RARα − /− and RARγ − /− male mice are also sterile. The double mutants of RARα/β and RARα/γ, in which both alleles of a receptor subtype are deleted to maximize phenotypic effects that otherwise may be masked by receptor redundancy, show many more of the expected phenotypes that are observed in vitamin A-deprived animals. RXRα − /− mutant mice exhibit embryonic lethality, due to defects in placenta and heart. These mice also have defects related to eye development. RXRβ − /− mice show male sterility due to improper spermatogenesis, and RXRγ − /− mice exhibit no major phenotypes although long-term memory may be affected. Both RXRβ − /− and RXRγ − /− are viable.

### B. Hematopoietic Differentiation

The observation that retinoic acid and its metabolite derivatives could induce differentiation of a number of human leukemic cell lines suggested that retinoids and their receptors would play an important role in physiological myeloid cell development and differentiation. RAR/RXR heterodimers function as modulators of granulocyte differentiation depending on the presence or absence of a ligand. In the presence of retinoic acid, RAR activity promotes granulocyte differentiation, whereas unbound RAR is an inhibitor of promyelocyte differentiation. The role of RARα as a regulator of myeloid differentiation is further supported by the inability of promyelocytes that harbor translocations between RAR and PML and/or PLZF to differentiate, resulting in acute promyelocytic leukemia. The cells with the PML-RAR fusion can be treated with RA and be induced to differentiate, indicating RAR activity as a primary mediator of cellular differentiation in the myeloid system. RAR activity has also been shown to be important in regulation of neutrophil maturation.

## C. Cell Proliferation/Apoptosis

The ability of retinoids to regulate cell cycle progression, profileration, and apoptosis has been well studied, although the mechanisms supporting the observations are not completely known. The inhibitory effect of the RARs and RXRs on cell growth can generally be attributed to their regulation of a number of proteins involved in the process of cell cycle and cell death. The targets of retinoid receptor activity include the AP-1 transcription factor, c-Myc, and retinoblastoma gene product Rb. These proteins are important in controlling the G1 to S phase of the cell cycle, and retinoid inhibition generally occurs at this transition stage.

A primary role for retinoid receptors in apoptosis was found when certain leukemic cells were treated with retinoic acid and other retinoid receptor-selective ligands. It was found that RAR and RXR ligands, when added together to HL60 cells, a promyelocytic cell line, induced apoptosis. These effects were receptor dependent and involved the down-regulation of certain anti-apoptotic proteins. In the development of myeloid cells from bone marrow progenitor cells, RXR also plays an important role through the regulation of apoptosis. In the absence of RXR activation, or down-regulation of RXR expression, myeloid cells undergo less apoptosis. RARs and RXRs have also been shown to regulate apoptosis in T and B lymphocytes. In T cells, rexinoids inhibit T-cell-receptor-mediated apoptosis by inhibiting the expression of the Fas ligand. Proper timing and regulation of cell death are critical in lymphocyte development to eliminate those cellular clones that would otherwise be self-reactive and lead to autoimmunity.

## D. Reproduction

Vitamin A has been shown to be important in spermatogenesis and maturation of spermatozoa. Vitamin A deficiency leads to defective spermatogenesis and subsequent sterility. In agreement with this finding, RARα −/−, RARγ −/−, and RXRβ −/− male mice are sterile. These data indicate that the primary mediators of retinoid signaling in the testis are likely to be the RARα and RXRβ receptors and that these receptors are not redundant for proper reproductive development. These mice are sterile due to testicular defects, including degeneration of the seminiferous epithelium, in the case of RARα −/− mice. In the RXRβ −/− mice, the male sterility is due to defective spermatozoa and lipid accumulation in Sertoli cells.

## E. Development

The importance of retinoid receptors to proper development and differentiation of tissues is well defined in experiments relating to limb formation as well as the finding that retinoids are teratogenic in humans. Studies in chickens show that placement of beads containing RA into developing limb bud sites can initiate novel developmental programs leading to a new limb structure. Consistent with these results, RAR/RXR antagonists can inhibit limb formation. Multiple limb abnormalities are also seen in RARα −/−, RXRα −/−, and double mutant RARα/RXRα, RARβ/RXRα, and RARα/RARγ mice. The mechanism of retinoid receptor function in limb development involves the induction of a cascade of gene expression, with induction of hox genes Hoxb-6 and Hoxb-8, which in turn lead to up-regulation of Sonic Hedgehog, which consequently up-regulates the bone morphogenic protein BMP-2. These proteins have been shown to be key factors in the process of limb development.

## F. Neural Development

Retinoids have been long associated with the differentiation of neurons and the development of the nervous system. The expression patterns of retinoid receptors in the central nervous systems throughout development and into adulthood suggested a role for these receptors in regulating certain neural functions. Data from a variety of sources including phenotypes associated with vitamin A deficiency, RAR/RXR mutant mice, and treatment of animals with retinoid receptor antagonist demonstrate an essential role for retinoid signaling in normal hindbrain development.

The results from RARβ/RXRβ, RARβ/RXRγ, and RXRβ/RXRγ double mutant mice also confirm a role for these receptors in the development of normal locomotor function. This defect in motor function is likely due to a reduction in expression levels of the dopamine receptors D1 and D2. This is consistent with RAR/RXR heterodimers binding to and regulating the promoter of the D2 receptor *in vitro*. These findings also hint at a link between retinoid receptor signaling and such neural disorders as Parkinson's disease and schizophrenia. Recent work that describes the search for endogenous RXR ligands has identified docosahexaenoic acid as a putative ligand. The ability of DHA to activate RXRs and the presence of learning abnormalities in DHA-deficient animals correlate well with the mutant mouse data and further support a role for retinoid receptors in neural development.

### G. Retinoid Receptors in Skin

The effects of vitamin A deficiency or excess on skin growth and differentiation have been known for a number of years. A lack of vitamin A leads to hyperkeratosis, which could be reversed upon addition of vitamin A. An excess of vitamin A, however, can result in inhibition of the process of keratinization. Since the levels of vitamin A were shown to be important regulators of skin processes, this led to the use of natural retinoic acids, including AtRA and 13-*cis*-RA, as a treatment for skin disorders including acne and psoriasis and for correcting sun-induced skin damage. The use of retinoic acid, unfortunately also has some undesired side effects including irritation, dryness and peeling of the skin, hair loss, and teratogenicity. Some of the skin-related side effects might be partly due to binding to the intracellular protein cellular retinoic acid-binding protein types I and II. Recent experiments *in vitro* and in animal models has established that the major isoforms of the receptors expressed in adult skin are RARγ and RXRα, although the other isoforms are expressed at varying levels during development. The ability to target RARγ specifically with synthetic agonist or antagonists has the promise of providing the benefits of retinoic acid treatment without the side effects. Indeed, the successful use of RARγ agonists in the treatment of acne and psoriasis has been reported.

## VI. RXR HETERODIMERIZATION

As introduced earlier, RXR functions as an obligatory heterodimeric partner for a large number of nuclear hormone receptors. Among the receptors that RXR can heterodimerize with are RARs, TR, VDR, PXR/SXR, PPARs, LXRs, FXR, CAR, and Nurr. Of these, PPARs, LXRs, FXR, PXR/SXR, CAR, and Nurr are permissive to the activation of the heterodimer via binding of an RXR ligand. The other receptors, RAR, TR, and VDR, are not permissive to activation by RXR ligands. The molecular mechanism for whether a heterodimer is permissive or not appears to depend on the recruitment of the co-repressor complex by the RXR partner in the absence of an agonist. In nonpermissive heterodimers such as RAR/RXR, even though an RXR ligand may bind, it is not sufficient to activate the complex, most likely due to an inability to displace the co-repressor from RAR. In this same complex, however, if an RAR agonist is added along with an RXR agonist, the net activation is higher than with the RAR compound alone. In permissive heterodimers, both receptors bind the co-repressor complex weakly so addition of an RXR agonist is sufficient to displace the co-repressor from the heterodimer and thus induce activity. Since these permissive heterodimers represent an important extension of the function of retinoid receptors, this section provides a general overview of the function of the PPAR, LXR, FXR, and xenobiotic receptor (PXR/SXR and CAR) heterodimers. The nonpermissive heterodimer receptors TR and VDR are not discussed, as other than mediating the target specificity through DNA binding of these heterodimers, there is as yet no other physiological role for RXR in these cases. RAR heterodimer function has previously been discussed and the Nurr receptors are also not included, as a physiological role for a Nurr/RXR heterodimer has not yet been established.

### A. PPAR/RXR

The PPARs have been shown to be important regulators of lipid metabolism and adipogenesis. There are three isoforms, α, δ, and γ, that activate transcription by binding to promoter elements as heterodimers with RXR. The PPAR/RXR heterodimers can bind primarily to DR1 elements. Natural and synthetic ligands have been identified for all three PPAR isoforms and, along with receptor mutant mice, have allowed for detailed study of the function of these receptors. PPARα is involved in the breakdown of fatty acids and PPARγ has been shown to be important in cellular differentiation of adipocytes and other cell types and to play a role in insulin sensitization, and thus diabetes, as well as atherosclerosis. PPARδ activity has also been linked to lipid metabolism. As PPARs are permissive to RXR ligand activation, it is possible to regulate the heterodimer through either a PPAR-selective agonist or an RXR ligand. Consistent with this, a combination of ligands for both PPAR and RXR leads to synergy in transcriptional activation. Acting presumably through the PPAR/RXR heterodimer, RXR agonists have been shown to have significant effects on insulin sensitization and therefore make RXR a potential therapeutic target for treatment of type II diabetes. Based on the finding that the DNA sequence can influence the activity of a compound, it is also possible that RXR ligands that potentiate the PPARγ/RXR heterodimer activity on specific target genes without activating RXR promiscuously can be found.

## B. LXR/RXR

Liver X receptors LXRα and LXRβ function to regulate cholesterol homeostasis and fatty acid metabolism. LXRα −/− mice have been shown to have defects in the process of converting dietary cholesterol to bile acids in the liver due to a lack of activation of expression of the cholesterol-7α-hydroxylase (CYP7A1) enzyme. Additional defects include a reduced expression of genes involved in lipogenesis such as sterol regulatory element-binding protein 1 (SREBP1) and fatty acid synthase. An additional target for LXR/RXR regulation in the macrophage and intestine is the ATP-binding cassette A1 (ABCA1) transporter protein, which is involved in reverse cholesterol transport. Activation of ABCA1 and other genes involved in cholesterol efflux by LXR agonists has been shown to lead to a reduction in cholesterol absorption. Furthermore, LXR agonists have also been demonstrated to increase the circulating levels of high-density lipoprotein cholesterol. Consistent with the various functions of LXRs in regulating cholesterol and lipogenesis, recent results also implicate LXR agonists as having anti-atherosclerotic effects.

As with PPARs, LXRs are permissive to RXR activation and RXR agonists can elicit similar biological responses to LXR-specific agonists including up-regulation of ABCA1 and SREBP1 and inhibition of atherosclerosis in animal models. Consistent with the action of LXR or RXR agonists, both LXR null mutant mice and mice with RXR deleted from hepatocytes exhibit an increase in triglycerides. Additional evidence for a role for retinoid receptors in fatty acid synthesis comes from the observation that patients treated with RXR agonists have elevated triglycerides. The function of the LXR/RXR heterodimer as determined through multiple studies therefore represents an important point of pharmacological intervention, through the LXR receptors and/or RXR receptor, for cholesterol regulation, lipid homeostasis, and control or prevention of atherosclerosis.

## C. FXR/RXR

The FXR was identified through homology screening and also by using RXR as bait in a yeast two-hybrid protein interaction assay. FXR functions as a permissive heterodimer with RXR and binds to an inverted repeat-1 DNA element. The finding that bile acids could function as ligands for FXR has established a role for FXR/RXR in bile acid metabolism. Targets for FXR regulation include ileal bile acid-binding protein and bile salt efflux pump in the intestine and CYP7A1 in the liver. FXR inhibits the expression of CYP7A1 and therefore opposes the function of LXR on this target, although the mechanism of how this repression occurs is not clear. Studies have demonstrated that an FXR agonist, when administered to rats, resulted in a lowering of triglycerides. The mechanism of how triglyceride production might be regulated by FXR is not yet known. Further experiments have demonstrated that guggulsterone, a steroid produced by the guggul tree and used for over 2500 years as a treatment for a number of disorders including high cholesterol, functions in the body primarily through FXR. The cholesterol-lowering effects of guggulsterone can be linked to its activity as an antagonist of FXR. The findings with FXR agonists and antagonists indicate that the FXR/RXR heterodimer also represents an important pharmacological intervention point for regulating cholesterol and triglyceride levels.

## D. PXR/SXR and CAR

The xenobiotic sensing receptors PXR/SXR and CAR have been shown to regulate responses to foreign chemicals in the body, as well as endogenous compounds such as bile acids. The target genes for these receptors include the cytochrome P450 CYP3 genes for PXR/SXR and CYP2 genes for CAR. There is some overlap between the targets and functions of these receptors, although important pharmacological and species differences exist. Implications for the regulation of these genes include mediating drug–drug interactions and elimination of toxic compounds from the body. As stated previously, these receptors bind to DNA-response elements (DR5) as heterodimers with RXR and are permissive to activation by RXR ligands. Although RXR ligands can activate the heterodimers on isolated response elements in transfection experiments, currently there is no conclusive evidence to suggest that synthetic or endogenous RXR ligands have a physiological role in regulating PXR/SXR or CAR target genes since it is unclear which heterodimer partner the compounds are working through.

As demonstrated in the previous sections, RXR heterodimers play important roles in many natural and disease states. A general statement regarding permissive versus nonpermissive heterodimers based on recent observations is that the RXR-permissive heterodimers appear to be "lipid" sensors that control many metabolic pathways including glucose, fatty acid, bile acid, and cholesterol signaling. Nonpermissive heterodimers alternatively regulate responses to classic endocrine hormones including steroids and nonsteroid hormones.

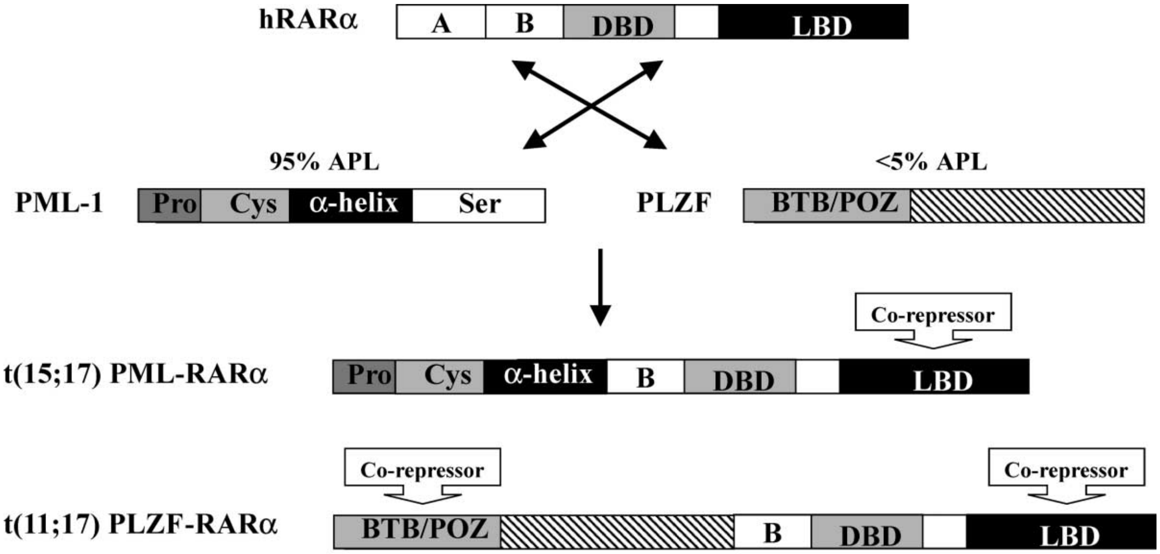
## VII. RETINOID RECEPTORS AND CANCER

### A. Acute Promyelocytic Leukemia

As described previously, the role of retinoids and retinoid receptors in regulating cell proliferation and differentiation is well established. As a result of these studies and the finding that translocations of the RARα gene are involved in certain leukemias, a clear link between retinoid signaling and cancer has been established. APL is characterized by abnormal proliferation of promyelocytes in the bone marrow and represents 10–15% of acute nonlymphoid leukemias. Experiments in which APL cells were treated with a variety of compounds demonstrated that AtRA could induce differentiation of these leukemic cells into granulocytes. This key finding has greatly improved the cure rate among patients with this form of leukemia and indicated that retinoid receptor signaling was involved. The majority of APL cases (95%) have been shown to develop as a result of a translocation between the RARα gene, which is on chromosome 17, and the PML gene, which is on chromosome 15. The resulting fusion proteins are shown in Fig. 6. The PML–RARα fusion proteins contain various lengths of the PML protein fused to a point in the second intron of RARα. The PML–RARα protein contains the N-terminus, the A domain of RARα, and the C-terminal parts of the PML protein. Experiments introducing a transgenic PML–RARα gene into mice result in development of leukemias that are similar to APL and confirm that this gene product is responsible for both the leukemia and the sensitivity of the cells to retinoic acid treatment.

The PML–RAR protein may inhibit myeloid differentiation in several ways. It is possible that PML–RAR inhibits the normal function of retinoic acid signaling by binding to and sequestering RXR, thus inhibiting the function of heterodimers reliant on RXR. Normal PML function may be inhibited through the disruption by PML–RAR of a nuclear structure known as promyelocytic organizing domains (PODs) or PML bodies. PODs have been implicated as sites of protein ubiquitination and turnover as well as storage of cellular factors involved in transcription. One important observation of PML–RAR leukemic cells is that upon treatment with RA, the normal nuclear organization of PODs is regained. Additional studies have demonstrated that the function of PML in regulating apoptosis is also impaired in the PML–RARα-expressing promyelocytes.

In addition to the PML–RARα translocation, APL has been characterized by two other translocations involving RARα that occur in 1–2% of APL cases. Translocation of the RARα gene with the PLZF gene on chromosome 11 results in a PLZF–RARα fusion protein, whereas a translocation with the nucleophosmin (NPM) gene on chromosome 5 results in a NPM–RARα fusion protein. The PLZF–RARα functions in a manner similar to PML–RARα to inhibit normal retinoid signaling and possibly to antagonize the activity of PLZF. Unlike PML–RARα cells, PLZF–RARα cells are unresponsive to retinoic acid treatment. Recent work has implicated the recruitment of a co-repressor protein complex, described in previous sections, to the fusion protein. The co-repressor SMRT has been shown to interact with both PLZF and RARα so that treatment with RA is not sufficient to disrupt the co-repressor–PLZF–RARα complex. Since PML does not interact with co-repressors, the addition of RA can lead to the displacement of the co-repressor complex from PML—RARα and restore transcriptional activity. These data highlight the importance of considering both the retinoid receptors and their cofactors when examining the normal physiology of retinoid signaling as well as the treatment of disease states.



| Fusion protein | RA responsive |
|---|---|
| t(15;17) PML-RARα | Yes |
| t(11;17) PLZF-RARα | No |

FIGURE 6 RAR translocations and APL. The fusion proteins resulting from translocations between the human RARα (hRARα), PML, and PLZF genes are shown. As indicated, 95% of acute promyelocytic leukemias are the result of a translocation between hRARα and PML-1, generating a fusion protein that contains up to the serine-rich region in PML and all but the A region of RARα. Since the fusion protein contains the RAR LBD, t(15;17) APL can be treated with retinoids such as AtRA. The co-repressor complex is shown bound to the RAR LBD, and presumably retinoids are effective through displacement of this complex. Another 5% of APL is due to a translocation with the PLZF gene. Binding of co-repressor to PLZF is not affected by retinoids, and thus t(11;17) APL patients are resistant to retinoids.

### B. Breast Cancer

Based on the role of retinoid receptors in regulating multiple cellular processes, another pharmacological target for retinoids has been the treatment of breast cancer. Both RARs and RXRs are expressed in normal and cancerous breast cells and regulate important functions in these cells including proliferation, differentiation, and apoptosis. Studies using the retinoids AtRA and 9-*cis*-RA have demonstrated that activation of the RAR/RXR heterodimer in both breast cell lines and in animal models of breast cancer results in an inhibition of tumorigenesis. Based on the promising results obtained with the natural retinoids, synthetic retinoids and rexinoids that have reduced toxicity and unwanted side effects have been tested for breast cancer prevention and treatment. The results from prevention of breast cancer with these compounds in animal models indicate that synthetic, receptor-selective compounds that regulate RAR/RXR will prove to be an important addition to the currently available treatments for this disease.

## VIII. SUMMARY

Retinoid signaling is an essential part of normal development and physiology. The discovery that RAR and RXR, members of the nuclear hormone receptor protein family, could mediate many of the *in vivo* effects of vitamin A and its metabolites has helped to uncover the mechanisms by which these effects are generated. The temporal and spatial expression of the retinoid receptors, isoforms, and many splice variants dictates the response to retinoids. It is clear that DNA binding to various response elements and the ability to recruit either co-activator or co-repressor complexes to the DNA are the ultimate result of natural or synthetic ligand addition.

The diversity of responses to retinoids and other physiological ligands is increased by the ability of RXR to function as a master regulator of nuclear receptor signaling through heterodimerization with a number of the receptors. The work identifying the retinoid receptors, characterizing their mechanism of action and role *in vivo*, has greatly influenced the knowledge of how retinoid function may be altered in disease states and how medicine can intervene to correct these defects.

## Glossary

**dimerization** The generation of a multiprotein functional unit comprising proteins of the same class. A monomer is a single unit, and a dimer is two units. In homodimers, the receptors are the same, whereas in heterodimers the receptors are different. This represents a central concept in the function of nuclear hormone receptors, as the majority work as heterodimers or homodimers.

**ligand agonist** A small molecule that upon binding to the receptor induces a conformation in the ligand-binding domain that results in activation of transcription, leading to an increase in gene expression.

**ligand antagonist** A small molecule that upon binding to the receptor induces a conformation in the ligand-binding domain that results in an inhibition of transcription activation by agonists, leading to a decrease in gene expression.

**ligand inverse agonist** A small molecule that upon binding to the receptor induces a conformation in the ligand-binding domain that results in an inhibition of the basal ligand-independent activity of the receptor, leading to a decrease in gene expression.

**nuclear hormone receptors** These proteins belong to a class of transcription factors that regulate gene expression in response to binding of small molecules called ligands. The ligand-bound receptor can activate or repress gene expression by binding to specific DNA-response elements. In the absence of ligand, these receptors generally repress gene transcription.

**response element** A short region of DNA that is bound by a transcription factor and the deletion or addition of which affects the transcription of genes containing such elements. For nuclear hormone receptors, most response elements consist of two 6 bp half-sites that have variable spacing and orientation with respect to each other.

**retinoids** Any of a number of small molecules that can bind to and regulate the activity of the nuclear hormone retinoic acid receptor.

**rexinoids** Any of a number of small molecules that can bind to and regulate the activity of the nuclear hormone retinoid X receptor.

**transcription** The process of synthesizing RNA from DNA directed by the activities of the enzyme RNA polymerase. RNA is subsequently made into protein through a process referred to as translation. The transcription process consists of three parts: initiation, elongation, and termination. Transcription factors are those proteins that can bind to specific DNA elements and regulate the process of transcription by activating or repressing RNA polymerase function through different mechanisms.

## See Also the Following Articles

**Glucocorticoid Receptor Structure and Function • Ligand Modification to Produce Pharmacologic Agents • Peroxisome Proliferator-Activated Receptors (PPARs)**

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# Regulators of G-Protein Signaling (RGS) Superfamily

David A. Sierra and Thomas M. Wilkie
*University of Texas Southwestern Medical Center, Dallas*

I. INTRODUCTION
II. RGS SUPERFAMILY: STRUCTURE AND PHYLOGENETIC RELATIONSHIPS
III. GAP ACTIVITY REGULATES SIGNALING
IV. SUMMARY

**G-protein signaling is dependent on a cycle of GTP binding on the G-protein α-subunit and GTP hydrolysis, which activate and inactivate signaling, respectively. Regulators of G-protein signaling (RGS) proteins are GTPase accelerating proteins for specific classes of G-protein α-subunits, serving as feedback inhibitors of G-protein signaling pathways by accelerating the rate of inactivation. RGS proteins may also facilitate signaling by serving as scaffolds and effector proteins. Thus, RGS proteins sharpen the kinetic response of both activation and termination of G-protein signaling in cells.**

## I. INTRODUCTION

G-protein signaling is regulated by the combined action of receptors and regulators of G-protein signaling (RGS) proteins to control intracellular responses to extracellular signals throughout an animal's lifetime. G-proteins regulate numerous essential functions in higher eukaryotes, including gametogenesis, fertilization, cell motility, feeding behavior, sleep, responses to light, and other environmental and hormonal cues. Signaling via heterotrimeric G-proteins initiates with agonist binding to heptahelical receptors. The role of heterotrimeric G-proteins, composed of α-, β-, and γ-subunits, is to transduce the signal received on agonist binding to the regulation of effector proteins and their downstream second-messenger systems. Signaling specificity is provided by the distinct receptor, G-protein α-subunit, and RGS protein that regulate the pathway.

## II. RGS SUPERFAMILY: STRUCTURE AND PHYLOGENETIC RELATIONSHIPS

RGS proteins are expressed in all higher eukaryotes, except for plants, together with all other components of G-protein signaling complexes, including agonists, heptahelical receptors, heterotrimeric G-protein α-, β-, and γ-subunits, and effector proteins. The first RGS protein was discovered in baker's yeast and found to be an important feedback inhibitor of the mating response to pheromone. Yeast of the opposite mating types recognize each other's proximity and initiate the first steps of conjugation via a G-protein signaling pathway. In the absence of the RGS protein termed Sst2 (supersensitive to pheromone), yeast could not recover from pheromone-induced cell cycle arrest in preparation for mating. Several years later, sequence similarity to the yeast Sst2 protein was independently discovered in several RGS proteins by three groups studying B-cell maturation, vesicular transport, and roundworm motility and egg laying. Similarity within these proteins was restricted to a region of approximately 130 amino acids, termed the RGS domain (Figs. 1 and 2). Subsequent experiments demonstrated that the RGS domain of RGS proteins displayed GTPase accelerating protein (GAP) activity toward the corresponding G-protein α-subunits expressed in yeast, worms, and mammals.

The superfamily of RGS proteins expressed in metazoan organisms now contains five families of RGS-like proteins, including RGS, rgRGS, RGS-PX, GPRK, and AKAP-RGS (Fig. 1 and Table 1). Proteins in the RGS and rgRGS families have very similar three-dimensional structures but the amino acid sequences are only distantly related between the families (Fig. 2). Interestingly, the GAP activity of proteins within each RGS-like family is restricted to a specific class of G-protein α-subunit, indicative of regulatory specificity within different G-protein signaling pathways (Table 1).

### A. The RGS Domain

Mammals express 22 RGS proteins that can be grouped into five subfamilies based on sequence

Until recently, the function of salicylates in plants was obscure; thus, they have traditionally been classified as secondary metabolites. By contrast, the pharmacological benefits of salicylates in humans are well documented. Aspirin (acetylsalicylic acid), for example, at various doses, protects against heart attack and stroke, inhibits blood clotting, and reduces pain, fever, and joint swelling. Only during the past 15 years have researchers discovered that SA serves important functions in plants. It is a signaling molecule that activates heat production, induces disease resistance, and, possibly, stimulates flowering.

## II. SA-REGULATED PROCESSES IN PLANTS

The first conclusive demonstration that SA functions as an endogenous signaling molecule came from Raskin and co-workers, who were studying thermogenesis in the voodoo lily (*Sauromatum guttatum*). On the day of flowering, there are two distinct periods during which the temperature of the spadix (floral spike) can increase by approximately 14°C. Prior to each thermogenic event, a large, transient increase in endogenous SA levels is detected. Confirming the role of SA as the signal for this phenomenon, it has been shown that exogenously supplied SA, as well as its derivatives acetylsalicylic acid (ASA) (Fig. 1) and 2,6-dihydroxybenzoic acid, can induce thermogenesis in explants of the voodoo lily spadix, whereas 31 structurally similar compounds do not have this effect.

SA also has been shown to play an important signaling role in plant disease resistance, and the mechanisms through which it activates this phenomenon have been studied extensively. The ability of a plant to recognize a pathogen is sometimes regulated by the direct or indirect interaction between the products of a plant resistance (*R*) gene and a pathogen avirulence (*avr*) gene. If either of these gene products is lacking, the plant fails to activate defenses in a timely and/or effective manner and the pathogen colonizes the plant. By contrast, when both gene products are present, a wide variety of resistance responses are activated in the inoculated leaf. These may include increases in reactive oxygen species (ROS), strengthening of cell walls, and synthesis/activation of various defense-associated proteins, such as the pathogenesis-related (PR) proteins. In addition, resistant plants frequently develop a hypersensitive response (HR) (Fig. 2). Subsequent to these local responses, uninoculated leaves usually exhibit increased *PR* gene expression and systemic acquired resistance (SAR).

It has been known for many years that treatment of tobacco with SA or ASA enhances resistance to tobacco mosaic virus (TMV) and induces PR protein accumulation. SA has more recently been shown to activate *PR* gene expression in several plant species and to enhance resistance to a variety of pathogens. The first direct evidence that SA is an endogenous signal for disease resistance came from studies of pathogen-infected tobacco and cucumber plants. In tobacco resisting infection by TMV, SA levels increased 20- to 50-fold in inoculated leaves, with these increases preceding or paralleling the accumulation of *PR* gene transcripts. SA levels also increased 2- to 10-fold in the uninoculated leaves, and this rise correlated with increased systemic *PR* gene expression. Similarly, in cucumber infected with *Colletotrichum lagenarium*, *Pseudomonas syringae*, or tobacco necrosis virus, 10- to 100-fold increases in SA levels were detected in the phloem sap of infected leaves prior to SAR development and activation of a defense-associated peroxidase in the uninoculated tissue.

Analyses of SA-deficient tobacco and *Arabidopsis* plants [due to inhibition of the SA biosynthetic pathway or expression of the salicylate hydroxylase (SH)-encoding *nahG* transgene] have further confirmed that SA plays an important role in activating disease resistance. These plants fail to develop SAR or express *PR* genes in the uninoculated leaves following pathogen infection. Furthermore, they are susceptible to infection by pathogens that they normally would resist and exhibit heightened susceptibility to those that cause disease. HR development is also delayed in TMV-infected SH-expressing tobacco and the lesions that form grow substantially larger than do those on comparable wild-type (wt) plants. Based on these results, SA may also regulate cell death and pathogen containment. Consistent with this possibility, inhibition of the SA biosynthetic enzyme phenylalanine ammonia lyase (PAL) in soybean suspension cells blocks pathogen-induced HR cell death. Additionally, genetic analyses of certain *Arabidopsis* mutants reveal that SA is required for expression of a constitutive cell death phenotype that mimics an HR.

Whether SA is a mobile signal that induces defense responses in uninfected tissues has been the subject of much debate. The timing at which SA levels increase and SAR develops in both tobacco and cucumber is consistent with this possibility. Furthermore, SA clearly is mobile; it has been detected in the phloem

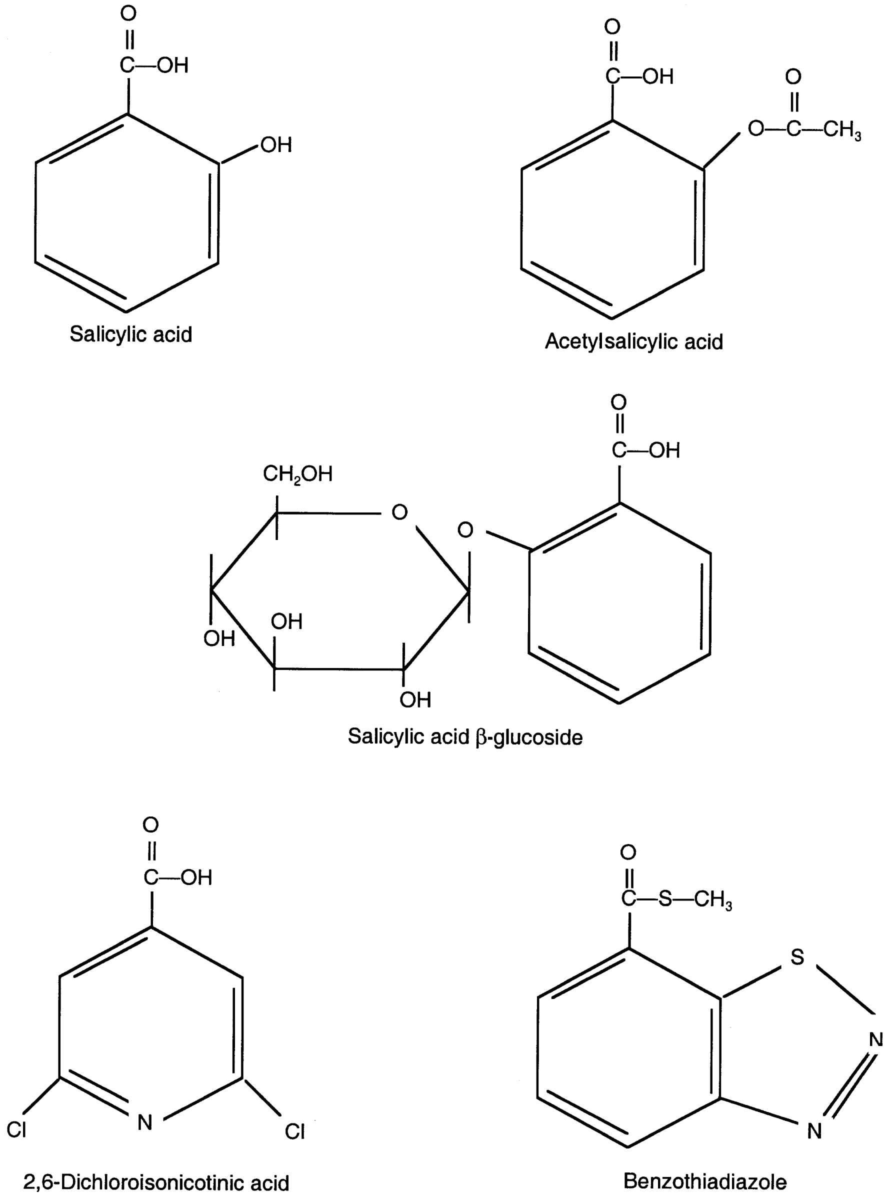

FIGURE 1 Salicylic acid and its derivatives or functional analogues. Salicylic acid and its derivative acetylsalicylic acid (aspirin) induce *PR* expression and systemic acquired resistance in plants; in contrast, salicylic acid β-glucoside is thought to be a storage form of salicylic acid. The synthetic compounds 2,6-dichloroisonicotinic acid and benzothiadiazole *S*-methyl ester, which exhibit structural similarity to salicylic acid, also induce defense responses in plants. In particular, benzothiadiazole *S*-methyl ester has been used commercially in the field to protect crop plants against certain pathogens.

sap of pathogen-infected cucumber and tobacco leaves. Tracer studies using $^{18}O_2$ or the SA precursor [$^{14}$C]benzoic acid (BA) also have suggested that much of the SA in the uninoculated leaves is transported there from the inoculated leaves. Alternatively, SA may be converted into methylsalicylate, a volatile compound that could serve as an airborne SAR signal.

A growing body of evidence, however, suggests that SA is not the long-distance SAR signal. In *P. syringae*-infected cucumber, the SAR signal is transmitted from the inoculated leaf several hours

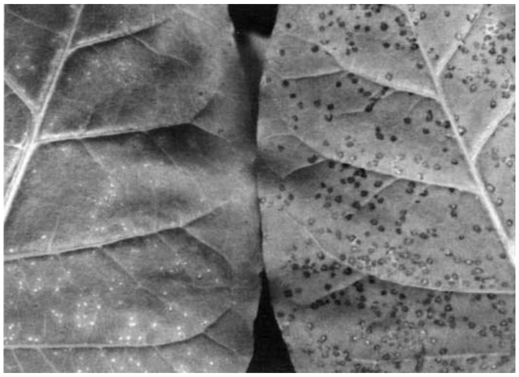

**FIGURE 2** The hypersensitive response (right) and systemic acquired resistance in tobacco following TMV infection. Tobacco plants resisting infection by TMV initially develop a hypersensitive response, characterized by necrotic lesions at the sites of pathogen entry. Subsequently, a broad-based, long-lasting systemic acquired resistance develops in uninoculated tissues. A secondary viral infection of the uninoculated leaves, occurring several days after the initial infection, results in much smaller lesions (left) as compared with those induced by the primary infection. The leaves are shown 4 days after infection. Reprinted from *Trends in Plant Science*, Vol. 2, pp. 266–274 (1997) (Durner *et al.*), with permission from Elsevier Science.

before an increase in SA levels can be detected in the petiole of the inoculated leaf. In addition, wild-type scions (the grafted tops of chimeric tobacco plants) develop SAR and express *PR* genes following TMV infection of SH-expressing or PAL-suppressed rootstocks. Because SA accumulation is not completely abolished in these rootstocks, it is possible that a small amount of SA travels to the scion and induces SAR. However, transgenic tobacco rootstocks that constitutively accumulate SA due to expression of the cholera toxin A1 subunit do not induce *PR* expression or SA accumulation in wt scions. Although these results suggest that SA is not the long-distance SAR signal, SA clearly plays an important downstream role in the defense pathway. SA-deficient scions fail to exhibit *PR* gene expression and/or SAR following infection of wt rootstocks.

Induction of flowering in various *Lemna* species, as well as in *Arabidopsis thaliana*, *Impatiens balsamina*, *Onacidium*, and *Pisita stratiotes* L, and flower bud formation in tobacco tissue cultures have been associated with SA treatment, thus SA has been proposed to be the inductive signal for flowering. However, other evidence does not support this conclusion. For example, SA levels are comparable in flowering and vegetative *Lemna* plants, and the ability of SA to stimulate flowering in several plant species appears to be a nonspecific effect. Thus, whether SA plays any role in flowering remains unclear.

## III. SA METABOLISM

A large body of evidence suggests that SA, many phytoalexins, and lignin are synthesized by the shikimate–phenylpropanoid pathway. The first step of this pathway, which is rapidly induced following pathogen infection, is the conversion of phenylalanine (Phe) to *trans*-cinnamic acid (*t*-CA) by PAL. Depending on the plant species, SA is then generated from *t*-CA through one of two intermediates, benzoic acid or *ortho*-coumaric acid. Both tobacco and cucumber appear to utilize the BA intermediate. The mechanism through which BA is generated from *t*-CA is poorly understood. An oxidative pathway, analogous to β-oxidation of fatty acids, and a nonoxidative pathway, involving the intermediate benzaldehyde, have both been proposed. Supporting the former possibility, no labeled benzaldehyde is detected in TMV-infected tobacco supplied with 3-[$^{13}C_1$]Phe. Once BA is formed, it can be converted to SA by BA 2-hydroxylase (BA2H). Characterization of partially purified BA2H reveals that its activity is strongly induced by exogenously supplied BA or TMV infection. Because this activation is blocked by cycloheximide (CHX), *de novo* synthesis of BA2H appears to be required. Alternatively, a recent study has suggested that BA is not the direct precursor of SA. Rather, a conjugate of BA, consisting of BA and a glucose (BAG) molecule, may be converted to SA.

Like BA, SA also can be conjugated to a glucose molecule. The predominant SA conjugate in many plant species is SA 2-O-β-D-glucoside (SAG); the BAG recently identified in tobacco is benzoylglucose. In tobacco and *Arabidopsis*, free SA and SAG can be detected at low levels. After infection, SA and SAG levels rise, with SAG predominating in both the inoculated and uninoculated leaves. The kinetics of accumulation for free BA parallel those of SA; however, there are conflicting reports as to whether BAG levels increase following infection or are constitutively elevated. The enzyme responsible for conjugating SA, and possibly BA and other phenolics, is uridine diphosphate (UDP)-glucose:SA glucosyltransferase. This enzyme has been characterized in several plant species, and genes encoding this enzyme have been recently cloned from tobacco.

The role of SAG during the resistance response is unclear. Chemically synthesized SAG activates *PR-1* expression in tobacco leaves. However, this induction probably is mediated by free SA, which is released from SAG by a nonspecific cell-wall-associated β-glucosidase. Thus, SAG does not appear to be the signal for defense responses. An alternative role for SAG is to serve as a storage form for SA. Conjugation of phenolic acids, as well as other phytohormones (e.g., auxin), to sugar molecules is a common and potentially reversible mechanism for the storage of various highly active or toxic compounds. In TMV-infected tobacco, deconjugation of SAG in the uninfected tissues of plants exhibiting SAR might therefore provide a rapid source of SA. In conjunction with other SAR-associated defenses, this SA might superinduce resistance, thereby ensuring the extremely rapid restriction of pathogen spread that is the hallmark of SAR.

In addition to the phenylpropanoid pathway, SA can be synthesized from isochorismate, which is derived from chorismate. This isochorismate pathway is utilized in microorganisms, and recent evidence suggests that it also functions in plants. Isoforms of isochorismate synthase have been purified and characterized from *Catharanthus roseus*. Furthermore, the *eds16* (also known as *sid2*) mutant of *Arabidopsis*, which exhibits enhanced disease susceptibility and fails to accumulate high levels of SA after pathogen attack, was recently found to contain a lesion in isochorismate synthase 1 (ICS1). Consistent with this possibility, transgenic tobacco and *Arabidopsis* expressing the two bacterial enzymes involved in the chorismate/isochorismate pathway exhibit elevated SA levels and enhanced disease resistance to viral, fungal, and oomycete pathogens.

## IV. MECHANISMS OF SA ACTION

The mechanisms through which SA signals thermogenesis and disease resistance have been studied intensely. In voodoo lilies, SA has been shown to induce alternative oxidase (Aox) expression, which in turn activates the alternative respiratory pathway, thereby generating heat. By contrast, SA appears to utilize multiple mechanisms to induce defense responses.

In tobacco, some defense-associated responses, such as expression of *PR* genes, enhancement of disease resistance, activation of the SA-inducible protein kinase (SIPK), which is a mitogen-activated protein kinase (MAPK), and increases in cytosolic calcium levels, are directly induced by exogenously supplied SA. Interestingly, the ability of SA to enhance disease resistance appears to be mediated by at least two pathways. One pathway, which regulates resistance to several viruses, including TMV, potato virus X, and cucumber mosaic virus, is sensitive to the Aox inhibitor salicylhydroxyamic acid (SHAM). By contrast, the pathway conferring resistance to a bacterial pathogen (*Erwinia caratovora*) and a fungal pathogen (*Botrytis cinerea*), as well as activating *PR-1* gene expression, is SHAM insensitive.

In addition to the defenses directly activated by SA, some responses, including generation of ROS, activation of HR-like cell death, and induction of certain defense genes, are poorly, if at all, activated by SA alone. Rather, SA potentiates these responses; they are induced more rapidly and/or to a greater extent when SA is supplied prior to or at the time of pathogen infection or treatment with elicitors (biotic or abiotic compounds/factors that induce various defense responses). The relationship between the SA-activated and SA-potentiated response pathways is unclear. However, they may be linked by a proposed positive feedback loop that involves SA, the ROS $H_2O_2$, and cell death.

In an effort to elucidate the mechanisms through which SA signals defense responses, several SA-interacting proteins or SA-binding proteins (SABPs) have been identified in tobacco. The first SABP identified was the $H_2O_2$-degrading enzyme catalase (CAT). Because SA and its functional analogues (i.e., those capable of inducing *PR* expression and enhanced resistance) inhibit CAT activity, it has been proposed that SA-mediated CAT inhibition leads to elevated levels of ROS, which might play a role in HR and SAR. Consistent with this possibility, SA also inhibits ascorbate peroxidase (APX), the other major $H_2O_2$-scavenging enzyme found in plant cells. However, subsequent studies suggest that $H_2O_2$ functions upstream, rather than downstream of SA in the defense pathway. These conflicting results may yet be reconciled by the presence of a putative self-amplifying feedback loop involving SA and $H_2O_2$. Alternatively, SA-mediated CAT inhibition might signal resistance through the generation of SA free radicals, which could initiate lipid peroxidation and thereby activate defenses.

To date, at least two other tobacco SABPs have been identified and characterized. SABP2 exhibits high affinity for SA ($K_d = 90$ n$M$) and has been recently purified. SABP3 exhibits moderate affinity for SA ($K_d = 3–4$ μ$M$) and has been shown to be the chloroplast carbonic anhydrase (CA). The ability of CA to reversibly convert carbon dioxide to bicarbonate is unaffected by SA, suggesting that its recently identified antioxidant activity is critical for defense signaling. It is striking that three of the five SA-interacting proteins currently identified (CAT, APX, and CA) exhibit antioxidant activity. In addition, cytosolic aconitase has been shown to interact with SA. The SA-mediated inhibition of this enzyme may increase citrate levels, which are known to induce *Aox* gene expression. This induction, along with the ability of SA to activate *Aox* expression directly, may play a role in activating the SHAM-sensitive pathway for viral resistance.

It is interesting to note that several SA-interacting proteins, notably CAT, APX, and aconitase, are also regulated by nitric oxide (NO). This small molecule, which signals a wide variety of processes in animals, has been recently shown to regulate aspects of plant growth, development, and disease resistance. In particular, NO is required for pathogen-induced defense gene expression in tobacco and *Arabidopsis*. NO is also needed for HR development in pathogen-infected *Arabidopsis* and it synergizes with ROS to induce cell death in pathogen-treated soybean suspension cells. These findings, combined with the observation that NO induces SA accumulation in tobacco and that SA induces NO accumulation in soybean, suggest that NO, SA, ROS, and cell death are all part of a self-amplifying feedback loop that regulates defense responses. Whereas NO induces *PR-1* expression in tobacco via an SA- and cyclic adenosine diphosphate-ribose (cADPR)-dependent pathway, it activates *PAL* expression via an SA-independent, but cADPR- and cyclic guanosine monophosphate (cGMP)-dependent pathway. Thus, both SA-independent and SA-dependent pathways mediate NO-induced defense responses. Moreover, several critical players in the animal NO signaling pathway are also utilized in plants.

Beyond regulating components of the SA signaling pathway, SA also cross-modulates the activity of the ethylene-mediated and jasmonic acid (JA)-mediated defense pathways. Some of the defenses regulated by ethylene and/or JA include expression of the defensin (*PDF1.2*) and thionin (*Thi2.1*) genes, resistance to certain pathogens, and development of induced systemic resistance (ISR) following infection by nonpathogenic root-colonizing rhizobacteria. Although these pathways do not require SA, there is growing evidence that SA influences them in either a positive or a negative manner. For example, SA antagonizes ethylene/JA-induced defense gene expression in pathogen-inoculated tobacco and *Arabidopsis*; it also antagonizes JA-mediated resistance to herbivorous insects in tobacco. By contrast, SA works in conjunction with ethylene or JA to superinduce *PR* gene expression in tobacco. Furthermore, SA, ethylene, and JA are all required for the induction of apoptotic (HR-like) cell death in *Arabidopsis* protoplasts treated with the fungal toxin fumonisin B1. All of these signals also may be involved in mediating resistance to *B. cinerea* in *Arabidopsis*. The mechanisms by which SA positively interacts with ethylene and/or JA to induce defenses are not known; however, the ability of SA (or certain SA analogues) to inhibit JA synthesis or action in several plant species provides at least one mechanism for the negative regulation.

## V. SA-INDUCED GENE EXPRESSION

SA treatment induces many of the same plant defense genes that are activated by pathogen attack. Traditionally, the SA-induced genes have been divided into one of two categories, immediate-early or late, depending on how rapidly they are activated by SA treatment and whether protein synthesis is required for their activation. Expression of the immediate-early genes, such as those encoding several glutathione *S*-transferases and the ethylene response element binding protein 1 (EREBP1), can be detected within 30 min of SA treatment. Expression of the *Agrobacterium tumefaciens* octopine (*ocs*) and nopaline (*nos*) synthase genes and activation of the cauliflower mosaic virus (CaMV 35S) promoter also are rapidly detected following SA treatment. Because induction of these genes is insensitive to the protein synthesis inhibitor CHX, their expression is probably regulated by preformed transcription factors. The promoters of genes belonging to this class contain an activator sequence-1 (*as-1*) or an *as-1*-like element; these cis-acting sequences partially mediate gene induction following SA, auxin, jasmonate, or $H_2O_2$ treatment. A family of basic leucine zipper (bZIP)-containing transcription factors that bind TGACG motifs, called TGA or OBF factors, binds these elements. SA treatment has been shown to enhance an *as-1* binding activity [presumed to be activation sequence factor-1 (ASF-1), a member of the TGA family] whereas phosphatase treatment of nuclear extracts decreases it. Based on these results, regulation of *as-1* binding activity by a phosphorylation event could provide a mechanism for the rapid, CHX-insensitive induction of immediate-early genes by SA. A MAPK (SIPK) that is rapidly activated in tobacco by TMV infection and SA treatment has been purified and characterized. Whether SIPK plays a role in activating the *as-1* binding activity following SA treatment is not known.

In contrast to the immediate-early genes, the late-response SA-induced genes are activated several hours after SA treatment and their induction is more sustained and is sensitive to CHX. Although the promoters for this class of genes, which includes the acidic *PR* genes, have been studied intensively, no common SA-responsive element has been identified. Analysis of the tobacco *PR-2d* promoter identified a TCA element that is common to several acidic *PR* promoters. However, this element is not required for SA inducibility of the *PR-2d* gene *in vivo*. Rather, a 25-bp element that contains sequences similar to W boxes is involved in SA-induced *PR-2d* expression. The W boxes are found in the promoters of several elicitor- and wound-induced genes. In addition, microarray analysis has revealed that they are found in the promoters of many genes induced during SAR development. The W boxes are bound by WRKY proteins; these proteins have been identified in parsley, tobacco, and *Arabidopsis*. They are a family of zinc-finger-type transcription factors, found exclusively in plants, that bind specifically to W box-type [(T)TGAC(C/T)] DNA sequence elements. The recent discovery that SA treatment and TMV infection induce a tobacco WRKY protein that binds an element in the basic class 1 chitinase (*PR-3*) gene provides an additional link between W boxes and SA-induced gene expression.

In the tobacco *PR-1a* promoter, researchers have identified an *as-1*-like motif that modulates, although is not obligately required for, SA-induced expression. Possibly, a second factor that is synthesized *de novo* after SA treatment works in conjunction with the *as-1* binding factor to induce this late class gene. By contrast, the *as-1*-like TGA binding site identified in the *Arabidopsis PR-1* promoter is required for SA-induced gene expression. The tobacco and the *Arabidopsis PR-1* genes also contain potential nuclear factor κB (NF-κB) binding sites. The significance of this element in tobacco is not known; however, mutations in the *Arabidopsis* sequence abolish inducibility of this promoter by the SA analogue 2,6-dichloroisonicotinic acid (INA). Analysis of the *Arabidopsis PR-1* promoter indicates that it also contains a W box. Because mutations in this box cause elevated levels of basal and induced gene expression, this element appears to regulate *PR-1* expression negatively. Finally, Myb binding sites have been identified in the tobacco *PR-1a* promoter. However, because overexpression or antisense expression of a TMV- and SA-inducible Myb gene (*myb1*) fails to affect SA-induced *PR-1a* expression, the importance of these sequences remains unclear.

## VI. GENETIC ANALYSIS OF THE SA SIGNALING PATHWAY

To elucidate the SA signaling pathway, numerous mutants of *Arabidopsis* have been generated. Based on their phenotype, these mutants can be divided into several broad categories; due to space constraints, only some are discussed here. The first category, which includes the *lsd* (lesion-simulating disease resistance response), *cpr* (constitutive expresser of *PR* genes), *acd2* (accelerated cell death), *cim3* (constitutive immunity), and *dnd1* (defense with no HR cell death) mutants, exhibits constitutive *PR* expression and enhanced disease resistance. These mutants also accumulate elevated levels of SA, and several develop spontaneous HR-like lesions. Whether all of these mutations represent genes in the SA signaling pathway is unclear. Many stimuli, such as ozone, ultraviolet light, and the over- or underexpression of various transgenes, also induce constitutive SA accumulation, spontaneous lesion formation, elevated *PR* expression, and enhanced resistance. Thus, activation of these defense responses may result from perturbations in cellular metabolism. Consistent with this possibility, the *acd2* mutation has been recently shown to alter red chlorophyll catabolite reductase and the *dnd1* mutation affects a cyclic nucleotide-gated ion channel.

By contrast, another class of mutants, named *npr1* (nonexpresser of *PR* genes) or *nim1* (noninducible immunity), fails to express *PR* genes or develop SAR following treatment with SA or its analogues. Cloning of the *Npr1* gene revealed that this critical SA signal transducer contains ankyrin repeats and a broad-complex, tamtrack, and bric-à-brac/potxvirus, zinc finger (BTB/POZ) domain. These domains, which are involved in protein–protein interactions, are required for NPR1 to be functional. NPR1 was subsequently shown to accumulate in the nucleus following SA treatment, and this nuclear localization is necessary but not sufficient for *PR* gene activation. A direct link between NPR1 and *PR* gene expression was established by the discovery that NPR1 differentially binds various members of the TGA transcription factor family.

In addition to the NPR1-dependent pathway, there is growing evidence that SA can signal defenses via an NPR1-independent pathway. For example, crossing an *npr1* mutant allele into enhanced disease resistance mutants (*cpr5*, *cpr6-1*, *cpr22,* or *acd6*) has either no effect or only a partial effect on constitutive defense gene expression and/or disease resistance. Furthermore, several suppressors of the *npr1* mutation have been identified. Some of these, including *ssi1*, *ssi2*, and *ssi4* (suppressor of SA insensitivity of *npr1–5*), constitutively express *PR* genes and exhibit enhanced disease resistance to bacterial and/or oomycete pathogens. By contrast, the *sni1* (suppressor of *npr1-1*, inducible) mutation restores the ability of INA to induce *PR* gene expression and resistance in the *npr1* background. Based on these observations, the SNI1 protein, which shares limited homology with the mammalian tumor suppressor retinoblastoma (Rb), may function as a negative regulator of SAR. Following SA treatment, SNI1-mediated suppression of the SAR signaling pathway would be alleviated by activated NPR1.

A category of mutants that affect SA synthesis has also been identified. These mutants, including *eds1*, *eds5*, and *eds16* [enhanced disease susceptibility; *eds5* and *eds16* correspond to *sid1* and *sid2* (SA induction deficient), respectively] and *pad4* (phytoalexin deficient), also exhibit increased pathogen susceptibility and/or depressed defense response activation following pathogen infection. The *EDS1* and *PAD4* genes encode proteins with some similarity to triacylglycerol lipases; their function in the SA signaling pathway is currently obscure. By contrast, *EDS16* encodes ICS1, which may catalyze SA synthesis via the chorismate/isochorismate pathway, as previously discussed.

## VII. SUMMARY

Over the past 15 years, our understanding of the role of SA in plants has been transformed from the assumption that SA mimics an endogenous signal to the realization that SA is a *bona fide* signal that regulates thermogenesis, disease resistance, and possibly flowering. In addition, it has been demonstrated that SA is synthesized via the phenylpropanoid pathway, and possibly the chorismate/isochorismate pathway. Analyses of the mechanisms of action of SA have revealed that SA induces thermogenesis by activating *Aox* gene expression. How SA activates disease resistance is less clear, but the discovery that CAT, APX, aconitase, and CA are SA-interacting proteins provides several interesting leads to follow. The observation that several SA-interacting proteins also are regulated by $H_2O_2$ and NO suggests there is significant interplay between these signals. Moreover, these findings argue that parallel defense signaling strategies are used in plants and animals. Combined with the mounting evidence revealing positive and negative cross talk between the SA-, JA-, and ethylene-mediated defense pathways, there are multiple avenues yet to be explored in our quest to understand how SA signals disease resistance in plants.

## Glossary

**alternative oxidase** The terminal oxidase of the alternative respiratory pathway; unlike the cytochrome respiratory pathway, which conserves energy from electron flow as chemical energy (ATP), the alternative respiratory pathway releases this energy as heat.

**hypersensitive response** Manifested by the formation of necrotic lesions at the site of pathogen entry; thought to play a role in reducing pathogen growth and spread.

**pathogenesis-related proteins** Encompass several families of proteins that are expressed first in the inoculated and subsequently in the uninoculated leaves of plants resisting pathogen attack; due to the correlation between *PR* gene expression and development of hypersensitive response and systemic acquired resistance, increased *PR* expression is frequently used as a marker for these phenomena.

**phytoalexins** Low molecular-weight compounds that exhibit antimicrobial activity.

**salicylate hydroxylase** Enzyme encoded by the bacterial *nahG* gene; converts salicylic acid into catechol, a compound that does not induce defense responses.

**systemic acquired resistance** Long-lasting enhanced resistance to a wide variety of pathogens; developed in the uninoculated tissues of a plant following a primary infection.

**thermogenesis** Generation of heat; occurs in certain plants during the flowering process; in voodoo lilies, the increased temperature of the spadix (the central column of the flower) volatilizes compounds, releasing a foul odor that attracts pollinating insects.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Jasmonates • Systemins**

## Further Reading

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# Secretin

Sebastian G. De La Fuente and
Theodore N. Pappas
*Duke University Medical Center*

I. INTRODUCTION
II. STRUCTURE AND CHEMICAL PROPERTIES
III. GENE COMPOSITION AND EXPRESSION
IV. SYNTHESIS OF SECRETIN
V. STIMULATORY RELEASE OF SECRETIN
VI. BIOLOGICAL AND PHYSIOLOGICAL ACTIONS
VII. CLINICAL APPLICATIONS OF SECRETIN/PATHOLOGICAL CONDITIONS

**Secretin, a 27-amino-acid peptide hormone produced by intestinal S cells, is released into the duodenum primarily in response to pH changes following food ingestion. Its major target organs include the pancreas, stomach, and gallbladder. Secretin affects pancreatic bicarbonate secretion, gastric acid secretion, and motility and augmentation of bile flow. Secretin has been found to be altered in some clinical conditions and is currently used as a diagnostic tool for assessing pancreatic function in chronic pancreatitis, in identification of the main pancreatic duct, and in diagnosis of gastrinoma (Zollinger–Ellison syndrome).**

## I. INTRODUCTION

The polypeptide hormone secretin was discovered in 1902 by Starling and Bayliss as a substance capable of stimulating pancreatic bicarbonate secretion. Based on purified intestinal mucosa extracts, secretin is structurally a linear sequence of 27 amino acids. The advent of highly sensitive radioimmunoassay techniques has help to identify the physiological stimulus that triggers cellular release of secretin as well as its major biological effects. The homology between secretins of different species has facilitated the understanding of its actions and roles in gastrointestinal physiology.

Secretin is synthesized by the intestinal S cells and is secreted into the bloodstream when the upper segments of the small intestine are exposed to the acidic contents of the emptying stomach. The main target organ of the hormone is the pancreas, which is stimulated to discharge bicarbonate into the intestinal lumen, neutralizing the contents of the intestines. In addition, secretin augments the release of digestive enzymes from the pancreas and inhibits further production of acids in the stomach.

The stimulatory properties of secretin that impact the pancreas are pharmacologically useful as a diagnostic tool to examine pancreatic exocrine function and to assist in the recognition of the main pancreatic duct in imaging studies. Secretin is also a valuable tool in the diagnosis of gastrinoma. Of clinical importance is the fact that altered levels of secretin have been found in various pathological conditions, thus making secretin a possible target for future therapeutic approaches.

## II. STRUCTURE AND CHEMICAL PROPERTIES

The linear 27-amino-acid secretin polypeptide has a molecular weight of 3055. The amino acid sequence has been determined in pigs, cows, dogs, chickens, humans, and rats (Fig. 1). Bovine secretin is identical to porcine secretin, whereas chicken and porcine secretins are similar at only 14 amino acid positions. The secretin precursor structure consists of the secretin sequence, a signal peptide, an amidation–cleavage sequence, a 72-amino-acid carboxyl-terminal extension peptide, and a short N-terminal peptide (Fig. 2). The $NH_2$-terminal sequence seems to be required for full bioactivity. Alteration of this segment results in significant loss of secretin capacity to stimulate exocrine pancreatic secretion.

Secretin shares structural homology with many other peptides present in the gut and brain. These include vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP), glucagon gene-related products (glucagon, GLP-1, GLP-2), and peptide histidine–isoleucine amide (PHI). Other compounds, such as growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP), and peptide HM (PHM), are also members of the secretin family. Prealbumin is somehow homologous to the secretin sequence. The similarities between some of these peptides are shown in Fig. 3.

Secretin is thermally stable at neutral pH, but at acidic pH it is labile and can be converted into β-aspartyl peptide. Secretin tends to be adsorbed by glass and plastic, thus it is necessary to add albumin to secretin solutions to avoid adsorption to these surfaces. Secretin has a short plasma half-life of 2–4 min and a metabolic clearance rate of approximately 13–15 $ml^{-1}\,kg^{-1}$. The kidneys are responsible for most of its clearance.

## III. GENE COMPOSITION AND EXPRESSION

The human secretin gene has been recently found to be located in chromosome 11p15.5. The locus is composed of four exons with a coding sequence spanning 713 bp of genomic DNA and encoding 123 amino acids. The human gene shares similarities to the gene in pigs and rodents, and all have a common four-exon structure. The first exon encodes a signal peptide and a segment of the N-terminal peptide; the bioactive secretin is encoded in the second exon. The third and four exons encode the C-terminal peptide of prosecretin. Although the posttranslational processing of secretin is not known in detail, a precursor has been isolated from the porcine intestine consisting of secretin plus 41 amino acids at the C terminus. The hormone-coding regions are highly comparable within secretin genes of different species; however, the C-terminal peptide differs substantially, suggesting that this portion is not of physiological importance. The secretin gene is also comparable to other genes of the secretin family. Among gene family members, VIP, glucagon, and secretin are all encoded in one exon. In contrast, GIP and growth hormone-releasing hormone peptides are encoded in separated exons.

## IV. SYNTHESIS OF SECRETIN

Immunoreactive methods have identified the small intestine of most species as the major site of secretin production. Immunoreactivity of secretin is highest in the duodenum and decreases along the horizontal axis, reaching insignificant levels in the ileum; however, low-level immunoreactivity has also been found in the porcine colon. Biosynthesis of secretin takes place in intestinal S cells. These are enteroendocrine cells that are interspersed among other epithelial cells, occurring at a frequency of approximately 6 cells per 1000 epithelial cells. A majority of the S cells are distributed within the intestinal villi, with small numbers in the

**Human synthetic secretin structure**

H-His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-***Glu-Gly***-Ala-Arg-Leu-
-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-$NH_2$

**Porcine secretin structure**

H-His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-***Asp-Ser***-Ala-Arg-Leu-
-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-$NH_2$

FIGURE 1 Amino acid sequences of synthetic human and porcine secretins. The two structures differ only at amino acids 15 and 16 (Glu-Gly vs Asp-Ser).

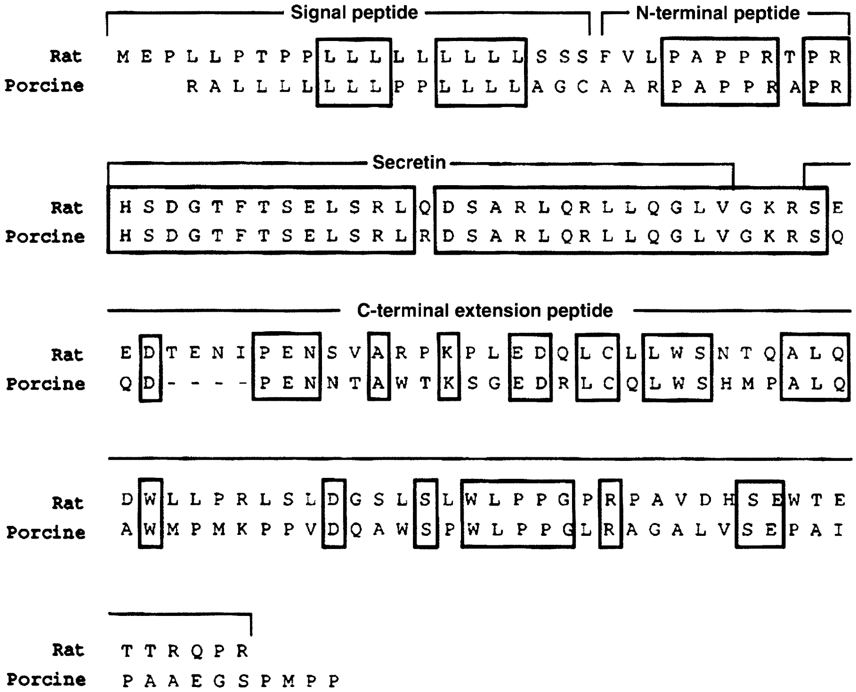
Signal peptide
N-terminal peptide
Rat M E P L L P T P P L L L L L L L L L S S S F V L P A P P R T P R
Porcine R A L L L L L L L P P L L L L A G C A A R P A P P R A P R
Secretin
Rat H S D G T F T S E L S R L Q D S A R L Q R L L Q G L V G K R S E
Porcine H S D G T F T S E L S R L R D S A R L Q R L L Q G L V G K R S Q
C-terminal extension peptide
Rat E D T E N I P E N S V A R P K P L E D Q L C L L W S N T Q A L Q
Porcine Q D - - - - P E N N T A W T K S G E D R L C Q L W S H M P A L Q
Rat D W L L P R L S L D G S L S L W L P P G P R P A V D H S E W T E
Porcine A W M P M K P P V D Q A W S P W L P P G L R A G A L V S E P A I
Rat T T R Q P R
Porcine P A A E G S P M P P

FIGURE 2 Comparison of amino acid sequences for the rat and porcine secretin precursors. Boxes enclose identical amino acids. Predicted functional domains are indicated above the amino acid sequences. Reproduced from "Comprehensive Endocrinology—Gut Peptides Biochemistry and Physiology" (J.H. Walsh and G.J. Dockray), p. 150 (1994), Raven Press, with permission from the publisher and the authors.

middle and upper crypt region. Ultrastructurally, S cells are characterized by a pear-shaped cytoplasm containing small, irregular granules of 200 nm displaying slight argyrophilia. Serotonin and substance P have been shown to be co-expressed in some duodenal S cells; however, this co-expression depends on the species studied and occurs in a small percentage of cells. The renewal time of S cells has been estimated to be 5 days. Guinea pigs and rodent secretin levels are relatively high at birth, falling in the postnatal period. Secretin cells have been identified in the rat duodenum as early as day 17 of gestation. Secretin mRNA is present in the developing pancreas, reaching maximal levels at day 19 of gestation.

Although extraintestinal sources of secretin have been experimentally located throughout the central nervous system, it is generally accepted that only the small intestine produces significant amounts.

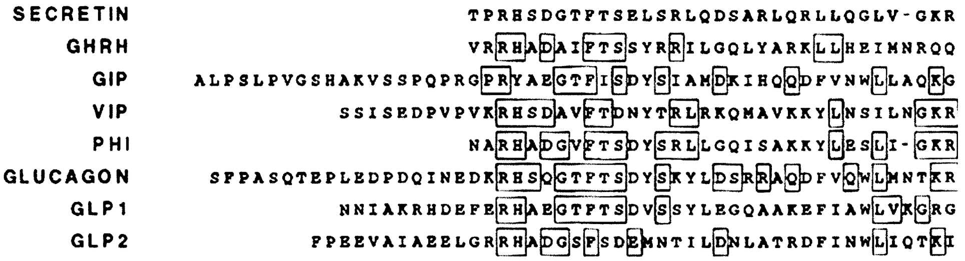
SECRETIN TPRHSDGTFTSELSRLQDSARLQRLLQGLV-GKR
GHRH VRRHADAIFTSSYRRILGQLYARKLLHEIMNRQQ
GIP ALPSLPVGSHAKVSSPQPRGPRYAEGTFISDYSIAMDKIHQQDFVNWLLAQKG
VIP SSISEDPVPVKRHSDAVFTDNYTRLRKQMAVKKYLNSILNGKR
PHI NARHADGVFTSDYSRLLGQISAKKYLESLI-GKR
GLUCAGON SFPASQTEPLEDPDQINEDKRHSQGTFTSDYSKYLDSRRAQDFVQWLMNTKR
GLP1 NNIAKRHDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
GLP2 FPEEVAIAEELGRRHADGSFSDEMNTILDNLATRDFINWLIQTKI

FIGURE 3 Alignment of the amino acid sequences encoded by the coding exons of the secretin family of genes. Amino acids identical to the corresponding exon in the secretin gene (top line) are enclosed in boxes. Sequences shown are encoded by the respective rat genes, with the exception of human gastric inhibitory peptide (GIP). GHRH, Growth hormone-releasing hormone; VIP, vasoactive intestinal peptide; PHI, peptide histidine–isoleucine amide; GLP, glucagon-like peptide. Reproduced from "Comprehensive Endocrinology—Gut Peptides Biochemistry and Physiology" (J. H. Walsh and G. J. Dockray), p. 152 (1994), Raven Press, with permission from the publisher and the authors.

Early experiments have demonstrated small concentrations in pituitary and pineal glands, hypothalamus, thalamus, and olfactory lobe. Secretin-like immunoreactivity has also been reported in the gastric antrum and pancreatic islets; however, these findings have not been corroborated. Measurements of secretin mRNA have failed to demonstrate significant concentrations in locations other than the intestines, but very low levels may simply be below the detection limits of Northern blot analysis techniques.

## V. STIMULATORY RELEASE OF SECRETIN

Among the known factors that trigger the release of secretin (Table 1), intralumenal acidification of the duodenum and proximal jejunum results in the strongest stimulus for secretion. Considerable amounts of hydrochloric acid (HCl) are normally produced in the stomach, facilitating digestion of large pieces of ingested foods. Most of this gastric acid production is buffered by the food and by alkaline secretions, but a considerable quantity of acid enters the duodenum, reducing duodenal intralumenal acidity to a pH range of 3.0–5.0. Experimental studies have calculated the pH threshold for secretin release to be approximately 4–5 in humans and 4.5 in dogs. Experimentally, infusing acid into the intestinal lumen stimulates secretin release. The increase in plasma secretin concentration levels depends on the acid load delivered to the duodenum and parallels the increase in secretion of bicarbonate from the pancreas.

Many substances stimulate the release of secretin (see Table 1), including bile salts, fatty acids, and herbal extracts. Intralumenal infusion of bile salts triggers the pancreas to release bicarbonate-rich secretions in animals and humans; however, little is known about the physiological role of endogenous bile in stimulating secretin release. Fatty acids, specifically oleic acid, also stimulate secretin release. Additionally, geranyl-geranyl acetone (a cyclic polyisoprenoid experimentally used to heal ulcers) and L-phenylpentanol, which is used as a condiment in some cultures, when administered intraduodenally, increase plasma secretin concentrations and pancreatic bicarbonate secretion.

**TABLE 1 Substances That Influence Sectretin Release**

| Stimulants | Inhibitors |
|---|---|
| Acid[a] | Oxethazine |
| Bile salts | Somatostatin |
| Long-chain fatty acids | Met-enkephalin |
| Oligopeptides (in rats) | |
| Sodium oleate | |
| Herbal extracts | |
| Geranyl-geranyl acetone | |

[a] Intraduodenal acid is the strongest stimulus for secretin release.

The role of the nervous system in controlling secretin secretion has long been a controversial issue. Based on animal studies, some investigators have proposed that secretin's actions depend heavily on cholinergic input; however, the fact that atropine (an anticholinergic drug) fails to inhibit secretin secretion in humans in response to duodenal acidification suggests a minor vagal control in secretin release. To date, the mechanisms by which a decreased duodenal pH stimulates secretin production remain unclear; nevertheless, *in vitro* studies have shown that secretin can be released after induction by $Ca^{2+}$ and cAMP.

Three substances are known to inhibit the release of secretin: oxethazine, somatostatin, and Met-enkephalin. Oxethazine is a substance with local anesthetic properties; when infused in the duodenum of dogs, it suppresses the plasma concentration increment of secretin and bicarbonate secretion in response to acidification. Little is known about the mechanism of action of this drug. In addition, both somatostatin and Met-enkephalin have been shown to reduce secretin levels and pancreatic response to duodenal acidification, but it is unclear if this inhibition occurs at physiological concentrations.

## VI. BIOLOGICAL AND PHYSIOLOGICAL ACTIONS

### A. Receptor Structure

Characterization of the secretin receptor in pancreatic acinar cells of guinea pigs has led to distinguish two different receptor types–high affinity and low affinity-depending on the receptor avidity for secretin. Secretin is a potent agonist for the high-affinity receptor whereas VIP weakly stimulates it. The low-affinity receptor shows much lower affinity for secretin and higher affinity for VIP. Both are G-protein-linked receptors that activate the adenylyl cyclase enzyme, resulting in augmentation of intracellular cyclic AMP (cAMP). The receptor is composed of seven putative hydrophobic transmembrane domains with a 427-amino-acid sequence; the molecular weight is 48,696.

Secretin receptor mRNAs have been detected in several tissues in addition to the pancreas, including heart, stomach, and nervous system. Autoradiographic examination of rat pancreatic tissue demonstrates high-affinity binding sites in both acinar and ductal cells, with no binding on islets and vascular structures.

## B. Secretin Effects and Mechanisms of Action

Secretin activity can be grouped into two categories: actions occurring at physiological levels and actions occurring at supraphysiological levels. Most of these actions are included in Table 2. Secretin effects can also be grouped into stimulatory or inhibitory actions.

Stimulation of pancreatic water and bicarbonate secretion represents the main physiologic action of secretin, resulting in neutralization of the acidic chyme entering the proximal intestines. Low intraduodenal pH triggers secretin secretion, which results in an incremental release of pancreatic water and bicarbonate. Among the components of the gastric chyme, titratable acid appears to be the main stimulant for secretin secretion. Immunoreactive levels of secretin are also significantly increased after ingestion of a meal of mixed types of foods. Additionally, fatty acids and the digestive by-products of fat cause release of significant amounts of immunoreactive secretin. It is well established that actions of secretin on the exocrine pancreas are potentiated by cholecystokinin (CCK) in dogs and humans. The concomitant release of CCK along with secretin produces a greater response, compared to the sum of their individual actions, because both hormones act together.

Animal studies have shown that secretin exerts inhibitory effects on gastric acid secretion and gastric motility, therefore acting as an enterogastrone. It is not clear whether these inhibitory actions result from a direct secretin effect on the stomach or as a consequence of various hormones and/or peptides acting in concert. Several hormones (serotonin, somatostatin, and peptide YY) are known to be released postprandially and modify acid secretion and gastric emptying rates. Interactions between such hormones and secretin may explain the inhibitory actions of secretin on gastric physiology. In rodents, for example, secretin strongly inhibits basal acid output by stimulating both somatostatin and prostaglandin release, thus indirectly influencing gastric secretion. However, these effects are less clear in humans; infusion of exogenous secretin at doses similar to levels seen following acidified meals fail to inhibit acid secretion. Furthermore, inhibition of acid output has not been achieved after administration of fivefold higher doses of exogenous secretin. On the other hand, both continuous and intermittent infusions of secretin have shown to retard gastric emptying of solid meals in humans. Pure secretin has shown to stimulate pepsin release in cats, dogs, and humans. Very small doses of secretin increase serum group pepsinogen I and pepsin output in young, healthy human volunteers. Gastric mucous secretion has been found to be increased by secretin in cats, dogs, and humans, but the physiological significance of these findings remains to be elucidated.

Biliary secretion is influenced by administration of supraphysiological doses of exogenous secretin. Pharmacological administration of secretin augments bile flow and bicarbonate concentration in bile, but it is unclear to date whether these effects are produced by endogenous secretin.

Other actions attributed to secretin include induction of bicarbonate secretion from duodenal

TABLE 2 Actions of Secretin

| Level | Stimulation | Inhibition |
|---|---|---|
| Physiological | Secretion of water and electrolytes from pancreas<br>Secretion of water and electrolytes in bile | Gastric emptying<br>Gastric acid secretion<br>Intestinal motility |
| Supraphysiological[a] | Bile flow<br>Lower esophageal sphincter<br>Release of insulin<br>Secretion from Brunner's glands in the duodenum<br>Renal excretion of water and electrolytes<br>Cardiac output<br>Splanchnic blood flow<br>Lipolysis in fat cells | |

[a] Pharmacological doses.

Brunner's glands, inhibition of lower esophageal sphincter tone, and lipolysis. Several studies have suggested a tropic effect of secretin on the pancreas. Pharmacological doses of secretin increase pancreatic weight, protein, DNA content, and thymidine incorporation in animals. These actions have been attributed to a costimulatory effect of secretin and CCK, which is known to have tropic properties. It is not known if theses actions take place in humans.

Secretin has also been found to mediate relaxation of intestinal smooth muscle in rats and to inhibit upper small intestinal motility in humans. Exogenous secretin influences the release of insulin, glucagon, parathyroid hormone, calcitonin, somatostatin, and pancreatic polypeptide (PP). Exogenous nonphysiologic secretin infusion rates can also produce incremental increases in pancreatic enzyme secretion. Most of these actions have been observed either at supraphysiological concentrations or in experimental models.

## VII. CLINICAL APPLICATIONS OF SECRETIN/PATHOLOGICAL CONDITIONS

### A. Pharmacological Uses of Secretin

Secretin has been used clinically for assessment of pancreatic exocrine function, identification of minor papilla during endoscopic retrograde cholangiopancreatography (ERCP), and diagnosis of gastrinoma (Zollinger–Ellison syndrome). Secretin is an adjunct to ultrasound and resonance magnetic imaging in inflammatory conditions of the pancreas, such as chronic pancreatitis. The periductal fibrosis present in the chronically inflamed pancreas limits the dilatation of the main duct after maximal secretin stimulation, which is used to distinguish chronic pancreatitis from the normal pancreas. The sensitivity, specificity, and efficacy of functional secretin studies have been estimated to be 67, 90, and 81%, respectively, as compared to histological evaluation. Pancreatic insufficiency can also be evaluated after administration of secretagogues (i.e., secretin and CCK). Secretin stimulatory effects are also used to collect pancreatic juices during ERCP. Concomitant utilization of secretin and ultrasound has been reported to be useful in selection of patients with pancreas divisum for accessory duct sphincteroplasty. In these patients, secretin is used to localize the site where the main pancreatic duct drains.

The differential effects of secretin on normal and gastrin-producing tumors are use to distinguish between hypergastrinemic conditions and patients with Zollinger–Ellison syndrome (ZES). ZES is characterized by gastrin-producing neuroendocrine tumors that are usually located in the pancreas or proximal duodenum; the tumors release excessive amounts of gastrin into the circulation. Intravenous administration of secretin in these patients produces an exaggerated gastrin response that is useful to identify ZES. Both synthetic porcine secretin and human synthetic secretin have been shown to be adequate testing agents. Additionally, secretin tests have been used to localize gastrinomas within the gut and to ensure a correct extirpation of the tumor during surgery. The two methods have been described for these purposes: selective intra-arterial secretin injection with successive recollection of venous hepatic sampling and intra-operative secretin testing to confirm resection of the tumor. Postoperatively, secretin tests have been used to detect subclinical disease recurrence. However, with the advent of octreotide scanning and intraoperative ultrasound, the secretin-dependent localization techniques are no longer commonly utilized. Secretin has recently elicited considerable attention due to alleged benefits of secretin in treating autism in children. Unfortunately, however, double-blind studies have found no evidence of such effectiveness.

### B. Pathophysiology of Secretin: Conditions of Excess or Deficiency

Plasma secretin concentration levels have been found altered in various clinical conditions. Hypersecretinemia (blood secretin levels above normal) is seen in ZES, in some cases of duodenal ulcers with marked hypersecretion of acid, and in patients with advanced renal failure. One case has been reported in the medical literature of a patient with a pancreatic tumor secreting five different hormones, one of which included secretin; however, no cases of isolated secretin production have been documented. Patients with ZES present abnormally elevated plasma secretin levels, with fasting concentrations above 15 pg/ml, which is rarely seen in healthy persons. In healthy subjects, plasma secretin concentrations are elevated after a prolonged fast or with extenuated physical exercise. In both instances, oral or intravenous administration of glucose reduces secretin blood levels.

Hyposecretinemia, or a blood secretin level below normal, has been found in patients with untreated adult celiac disease or achlorhydia. Secretin concentrations in patients with celiac disease fail to increase after exogenous duodenal acidification or after a mixed meal. In contrast, patients with achlorhydia present reduced secretin levels after a mixed meal, although the response to duodenal acidification remains normal.

## Glossary

**chyme** Semifluid mixture consisting of partly digested food and gastric juices; passes from the stomach into the small intestine.

**exon** Portion of DNA that encodes a section of the mature messenger RNA.

**intron** Portion of DNA that lies between two exons; it is transcribed into RNA, but is not expressed in the final product.

**locus** Position in a chromosome of a particular gene or allele.

**peptide** Compound formed by two or more amino acids, in which a carboxyl group of one is united with the amino group of another.

## See Also the Following Articles

**Cholecystokinin (CCK) • Gastrin • Gastrointestinal Hormone (GI) Regulated Signal Transduction • Gastrointestinal Hormone-Releasing Peptides • Glucagon Gene Expression • Glucagon-like Peptides: GLP-1 and GLP-2 • Peptide YY • Vasoactive Intestinal Peptide (VIP)**

## Further Reading

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# Selective Estrogen Receptor Modulators

See *SERMs*

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# SERMs (Selective Estrogen Receptor Modulators)

DONALD P. MCDONNELL
*Duke University Medical Center*

I. INTRODUCTION
II. THE EMERGENCE OF THE SERM CONCEPT
III. THE MECHANISM OF ACTION OF SERMs

**Although estrogen was initially considered solely a reproductive hormone, extensive clinical findings have indicated that its influence extends to a variety of target tissues not generally considered to be involved in reproduction. Specifically, estrogen has positive actions in the skeleton, the cardiovascular system, and the central nervous system. Interestingly, the mechanism by which this hormone manifests its biological activities in different tissues is dissimilar, enabling the development of selective estrogen receptor modulators (SERMs), compounds whose relative estrogenic/antiestrogenic activities vary between cells.**

## I. INTRODUCTION

Despite the medical benefits afforded by estrogen replacement therapy, the number of women who initiate or remain on therapy for greater than 1 year is relatively small. This is due in part to the fear that estrogens increase the risk of developing breast cancer. Consequently, it was anticipated several years ago that there was an unmet medical need for novel estrogen receptor modulators that would retain the beneficial effects of estrogens in most target organs but which would be inactive in the breast.

The emergence of selective estrogen receptor modulators (SERMs), compounds whose agonist/antagonist activities are manifest in a cell-selective manner, indicates that considerable progress toward this goal has been made. It is likely that a clearer understanding of the mechanisms that determine the pharmacological activities of the currently available SERMs will assist in the discovery and development of additional compounds of this class for use in the treatment of conditions associated with long-term estrogen deprivation.

With few exceptions, the clinical studies supporting nonreproductive actions of estrogens have not been performed in a double-blinded, placebo-controlled manner. Therefore, it has been difficult to establish, in a definitive manner, whether estrogens have clinically important, beneficial activities in postmenopausal women outside of their utility to treat climacteric symptoms. In the absence of these definitive studies, there has been some reluctance on the part of the pharmaceutical industry to invest in the development of improved "estrogens." Some resolution to this issue was provided by the recent publication of the interim results of the National Institutes of Health-sponsored Women's Health Initiative (WHI). This trial was set up to examine the long-term effects of the most popular hormone replacement regimens in the cardiovascular system and in breast although many other secondary outcomes were considered. One arm of this trial, a combination of conjugated equine estrogens and the progestin medroxyprogesterone acetate (HRT), was terminated early because an interim analysis demonstrated a clear increase in the incidence of breast cancer and cardiovascular events. This was a disappointing result considering the general belief, based on multiple retrospective studies, that postmenopausal estrogen supplementation would be beneficial in the cardiovascular system. Although overshadowed by an enormous amount of negative media coverage, this study also proved in a definitive manner that estrogens reduced the incidence of hip fractures by approximately 40% and significantly reduced the incidence of colorectal cancer. However, aside from the established effects of estrogen on the quality of life of menopausal women, the additional positive and negative effects highlighted by the WHI study appear to cancel each other out and do not support long-term use of HRT. It remains to be determined whether estrogen alone (the second ongoing arm of the WHI study) provides an improved risk–benefit profile in women without a uterus.

From the perspective of drug discovery, the results from the WHI are very useful. It demonstrated conclusively that estrogens were beneficial in the skeleton and that they reduced the incidence of colorectal cancer. Furthermore, it made it clear that compounds that could manifest estrogenic activities in a tissue-selective manner would have utility in menopausal medicine. Although far from optimal, the clinical profiles of the first-generation SERMs have suggested that it will be possible to generate compounds that provide only the beneficial effects of estrogens and which significantly improve the health of postmenopausal women. As will be discussed below, the currently available SERMs arose from the accidental discovery that compounds designed as anti-estrogens could exhibit estrogenic activity in some tissues. It is likely that elucidation of the mechanisms of this tissue selectivity will enable the rational development of improved SERMs. Thus, although some consider the findings of the WHI study to signal the end of HRT, others see it as an impetus to develop compounds with improved specificity and share in the expectation that such medicines will have a positive impact on the health of menopausal women.

## II. THE EMERGENCE OF THE SERM CONCEPT

Considering the classical models of estrogen action, it was initially difficult to understand how, beyond taking advantage of fortuitous pharmacokinetic properties, it would be possible to develop therapeutically useful SERMs. However, in the early 1990s a landmark study demonstrated that the "anti-estrogen" tamoxifen could actually function as an estrogen in the lumbar spine. Specifically, this placebo-controlled trial demonstrated that like estrogen, tamoxifen could increase bone mineral density in the lumbar spine of postmenopausal women who were being administered tamoxifen as adjuvant therapy for breast cancer. Indeed, were it not for the fact that this drug also functioned as an estrogen in the uterus and that this activity has been associated with an increased risk of endometrial cancer, it may have been used as a treatment for osteoporosis. Regardless, it was this finding that birthed the field of SERMs and provided the impetus to search for compounds that functioned like tamoxifen in bone but lacked uterotrophic activity. To date, these efforts have led to the development of one compound, raloxifene, which is approved for the treatment and prevention of osteoporosis. Several additional SERMs, with pharmaceutical properties superior to

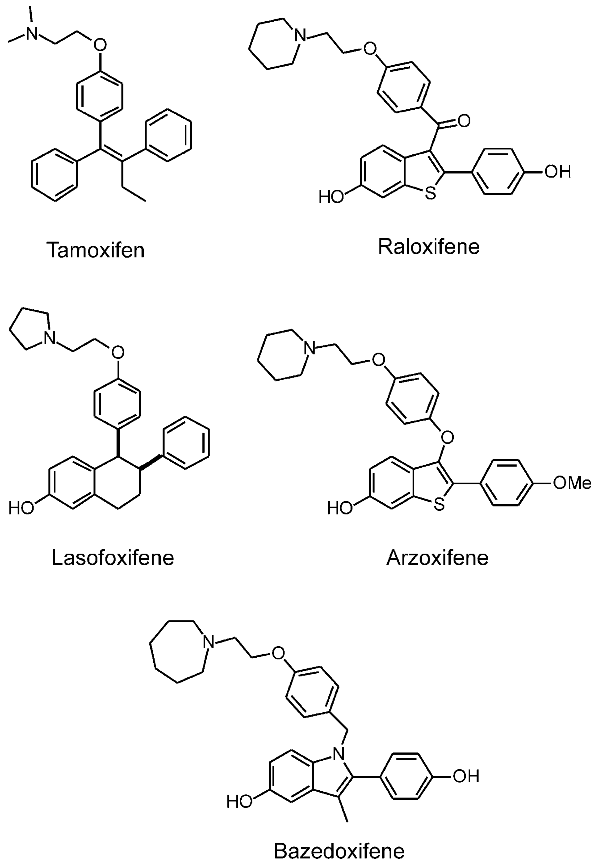

**FIGURE 1** Structures of the most clinically important SERMs.

those of raloxifene, i.e., lasofoxifene, bazedoxifene, and arzoxifene, are in the late stages of clinical development and will likely emerge on the market in the near future (Fig. 1).

## III. THE MECHANISM OF ACTION OF SERMS

The observation that the SERM tamoxifen could manifest estrogenic activities in some cells but oppose estrogen action in others suggested that a reevaluation of the classical models of ER pharmacology was needed. In the established models of estrogen action, the unoccupied nuclear estrogen receptor (ER) resides in the nuclei of target cells in an inactive form. Upon binding to an agonist, such as estradiol, the biochemical properties of the ER are altered in a way that allows the interaction of a receptor dimer with specific DNA sequences [estrogen-response elements (EREs)] within the promoters of responsive genes. The DNA-bound ER can then regulate target gene transcription, either positively or negatively. In this model, agonists function as "switches" that facilitate the conversion of ER from an inactive to an active form, whereas antagonists function by competitively inhibiting the binding of agonists. However, the realization that the relative agonist/antagonist activity of ER ligands can differ between cells and even between different promoters in the same cell suggests that the pharmacology of this class of compounds is more complex than originally anticipated. This complexity has allowed the development of SERMs, compounds whose agonist and antagonist activities are manifest in a cell- and promoter-restricted manner. Although SERMs are a relatively new classification, the observation that the biological character of ER ligands could differ between cells was first observed in the late 1960s when it was demonstrated that tamoxifen, then classified as an ER antagonist, could function as a partial agonist in the reproductive systems of rodents. Since that time, other SERMs that display unique agonist/antagonist profiles have emerged, leading to the realization that different compounds acting through the same receptor can manifest different activities in different cells. The explanation for this cellular discrimination is slowly being unraveled, providing a molecular mechanism to explain the differential activity of SERMs and suggesting ways to develop improved compounds of this class in the future.

A major advance in understanding estrogen action was the discovery and cloning in 1996 of a second estrogen receptor (ER-β) that is genetically distinct from ER-α, which was cloned in 1986. The expression patterns of these receptors and the phenotypic consequences of their disruption differ, indicating that each has a distinct role in ER pharmacology. It has been shown that ER-β can heterodimerize with ER-α and inhibit ER-α action *in vitro*, suggesting that differences in the relative expression levels of the two receptors may dictate cellular sensitivity to estrogens. Although the roles of these two receptors in SERM action remain to be established, raloxifene and tamoxifen have been shown to bind to both receptors. On classical ERE-containing genes, these SERMs function as pure antagonists when acting through ER-β but can function as partial agonists when acting through ER-α. However, it now appears that in certain circumstances tamoxifen and other SERMs, acting through ER-β, may manifest robust agonist activity. For instance, on some promoters ERs do not interact directly with EREs but rather are tethered to promoters through protein–protein interactions with transcription factors prebound to promoters.

When ER-β interacts with promoters in this manner, its transcriptional activity is suppressed by estrogens and activated by SERMs, and the reverse is true for ER-α. These indirect ER–promoter interactions have been demonstrated conclusively only *in vitro*; thus, although these findings are intriguing, the physiological significance of these alternate pathways remains to be established. Regardless, it is clear that the actions of estrogens and SERMs *in vivo* represent their composite activities through these two receptors as homo- or heterodimers.

In the uterus, a tissue that predominantly expresses ER-α, tamoxifen but not raloxifene (a second-generation SERM) manifests agonist activity, indicating that SERM pharmacology cannot be explained solely by differential receptor activation. This finding, which has been confirmed in several *in vitro* systems, suggests that some cells are able to distinguish between ER-α–tamoxifen and ER-α–raloxifene complexes. Using protein crystallography and techniques that evaluated the surface changes of the receptor that occur upon ligand binding, it was demonstrated that the structure of the ER was influenced by the nature of the bound ligand. Cumulatively, these studies indicate that the conformations of the tamoxifen–ER, raloxifene–ER, and estradiol–ER complexes are different from one another and from the unbound receptor. The relevance of receptor conformation to biological activity was strengthened recently by the observation that the structure of ER-α in the presence of the pure antagonist ICI164,384 is distinct from that observed in the presence of agonists or SERMs. Thus, receptor conformation appears to be the primary determinant of the pharmacological activity of SERMs, estrogens, and anti-estrogens.

The full significance of the differential effect of estrogens and SERMs on ER structure was not realized until the discovery of co-activators and co-repressors, proteins that interact with and modulate ER function. Co-activators such as SRC-1 (steroid receptor co-activator-1), GRIP1 (glucocorticoid receptor interacting protein-1), and AIB-1 (amplified in breast cancer-1) (p160 family), preferentially interact with agonist-activated ER and potentiate transcriptional activation by coupling the receptor to the transcription apparatus and by altering the architecture of the target promoter, thus facilitating transcription. Much of what is known about the role of the known co-activators in SERM action has come from studies of tamoxifen. Some studies have demonstrated that overexpression of the p160 co-activators (SRC-1, GRIP-1, and AIB-1) in target cells can convert tamoxifen from an antagonist into an agonist. Additional studies have indicated that tamoxifen-activated ER is capable of interacting, in an ectopic manner, with cofactors with which estradiol–ER would not normally couple. Whether these types of interactions occur when ER is occupied by other SERMs remains to be determined. In contrast to co-activators, co-repressors like nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) interact preferentially with antagonist-activated ER and suppress activation by altering the structure of the responsive promoter in such a way as to reduce its ability to be transcribed. The importance of co-repressors in SERM action was confirmed in studies demonstrating that tamoxifen could function as a full agonist in cells derived from mice bearing a genetic disruption of NCoR. It is not yet clear whether the co-repressors are physiologically important regulators of ER action or whether they are engaged only when the receptor is bound to a synthetic antagonist. To date, over 20 different ER-interacting co-activators and co-repressors have been identified. These have different relative and absolute expression levels among cells and display distinct preferences for different ER–ligand complexes. It appears that differential cofactor recruitment is a key determinant of SERM pharmacology and that progress will be made in the near future in ascribing the different activities of these compounds to specific ER–cofactor interactions.

It is not likely that differential cofactor recruitment alone is the only determinant of the agonist/antagonist activity of SERMs. For example, in cultured cells, ligand binding to ERs can activate extranuclear signaling pathways in a transcription-independent manner. Although the physiological importance of these activities is unclear, they highlight the extreme complexity of ER action and the multiplicity of the systems and signaling pathways that may be modulated by the ERs and their ligands.

To summarize, the complex pharmacological activities of SERMs are the result of at least three different factors (Fig. 2): (1) differential receptor expression; (2) receptor conformation (an activity that influences cofactor recruitment); and (3) differential expression of cofactors. Globally, these activities explain most of the tissue-selective activities of SERMs, although the specific contribution of each activity and the particular components that are important for a given tissue remain to be determined.

Upon binding to an agonist or an antagonist, the ER (α- and/or β-isoforms) undergoes a conformational change that permits its spontaneous dimerization

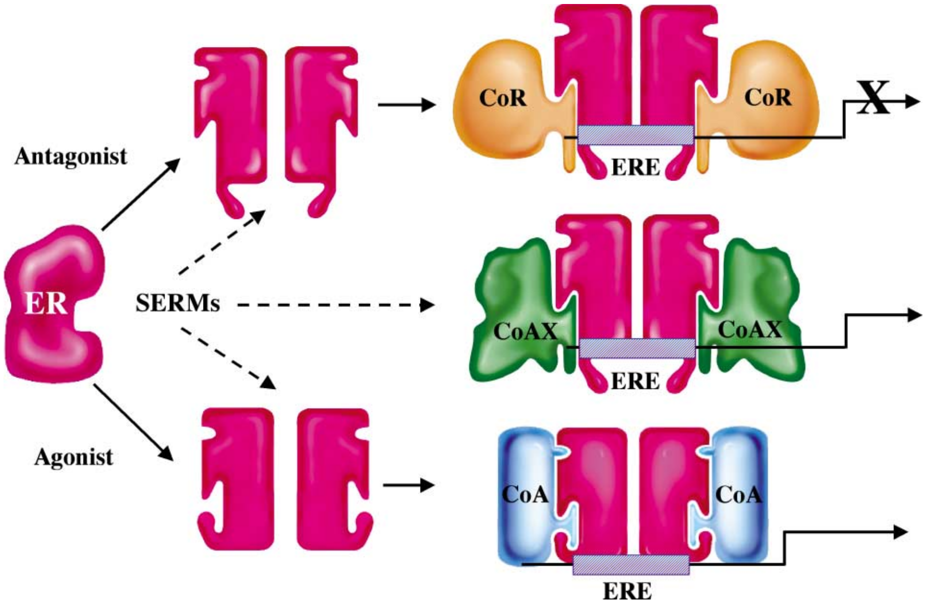

FIGURE 2 An updated model of estrogen receptor action may help to explain the activity of SERMs. CoA, co-activator; CoAX, co-activator X (yet to be identified); CoR, co-repressor.

and facilitates the subsequent interaction of the dimer with specific DNA sequences, EREs, located within the regulatory regions of target genes. It has now been determined that receptors activated with full agonists, such as 17β-estradiol, can couple with any one of a number of transcription co-activators. The resulting biological response will be determined by the cofactors that are available in a given cell and which of these proteins actually dock with the receptor. In the presence of a pure antagonist such as ICI182,780, it has been demonstrated that ER interacts preferentially with a co-repressor protein. Upon binding SERMs, however, the receptor has been shown to adopt overall conformations that make these complexes distinct from agonist- or antagonist-activated receptor. These complexes can interact with either co-activators or co-repressors and are very sensitive to the expression levels of these proteins. When co-repressors dominate, SERMs function as antagonists, whereas when co-activators dominate, partial agonist activity is observed. To complicate things further, it has also been shown that in certain circumstances SERM-activated ERs can interact in an ectopic manner with co-activators with which the ER–estradiol complex does not normally interact. The implication of this model is that the major determinants of SERM activity are receptor shape and the relative and absolute levels of co-activators in target cells.

## Glossary

**co-activators** Proteins that interact with activated transcription factors at target gene promoters and facilitate their contact with the general transcription apparatus in cells. In addition, co-activators nucleate the assembly of a large complex of proteins at target gene promoters that enhance transcriptional activation by enzymatically modifying histones and effecting a local decondensation of chromatin.

**co-repressors** Proteins that interact with inactive transcription factors and help to inhibit the activity of these proteins by nucleating a large complex of proteins, which functions to condense chromatin structure and repress transcription.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Environmental Disruptors of Sex Hormone Action • Estrogen and Progesterone Receptors in Breast Cancer • Estrogen Receptor-α Structure and Function • Estrogen Receptor-β Structure and Function • *In Vitro* Fertilization • Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology**

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# Sex Hormone-Binding Globulin (SHBG)

GEOFFREY L. HAMMOND AND KEVIN N. HOGEVEEN
*University of Western Ontario, Canada*

I. STRUCTURE AND FUNCTION OF PLASMA SEX HORMONE-BINDING GLOBULIN AND TESTICULAR ANDROGEN-BINDING PROTEIN
II. CONTROL OF *SHBG* GENE EXPRESSION
III. SIGNIFICANCE OF ALTERNATIVE *SHBG* TRANSCRIPTS AND *SHBG* VARIANTS
IV. ROLE(S) OF *SHBG* GENE EXPRESSION IN MALE REPRODUCTION

**Sex hormone-binding globulin (SHBG) transports androgens and estrogens in the blood and regulates the access of these sex steroids to their target tissues. Plasma SHBG is produced by the liver and is structurally identical to the androgen-binding protein (ABP) produced in Sertoli cells. A single *SHBG* gene encodes both SHBG and ABP, and its expression in the liver and testis responds differently to developmental, hormonal, and external cues. The *SHBG* gene is expressed in several other tissues, as well as in cancer cell lines that contain alternatively spliced *SHBG* transcripts of unknown function. Genetic variants of human *SHBG* that influence its expression and/or the plasma levels of SHBG have been identified. The biological significance of *SHBG* expression in the testis may vary between species, and this article will review the current understanding of the structure and function of SHBG in the context of its proposed roles in male reproduction.**

## I. STRUCTURE AND FUNCTION OF PLASMA SEX HORMONE-BINDING GLOBULIN AND TESTICULAR ANDROGEN-BINDING PROTEIN

Plasma sex hormone-binding globulin (SHBG) and testicular androgen-binding protein (ABP) are homodimeric glycoproteins, and each monomeric subunit comprises two laminin G-like (LG) domains (Fig. 1). These two laminin G-like domains are also found in several other proteins with diverse and unrelated functions, including protein S, GAS 6 (product of the growth arrest-specific gene 6), and several extracellular matrix-associated proteins (e.g., laminin, merosin, and agrin). The significance of these structural similarities is unknown but several of these proteins are ligands for plasma membrane receptors or interact in some way with other proteins. The amino-terminal LG domain of each subunit contains a steroid-binding site, the dimerization domain, and several metal-binding sites. Contrary to previous assumptions, it is now known that each subunit of the SHBG homodimer contains a functional steroid-binding site that may exist in different states of occupancy by various ligands. The structure and functional properties of the carboxyl-terminal LG domain of SHBG are less well understood but it normally contains two consensus sites for N-linked glycosylation, one of which is conserved phylogenetically and likely provides a specialized function. Although glycosylation is not necessary for steroid binding or dimerization, it may influence the biological half-life of the protein and/or its partitioning

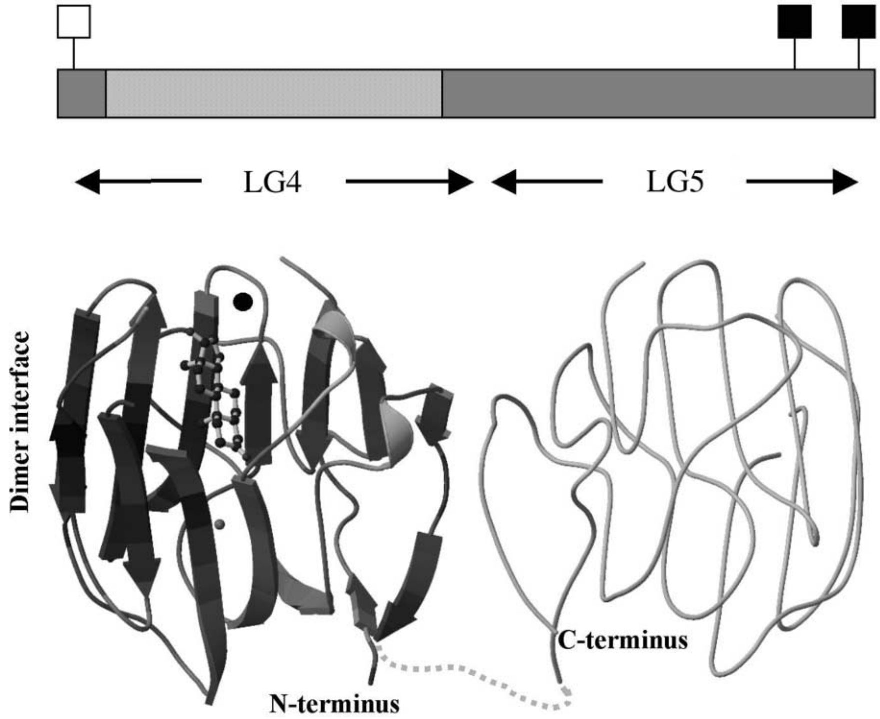

FIGURE 1 Structure of human sex hormone-binding globulin. Linear diagram of the 373 amino acid residues that constitute the mature human SHBG monomer (top) showing the positions of one O-linked (open box) and two N-linked (solid boxes) carbohydrate chains. The internal shaded region corresponds to the minimal sequence required to produce a truncated form of SHBG that contains a fully active steroid-binding site and dimerization domain. Each human SHBG monomer includes two laminin G-like domains (LG4 and LG5). The crystal structure of the amino-terminal LG4 domain (bottom) shows 5α-dihydrotestosterone in the steroid-binding site, the position of the homodimer interface, and a $Ca^{2+}$ ion (small shaded ball). Also shown is a $Zn^{2+}$ ion (solid ball) that occupies a potential zinc-binding site within a region (between residues P130 and P137) of disorder (not visible), which lies over the entrance of the steroid-binding site. Occupancy of this zinc-binding site specifically reduces the binding affinity of estradiol without changing the binding affinity of androgens. The predicted structure and relative position of the carboxy-terminal LG5 domain within the context of the overall SHBG monomer tertiary structure are also shown, with a dashed line corresponding to a short 5-residue sequence of unresolved structure connecting the LG4 and LG5 domains.

within extravascular compartments of sex steroid hormone-sensitive tissues.

In the blood, human SHBG binds biologically active androgens and estrogens with high affinity, and the plasma concentrations of SHBG play a key role in regulating the distribution of these steroids between the protein-bound (primarily SHBG-bound and albumin-bound) and the non-protein-bound or "free" fractions. This finding is considered to be important because only free steroids are generally considered to be available to target tissues. However, there is evidence that SHBG can enter the extravascular compartments of some tissues and may therefore exert a local effect on the access of steroids to their target cells. Human SHBG is also a zinc-binding protein, and occupancy of a zinc-binding site within the amino-terminal LG domain influences its steroid-binding specificity. This may be important in tissues where zinc concentrations are particularly high and where SHBG may accumulate, such as in the male reproductive tract.

Plasma SHBG and ABP interact with cell membranes of steroid-dependent tissues within the male reproductive tract. Although the significance of these interactions is not well understood, they may serve different functions. For instance, after being secreted into the seminiferous tubule fluid of male rats, ABP is actively taken up by epithelial cells within the epididymis. This is believed to facilitate the entry of testosterone into these cells and enhance the activation of androgen-responsive genes encoding proteins involved in sperm development. By contrast, only unliganded SHBG is capable of binding specific "receptor" sites within human prostate plasma

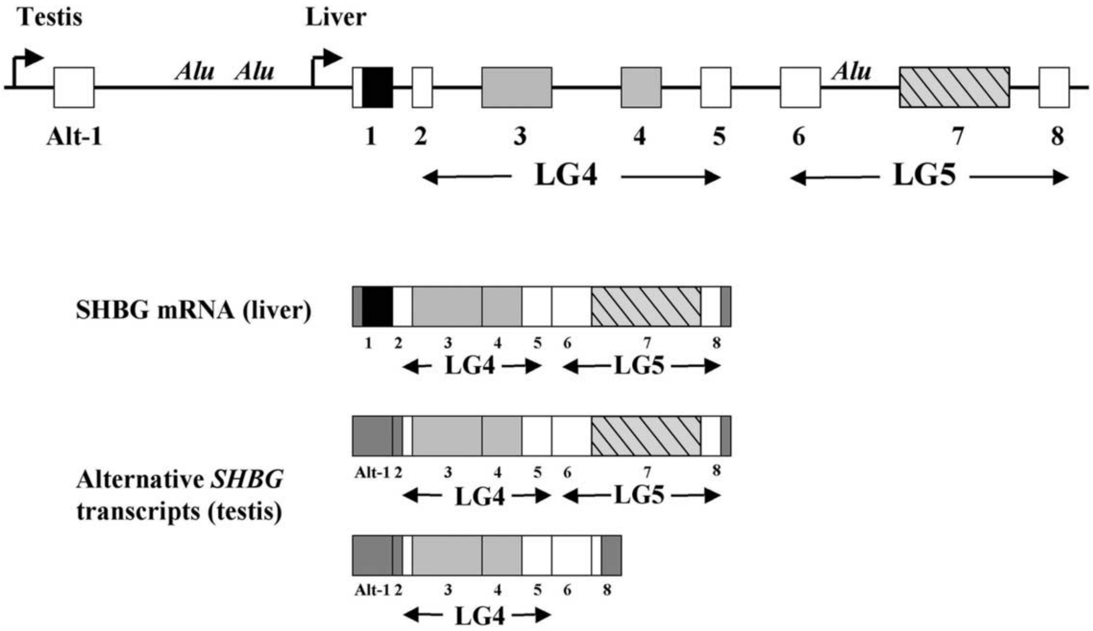

**FIGURE 2** Diagrams of the human sex hormone-binding globulin (*SHBG*) gene and the major human *SHBG* transcripts in the liver and testis. The structure of the human *SHBG* gene (top) shows the positions of repetitive *Alu* sequences and the major transcription start sites (arrows) utilized in the liver and testis. The exons that produce SHBG mRNA in the liver and the alternative *SHBG* transcripts found in the testis are shown below the diagram of the *SHBG* gene structure with respect to sequences that encode the LG4 and LG5 domains of human SHBG. Boxes within the diagrams of these transcripts correspond to exon sequences. Exons (3 and 4) that encode sequences that constitute the steroid-binding site and dimerization domain are shaded in the gene sequence (top) and transcript sequences (bottom). The exon 1 sequence included within the SHBG mRNA found in the liver shows the sequence that encodes the secretion signal polypeptide (black area), which is removed during production of the mature form of plasma SHBG. In the testis, the two major *SHBG* transcripts contain an alternative exon 1 (Alt-1) sequence that replaces the exon 1 sequence present in the SHBG mRNA found in the liver, and they either contain or lack sequences corresponding to exon 7 (diagonally striped rectangles). The dark gray shaded areas within the exon 1 and exon 8 sequences present within the liver SHBG mRNA correspond to 5′ and 3′ noncoding sequences, respectively. Similarly, the dark gray shaded areas within Alt exon 1 and exon 2 sequences within the alternative transcripts found in the testis represent 5′ noncoding regions, whereas the dark gray shaded regions in exon 8 sequences correspond to the 3′ sequences that follow the termination of the major open reading frames.

membranes. In these circumstances, the membrane-bound SHBG can interact with biologically active androgens or estrogen, and if this occurs, SHBG appears to dissociate from the receptor, resulting in an increase in intracellular cyclic AMP levels and the activation of a signal transduction cascade. Although this receptor system has been characterized extensively at the biochemical level, the receptor itself has not been identified and its biological significance remains obscure.

## II. CONTROL OF *SHBG* GENE EXPRESSION

Plasma SHBG and testicular ABP are encoded by eight exons within a single 4 kb gene (*SHBG*), which is located on the short arm (p12–p13) of human chromosome 17 (Fig. 2). The temporal and spatial expression of *SHBG* varies considerably between species, as does its sensitivity to various hormonal stimuli. Little is known about how *SHBG* is regulated during fetal life but it is expressed transiently in the livers of fetal rodents, resulting in fluctuations of plasma SHBG levels during critical periods of sexual development, and this likely occurs in other species including humans. In rats and mice, however, *SHBG* is not expressed in the liver during postnatal life and the very small amount of SHBG in the blood of mature rats most likely originates from gonadal sources. By contrast, *SHBG* expression in the livers of most other mammalian species increases after birth and represents the major source of plasma SHBG throughout life. In humans, changes in plasma SHBG levels occur during puberty, leading to approximately twofold higher levels in women than in men. Plasma SHBG levels in humans are also under complex hormonal and metabolic control. For instance, exogenous estrogens and thyroid hormone markedly increase plasma SHBG levels, whereas anabolic androgens and androgenic progestins reduce plasma SHBG levels. Low plasma SHBG levels are found in obese men and women and this may in some way be related to the effects of insulin on reducing SHBG production by hepatocytes. The latter are of interest because several reports have indicated that low plasma SHBG levels are an early indicator of predisposition to diabetes and associated cardiovascular disease. However, it is not known whether any of these effects are mediated at the level of gene expression or reflect changes in the plasma half-life of SHBG.

The expression of *SHBG* in rat Sertoli cells has been studied extensively and is controlled directly by follicle-stimulating hormone and indirectly by testosterone through intermediary effects involving factors produced by the myoid cells surrounding the seminiferous tubules. The promoter responsible for controlling the production of ABP mRNA in Sertoli cells has been examined but the molecular mechanisms responsible for its regulation remain to be defined.

When the human *SHBG* proximal promoter sequence is compared with the corresponding regions of *SHBG* proximal promoter sequences in several other mammalian species, there is one major difference. The human *SHBG* promoter includes a region that contains two nuclear factor-binding sites that are not present in the promoters of other species. One of these sites binds hepatocyte nuclear factor-4, whereas the other binds the upstream stimulatory factor, and it is possible that this difference contributes to some of the species differences in *SHBG* expression.

## III. SIGNIFICANCE OF ALTERNATIVE *SHBG* TRANSCRIPTS AND *SHBG* VARIANTS

The rat and human *SHBG* genes produce several other transcripts in addition to those encoding plasma SHBG and testicular ABP. These transcripts invariably contain alternative noncoding exon 1 sequences that replace the exon 1 that contains the translation initiation codon for the SHBG and ABP precursor polypeptides. The human alternative *SHBG* transcripts also often lack exon 7 sequences, and repetitive *Alu* sequences are located upstream of both the exon 1 and the exon 7 sequences that are either replaced or removed from these transcripts (Fig. 2). This may be significant because exons within the rat ABP that are differentially utilized are also preceded by repetitive DNA sequence elements. A rat *SHBG* transcript with an alternative exon 1 sequence has been shown to encode an ABP-related product containing a localization signal that directs it to the nucleus. However, the coding sequence for the unique amino-terminal sequence associated with this protein is not conserved in the alternative human *SHBG* transcripts identified to date. It has also been reported that *SHBG* is expressed in a variety of other tissues and cell types including rodent kidney, gut, and brain, the human uterus, placenta, and prostate, and several human cancer cell lines. The identity of the *SHBG* transcripts in most of these tissues and cell lines has not been defined but most of them are alternatively spliced transcripts with alternative exon 1 sequences.

Several human *SHBG* variants have been reported. The most common is a single-nucleotide polymorphism in exon 8, which results in a D327N substitution and introduces an additional N-glycosylation site in the carboxy-terminal laminin G-like domain. Although the steroid-binding affinities of this variant protein are normal, it may have an increased plasma half-life. However, even homozygous carriers of this allele display no obvious phenotype. A $(\mathrm{TAAAA})_n$ polymorphism in the human *SHBG* proximal promoter has been identified. The number of these repeats influences its transcriptional activity but it remains to be determined whether this polymorphism is associated with clinical disorders attributed to low plasma SHBG levels. It is likely that other variations in the coding and regulatory sequences of human *SHBG* exist and may explain the interindividual variations in plasma SHBG levels that may contribute to the etiology of diseases associated with inappropriate exposure to either androgens or estrogens.

## IV. ROLE(S) OF *SHBG* GENE EXPRESSION IN MALE REPRODUCTION

The ABP produced by rat Sertoli cells is secreted into the lumen of the seminiferous tubule and accompanies the developing sperm as they migrate to the *caput* epididymis, where it is internalized by lumenal epithelial cells. Although it has been assumed that this plays an important role in maintaining a highly androgenic environment during sperm maturation, there is no evidence that this role is conserved across species, and it may simply be restricted to mammals that lack SHBG in their blood circulation. Moreover, the mouse testis produces much less ABP than the rat testis, and transgenic mice that overexpress rat ABP in their Sertoli cells tend to become infertile due to meiotic arrest and a marked increase in germ cell apoptosis. The reason for this is not known but there is evidence that immunoreactive ABP produced in Sertoli cells is internalized by germ cells.

Early reports indicated that SHBG isolated from human testis homogenates differed from plasma SHBG in terms of its carbohydrate composition. However, it is virtually impossible to exclude the plasma contamination of SHBG from such extracts and there is evidence that plasma SHBG can readily enter the interstitial compartments of the testis. Thus, this difference in SHBG glycoforms between plasma and testis extracts might simply reflect the sequestration of a particular glycoform of plasma SHBG by the testis. Moreover, although *SHBG* transcripts can be readily detected in the human testis, what proportion of these transcripts encode an SHBG molecule with a leader sequence for secretion is not known. In fact, all of the near-full-length SHBG cDNAs isolated from human testis libraries contain alternative exon 1 sequences, and the testes of mice that express human *SHBG* transgenes contain only these same alternative transcripts. The biological significance of the alternative transcripts is obscure, but their abundance is tightly regulated throughout the spermatogenic cycle. These observations raise obvious questions about whether SHBG and ABP play distinct species-specific roles in controlling sex steroid hormone action in the testis and accessory reproductive tissues, and whether *SHBG* transcripts with alternative exon 1 sequences have some other function that remains to be defined.

In some human reproductive tissue, such as the prostate, there is evidence that plasma SHBG accumulates within extravascular compartments and within the stroma in particular. The mechanism responsible for this is unknown but SHBG in the extravascular compartments of these tissues might play a more direct role in regulating either the entry of active steroids into their target cells or the removal of metabolites. For instance, human SHBG not only binds testosterone with high affinity but also binds its major metabolites and with either higher (5α-dihydrotestosterone) or equal (5α-androstanediols) affinity. Furthermore, the possible interaction between SHBG and specific extracellular components within these locations may represent an additional means of modulating the actions of androgens and/or estrogens in reproductive tissues.

## Glossary

**androgen-binding protein** A protein identical in sequence to the plasma sex hormone-binding globulin that is produced in the Sertoli cells of the testis. In rats, this protein displays a preference for biologically active androgens.

**sex hormone-binding globulin** A homodimeric plasma glycoprotein composed of two 42 kDa polypeptide subunits encoded by the *SHBG* gene. Each subunit contains a high-affinity steroid-binding site, which in humans binds both biologically active androgens and estrogens.

## See Also the Following Articles

**Androgen Effects in Mammals • Androgen Receptor-Related Pathology • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Phytoestrogens • Sex Hormones and the Immune System**

## Further Reading

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TABLE I Effects of Estrogen on the Immune System (Animal Model Data)

**On sites of immune cell development**
- On bone marrow
  - Increased estrogen levels reduce bone marrow mass and increase death of pre-B cells
  - Hypogonadal mice (HPG/Bm–hpg/hpg or ovariectomized) have increased bone marrow cellularity with accumulation of B-cell precursors that reverts with estrogen treatment
- On thymus
  - Increased estrogen leads to thymus atrophy with apoptosis of CD4$^{+}$ CD8$^{+}$ T-cell precursors
  - Prepubertal gonadecomy leads to thymus hypertrophy
- Chronic administration of estrogen leads to development of alternative sites of lymphopoiesis in liver and spleen

**In peripheral blood**
- On B cells
  - Increased percentage of B cells in S and G2/M phases
  - Reduced susceptibility to apoptosis, partly due to increased expression of Bcl/2
  - Increased number and activity of plasma cells in peripheral blood and bone marrow, leading to elevated antibody levels
  - Increased expression of autoantibodies (e.g., anti-dsDNA, cardiolipin phosphatidyl serine)
- On T cells
  - Decreased delayed-type hypersensitivity response in mice
  - Reduced proliferation when stimulated *in vitro* with PHA or Con A
- On the cytokine milieu
  - Hormone-responsive elements identified upstream of IL-6 and IFN-γ-encoding genes
  - Elevated estrogen levels increased frequency of IL-6 and IL-10 and reduced TNF-α-secreting cells in spleen
  - Both increased and reduced IFN-γ and IL-2 reported in different mouse models
  - Reduced IL-1 and TNF-α reported with anti-estrogens
- On innate immunity
  - Increased estrogen levels reduce NK-cell activity
  - Increased estrogen levels reduce phagocytic and cytostatic activity of macrophages
  - Elevated serum estrogens reduce macrophage production of reactive oxygen intermediates and IL-1
  - At low concentrations, estrogen increased, TNF-α release by LPS-stimulated macrophages, whereas the opposite was evident at higher concentrations

high concentrations reduce—mixed lymphocyte reactions. Estrogens inhibit proliferation and facilitate apoptosis (by down-regulating Bcl-2) of CD4$^{+}$ T cells in a dose-dependent manner.

Cytokines have dramatic effects on the regulation of immune responses and the pathogenesis of a variety of diseases. Pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNFα), are produced primarily by phagocytic monocytes in response to an infectious agent. The cytokines secreted by T cells have been categorized as type 1 or Th1 [secreting interferon-γ (IFN-γ)] and type 2 or Th2 (secreting IL-4, IL-5, and IL-10) and promote an adaptive response to the pathogen that is mainly cellular (type 1) or antibody (type 2) mediated. Estrogens favor a type 2 immune response by increasing the secretion of type 2 cytokines and by protecting type 2 CD4$^{+}$ T cells from death by apoptosis. Premenopausal women have larger numbers of cells secreting TNFα and IFN-γ than postmenopausal women and estrogen levels in their sera correlate with the number of cells secreting IL-4 on stimulation with phytohemagglutinin (PHA). In human CD4$^{+}$ Jurkat T-cell lines, estrogens suppress IL-2 secretion and receptor expression. This reduction is associated with decreased nuclear binding of nuclear factor kappa B and activator protein 1.

Estrogens modulate the innate immune response by altering the phagocytic and antigen-presenting properties of macrophages and dendritic cells. At low concentrations, estrogen increases TNFα release by lipopolysaccharide-stimulated macrophages, whereas the opposite is evident at higher concentrations. In addition, estrogens modulate the maturation of CD14$^{+}$ monocytes into dendritic cells by granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4. With regard to natural killer (NK) cells, in mice, treatment with estrogens leads to stimulation of NK-cell activity during the first month, but prolonged exposure reduces NK-cell activity, both *in vivo* and *in vitro*. A similar reduction in NK-cell activity is observed in prostate cancer patients treated with estrogens.

## III. PROLACTIN

A variety of immune cells express receptors for prolactin. Estrogen stimulates pituitary production of prolactin through its inhibition of hypothalamic dopaminergic suppression. In addition, several lines of evidence suggest that lymphohematopoietic cells in the periphery secrete prolactin. Prolactin stimulates both cellular and humoral immunity. On T cells, it promotes type 1 responses by increasing the secretion of IL-2 and IFN-γ and the expression of IL-2 receptors. On B cells, prolactin appears to synergize with estrogen to promote Ig production. Hyperprolactinemia, created by the transplantation of syngeneic pituitary glands, accelerates the mortality from immune complex glomerulonephritis in autoimmune B/W [(New Zealand Black × New

Zealand White)F1] mice, suppresses T-cell proliferation, and increases autoantibody concentrations.

## IV. PROGESTERONE

Studies have shown that high levels of progesterone *in vivo* prolong the survival of allogeneic skin grafts as well as xenogenic tumor cell implants in hamster uteri. Progesterone, in contrast with estrogen or testosterone, inhibits spontaneous or glucocorticoid-induced thymocyte apoptosis *in vivo* and *in vitro*. In addition, treatment of T cells with pharmacological concentrations of progesterone blocks T-cell activation and killing by PHA or concanavalin A (Con A).

The identification of progesterone receptors on B cells, plasma cells, and macrophages suggests a direct effect on lymphocytes. Interestingly, the progesterone receptors are at low to undetectable levels in lymphocytes from nonpregnant women. Lymphocyte expression of progesterone receptors is up-regulated on cell activation either by a mitogen (e.g., PHA) or by allogeneic stimuli (such as the fetus). In addition, progesterone induces the secretion of progesterone-induced blocking factor, which blocks NK-cell activity, augments the secretion of IL-3, IL-4, IL-5, and IL-10, and inhibits the secretion of IL-12 by $CD4^+$ and $CD8^+$ T cells. The resulting shift in the cytokine balance toward type 2 is required for a successful pregnancy (see below).

## V. ANDROGENS

Expression of the androgen receptor has been documented in lymphoid and nonlymphoid cells of thymus and bone marrow, but its expression in mature peripheral lymphocytes remains controversial. This expression pattern suggests that the major impact of androgens must be on the developmental maturation of T and B lymphocytes rather than on the mature effector cells. Like estrogens, androgens induce thymus involution with apoptosis of $CD4^+CD8^+$ (DP) thymocytes that is mediated by increased local TNFα and reduced IL-3. Prepubertal castration of male mice induces hypertrophy of the thymus. The resulting thymus retains its normal architecture but has increased cellularity (mainly due to proliferation of DP immature thymocytes). Administration of androgens blocks the proliferation of immature T cells. Castration of male mice also results in expansion of the pre-B-cell population in the bone marrow that may be reversed by testosterone or dehydrotestosterone treatment. Despite the apparent lack of androgen receptor in peripheral mature immune cells, a 40% increase in spleen weight is observed in castrated male mice.

*In vitro*, androgens enhance the activity of $CD8^+$ T cells as demonstrated by the reduced proliferation of spleen cells in response to PHA. In addition, higher levels of IFN-γ and IL-2 and lower levels of IL-4 and IL-10 are secreted by phytohemagglutinin-stimulated lymphocyte culture supernatants of men compared with women. In mice, treatment *in vivo* or direct exposure of lymphocytes to androgens leads to increased production of IL-2. Androgens also reduce at high doses (or increase at low doses) TNFα secretion and nitric oxide synthesis by macrophages. On B cells, androgens reduce Ig secretion and proliferative responses to pokeweed mitogen. This effect is partially dependent on IL-10 secretion but independent of the hormonal status of the cell donor (male or female; luteal or follicular phase of menstrual cycle; postmenopausal).

In contrast to estrogens, androgens have favorable effects on the course of several autoimmune diseases. This has been shown in animal models of experimental autoimmune encephalitis, adjuvant arthritis, and systemic lupus erythematosus (SLE). The protective effects are mediated by the shift toward a type 1 immune response, the reduced B-cell activity with lower Ig secretion, and the reduced secretion of pro-inflammatory cytokines by macrophages. These encouraging results have led to several ongoing clinical trials in patients with multiple sclerosis and SLE.

## VI. PREGNANCY AND THE IMMUNE SYSTEM

A special event in hormonal status, pregnancy, is known to down-regulate cell-mediated immune responses, especially of the Th1 type, to safeguard the fetus. The cytokine milieu is important during implantation and contributes to the maintenance of the feto-placental unit. Low doses of IL-4, IL-5, GM-CSF, IL-3, or anti-TNFα improve implantation and reduce resorption rates whereas administration of TNFα, IFN-γ, or IL-2 causes abortion. In addition, during pregnancy there is an increase in γ/δ T cells and reduced NK-cell activity. These changes in the immune milieu are partly mediated by sex hormones. As a result, the pregnant woman has reduced DTH and allograft rejection, increased susceptibility to intracellular infections, and higher immunoglobulin levels than nonpregnant women.

## VII. SEX HORMONES AND AUTOIMMUNE DISEASE

Autoimmune diseases are disorders in which the immune system attacks self in an uncontrolled manner. The phenomenon of gender bias in the susceptibility to autoimmune diseases has been recognized for many years. The data on the incidence of human autoimmune disease in adulthood show that females account for 65–75% of rheumatoid arthritis, Addison's disease, and myastenia gravis patients; for 85% of Hashimoto thyroiditis and Grave's disease patients; and for >90% of SLE patients. Changes in sex hormone levels such as during puberty, pregnancy, and menopause impact on the course of these diseases. Although sex hormones alone do not cause autoimmune disease, abnormal hormone levels or an altered response to these sex hormones may provide fertile ground for other factors (genetic, infectious) to trigger disease. Abundant clinical evidence connects estrogen to the pathogenesis of SLE; SLE patients, in general, have increased levels of estrogen or active estrogen metabolites and reduced levels of androgens in sera. The majority of the symptomatic episodes in women with regular menstrual cycles occur during the luteal phase of the menstrual cycle. Flares commonly take place during or immediately after pregnancy. In postmenopausal women, the impact of hormonal replacement on the incidence and course of SLE remains controversial and has led to a large ongoing multicenter trial. Additional evidence for the pathogenic role of steroid hormones on SLE includes a report describing three cases of previously healthy women who developed SLE after repeated cycles of ovulation induction. Moreover, repeated cycles of ovulation induction therapy in SLE patients have resulted in severe (even fatal) flares in SLE patients with anti-cardiolipin antibodies. It should be noted, however, that SLE is a multifactorial disease and hundreds of women undergo repeated cycles of ovulation induction every year without developing autoimmune diseases.

Several lines of investigation suggest a pathogenic role for the reduced levels of dehydroepiandrosterone sulfate (DHEAS) in female SLE patients, including an association with reduced levels of IL-2 and IFN-$\gamma$ that are characteristic of SLE. Recent clinical trials showed that supplementation with exogenous DHEAS resulted in a modest clinical improvement in patients with moderate disease. The proposed mechanistic basis for their therapeutic success is the modulation of T-cell activity and cytokine secretion *in vivo*.

## Glossary

**adaptive immunity** Branch of the immune system that mediates antigen-specific immune responses and immunologic memory. It consists of two limbs, cellular immunity and humoral immunity, which are mediated respectively by T and B cells expressing rearranged clonotypic antigen receptors capable of specifically recognizing the diverse antigens of the myriad infectious agents in the environment.

**cytokines** Soluble proteins that are involved in the regulation of the growth and activation of immune cells and mediate normal and pathologic inflammatory and immune responses.

**innate immunity** Branch of the immune system that is activated by conserved pathogen-associated molecular patterns, mediates immediate pathogen elimination, and presents the pathogen's antigens to the adaptive immune system. Its effector cells include natural killer cells, monocytes/macrophages, dendritic cells, neutrophils, basophils, eosinophils, tissue mast cells, and epithelial cells.

## See Also the Following Articles

**Androgens: Pharmacological Use and Abuse • Corticotropin-Releasing Hormone, Stress, and the Immune System • Glucocorticoids and Autoimmune Diseases • Placental Immunology • Progesterone Action in the Female Reproductive Tract • Prolactin (PRL) • Sex Hormone-Binding Globulin (SHBG)**

## Further Reading

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**Subsequent sexual development of the genitalia and the brain is dependent on the influence of gonadal hormones.**

## I. INTRODUCTION

Males and females have evolved to have distinct reproductive potentials marked by profound gender differences in the control of gonadal function and behavior by the central nervous system. Females exhibit cyclic ovarian cycles characterized by fluctuating levels of gonadotropins and gonadal steroids that lead to periodic ovulation. In many species, the expression of female reproductive behaviors is synchronized to occur at or around the time of ovulation by the sequential secretion of high levels of estradiol followed by progesterone. Males, on the other hand, exhibit tonic steady-state levels of gonadotropins and gonadal steroids that maintain an uninterrupted production of sperm and an unconstrained potential for fertilization.

## II. INFLUENCE OF TESTOSTERONE ON THE DEVELOPING BRAIN: GONADOTROPIN SECRETION

Specific regions of the brain play important roles in controlling reproductive behavior, gonadal function, and ovulation. The concept of sexual differentiation of the brain began with studies of reproductive functions. Early studies on rats by Carroll Pfeiffer showed that testicular implants into neonatal female rats permanently blocked ovulation. Pfeiffer concluded, incorrectly, that anterior pituitary function was altered by exposure to testicular secretions. It was later shown through transplantation experiments that the pituitary from a male rat could support ovulation and that the sex difference resides in the brain, not in the pituitary. Subsequent studies demonstrated unequivocally that the ability of rats to ovulate is related to the absence of testes in neonatal life. Males castrated within the first few days of life are able to sustain estrogen-stimulated gonadotropin secretion necessary for cyclic gonadotropin secretion and ovulation. Females exposed to exogenous testosterone within a week of birth become permanently anovulatory. These studies in rats demonstrate that the secretion of gonadotropins is regulated by both a tonic and a cyclic neural feedback system. Both genetic males and neonatally androgen-exposed (androgenized) females lack the neural mechanisms that control the ovulatory discharge or surge of gonadotropin. In contrast, these neural mechanisms are functional in genetic females and in neonatally castrated males.

The surge mechanism for the control of gonadotropin is not sexually differentiated in nonhuman primates, as it is in rodents. Castrated male monkeys secrete surge levels of luteinizing hormone in response to an estrogen challenge, and ovulation can be sustained when ovarian implants are placed subcutaneously into castrated male monkeys.

## III. INFLUENCE OF TESTOSTERONE ON THE DEVELOPING BRAIN: SEXUAL BEHAVIOR

Gonadal hormone exposure during development is also responsible for dimorphisms in neural mechanisms that regulate sexual behavior. Charles Phoenix and colleagues demonstrated that the exposure of fetal female guinea pigs to testosterone permanently suppressed the expression of female sexual receptivity and facilitated malelike mounting behavior in adulthood. Similar studies performed on rats demonstrate that exposing neonatal females to testosterone will suppress feminine sexual behavior and enhance masculine sexual behavior. Conversely, castration of male rats soon after birth permanently suppresses masculine sexual behavior and enhances feminine sexual behavior. Exogenous testosterone treatment during early pregnancy masculinizes the genitalia and behaviors of female rhesus monkeys in infancy and adulthood. These studies suggest that gonadal steroids influence the differentiation of the brain in nonhuman primates as well as in rodents.

Phoenix referred to the permanent developmental effects of gonadal steroids as organizational effects to distinguish them from the activational effects that steroids exert to influence behavior temporarily during adulthood. At the time that this terminology was proposed, there was no evidence for hormone-dependent structural or chemical sex differences in the central nervous system. However, scientists have now identified many hormone-dependent sex differences in the anatomy and chemistry of the brain, some of which may underlie the differences in neuroendocrine function and sexual behavior that have been described. Any sex difference in brain structure or function established during sexual differentiation should remain after gonadectomy in adulthood or after adults of either sex are exposed to equivalent hormone doses. On the other hand, sex differences that are the result of activational steroid effects should be eliminated after gonadectomy or in the presence of similar hormone environments.

## IV. CRITICAL PERIODS

Steroid hormones have an organizational effect only when present during a sensitive developmental period, commonly referred to as the critical period for sexual differentiation. The critical period is an empirical concept that differs depending on the species and the various brain functions being considered. For rats, which are born in an immature state, androgens given just after birth can affect adult sexual behavior. Other species, such as guinea pigs and nonhuman primates, must be exposed to androgens prenatally for adult behaviors to be modified.

These landmark studies established the thesis that the sexual phenotype of the brain is caused by the differentiating actions of testosterone during early development. In this way, sexual differentiation of the brain is comparable to that of other components of the reproductive system. Moreover, like the reproductive system, the female-typical brain appears to develop in the relative absence of hormonal exposure. It is increasingly apparent that a diverse set of brain functions is sexually differentiated. Some, such as regulation of gonadotropin and prolactin secretion, sexual behavior, maternal behavior, and aggression, are closely related to reproductive function. Others, such as taste preference, play behavior, and learning behavior, are not directly related to reproduction but are influenced by early hormone exposure.

## V. ROLE OF ESTROGENIC METABOLITES OF TESTOSTERONE

When newborn female rats are treated with estradiol they develop a pattern of anovulatory sterility in adulthood that is very similar to that observed with perinatal testosterone exposure. In fact, the neonatal brain is much more sensitive to estrogen than to androgen and the effects of neonatal androgen exposure can be blocked by antiestrogens. Although these results were at first puzzling, it is now known that the developing rat brain has the capacity to convert circulating androgen to estrogen because of the presence of cytochrome P450 aromatase within neurons. This evidence led to the formulation of the aromatization hypothesis, which suggests that circulating androgens are converted into estrogens by aromatase in the brain, and that these estrogens are responsible for masculinizing the developing nervous system. If estrogen is the masculinizing hormone, then what protects the fetal brain from maternal estrogens? In rats, a protein found in the blood, called alphafetoprotein, binds estrogen but not testosterone in the first few weeks after birth. Alphafetoprotein traps circulating estrogen and thus protects the female brain from steroid exposure. In males, testicular testosterone is not bound by alphafetoprotein. Testosterone readily enters the neonatal brain of the male and is converted to estradiol to exert masculinizing effects.

There is little evidence that aromatized metabolites of testosterone play a role in sexual differentiation in other animal models, including guinea pigs and nonhuman primates. In these species, testosterone or its major androgenic metabolite, dihydrotestosterone, masculinizes the central nervous system and behavior. Nonetheless, high levels of aromatase are present in developing brains of every species so far studied, suggesting that locally produced estrogen may be needed for neural growth. Alphafetoprotein does not protect the fetus from maternal steroids in nonrodent species; instead, the placenta is thought to be important as a barrier that regulates the exposure of the fetus to maternal as well as exogenous steroids.

## VI. SEXUAL DIMORPHISMS IN THE MAMMALIAN BRAIN

Sex differences in brain function are thought to derive, in part, from structural or morphological differences in the central nervous system. Sex differences in neural morphology are referred to as sexual dimorphisms. Over the past 30 years, sexual dimorphisms in the nervous system have been described at virtually every anatomical level—molecular, ultrastructural, cellular, and neural. Moreover, structural and cellular differences leading to sex differences in neural function and behavior appear to be distributed throughout the nervous system rather than concentrated in a single structure or circuit.

The first demonstration of a sexual dimorphism in the brain was the discovery by Raisman and Field that the number of synapses in the preoptic area was greater in male rats than in females. More apparent sexual dimorphisms in the number, size, and shape of neurons were soon described in the song control circuits of zebra finches. The song control nuclei are five to six times larger in males than in females. Moreover, it is believed that early exposure to androgen or estrogen organizes the larger masculine song system.

In rats, there is an obvious sex difference in a group of neurons called the sexually dimorphic nucleus of the preoptic area (SDN-POA). Roger Gorski was first to note that the volume of the

SDN-POA is about five times larger in the male than in the female. Most of this difference arises because there are greater numbers of neurons in the male nucleus. Like the song control nuclei in zebra finches, the volume of the SDN-POA is controlled by perinatal exposure to gonadal steroids but is not affected by circulating hormones in adulthood. Males castrated at birth have much smaller SDN-POAs in adulthood, whereas females treated with testosterone perinatally have larger malelike SDN-POAs as adults. Thus, perinatal exposure to androgens permanently alters the structure of the SDN-POA. Moreover, development of the nucleus conforms to the aromatase hypothesis because it has been demonstrated that conversion of testosterone to estradiol in the brain is required to masculinize the SDN-POA. Further support for this hypothesis was provided when it was shown that the SDN-POA in androgen-insensitive rats is masculinized. These rats have nonfunctional androgen receptors and thus masculinization cannot be mediated through this pathway. However, androgen-insensitive neonatal rats have normal levels of aromatase and estrogen receptor function, suggesting that the aromatization pathway mediates sexual differentiation of the SDN-POA.

Perinatal steroid hormones seem to act, in part, by preventing apoptotic cell death in the SDN-POA. Studies have shown that the incidence of apoptosis in SDN-POA between postnatal day 7 (P7) and P10 is higher in female rats than in male rats. Administration of testosterone or estradiol reduces the incidence of apoptosis in the SDN-POA of female rats or neonatally castrated male rats. The exact function of the SDN-POA is not yet completely understood. An indication of its function is the positive correlation between volume of the SDN-POA of rats and quantitative measures of adult male copulatory behavior. However, lesions of SDN-POA in rats cause only a transitory decline in male copulatory behavior. The function of the SDN-POA is much better understood in gerbils, which have a similar sexually dimorphic area (SDA) within the preoptic hypothalamus. Bilateral lesions of this region eliminate mating behavior in male gerbils.

Sexual dimorphisms have been identified in homologous medial preoptic nuclei of many species, including rats, gerbils, mountain voles, guinea pigs, ferrets, quail, macaques, and humans (see Section VII). In each case, males exhibit a larger volume and cell density than do females. The presence of sexually dimorphic nuclei within the medial preoptic area of so many species supports the idea that these structures are evolutionarily homologous. Other sexual dimorphisms are evident in several regions of the rat nervous system that are associated with sexual behavior and gonadotropin secretion. Most, but not all, of these nuclei are larger in males than in females. In particular, a cell group in the preoptic area of the hypothalamus known as the anteroventral periventricular preoptic nucleus (AVPv) is larger and has more cells in females than in males. The AVPv has extensive projections throughout the hypothalamus and is thought mediate the feedback of ovarian hormones to control the release of gonadotropin-releasing hormone by the hypothalamus. The development of sex differences in AVPv appears to be due to the organizational effects of perinatal hormone exposure. The nuclear size differences can be reversed by castration of newborn males or administration of testosterone or its aromatized metabolites to females within the first week of life. In contrast to SDN-POA, testosterone and estradiol increase cell death in AVPv during perinatal development, accounting for the smaller nuclear size in males.

Another well-studied model of sexual differentiation is found in the spinal cord. A small cluster of motoneurons in the lower lumbar spinal cord form the spinal nucleus of the bulbocavernosus (SNB). These motoneurons innervate the striated bulbocavernosus muscle at the base of the penis and play a role in erection and ejaculation. This nucleus and its target musculature are absent in the adult female. The musculature and SNB are present in both sexes at birth. The sexual dimorphism arises as the result of perinatal steroid hormone exposure. The results of several studies show that exposure to testosterone or the nonaromatizable androgen dihydrotestosterone maintains the bulbocavernosus musculature. Moreover, estrogen is not effective, so aromatization seems to be unimportant for the masculine development of this system. In the absence of androgen exposure, the muscles and the motoneurons in females begin to die. Androgens, either endogenous or exogenously administered, enhance muscle differentiation, which in turn appears to promote the survival of SNB motoneurons. No one knows yet what neurotropic substance the bulbocavernosus muscles provide to keep the SNB neurons alive.

Aside from the critical role of steroid hormones and programmed cell death, other factors may contribute to sexual differentiation of the nervous system. The behavior of rat dams with their male pups constitutes an example of how experience might interact with steroids to alter neural systems and behavior. During the postnatal period, the dam grooms the anogenital region of her pups to stimulate urination and defecation. Male pups are groomed more often than female pups. Injecting females with testosterone attracts more attention from the dam, suggesting that the dam may detect androgenic metabolites in the pup's urine. This maternal attention contributes directly to the expression of normal male sexual behavior and the size of the SNB in adult males.

In summary, steroid hormones play a pivotal role in the sexual differentiation of the nervous system. In most models that have been studied, one can manipulate the sexual dimorphism in the nervous system by manipulating steroid hormones. Steroids, powerful regulators of gene expression and cellular function, act during early life to control cell survival, synapse formation, cell migration, and cell differentiation within the nervous system. Most likely steroid hormones act together with neurotransmitters and growth factors to sculpt neural development. In addition, social and environmental factors have been shown to play a role in shaping sex differences in the nervous system. Collectively these mechanisms act together to determine the final sexual phenotype of the brain. Recent evidence suggests that steroids also produce temporary structural changes in the brain of the adult, highlighting the notion that some neural sexual dimorphisms require steroid hormone both during development and in adulthood to display sex differences fully in neuroendocrine function and behavior.

## VII. SEXUAL DIFFERENTIATION OF THE HUMAN BRAIN

Male and female humans, like other animals, are exposed to different hormonal environments during early development. Males have elevated levels of testosterone toward the end of the first trimester and into the first few weeks of the second trimester and again during the first 6 months after birth. Females probably produce estrogens prenatally, although it is not clear that this contributes to development. The question of whether these hormonal differences influence sexual differentiation of the human brain and behavior cannot yet be answered unequivocally. This results, in part, from obvious ethical restrictions on hormonal manipulations in human fetuses. In addition, human males and females experience a very different process of socialization that undoubtedly contributes to behavioral sex differences. Consequently, it is difficult to determine the relative contribution that hormones and social environment make to psychosexual development in humans.

Sex differences have been reported in a variety of human behaviors. Males are typically more aggressive, exhibit more rough-and-tumble play behavior, and perform better on visuospatial tasks. Females typically excel at verbal skills, especially verbal fluency and perceptual speed. Although these differences are statistically significant, they are based on a large number of subjects and there is usually more variation within each sex than between sexes for most human behaviors. The largest behavioral sex differences are seen in childhood play, sexual orientation, and gender identity.

A number of structural sexual dimorphisms in the human brain have been described, but many of the reports are controversial and their functional significance is less well established than in animal models. The sexually dimorphic nucleus identified in the preoptic area of humans was named the SDN-POA because it was thought to be the analogue of the SDN-POA found in rats and is larger in males than in females. Two subsequent studies have failed to replicate this finding, although both reported a sex difference in a separate subregion of the preoptic area, i.e., the anterior hypothalamus, known as the third interstitial nucleus of the anterior hypothalamus (INAH-3). The INAH-3 is larger in males than in females. Although controversial, the size of INAH-3 has also been related to sexual orientation, with homosexual and bisexual men having smaller (i.e., female-typical) INAH-3s than presumed heterosexual men. Other hypothalamic regions reported to show sex differences in humans include the suprachiasmatic nucleus and the bed nucleus of the stria terminalis. Midline brain structures showing sex differences include portions of the corpus collosum, anterior commissure, and massa intermedia. The anterior commissure has been related to sexual orientation and the corpus collosum has been related to language lateralization.

An informative approach for asking whether fetal steroids affect neural development in humans is to study individuals with clinical conditions that disturb the normal relationship between genetic sex and hormonal sex. It must be remembered, however, that these studies are not true experiments, and data derived from them are not conclusive on their own. Nonetheless, one interesting set of patients is females that are exposed to excessive androgens *in utero*, typically due to congenital adrenal hyperplasia (CAH). Individuals with CAH lack synthetic enzymes needed to produce corticosteroids and as a consequence large amounts of precursor androgens are produced prenatally. The external genitalia may be mildly or extensively virilized. CAH girls behave more like boys, showing a pattern of behavior consisting of rough active outdoor play and interest in toys generally preferred by boys, with less interest in feminine clothing and doll play. In adulthood, CAH women are usually attracted to men, but are more likely to be homosexual or bisexual than are non-CAH women. Although some researchers attribute the behavioral masculinization in CAH girls to influences of androgens on brain development, others have noted that many other influences exist in this condition, such as psychological and social issues associated with chronic illness, ambiguous genitalia, and reduced fertility.

A clinical condition that has provided information on the behavioral consequences of androgen deficiency is androgen insensitivity. Genetic males with androgen insensitivity produce androgens but exhibit a defect in the androgen receptor that makes the receptor incapable of responding. Completely androgen-insensitive individuals look like normal females at birth, are raised as females, and develop feminine secondary sexual characteristics at puberty; however, they fail to menstruate because they lack female internal reproductive organs. These individuals act like normal females, displaying feminine spatial learning behavior and verbal behavior, and are sexually attracted to men. The fact that androgen-insensitive humans exhibit unambiguous feminine behaviors and gender identity argues that, unlike the case of androgen-insensitive rats, aromatized metabolites of androgens cannot be playing a major role for masculinizing the human brain. In agreement, it has been reported that males having congenital estrogen deficiency due to mutation of the aromatase gene assume a heterosexual sexual orientation and male sexual identity. There is evidence that *in utero* exposure of females to the synthetic estrogen diethylstilbestrol (DES) can masculinize the development of language lateralization, but this effect is small. Taken as a whole, these observations suggest that estrogen does not have the critical effect on nervous system sexual differentiation described in some rodent models.

## VIII. SUMMARY

Gonadal steroids have profound effects on the sexual development of the brain in many nonhuman species. Testosterone secretion by fetal testes in males during the critical period is both necessary and sufficient to masculinize and defeminize gonadotropin feedback and the expression of adult sexual behaviors. In some rodent species, the intracellular aromatization of testosterone to estradiol is critical for these events to occur. In contrast, females develop in the absence of high androgen exposure. Developmental exposure to androgens has a permanent influence over the cell morphology and circuitry of the brain, i.e., the size of specific regions, the patterns of synaptic connections, the distribution of various neurotransmitters, and the expression of steroid receptors and signaling molecules. However, in some cases the functional significance of these sexual dimorphisms remains to be established. The cellular and molecular processes by which androgens and estrogens act to organize sex specific brain functions are not completely understood, but have been shown to include the regulation of cell migration, neuronal growth and axonal branching, and programmed cell death. Steroid hormones have also been shown to interact with social and environmental factors ultimately to determine the final sexual phenotype of the brain. Because of the dramatic effects of perinatal androgens in lower species, it has been generally assumed that similar processes occur in humans. Indeed, a number of behaviors and morphological brain features are sexually dimorphic in humans. Studies of humans with clinical syndromes in which fetuses are exposed to too much or too little androgen provide some support for these suppositions. However, because social environment plays such a crucial role in human psychosocial development, the question of whether and to what degree hormones influence the sexual differentiation of the human brain and behavior cannot yet be answered.

## Glossary

**activational effect** Postpubertal process by which sex steroids exert an effect that transiently activates preexisting hormone-sensitive neural circuits.

**aromatase** Cytochrome P450 enzyme that converts androgens to estrogens; an important signaling pathway by which testosterone affects neural development and function.

**critical period** Time during development when sex steroids exert their organizational effects.

**organizational effect** Early developmental process by which sex steroids exert an effect that causes permanent, hard-wired differences in the structure and function of the central nervous system.

**sexual dimorphism** Sex difference in brain structure or function.

**sexual differentiation** Developmental process by which the two sexes become different.

## See Also the Following Articles

**Androgen Effects in Mammals • Aromatase and Estrogen Insufficiency • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Environmental Disruptors of Sex Hormone Action • Estrogen in the Male: Nature, Sources and Biological Effects • Oxytocin • Sexual Differentiation, Molecular and Hormone Dependent Events in • Spermatogenesis, Hormonal Control of • Testis Descent, Hormonal Control of**

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# Sgk Protein (Serum- and Glucocorticoid-Inducible Protein Kinase)

Gary L. Firestone
*University of California, Berkeley*

I. INTRODUCTION
II. Sgk PROTEIN STRUCTURE–FUNCTION
III. CELLULAR AND PHYSIOLOGICAL FUNCTIONS OF STIMULUS-INDUCED Sgk
IV. REGULATION OF Sgk EXPRESSION DURING DEVELOPMENT AND IN ADULT TISSUE
V. STIMULUS REGULATION OF Sgk PROMOTER ACTIVITY AND GENE TRANSCRIPTION
VI. REGULATION OF Sgk PHOSPHORYLATION AND ENZYMATIC ACTIVITY THROUGH THE GROWTH FACTOR-ACTIVATED PI3-KINASE PATHWAY
VII. Sgk SUBSTRATE SPECIFICITY AND TARGET PROTEINS
VIII. HORMONE AND STIMULUS-DEPENDENT CONTROL OF Sgk SUBCELLULAR COMPARTMENTALIZATION
IX. POTENTIAL CONNECTIONS OF Sgk WITH HUMAN DISORDERS

**Serum- and glucocorticoid-inducible protein kinaseSerum- and glucocorticoid-inducible protein kinase (Sgk) is a unique point of crosstalk that is targeted by diverse hormone signaling cascades. Cell surface receptor, nuclear receptor, and cellular stress signal transduction pathways converge on Sgk to alter cellular function, control cell proliferation, activate osmoregulatory pathways, and/or determine whether a cell survives or undergoes apoptosis. An important biological feature of Sgk is that this protein kinase is regulated at three distinct levels of cellular control. Unlike most other protein kinases, the transcription, enzymatic activity, and subcellular localization of Sgk can be acutely controlled in a stimulus-dependent manner. Sgk mediates its downstream effects through phosphorylation of specific proteins and by its specific binding to certain target proteins, although only a limited number of Sgk substrates and nonsubstrate target proteins have been identified.**

TABLE 1 Genes Involved in Sexual Determination

| Gene | Gene localization | Protein | Protein function | Affected pathway/metabolite synthesized |
|---|---|---|---|---|
| *WT1* | 11p13 | WT1 | Transcription factor | Development of bipotential gonad |
| *FTZ1-F1* | 9q33 | Steroidogenic factor-1 | Receptor | Gonadal development; steroidogenesis |
| *DAX-1* | Xp21 | DAX-1 | Transcription factor | Adrenal and gonadal development |
| *SRY* | Yp11.3 | TDF | Transcription factor | Testicular development |
| *SOX-9* | 17q24 | SOX-9 | Transcription factor | Testicular and cartilage development |
| *DMRT1* and *DMRT2* | 9p24 | DMRT1 and DMRT2 | Unknown | Testicular development |
| *Wnt-4* | 1p31 | Wnt-4 | Growth factor | Ovarian development |
| *AMH* | — | Amh | Peptide hormone | Regression of Müllerian ducts |
| *AMH II R* | 12 | AMH receptor type 2 | Receptor | Regression of Müllerian ducts |
| *LHR* | 2p21 | LH receptor | Receptor | Induction of testosterone synthesis |
| *StAR* | 8p11 | Steroidogenic acute regulatory protein | Transporter protein | Regulator of rapid steroid synthesis |
| *CYP11A* | 15q23–24 | P450 side chain cleavage | Enzyme; gonads and adrenals | Pregnenolone |
| *CYP17* | 10q24 | P450c17 | Enzyme; gonads and adrenals | 17α-OH pregnenolone, 17α-OH progesterone, dehydroepiandrosterone, androstenedione |
| *HSD3B2* | 1p11 | 3β-Hydroxysteroid dehydrogenase type 2 | Enzyme; gonads and adrenals | Progesterone, 17α-OH, progesterone, androstenedione |
| *CYP21B* | 6p21 | 21-Hydroxylase | Enzyme; adrenals | 11-Deoxycortisol, deoxycorticosterone |
| *HSD17B3* | 9p22 | 17β-Hydroxysteroid dehydrogenase type 3 | Enzyme; testis | Testosterone |
| *SRD5A2* | 2p23 | 5α-Reductase type 2 | Enzyme; androgen target tissues | Dihydrotestosterone |
| *CYP19* | 15q21 | Aromatase | Enzyme | Estradiol formation |
| *AR* | Xq12 | Androgen receptor | Receptor | Androgen action |

Another gene involved in the development of the bipotential gonad and the kidneys is the recently cloned *LIM1* gene. Homozygous deletions in this gene in mice lead to developmental failure of both gonads and kidneys. To date, no human mutations in this gene have been described, although a phenotype of renal and gonadal developmental defects in association with brain abnormalities might be anticipated. The role of steroidogenic factor-1 (SF-1) in gonadal formation is not yet clear. SF-1 is the product of the *FTZ1-F1* gene and is believed to be a nuclear orphan hormone receptor due to the presence of two zinc fingers and a ligand-binding domain in its molecular structure. *FTZ1-F1* mRNA is expressed in the urogenital ridge, which forms both the gonads and the adrenals, and is also found in developing brain regions. Mice lacking SF-1 fail to develop gonads, adrenals, and the hypothalamus. In humans, SF-1 mutation leads to adrenal insufficiency and sex reversal in 46,XY individuals. However, SF-1 is probably also involved in other aspects of sexual development, because it regulates expression of steroidogenic enzymes as well as transcription of the anti-Müllerian hormone (AMH).

Progession of gonadal differentiation beyond the bipotential gonad stage is mediated through gonosomal and autosomal genes. It was long believed and has been proved that a specific testis-determining factor (TDF) is essential for testicular development and that the encoding gene is located on the Y chromosome. This gene, named *sex-determining region of the Y chromosome* (*SRY*), is a single-exon gene; it encodes a protein with a DNA-binding motif that acts as a transcription factor and in turn regulates the expression of other genes. There is evidence that *SRY* binds to the promoter of the *AMH* gene and also controls expression of steroidogenic enzymes. Thus, *SRY* probably induces expression of *AMH*, preventing the formation of Müllerian duct derivatives. Genetically engineered mice with a normal male phenotype have been produced by introducing the mouse homologue *sry* into female mouse embryos, evidence that *SRY* is the TDF. Furthermore, naturally occurring mutations of *SRY* have been described in humans.

Autosomal genes that are structurally related to *SRY* genes have been described. These "*SRY*-box-related," or *SOX*, genes are to some extent involved in testicular development. *SOX-9* is connected with chondrogenesis and gonadal differentiation. This gene is transcribed especially following *SRY* expression in male gonadal structures. Additionally, *SOX-9* is an activator of the type II collagen gene, which is essential for formation of the extracellular matrix of cartilage. Defects in *SOX-9* therefore lead to skeletal malformations known as campomelic dysplasia as well as to sex reversal in 46,XY species.

The *DAX-1* gene is involved in adrenal, ovarian, and testicular development. This gene is located on the X chromosome; the name *DAX-1* derives from the gene location: the dosage-sensitive sex reversal locus (adrenal hypoplasia congenital critical region) on X gene 1. *DAX-1* is expressed during ovarian development but is silent during testis formation, implying a critical role in ovarian formation. Interestingly, *DAX-1* is repressed by *SRY* during testicular development. However, if a duplication of the *DAX-1* region on Xp21 is present in a 46,XY patient, and, thus, the activity of its gene product is enhanced, testicular formation is impaired. In contrast, mutations in *DAX-1* diminishing its activity lead to a lack of adrenal formation and also to hypogonadal hypogonadism in congenital adrenal hypoplasia. Additional genes involved in testicular differentiation have been localized on chromosome 10 and on chromosome 9 (*DMRT1* and *DMRT2*).

Until recently, the genetic events leading to ovarian development have not been well understood. Ovarian differentiation has been presumed to be a passive event, dependent on the absence of the dominant effects of the genes involved in testicular differentiation. However, there is a role for an active genetic pathway mediating ovarian development. Wnt-4 is an example of a member of the Wnt family of locally acting secreted growth factors. The corresponding gene is located on chromosome 1. In mice, deletion of *Wnt-4* causes virilization of XX animals. In contrast, it has been recently demonstrated that a duplication of 1p31–p35, which includes the *Wnt-4* locus in a 46,XY human, is associated with sex reversal and hence a female phenotype. *Wnt-4* is known to up-regulate *DAX-1,* which in turn antagonizes *SRY.* These findings suggest that *Wnt-4* has a key role in gonadal development and is also actively involved in ovarian differentiation.

## III. SEXUAL DIFFERENTIATION

In early gestation, the anlagen for the Wolffian and Müllerian ducts are present in the fetus, regardless of the karyotype. If testicular formation is unhindered, the Sertoli cells will produce AMH. High concentrations of AMH and active binding to membrane receptors in the mesenchymal cells surrounding the Müllerian ducts are necessary to exert the action of AMH. Reduced AMH excretion due to reduced Sertoli cell numbers is thus responsible for partial uterus formation in sex determination disorders. The AMH gene is under transcriptional control of several proteins involved in sexual differentiation. SF-1 binds directly to the AMH gene promoter and activates its transcription in Sertoli cells. A regulatory effect of SRY on AMH receptor expression has also been reported. Both lack of AMH and insensitivity to this hormone have been described in human disease. In persistent Müllerian duct syndrome (PMDS), 46,XY males are characterized by the presence of fallopian tubes and a uterus. The external genitalia are unequivocally male because steroid hormone formation is normal. Mutations within the AMH gene have been demonstrated in AMH deficiency. Although patients with AMH deficiency may have low serum AMH levels, in approximately 50% of the cases, AMH is within the normal limit or even elevated. This is assumed to be due to a defect of the type II AMH receptor, which is necessary for binding of ligand and exertion of AMH action. This receptor has been cloned and functionally relevant mutations have been demonstrated in patients with PMDS. The AMH type II receptor gene has been localized on chromosome 12. PMDS due to both AMH and AMH type II receptor gene mutations is inherited in an autosomal recessive fashion.

## IV. ENZYMATIC PATHWAYS OF SEX STEROID SYNTHESIS

Unhindered steroid hormone formation and action are necessary for normal development of the external genitalia. Furthermore, defects in cholesterol synthesis may also lead to distinct sex phenotypes, including defects in genital development. The Smith–Lemli–Opitz syndrome is an autosomal recessive disorder with several congenital abnormalities and mental retardation. In addition to genital malformations, polydactyly, cardiac abnormalities, and growth disorders are noted. This syndrome is caused by defects in 7-dehydrocholesterol reductase, with elevated levels of the cholesterol precursor 7-dehydrocholesterol and low serum cholesterol levels. Analysis of mutations in the 7-dehydrocholesterol reductase gene in patients with biochemically proved Smith–Lemli–Opitz syndrome has for the first time demonstrated the importance of intact cholesterol synthesis for steroid production.

The first steps of steroid biosynthesis are common pathways for glucocorticoids, mineralocorticoids, and sex steroids. Testosterone synthesis in the developing testes is controlled during early fetal life by human chorionic gonadotropin (hCG) and only later by the fetal luteinizing hormone (LH). Both hCG and LH stimulate testosterone synthesis via the LH receptor (LHR). The LHR belongs to a family of G-protein-coupled receptors with seven transmembrane helices. It has a long extracellular domain involved in ligand binding, in contrast to other receptors of this family, e.g., the thyroid-stimulating hormone (TSH) receptor. The genetic organization of LHR was elucidated in 1995. The gene is localized on chromosome 2p21 and spans over 90 kb, with a coding region divided into 11 exons. Naturally occurring mutations within the LHR, depending on their localization, have been demonstrated to result both in loss as well as gain of function. Inactivating mutations of the LHR are associated with gonadotropin unresponsiveness and lead to Leydig cell agenesis and subsequently to defective sexual differentiation. More often the result is a completely female phenotype, but incomplete virilization due to partial receptor responsiveness with subnormal androgen synthesis has been described. These mutations are typically located in the transmembrane domain of the receptor, but mutations within the extracellular domain have also been reported to result in loss of function. These molecular abnormalities imply an active role of the LHR in Leydig cell growth and differentiation. In contrast, constitutive activation of the LHR leads to normal male phenotype, although precocious pseudopuberty occurs due to mutation of the LHR, with excessive secretion of testosterone by the Leydig cells (familial testotoxicosis). Microscopically, Leydig cell hyperplasia is evident in the testes of these patients. Activating mutations of the LHR are also located in the transmembrane domain, frequently near the third intracellular loop of the receptor.

Defects within the first steps of steroid synthesis will affect either all or at least two of the final metabolites within the gonads and the adrenals. Steroid hormones are synthesized from cholesterol within the mitochondria. The acute stimulation of steroid synthesis is mediated by the steroidogenic acute regulatory (StAR) protein, which is an active transporter of cholesterol through the inner mitochondrial membrane. Mutations within StAR lead to severe lack of adrenal steroidogenesis as well as lack of virilization in 46,XY individuals in lipoid congenital adrenal hyperplasia. Intrauterine survival of affected embryos is possible, because placental steroidogenesis is not StAR dependent. Due to accumulation of cholesterol, the adrenals and testes are further damaged and the residual non-StAR-dependent steroid synthesis is also diminished. Therefore, at birth, low levels of steroids may be measurable, but these may be depleted later. The StAR gene has been cloned and several mutations have been characterized in patients with lipoid congenital adrenal hyperplasia. StAR mutations, as are all other genetic defects of steroid biosynthesis, are inherited in an autosomal recessive fashion, and both genetic female and male individuals can be affected.

The first enzymatic step in steroid synthesis from cholesterol to pregnenolone is mediated by the mitochondrial cytochrome P450 enzyme, which cleaves the cholesterol side chain. Until recently, only one naturally occurring mutation in this enzyme had been described in association with human disease. It was hypothesized that although the mutation did not exert a dominant negative effect, it would still cause 46,XY sex reversal and adrenal insufficiency because haploinsufficiency would lead to slow accumulation of cholesterol in the target organs, and hence to their destruction. It has been postulated that homozygous P450 side chain cleavage (P450scc) mutations would also—in contrast to defects in StAR—affect placental steroid synthesis and therefore these fetuses would not survive. This does not hold true for the other enzymes of early androgen biosynthesis. The P450c17 enzyme is a qualitative regulator of steroid synthesis with two distinct activities. The activities of both 17α-hydroxylase and 17,20 lyase can be differentially regulated. Although 17α-hydroxylase catalyzes the conversion of pregnenolone to 17-OH pregnenolone and progesterone to 17-OH progesterone, the 17,20 lyase activity is necessary for the enzymatic reaction from 17-OH pregnenolone to dehydroepiandrosterone and 17-OH progesterone to androstenedione. P450c17 is encoded by a single-copy gene on chromosome 10q24.3 and mutations within this gene can inhibit both functions, or, selectively, only the 17,20 lyase activity, of the resulting protein. Patients with isolated 17,20 lyase deficiency have been described only recently. The underlying molecular abnormalities within the P450c17 protein result in a severely diminished 17,20 lyase activity but only moderately inhibited 17α-hydroxylase function. Interestingly, 17,20 lyase activity depends largely on phosphorylation of the protein, and dephosphorylation of P450c17 may be a major factor in isolated 17,20 lyase deficiency. To date, only two mutations in the P450c17 gene fulfill the criteria for causing isolated 17,20 lyase deficiency. Differentiation of the two enzyme activities is important in diagnosing human disorders of P450c17 deficiency, because a combined defect will cause hyperaldosteronism and endocrine hypertension, whereas patients with isolated 17,20 lyase deficiency will have normal glucocorticoid synthesis.

The third important enzyme of ubiquitous steroidogenesis is 3β-hydroxysteroid dehydrogenase (3β-HSD), which also plays a major role in androgen biosynthesis. 3β-HSD catalyzes the formation of $\Delta^4$ steroids, thus also the synthesis of androstenedione, the major precursor of testosterone. Two isoforms of the human enzyme have been cloned. The type 1 enzyme catalyzes the formation of progesterone, androstenedione, and 17-OH progesterone from pregnenolone, dehydroepiandrostenedione, and 17-OH pregnenolone. Type 2 3β-HSD shares a 90% sequence homology with the type 1 form, but has a lower catalytic efficiency. Genes for both types are located on chromosome 1p13.1 and consist of four exons. In 3β-HSD deficiency, mutations within the gene encoding for the type 2 enzyme have been found. Type 2 3β-HSD is predominantly expressed in the adrenals and gonads, hence its blockade results in congenital adrenal hyperplasia. In males, defective virilization due to diminished testosterone synthesis is also noted. Females may demonstrate signs of excessive virilization due to elevated adrenal androgens.

The classic form of congenital adrenal hyperplasia and the most common cause of intersex disorders in 46,XX individuals is 21-hydroxylase deficiency. The enzymatic step from 17α-hydroxyprogesterone to 11-deoxycortisol is inhibited in the glucocorticoid pathway, and this inhibition occurs in part also in the synthesis of deoxycorticosterone from progesterone in mineralocorticoid synthesis. Patients with severe 21-hydroxylase deficiency will have excessively high adrenal androgen levels and 46,XX children will display severe virilization of the external genitalia. In children with the 46,XY karyotype, the phenotype is not altered. The *CYP21* gene has been determined to be involved; *CYP21* is localized on chromosome 6p21.3 and direct genetic analysis is performed on a regular basis for diagnostic purposes. This is the prerequisite not only for genetic counseling, but also for approaches for experimental prenatal maternal therapy with glucocorticoids.

Late sex steroid synthesis defects comprise enzymatic reactions that do not inhibit glucocorticoid and mineralocorticoid formation. Although 17β-hydroxysteroid dehydrogenase (17β-HSD) converts androstenedione to testosterone within the testes, 5α-reductase (5α-R) catalyzes the conversion of testosterone to dihydrotestosterone (DHT) in the peripheral target cells. At least five different 17β-HSD isoenzymes exist. Only mutations in the type 3 enzyme have been demonstrated to be responsible for defective sex differentiation in patients with 17β-HSD deficiency. This disorder is characterized by a severe virilization defect in 46,XY individuals, although they show strong signs of virilization during puberty, with marked phallic enlargement. The type 3 17β-HSD gene is located on chromosome 9p22, spanning over 11 exons and encoding a protein of 310 amino acids. This enzyme is expressed only in the testes, compatible with its important role in testicular androgen formation. However, no strict genotype–phenotype correlation has been demonstrated in 17β-HSD deficiency. It remains to be investigated whether the other 17β-HSD isoenzymes play a critical role in the phenotypic expression of 17β-HSD deficiency due to decreased peripheral conversion of testosterone to androstenedione.

Further conversion of testosterone to DHT is catalyzed in the peripheral target tissues and not within the gonads. The two isoenzymes of 5α-reductase are expressed in diverse tissues, but type 2 5α-reductase is more abundant in genital structures. The type 1 enzyme is necessary for reduction of androgens to inhibit excess formation of estrogens, and thus mice lacking this enzyme fail to maintain normal pregnancies. A specific role of the 5α-reductase type 1 enzyme in male sexual differentiation has not been demonstrated. In contrast, several mutations have been described in the type 2 enzyme in patients with defective virilization. The underlying gene has been localized to chromosome 2p23 and is divided into five exons. In 5α-reductase deficiency, DHT formation is severely diminished. However, testosterone levels are normal or even elevated. Affected 46,XY individuals are usually born with ambiguous external genitalia, but the phenotype may be highly variable. The differentiation of Wolffian structures, which is largely dependent on testosterone, is not impaired. At the time of puberty, strong virilization may occur due to high endogenous testosterone levels. Gynecomastia due to estrogen excess has rarely been described.

In 46,XX patients, a rare cause for virilization is a defect in the aromatization of estradiol from testosterone. The aromatase complex belongs to the cytochrome P450 enzymes and is expressed in the gonads and in a variety of other tissues. There is only one corresponding gene, localized on chromosome 15q21. The mother carrying a fetus with aromatase deficiency may suffer from excessive masculinization. Biochemical analysis showing low or absent estradiol in conjunction with elevated androstenedione and testosterone is diagnostic. In males, aromatase deficiency will not lead to a genital abnormality, but due to the diminished estradiol, skeletal maturation is delayed extensively.

## V. MECHANISMS OF ANDROGEN ACTION

The final biological steps in the cellular cascade of normal male sexual differentiation are initiated by the molecular interaction of testosterone and dihydrotestosterone with the androgen receptor (AR) in androgen-responsive target tissues. The AR is a ligand-activated transcription factor of androgen-regulated genes. It is commonly assumed, though not experimentally proven to date, that a controlled temporal and spatial expression of androgen-regulated genes during early embryogenesis provokes a distinct spectrum of functional and structural alterations of the internal and external genitalia, ultimately resulting in the irreversible formation of the normal male phenotype. However, the AR is also expressed in females during embryogenesis, and elevated androgens during this stage, as in congenital adrenal hyperplasia, will lead to virilization in genetic females.

The AR belongs to the intracellular family of structurally related steroid hormone receptors. Transcriptional regulation through the AR is a complex multistep process involving androgen binding, conformational changes of the AR protein, receptor phosphorylation, nuclear trafficking, DNA binding, cofactor interaction, and, finally, transcription activation. The human AR gene was cloned more than a decade ago by several groups and has been mapped to Xq11–q12. It spans approximately 90 kilobases (kb) and comprises eight exons(1–8, or A–H). Transcription of the AR gene and subsequent splicing usually results in distinct AR mRNA populations in genital fibroblasts. Translation of the mRNA to the AR protein usually leads to a product migrating at about 110 kDa in Western immunoblots, comprising between 910 and 919 amino acids.

The AR shares its particular modular composition of three major functional domains with the other steroid hormone receptors. A large N-terminal domain precedes the DNA-binding domain, followed by the C-terminal ligand-binding domain. Additional functional subdomains can be identified by *in vitro* studies of artificially truncated, deleted, or point-mutated ARs. Upon entering target cells, androgens interact very specifically with the ligand-binding pocket of the AR. This initiates an activation cascade with conformational changes and nuclear translocation of the AR. Prior to receptor binding to target DNA, homodimerization of two AR proteins occurs in a ligand-dependent manner. This is mediated by distinct sequences within the second zinc finger of the DNA-binding domain as well as through specific structural N–C-terminal interactions. The AR homodimer binds to hormone response elements (HREs) that usually consist of two palindromic (half-site) sequences within the promoter of androgen regulated genes. Through chromatin remodeling, direct interaction with other transcription factors, and specific coactivators and corepressors, a steroid receptor-specific modulation of the assembly of the preinitiation complex is achieved, resulting in specific activation or repression of target gene transcription.

Defective androgen action due to cellular resistance to androgens causes the androgen insensitivity syndrome (AIS). The end-organ resistance to androgens results in a wide clinical spectrum of defective masculinization of the external genitalia in 46,XY individuals. Müllerian duct derivatives are usually completely absent because of the normal ability of the fetal testes to produce AMH. Cloning of the AR gene made it obvious that inactivating mutations of the AR gene represent the major molecular genetic basis of AIS. Due to the X-chromosomal recessive inheritance, female carriers may typically be conductors. Partial impairment of AR function is usually associated with partial androgen insensitivity syndrome (PAIS). The considerable variability in the degree of impaired AR activity accounts for a wide clinical spectrum of external undervirilization observed in PAIS. In the complete androgen insensitivity syndrome (CAIS), any *in vivo* androgen action is abolished due to complete inactivation of *in vivo* AR signaling. Therefore, these patients have normal female external genitalia with a short and blind-ending vagina. At puberty, CAIS patients acquire a normal female body shape and they show normal breast development. This is due to increasing estradiol levels that accompany elevated pubertal testosterone biosynthesis and conversion to estradiol by aromatization. Usually, pubic or axillary hair is absent.

More than 300 different mutations have been identified in AIS to date. Extensive structural alterations of the AR can result from complete or partial deletions of the AR gene. Smaller deletions may introduce a frameshift into the open reading frame, leading to a premature stop codon downstream of the mutation. Similar molecular consequences arise from the direct introduction of a premature stop codon due to point mutations. Such alterations usually lead to severe functional defects of the AR and are associated with CAIS. Extensive disruption of the AR protein structure can also be due to mutations leading to aberrant splicing of the AR mRNA. However, because aberrant splicing can be partial, thus enabling expression of the wild-type AR, the AIS phenotype is not necessarily CAIS but may also present as PAIS. The most common molecular defects of the AR gene are missense mutations. They may result either in CAIS or in PAIS because of complete or partial loss of AR function. Mutations within the ligand-binding domain may alter androgen binding but may also influence dimerization due to disruption of N–C-terminal structural interactions. Mutations within the DNA-binding domain can affect receptor binding to target DNA. The functional role of mutations within the N-terminal domain is not completely understood. Moderate extension of the polyglutamine trinucleotide segment ($>30$) may cause a moderate inhibition of transcriptional activity and therefore result in mild androgen insensitivity, i.e., gynecomastia. Isolated male infertility due to impaired spermatogenesis may be a symptom of mild AIS as well. Increasing knowledge about the cofactors of AR signaling continue to influence the concept of the pathogenesis of AIS on the molecular level. Recently, the first female patient with complete AIS without an AR gene mutation, but with clear experimental evidence for an AR co-activator deficiency as the only underlying molecular mechanism of defective androgen action, has been reported. Cofactors of the AR will presumably play a pivotal role in understanding the phenotypic variability in AIS. So far, only a few mechanisms contributing to the phenotypic diversity in AIS have been identified in affected individuals. A striking phenotypic variability in a family with partial AIS has been attributed to differential expression of the 5α-reductase type 2 enzyme in genital fibroblasts. Another mechanism may be the combination of varying androgen levels during early embryogenesis and partially inactivating mutations of the ligand-binding domain.

Moreover, postzygotic mutations of the AR gene resulting in a somatic mosaicism of mutant and wild-type AR genes can contribute to modulation of the phenotype. This can result in a higher degree of virilization than expected from the AR mutation alone, because of the expression of the wild-type AR in a subset of somatic cells. Because at least one-third of all *de novo* mutations of the AR gene occur at the postzygotic stage, this mechanism is not only important for phenotypic variability in AIS but also crucial for genetic counseling.

Further decoding of the molecular and biochemical pathways is necessary for a comprehensive understanding of normal and abnormal sexual determination and differentiation. Based on the known molecular defects involved in impaired human sexual development, recent achievements in the fields of functional genomics and proteomics offer unique opportunities to identify the genetic programs downstream of these pathways, which are ultimately responsible for the structure and function of a normal or abnormal genital phenotype.

## Glossary

**gonads** Testes and ovaries; develop from the urogenital ridge through a bipotential gonad. This process is mediated through differentiated genetic control.

**hormone receptor** A mediator for hormone action. In sexual differentiation, receptors for peptide hormones and steroid hormones play an active role.

**sexual determination** Gonadal development via genetically determined pathways.

**steroid biosynthesis** Synthetic pathways that start with cholesterol; defined enzymatic steps produce the final products, glucocorticoids, mineralocorticoids, and sex steroids. Intermediate products may induce distinct hormonal actions, as is seen in enzymatic pathway defects that lead to abnormal hormonal profiles and defined disorders in humanz.

## See Also the Following Articles

**Androgen Effects in Mammals • Anti-Müllerian Hormone • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Environmental Disruptors of Sex Hormone Action • Gonadotropin-Releasing Hormone and Puberty • Gonadotropin-Releasing Hormone Ontogeny • Sexual Differentiation of the Brain • Spermatogenesis, Hormonal Control of • Testis Descent, Hormonal Control of**

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# Sexual Differentiation of the Brain

CHARLES E. ROSELLI
*Oregon Health and Science University*

I. INTRODUCTION
II. INFLUENCE OF TESTOSTERONE ON THE DEVELOPING BRAIN: GONADOTROPIN SECRETION
III. INFLUENCE OF TESTOSTERONE ON THE DEVELOPING BRAIN: SEXUAL BEHAVIOR
IV. CRITICAL PERIODS
V. ROLE OF ESTROGENIC METABOLITES OF TESTOSTERONE
VI. SEXUAL DIMORPHISMS IN THE MAMMALIAN BRAIN
VII. SEXUAL DIFFERENTIATION OF THE HUMAN BRAIN
VIII. SUMMARY

**The hormonal environment that exists during perinatal development is thought to be the predominant determinant for sex differences in reproductive endocrinology and behavior. In this way, the brain and the reproductive system appear to follow the same rules of sexual differentiation, and, as such, the brain conforms to the hormonal theory of sexual differentiation that was first elaborated by the classic work of Alfred Jost. Specifically, in mammals, sex chromosomes specify differentiation of the bipotential gonads into either testes or ovaries.**

**The list of hormone- and stimulus-dependent cascades that include Sgk as a key integrator of receptor signaling events continues to expand, and Sgk has been implicated in the pathology of several human endocrine disorders.**

## I. INTRODUCTION

In vertebrates and other multicellular organisms, individual cells are inundated with many types of hormonal cues and other extracellular stimuli that activate a diverse array of intracellular signal transduction pathways. The ability of cells to sense dynamic changes in their environment and then mount physiologically appropriate responses requires communication with regulatory molecules that can coordinately integrate the intracellular signals that emanate from different receptor signaling cascades. One such critical intracellular component is the serum- and glucocorticoid-inducible protein kinase, Sgk. This protein kinase was originally isolated in a differential screen for glucocorticoid-inducible transcripts from rat mammary epithelial tumor cells as a novel protein kinase that is under acute transcriptional control by serum and glucocorticoids.

Emerging evidence implicates Sgk as an important focal point by which cell surface receptors, nuclear receptors, and cellular stress signaling pathways converge to alter cellular function and the proliferative state and/or determine whether a cell survives or undergoes apoptosis. For example, in different cell and tissue types, Sgk can act as a stimulus-dependent switch in cellular responses to glucocorticoids, mineralocorticoids, follicle-stimulating hormone, growth factor-/insulin-activated cell survival and proliferative pathways, hyperosmotic shock, and transforming growth factor-β (TGF-β). Furthermore, Sgk has been implicated in the pathology of endocrine disorders such as diabetic nephropathy and in physiological abnormalities associated with alterations in the mineralocorticoid control of renal sodium transport.

The various hormone receptor-activated cascades regulate Sgk availability and function at three distinct levels of cellular control. First, an expanding set of hormonal and nonhormonal extracellular cues strongly stimulate Sgk gene expression. Second, Sgk is phosphorylated and enzymatically activated as a downstream component of the phosphatidylinositol 3-kinase (PI3-kinase) signaling cascade that mediates the mitogenic and cell survival response to many growth factors and insulin. Finally, the nuclear–cytoplasmic shuttling of Sgk is controlled by the cell cycle, as well as by exposure to specific hormones and to environmental stress. Thus, a biologically significant feature of Sgk is the simultaneous and stringent stimulus-dependent regulation of its transcription, subcellular localization, and enzymatic activity (Fig. 1).

## II. Sgk PROTEIN STRUCTURE–FUNCTION

The *sgk* gene encodes a 431-amino-acid, 50 kDa protein, and its catalytic domain shows strong homology (45–55% identity) to the catalytic domains of several well-characterized members of the "AGC" family of serine/threonine protein kinases that are constitutively expressed. The protein kinases most closely related to Sgk are Akt/protein kinase B (PKB), protein kinase A, protein kinase C-ζ, and the rat $p70^{S6K}/p85^{S6K}$ kinases. In addition, two other Sgk isoforms have been uncovered (Sgk-2 and Sgk-3), although their cellular functions have not been well studied. This article will restrict its discussion to the originally isolated isoform of Sgk. Members of the related family of mammalian protein kinases that includes Sgk propagate cell signaling cascades associated with the control of cell growth, differentiation, and cell survival and are highly conserved between metazoans and mammals. For example, in addition to humans, rats, and mice, Sgk homologues have been identified in the genomes of diverse species such as frogs, the nematode *Caenorhabditis elegans*, and the budding yeast *Saccharomyces cerevisiae*. One rationale for the evolutionary conservation of Sgk is its role in allowing unicellular and multicellular organisms to adapt and survive environmental stresses such as extreme changes in nutrient levels, temperatures, or osmolarity. In this regard, the two yeast Sgk homologues can be functionally complemented with mammalian Sgk, which strengthens the notion of an evolutionarily important role for Sgk in integrating hormone-activated cellular signals.

As shown in Fig. 2, Sgk has several distinctive structural features that are likely to control aspects of Sgk function in a stimulus-specific context. Sgk has three general regions, a relatively short (70 amino acids) unique amino-terminal domain, a central region containing the catalytic domain, and a unique carboxy-terminal domain. Each of these subregions contains important structural features required for the cellular regulation of Sgk. The N-terminal domain contains a phosphorylation site at serine 78 (P-P-S78-P) that fits the P-x-S/T-P consensus requirements for certain proline-directed kinases and has been shown to be phosphorylated by the big mitogen

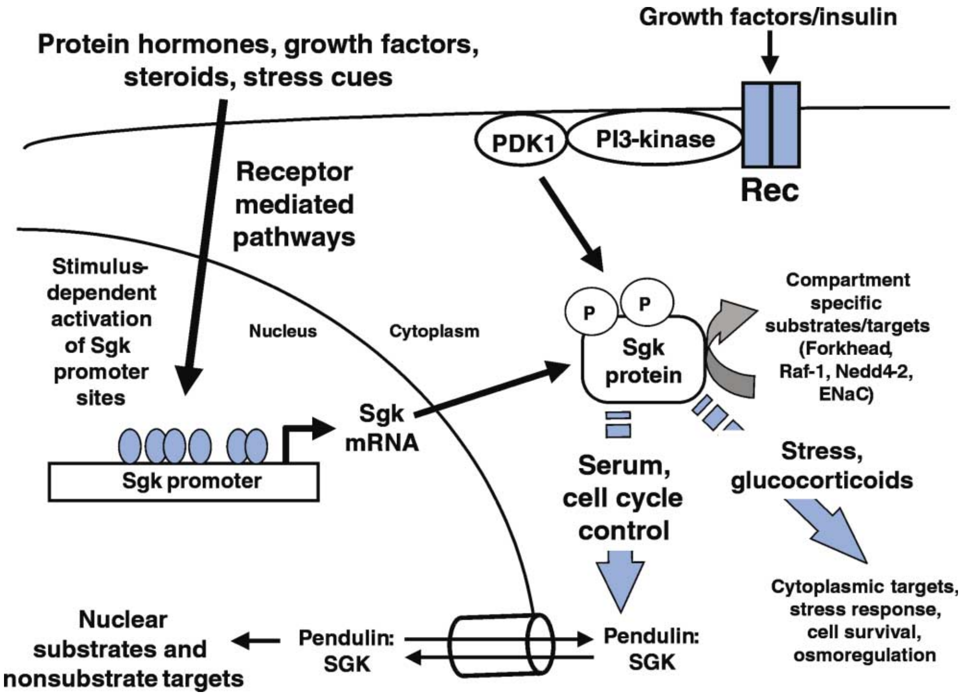
Growth factors/insulin
Protein hormones, growth factors, steroids, stress cues
PDK1
PI3-kinase
Rec
Receptor mediated pathways
Stimulus-dependent activation of Sgk promoter sites
Nucleus
Cytoplasm
P
P
Sgk protein
Compartment specific substrates/targets (Forkhead, Raf-1, Nedd4-2, ENaC)
Sgk mRNA
Sgk promoter
Serum, cell cycle control
Stress, glucocorticoids
Cytoplasmic targets, stress response, cell survival, osmoregulation
Nuclear substrates and nonsubstrate targets
Pendulin: SGK
Pendulin: SGK

FIGURE 1 Hormone and stimulus control of Sgk expression, enzymatic activity, and subcellular localization. Sgk is regulated at three distinct levels of cellular control. The activation of hormone receptor-dependent cascades, as well as environmental stress, regulates Sgk expression by targeting distinct elements in the Sgk promoter. The growth factor/insulin activation of the PI3-kinase pathways results in the phosphorylation of Sgk and the generation of an enzymatically active Sgk. Under proliferative conditions, Sgk is imported by the actions of pendulin/importin-α into the nucleus, where Sgk has access to its nuclear target proteins. After treatment with glucocorticoids or exposure to environmental stress, Sgk resides in a cytoplasmic compartment and mediates the cell survival response.

activative protein kinase 1 (BMK1)/extracellular signal-related kinase 5 (ERK5) member of the mitogen-activated protein kinase (MAPK) family. There are several proline-rich tracts in the N-terminal domain that could conceivably be recognition sites for Sgk target proteins, as well as a putative mitochondrial import signal that may localize cytoplasmic Sgk to this organelle as part of the hormone-dependent cell survival response.

The central domain of Sgk contains all of the essential amino acid sequences necessary to be a functional serine/threonine protein kinase including lysine 127 in the ATP-binding region and threonine 256 in the activation loop. Mutation of lysine 127 (to methionine) forms a kinase-dead version of Sgk. As described in more detail in Section VI and diagrammed in Fig. 2, Sgk is enzymatically activated by phosphorylation of threonine 256 in the activation loop of Sgk, as well as by phosphorylation of serine 422 in the carboxy-terminal domain, by the PI3-kinase-dependent pathway through the direct actions of phosphoinositide-dependent protein kinase 1 (PDK1). The central domain also contains a nuclear localization signal between amino acids 131 and 138 in the central domain (see Section VIII) that controls the signal-dependent nuclear import of Sgk, as well as specific recognition sites for other proteins such as Nedd4-2 at a PY domain (see Section III). Similarly, the carboxy-terminal domain contains a putative PDZ-binding motif that is likely to be involved in Sgk recognition of target proteins. The Sgk structure domains suggest that this protein kinase acts through phosphorylation of specific substrates (see Section VII) and through specific sets of regulated protein–protein interactions.

## III. CELLULAR AND PHYSIOLOGICAL FUNCTIONS OF STIMULUS-INDUCED Sgk

### A. Role of Sgk in Glucocorticoid and Growth Factor Regulation of Cell Proliferation

Sgk is a transcriptionally induced component of glucocorticoid- and growth factor-regulated gene

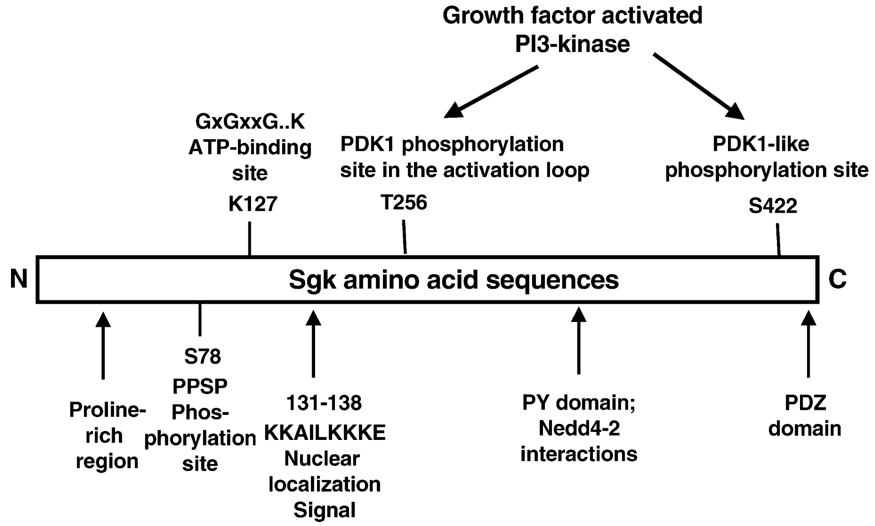

FIGURE 2 Sgk protein structure and functions. The Sgk protein contains three phosphorylation sites. Serine 78 is a proline-directed phosphorylated site that is recognized by members of the mitogen-activated protein kinase family. Threonine 256 and serine 422 are phosphorylated by PDK1 through a PI3-kinase-dependent cascade and are required for the growth factor/insulin activation of Sgk enzymatic activity. Lysine 127 is needed for ATP binding and protein kinase activity. Sgk also contains a proline-rich region in the N-terminal domain, a nuclear localization signal at 131–138, a PY domain required for the Sgk interactions with Nedd4-2, and a putative PDZ site that likely is involved in the recognition of Sgk target proteins.

networks that are highly associated with the control of cellular proliferation. In mammary tumor cells, the serum-induced expression and S-phase nuclear import of Sgk (see Section VIII) are necessary for cell cycle progression of mammary epithelial cells. The BMK1/ERK5 member of the mitogen-activated protein kinase gene family was shown to induce cell proliferation through its interaction with and phosphorylation of Sgk. Consistent with a role for Sgk in proliferative control in normal cells, the sgk gene resides in a single chromosomal locus assigned to band 6q23, a region frequently affected by deletion in various human neoplasias. Sgk appears to also be involved in certain hormone-regulated growth inhibitory responses. The glucocorticoid-induced G1 cell cycle arrest of mammary tumor cells is accompanied by and partially dependent on the stimulated expression of cytoplasmic localized Sgk in mammary tumor cells. Ectopic expression of Sgk in this cell system inhibits cell proliferation. Given that glucocorticoids are considered a physiological stress hormone, it is tempting to consider that the induction of Sgk by this steroid allows cells to mount physiologically appropriate responses to changes in the external milieu resulting from mitogenic cues and/or stress stimuli.

## B. Role of Sgk in the Cellular Stress Response and in Cell Survival Cascades

Sgk has been shown to be a cell survival component of the responses to cellular stressors such as hyperosmotic shock in various epithelial cells. Subsequent to the original cloning of Sgk, this protein kinase was revealed in a screen for an osmotic shock-inducible gene from hepatocytes, and sgk transcripts have been shown to be induced by hyperosmolarity and secretagogues in shark rectal gland. Hyperosmotic shock strongly induces Sgk transcription in mammary epithelial cells by a pathway that utilizes the p38/MAPK cascade, which is the mammalian homologue of the stress-activated *S. cerevisiae* HOG1 proline-directed protein kinase. Expression of the wild-type enzymatically active Sgk, but not the kinase-dead forms of Sgk, protects the transfected cells from stress-induced apoptosis, whereas expression of kinase-dead forms of Sgk had no protective effects. Sgk was also shown to be an important component of the glucocorticoid cell survival response to growth factor deprivation in a human breast cancer cell line. The Sgk-3 isoform has been implicated in the interleukin-3-mediated survival of hematopoietic cells, suggesting that a subset of cellular responses are likely be Sgk isoform-specific.

### C. Role of Sgk in Mineralocorticoid and Insulin Regulation of Sodium Homeostasis and in Osmotic Control of Cell Volume

Sgk stimulates epithelial sodium channel (ENaC) activity and enhances its membrane abundance in co-injected *Xenopus laevis* oocytes, suggesting that one function of Sgk is to control cell volume and sodium homeostasis following osmotic stress of cells. Evidence from several studies indicates that Sgk plays a central role in integrating mineralocorticoid and insulin signals as part of the osmoregulatory mechanism in the kidney. The current viewpoint is that aldosterone regulates sodium homeostasis by stimulating the expression of Sgk, which in turn causes an increase in total renal cell membrane ENaC activity. Sgk directly binds to the ENaC β-subunit, but does not phosphorylate this ion channel. Recent evidence shows that Sgk does phosphorylate the ubiquitin ligase Nedd4-2, which reduces the binding of Nedd4-2 to the C-terminal tail domain of the ENaC β-subunit. The Sgk-dependent phosphorylation of Nedd4-2 appears to require a PY domain recognition site within Sgk (see Fig. 2). As a result, the degradation of ENaC is reduced, with the net effect being an elevation in the steady state level of membrane-associated ENaC and subsequent stimulation of sodium transport in response to mineralocorticoids. Insulin signaling plays a role in the process, and in renal cells insulin can synergize with mineralocorticoids to regulate sodium transport. Insulin has been proposed to activate Sgk enzymatic activity through the PI3-kinase cascade (see Section VI), and consistent with this notion, an inhibition of PI3-kinase activity blocks insulin-stimulated sodium transport. This response, which has been generally observed in cell systems, has a physiological impact because in one study, decreased sodium excretion during sodium depletion was observed in Sgk knockout mice compared to littermate controls.

### D. Other Biological Functions of Sgk

Although not directly linked to stimulus-regulated mechanisms, Sgk has been implicated in a variety of cellular functions that may eventually prove to be associated with hormonal mechanisms of action. For example, Sgk has been linked to the function and cellular utilization of several other ion channels, such as the cystic fibrosis transmembrane regulator-dependent chloride channel and certain voltage-gated potassium channels. The control of potassium ion channel activity suggests a role for Sgk in neuronal excitability. Consistent with this concept, a recent study has implicated Sgk in facilitating memory consolidation of spatial learning in rats. Sgk transcripts were shown to be expressed at significantly higher levels in the hippocampus, an area of the brain with a high level of glucocorticoid receptors, of fast learners compared to slower learners. Thus, it is likely that many functions of Sgk will eventually be revealed, beyond the stimulus-dependent control of proliferation, cell survival, and osmoregulatory processes.

## IV. REGULATION OF Sgk EXPRESSION DURING DEVELOPMENT AND IN ADULT TISSUE

Sgk expression is under stringent developmental control during mouse embryogenesis. Sgk transcripts are first observed at embryonic day 8.5 (E8.5) in the decidua and yolk sac, and then during developmental stages E9.5 through E12.5, this kinase is highly localized in the heart chamber, otic vesicle, blood vessels surrounding the somites, and lung buds. At later stages of mouse embryogenesis, E13.5 through E16.5, sgk expression becomes highly concentrated in brain (choroid plexus), distal epithelium, terminal bronchi/bronchioles, adrenal gland, liver, thymus, and intestines, remains high in heart tissue, and is expressed at a low level in the other embryonic tissues. In the adult, Sgk is concentrated in the choroid plexus of the brain, which is involved in osmotic and pH regulation of cerebral spinal fluid. Sgk is also concentrated in specific areas of the adult kidney (glomeruli and nephrons in the cortical region, as well as in the medulla, papilla, and calyces), suggesting a role in regulating osmotic balance. Consistent with this concept, Sgk is induced by the mineralocorticoid aldosterone in the cortical collecting ducts of the rodent kidney. In adult rat tissue, Sgk is also highly expressed in the thymus, ovary, and lung. A high level of Sgk has been reported in phagocytes, which is consistent with Sgk being involved in the inflammatory response. The tissue-specific and temporal pattern of *sgk* expression during mouse embryogenesis suggests that *sgk* has a potential role in heart development and/or vasculogenesis at early developmental stages and may function in tissues involved in osmoregulation and other physiological stress pathways at later developmental stages and in the adult.

## V. STIMULUS REGULATION OF Sgk PROMOTER ACTIVITY AND GENE TRANSCRIPTION

Sgk expression can be acutely regulated by a variety of hormones and extracellular stress cues that are known to target specific DNA elements in gene promoters. Only a few studies have used the Sgk promoter to rigorously establish the transcriptional control of Sgk gene expression. Glucocorticoids, the p53 tumor suppressor gene, follicle-stimulating hormone, and hyperosmotic stress have been shown to induce Sgk gene products by pathways that target specific DNA elements in the promoter (Fig. 3). In addition to these stimuli, Sgk transcript levels have been to shown to be stimulated in a tissue-specific manner by mineralocorticoids, TGF-β, cytokines such as granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α, ischemic injury of the brain, growth factors such fibroblastic growth factor and platelet-derived growth factor, diabetic nephropathy, changes in hepatocyte cell volume, inflammatory disease, and fibroblast wound repair, whereas heparin suppresses Sgk expression. Although these studies have not characterized the precise level of regulation, it is likely that these responses involve selective changes in Sgk transcription through the control of Sgk promoter activity. In this regard, based on sequence analysis, a variety of intriguing putative DNA elements exist in this promoter for transcription factors known to play a role in cellular differentiation, proliferation, and stress responses and may thereby account for hormone-regulated changes in Sgk expression.

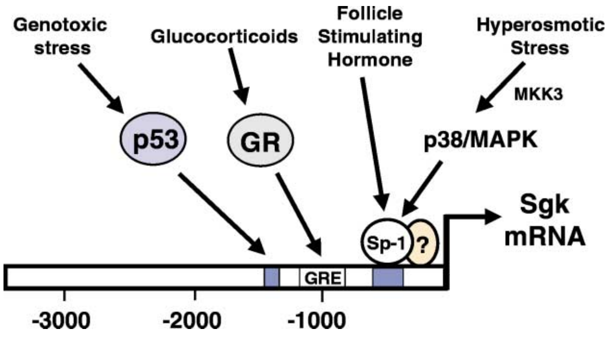

FIGURE 3 Hormone and environmental stress pathways target distinct elements in the Sgk promoter. Sgk is a primary glucocorticoid-responsive gene and contains a functional glucocorticoid-response element (GRE) in its promoter. The Sgk GRE is similar to consensus GREs in other systems and binds to the glucocorticoid receptor (GR) with high affinity. The Sgk promoter is also targeted by the p53 tumor suppressor gene as a downstream response to genotoxic stress. Follicle-stimulating hormone activates a receptor signaling pathway that simulates Sgk transcription through a Sp-1 transcription factor. Hyperosmotic stress activates a p38/mitogen-activated protein kinase (p38/MAPK) pathway through the actions of MKK3 (MAPK kinase kinase-3), which also targets Sp-1 in the Sgk promoter. Sp-1 interacts with other transcription factors in a tissue-dependent manner to stimulate Sgk transcription. The Sgk promoter contains many other putative transcription factor sites that likely account for the regulation of Sgk gene expression of other hormones and extracellular stimuli.

### A. Sgk Is the Primary Glucocorticoid-Responsive Gene Containing a Glucocorticoid-Response Element in Its Promoter

Sgk expression is stimulated by glucocorticoids in a variety of cells and tissues, and the Sgk promoter contains a functional glucocorticoid-response element (GRE) that accounts for its glucocorticoid inducibility. A combination of mutagenesis, transfection of Sgk promoter-driven reporter plasmids, and DNA–glucocorticoid receptor-binding assays revealed the presence of the Sgk GRE, which is located at approximately −1000 bp of the Sgk promoter (Fig. 3). The Sgk GRE is highly homologous to the consensus glucocorticoid-response element and is sufficient to confer glucocorticoid responsiveness to a heterologous promoter in a manner that requires a functional receptor. Mutation of the Sgk GRE eliminates glucocorticoid responsiveness of the Sgk promoter. These decisive promoter studies support the notion that Sgk is a primary glucocorticoid-responsive gene. Given that glucocorticoid and mineralocorticoid receptors bind to the same DNA element, it is likely that the GRE in the Sgk promoter accounts for the aldosterone induction of Sgk transcripts in mammalian and amphibian renal cells.

### B. Regulation of Sgk Promoter Activity by the p53 Tumor Suppressor Protein, Hyperosmotic Stress, and Follicle-Stimulating Hormone

The Sgk promoter is a transcriptional target of the p53 tumor suppressor protein, a known target of genotoxic stress. Using both functional and DNA-binding strategies, the wild-type p53 tumor suppressor protein was shown to strongly stimulate Sgk promoter activity in mouse mammary epithelial cells, but repressed Sgk promoter activity in Rat2 fibroblasts. The Sgk p53 sequences at −1380 bp and at −1345 bp were sufficient to confer p53-dependent transactivation or transrepression to a heterologous promoter in a cell type-specific manner. Interestingly, both the murine and the human wild-type p53 tumor suppressor proteins, but not a mutant p53, can functionally interfere with the glucocorticoid receptor transactivation of the Sgk promoter through events converging on the Sgk GRE.

Sgk promoter activity can be stimulated by follicle-stimulating hormone in adult rat ovarian granulosa cells through activation of the Sp-1 transcription factor in the Sgk promoter. This response is also dependent on activation of the cyclic AMP pathway, although the precise upstream pathways that target Sp-1 have not characterized. A systematic mutagenic analysis of the Sgk promoter activity revealed a hyperosmotic stress-regulated element that is located between positions −50 and −40 in the Sgk promoter and that mediates the hyperosmotic response to the organic osmolyte 0.3 M sorbitol. This region of the Sgk promoter contains a consensus Sp-1 DNA element, and the stimulus-regulated binding of the Sp-1 transcription factor to this site accounts for the stimulation of Sgk transcription. The hyperosmotic stress cascade targets Sp-1 in the Sgk promoter by activating the p38/MAPK stress kinase. For example, incubation with the SB202190- or SB203580-specific inhibitors of p38/MAPK dampens the hyperosmotic stress stimulation of the Sgk promoter and Sgk protein production. Active MAPK kinase kinase-3 acts upstream of p38/MAPK to stimulate Sgk promoter activity in a sorbitol-dependent manner (Fig. 3). Sp-1 is known to interact with other transcription factors, and it is likely that the follicle-stimulating hormone and the hyperosmotic stress control of Sgk promoter activities require different Sp-1 protein–protein interactions.

## VI. REGULATION OF Sgk PHOSPHORYLATION AND ENZYMATIC ACTIVITY THROUGH THE GROWTH FACTOR-ACTIVATED PI3-KINASE PATHWAY

The stimulus-dependent control of Sgk phosphorylation and enzymatic activity through a PI3-kinase cascade represents a second level of Sgk regulation in the cell (Fig. 1). The PI3-kinase-activated signal transduction pathway enhances cell survival and proliferation in response to a variety of growth factors. PI3-kinase directly binds to tyrosine kinase receptors (or to insulin substrate-1) via a SH2 domain in its p85 subunit. PI3-kinase converts phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate, which binds to the pleckstrin homology domain contained within PDK1, resulting in its activation. Active PDK1 then directly phosphorylates and enzymatically activates several serine/threonine protein kinases, including Sgk and several protein kinases most homologous to Sgk, such as Akt/PKB. Full stimulation of Sgk enzymatic activity requires the PDK1-dependent phosphorylation at threonine 256, which is located within the activation loop of Sgk, and at serine 422, in the carboxy-terminal domain. Ablation of both phosphorylation sites by mutation of threonine 256 and serine 422 to alanines inhibits Sgk enzymatic activity and in some systems forms a potent dominant negative form of Sgk. Furthermore, substitution of the PI3-kinase-dependent phosphorylation sites with aspartate to mimic the charge effects of phosphorylation generates a constitutively active protein kinase. Treatment of cells with the LY294002 chemical inhibitor of PI3-kinase abolishes the hormone activation of Sgk enzymatic activity and prevents the production of the hyperphosphorylated forms of Sgk in the cell systems that have been examined.

Sgk phosphorylation and enzymatic activity have been shown to be regulated as a downstream component of the PI3-kinase cascade in response to a variety of extracellular signals, such as serum growth factors, insulin-like growth factor-I, insulin, oxidative stress, and hyperosmotic stress. Importantly, the disruption of PI3-kinase-dependent phosphorylation and activation of Sgk abolishes many Sgk cellular functions, such as insulin-stimulated sodium transport, glucocorticoid-dependent effects on cell proliferation and cell survival, mitogenic cell survival responses, and the cellular response to osmotic changes. The precise role of Sgk in many of these cellular functions has not been established because the same sets of extracellular signals that activate the stimulus-induced Sgk enzymatic activity also activate constitutively expressed Akt/PKB, which is highly related to Sgk and contains analogous PDK-1-dependent phosphorylation sites. In studies examining its downstream targets, Akt/PKB, like Sgk, has been indicated to play roles in maintaining cell survival in response to growth factors, in insulin-regulated glucose metabolism, in transcriptional control, and in regulation of apoptosis after environmental stress in many different cell types. Although Sgk and Akt/PKB are highly homologous in their catalytic domains and have similar activation profiles, they display unique features that suggest that they provide complementary rather than redundant cell functions.

## VII. Sgk SUBSTRATE SPECIFICITY AND TARGET PROTEINS

By screening of a peptide library, consensus Sgk substrate sites that are generally similar to the Akt/PKB enzymatic specificity (RXRXXS/T) and that suggest some overlap in protein substrates have been identified. Sgk phosphorylates several peptides, and the most selective peptide substrate for Sgk is KKRNRRLSVA, which was named Sgktide. Arginines at the − 2/− 3 and the − 5/− 6 positions, relative to the phosphorylated serine, were found to be required for Sgk activity. In mostly *in vitro* assays, several substrates for Sgk have been revealed. Both Sgk and Akt/PKB have been shown to phosphorylate *in vitro* glycogen synthase kinase 3, the apoptotic component Bad, the forkhead transcription factor FKHRL1, and the Raf-1 component of mitogen signaling cascades. In transfected cells, the T256A/S422A mutant Sgk acts as dominant negative for phosphorylation of the forkhead transcription factor and attenuates the cell survival response to extracellular stress. These results directly implicate Sgk in the hormone-dependent control of cell survival. There are subtle differences in the substrate specificity of Sgk and Akt/PKB that are likely to be biologically significant. The best example with an endogenous substrate is with the FKHRL1 forkhead transcription factor in that Sgk and Akt/PKB display preferences for different phosphorylation sites in this protein. Given the important role of forkhead transcription factors in controlling cell survival and apoptotic responses, one viewpoint is that this dual phosphorylation causes a more enhanced cell survival response. It is likely that hormone-regulated activation of both Sgk and Akt/PKB, for example, by insulin and other growth factors, similarly enhances the corresponding downstream cascades through the complementary actions of Sgk and Akt/PKB.

As mentioned earlier, Sgk phosphorylates the ubiquitin ligase Nedd4-2, which causes a significant reduction in the interaction of this ligase with the ENaC, resulting in a net mineralocorticoid-dependent elevation in ENaC levels in the membrane of renal cells. ENaC is a direct protein-binding target of Sgk, and although the precise mechanism underlying these interactions has not been established, it is tempting to consider that Sgk–ENaC protein–protein interactions allow the efficient access of Sgk to its Nedd4-2 substrate. Sgk was also shown to bind to, but not phosphorylate, the nuclear import receptor pendulin/importin-α, and the hormone-regulated implications of this observation are described below. Only a limited number of Sgk substrates and nonsubstrate target proteins have been identified, and a key future direction for the field will be to identify the many unknown Sgk target proteins that account for the stimulus-dependent responses associated with Sgk.

## VIII. HORMONE AND STIMULUS-DEPENDENT CONTROL OF Sgk SUBCELLULAR COMPARTMENTALIZATION

Emerging evidence suggests that the regulated subcellular localization of Sgk, a third level of cellular control of Sgk, is a physiologically important process that helps the cells integrate extracellular proliferative, stress, and differentiation signals. The control of Sgk compartmentalization provides Sgk access to its critical targets and is particularly important for integrating intracellular signaling pathways when different hormonal cues with opposite cellular functions can induce enzymatically active Sgk. Depending on the extracellular stimuli, the regulated compartmentalization of Sgk can be viewed as controlling the accessibility of this protein kinase to its substrates and nonsubstrate protein targets.

### A. Stimulus and Cell Cycle Regulation of Sgk Localization to the Nucleus or the Cytoplasmic Compartment

The subcellular distribution of Sgk between the nucleus and the cytoplasm is stringently controlled in a stimulus-dependent manner in mammary epithelial cells and in ovarian cells. In serum-stimulated proliferating mammary tumor cells, a nuclear form of Sgk can be detected, whereas in glucocorticoid growth-arrested cells or in hyperosmotically stressed cells, enzymatically active Sgk is localized exclusively to the cytoplasmic compartment. During the *in vivo* transition from granulosa cells in the proliferative stage of growing follicles in the ovary to terminally differentially nongrowing luteal cells, the subcellular distribution of Sgk changes from being predominately nuclear to almost entirely cytoplasmic.

Laser scanning cytometry, which simultaneously monitors Sgk localization and DNA content in individual mammary tumor cells of an asynchronously growing population, revealed that Sgk actively shuttles between the nucleus and the cytoplasm in synchrony with the cell cycle. Sgk is predominantly nuclear in S and G2/M phase cells and resides in the cytoplasmic compartment during the G1 phase of the cell cycle. Immunofluorescence and biochemical studies showed that treatment with glucocorticoids, which induce a G1 cell cycle arrest, or exposure to hyperosmotic stress results in a cytoplasmic form of Sgk. The precise cytoplasmic compartment in which Sgk resides has not been fully characterized, although some evidence indicates a mitochondrial location for the stressed-induced Sgk. In cells synchronously released from the G1/S boundary, Sgk exclusively localizes to the nucleus during progression through the S phase. The forced retention of exogenous Sgk in either the nucleus or the cytoplasm suppresses the growth and DNA synthesis of serum-stimulated cells. This result indicates that a key proliferative signal in mammary tumor cells is the continuous shuttling of Sgk between the nucleus and the cytoplasm. In the ovarian system, on treatment with FSH, Sgk resides in the nucleus of proliferating granulosa cells, and in terminally differentiated luteal cells, Sgk is located in the cytoplasmic compartment.

Taken together, these results suggest that the spatial and temporal regulation of Sgk is vital for executing complex growth and differentiation programs, which suggests the existence of specific regulatory mechanisms for localizing Sgk to distinct cellular compartments. Conceivably, the signal-dependent sequestration of Sgk in different subcellular locations also entails interactions with particular cellular proteins that target Sgk to specific intracellular destinations and thereby control accessibility to its protein targets.

### B. Mechanism of Stimulus Regulation of Sgk Nuclear-Cytoplasmic Shuttling

A yeast two-hybrid screen demonstrated that the nuclear receptor pendulin/importin-α is a highly specific Sgk-interacting protein. *In vitro* binding assays and co-immunoprecipitations of cell extracts demonstrated that pendulin/importin-α strongly binds to Sgk. Pendulin/importin-α recognizes nuclear localization signals in its cargo proteins and then through the interactions with importin-β, the receptor–cargo protein complex is imported into the nucleus through nuclear core complexes. Mutagenesis of Sgk identified a "bipartite-like" nuclear localization signal sequence, KKAILKKKE, between amino acids 131 and 139 in Sgk. This sequence mediates the *in vitro* binding of Sgk to pendulin/importin-α and the nuclear import of Sgk. For example, mutation of the Sgk nuclear localization signal by amino acid substitutions (K to A) in the context of the full-length Sgk ablates the *in vitro* interaction of Sgk with pendulin/importin-α.

Subcellular localization studies documented that pendulin/importin-α co-localizes with Sgk to the nucleus in serum-stimulated cells and to the cytoplasmic compartment in dexamethasone-treated mammary tumor cells. One viewpoint is that the selective interactions between Sgk and pendulin/importin-α control the cell cycle-dependent nuclear localization of Sgk in serum-treated cells and play a role in distributing Sgk to the cytoplasm in glucocorticoid-treated or stressed cells. Thus, the Sgk–pendulin/importin-α interactions provide a mechanistic basis for the stimulus-regulated compartmentalization.

## IX. POTENTIAL CONNECTIONS OF Sgk WITH HUMAN DISORDERS

The central role of Sgk in integrating the cross talk between nuclear receptor and plasma membrane receptor signals would predict that many physiological abnormalities would be associated with the dysfunctional regulation of expression, activity, and subcellular localization of Sgk. Indeed, recent evidence has indicated that Sgk plays a role in the pathology of certain human disorders. Alterations in Sgk expression, in combination with its cellular role in osmoregulation, suggest a role for Sgk in the nephropathy that is associated with the diabetic disease state, conceivably as part of the hyperosmotic response in the kidney to high plasma glucose levels. Similarly, Sgk has been implicated in the salt-sensitive hypertension associated with the insulin-resistance syndrome. Transforming growth factor-β stimulates Sgk expression, and this response suggests that Sgk plays a role in the fibrogenic actions of this hormonal cue and by extension is involved in fibrosing disease. Consistent with this notion, Sgk expression is elevated in fibrosing pancreatitis and in inflammatory bowel disease. The stimulus-dependent cell survival and proliferative functions of Sgk directly suggest that defects in the cellular control of Sgk signaling may be associated with a subset of human disorders with dysfunctional growth control and apoptotic mechanisms, such as cancer. The control of Sgk nuclear–cytoplasmic shuttling may be a particularly important facet of Sgk control because the nuclear form of Sgk is associated with proliferative conditions, whereas after cellular stress the cytoplasmic form of Sgk is important for cell survival and antiproliferative responses. Thus, it is tempting to consider that the nuclear Sgk substrates could provide potential targets for therapeutic intervention to selectively dampen cell proliferation or to modulate pathological states of cells associated with the nuclear localization of Sgk. In a complementary manner, small-molecule inhibitors of Sgk kinase activity have the potential to be developed as novel therapeutic agents to control certain neoplasias by selectively regulating proliferative, survival, and/or apoptotic signaling pathways that are dependent on Sgk.

## Glossary

**hormone receptor signaling cascade** The chain of intracellular events beginning from the activation of a hormone receptor that ends in the final response to the hormone. Also known as a signal transduction pathway.

**phosphatidylinositol 3-kinase (PI3-kinase)** Kinase that directly binds to tyrosine kinase receptors (or to insulin substrate-1) via a SH2 domain in its p85 subunit. PI3-kinase converts phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate. The PI3-kinase signal transduction pathway mediates cell survival and proliferative responses to a variety of growth factors and insulin.

**phosphoinositide-dependent protein kinase-1** Protein kinase that is activated by phosphatidylinositol 3-kinase and in turn directly phosphorylates and enzymatically activates serum- and glucocorticoid-inducible protein kinase.

**serum- and glucocorticoid-inducible protein kinase** Protein kinase that is regulated by hormones and other extracellular signals at three distinct levels of cellular control and is a unique point of cross talk in hormone signaling cascades.

**stress response** The ability of cells to survive or undergo apoptosis in response to environmental stress conditions, such as changes in osmolarity, nutrient deprivation, or extreme temperatures. Generally considered to be initiated by receptor-mediated events.

## See Also the Following Articles

**Apoptosis • Membrane Receptor Signaling in Health and Disease • Receptor–Receptor Interactions • Signaling Pathways, Interaction of • Stress**

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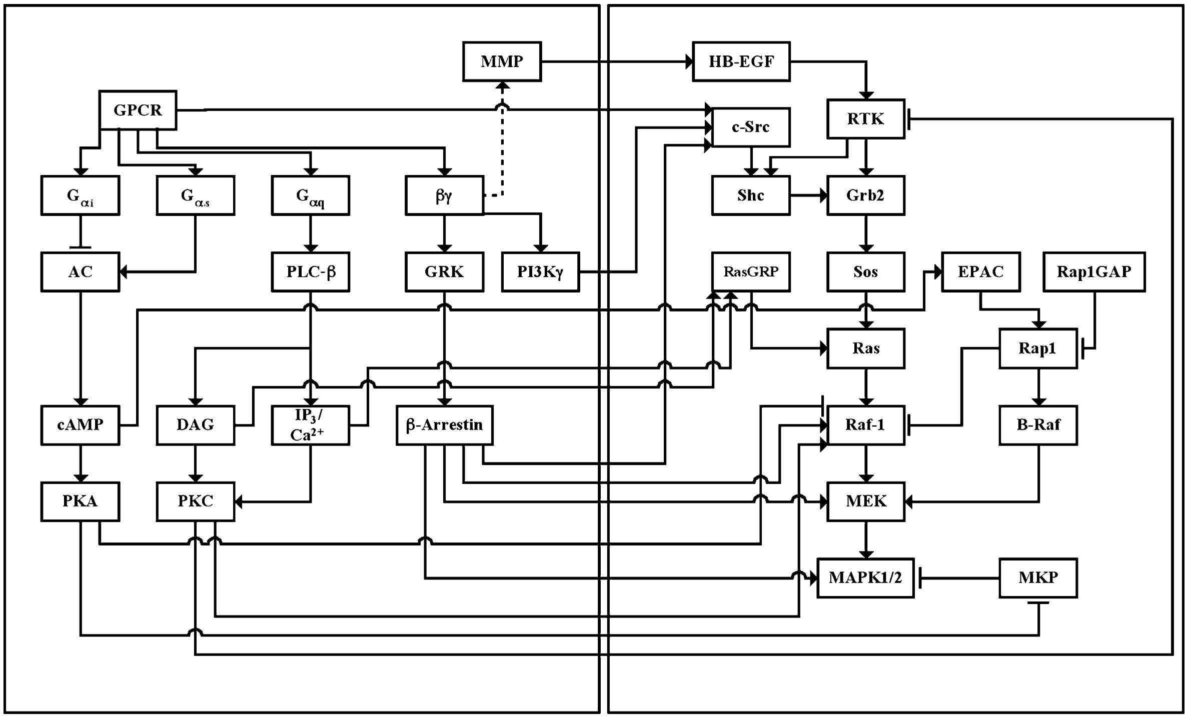

FIGURE 1 Regulation of the RTK–MAPK cascade by GPCR signaling pathways. In the right box are the components and related regulators of the RTK–MAPK cascade, and in the left box are the effectors and components of GPCR and heterotrimeric G-proteins. Within the signaling circuits, lines with arrows represent stimulation and lines with vertical bars at the end represent inhibition. Dotted lines indicate indirect regulation or regulation by unknown mechanisms. GPCR, G-protein-coupled receptor; MMP, matrix metalloproteinase; AC, adenylyl cyclase; PLC-β, phopholipase C-β; GRK, G-protein-coupled receptor kinase; PI3Kγ, phosphatidylinositol 3-kinase γ; DAG, diacylglycerol; $IP_3$, inositol 1,4,5-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; HB-EGF, heparin-binding epidermal growth factor; RTK, receptor tyrosine kinase; Shc, Src homology and collagen; Grb2, growth factor receptor-binding protein 2; RasGRP, guanyl nucleotide-releasing protein for Ras; Sos, son of sevenless; EPAC, exchange protein directly activated by cAMP; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase/ERK kinase; MKP, MAPK phosphatase.

(pleckstrin homology) domain of Sos is also essential for membrane translocation and for complete activation of Ras.

Membrane recruitment of Sos can be also accomplished by binding of Grb2/Sos to Shc (Src homology and collagen), another adapter protein that possesses a phosphotyrosine-binding (PTB) domain, a SH2 domain, and a SH3 domain. Shc forms a complex with many receptors through its PTB domain. Shc is a substrate for the RTKs and, on tyrosine phosphorylation, binds to the SH2 domain of Grb2.

Alternatively, Grb2/Sos complexes can be recruited to the cell membrane by binding to membrane-linked docking proteins, such as insulin receptor substrate 1 or fibroblast growth factor (FGF) receptor substrate 2, which become tyrosine phosphorylated in response to activation of certain RTKs.

Once in the active GTP-bound state, Ras stimulates MAP kinase kinase kinases (MAPKKKs) Rafs, which include Raf-1, A-Raf, and B-Raf. Activated Raf stimulates MAPK kinase (MAPKK), which is also called MEK (MAPK kinase/ERK kinase), by phosphorylating a key Ser residue in the activation loop. MEK then phosphorylates MAPK on Thr and Tyr residues at the activation loop, leading to its activation. Activated MAPKs phosphorylate and regulate the activity of key enzymes and nuclear proteins, which can ultimately regulate the expression of genes essential for cell proliferation.

## III. G-PROTEIN-COUPLED RECEPTORS AND CELL PROLIFERATION

GPCRs are involved in diverse important biological activities, from cellular functions (cell proliferation, cell transformation, cell differentiation, endocytosis, exocytosis, neurotransmission, chemotaxis, etc.), to physiological functions (photo- and chemoreception, secretion from endocrine and exocrine glands, blood pressure control, etc.), to morphogenesis (embryogenesis, angiogenesis, tissue regeneration, etc.).

The proliferative effect of GPCRs was recognized by the initial finding that the serotonin and muscarinic acetylcholine receptors can trigger the transformation of fibroblast cells. It also has been shown that DNA viruses encoding functional GPCRs, including human cytomegalovirus, Herpesvirus saimiri, and Kaposi's sarcoma-associated herpes virus, can contribute to malignant transformation and ultimately to human cancer due to their persistent activity. The ability of GPCRs to affect cell growth was further confirmed by the identification of the naturally occurring activated mutants of $G_\alpha$ in various cancers. The activated mutants $G_{\alpha s}$, $G_{\alpha i2}$, and $G_{\alpha 12}$ are referred to as the *gsp*, *gip2*, and *gep* oncogenes, respectively.

The signaling pathways mediating the proliferative effects of GPCRs and heterotrimeric G-proteins are still under intensive study. The RTK–MAPK pathway appears to play an essential role in cell proliferation. The emerging picture is that GPCRs achieve their mitogenic effect through the regulation of the RTK–MAPK pathway at different levels by multiple mechanisms.

## IV. HETEROTRIMERIC G-PROTEINS AND THEIR SECOND MESSENGER-GENERATING SYSTEMS

Approximately 20 mammalian G-protein α-subunits have been identified. Based on their primary sequence similarity, they are divided into four families: $G_s$, $G_i$, $G_q$, and $G_{12}$. These G-protein α-subunits regulate the activity of several second-messenger-generating systems. The members of the $G_q$ family control the activity of phosphatidylinositol-specific phospholipases, such as phospholipase C-β, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (to generate two second messengers, inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ and DAG in turn lead to an increase in the intracellular concentrations of free calcium $[Ca^{2+}]_i$ and the activation of a number of protein kinases, including protein kinase C (PKC). The members of the $G_s$ family activate adenylyl cyclases, whereas $G_i$ family members can inhibit a subset of these enzymes, thereby controlling the intracellular concentrations of cyclic AMP (cAMP). cAMP can further activate protein kinase A (PKA) and the recently identified guanine nucleotide exchange factor for small GTPase Rap1, EPAC (exchange protein directly activated by cAMP). $G_\alpha$ subunits of the $G_i$ family, which includes $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3,}$ $G_{\alpha o}$, transducin ($G_{\alpha t}$), and gustducin ($G_{\alpha gust}$), also activate a variety of phospholipases and phosphodiesterases and promote the opening of several ion channels. The members of the $G_{\alpha 12}$ family stimulate small GTPase Rho through Rho guanine nucleotide exchange factors (RhoGEF), which include p115-RhoGEF, PDZ-RhoGEF, and leukemia-associated RhoGEF.

Thus far, 6 G-protein β-subunits and 12 G-protein γ-subunits have been cloned. On GPCR activation, dimers are released from the heterotrimeric complex and regulate the activity of many signaling molecules, including ion channels, phosphatidylinositol 3-kinases (PI3Ks), phospholipases, adenylyl cyclases, and receptor kinases. Distinct pools of $G_{\beta\gamma}$ subunits may play different roles in signal transmission.

## V. SIGNALING BY GPCRs TO MAPK PATHWAY THROUGH SECOND MESSENGERS

### A. PKA

In NIH 3T3 cells, activated $G_{\alpha s}$ and 8-Br-cAMP inhibit H-Ras-stimulated DNA synthesis and MAPK activity. Phosphorylation of Raf-1 at multiple serine residues by PKA on cAMP activation reduces the affinity of Raf for Ras and thus decreases Raf kinase activity. Also, PKA-mediated phosphorylation of a negative regulatory phosphatase of MAPKs is required for agonist-induced activation of MAPKs in T cells.

### B. PKC

Activation of PKC by G-protein-coupled receptors results in EGFR phosphorylation on multiple Ser and Thr residues, including Thr-654 in the juxtamembrane domain of EGFR. PKC-induced phosphorylation of EGFR results in an inhibition of its PTK activity and in strong inhibition of EGF binding to the extracellular ligand-binding domain. PKC-mediated phosphorylation of the juxtamembrane domain of EGFR thus appears to provide a negative feedback mechanism for the control of receptor activity. Moreover, PKC can phosphorylate and activate Raf-1 and thus stimulate MAPK activity. Thus, PKC can have different effects in connecting the GPCR to RTK pathways, depending on its sites of action.

## C. RasGRP

In addition to the catalytic domain, RasGRP, a guanyl nucleotide-releasing protein for Ras, consists of an atypical pair of "EF hands" that bind calcium and a DAG-binding domain. RasGRP activates Ras and causes transformation in fibroblasts. ERK1 and ERK2 are activated in rat2 cells expressing RasGRP in response to increases in membrane DAG and free cytoplasmic calcium induced by GPCR agonist endothelin-1. Sustained ligand-induced signaling and membrane partitioning of RasGRP are absent when the DAG-binding domain is deleted. RasGRP is expressed in the nervous system, where it may couple changes in DAG and possibly calcium concentrations to Ras activation.

## D. Rap1 and EPAC

### 1. *Control of the MAPK Pathway by Rap1*

Ras-related protein Rap1 was identified in a screen for proteins that can suppress the transformed phenotype of fibroblasts oncogenically transformed by one of the mutated Ras genes, *K-ras*. Rap1 can interact with members of the Raf subfamily, Raf-1 and B-Raf. Its effect on the MAPK pathway depends on the interaction partners.

### 2. *Inhibition of Raf-1 by Rap1*

Rap1 binds to the serine/threonine kinase Raf-1 *in vitro*, through an interaction with both the Ras-binding domain and the adjacent cysteine-rich region of Rap1 and, in addition, the two proteins co-immunoprecipitate. It has been proposed that Rap1 inhibits Ras/ERK signaling by trapping Raf-1 in an inactive complex.

### 3. *Activation of B-Raf by Rap1*

In some cell types, Rap1 is implicated in the activation of the MAPK pathway. The mechanism may be that Rap1 binds to and activates the Raf family member B-Raf. The evidence for this interaction includes the direct binding of Rap1 to, and activation of, B-Raf *in vitro* and the fact that inhibitors of Rap1, such as Rap1 GTPase-activating protein (Rap1GAP) and Rap1N17, abolish the activation of the B-Raf–MAPK pathway. The different effect of Rap1 on B-Raf (activating) and Raf-1 (inactivating) is thought to reside in the interaction of Rap1 with the cysteine-rich region of the two kinases, as swapping the two domains reverses the effect of Rap1 on the two proteins.

### 4. *EPAC*

Rap1 is activated in response to a range of stimuli through a number of second-messenger molecules, including cAMP, $Ca^{2+}$, and diacylglycerol. PKA is activated by cAMP. However, by using inhibitors and mutants, it was found that PKA is not involved in the cAMP-induced activation of Rap1. Indeed, although PKA phosphorylates Rap1 near its carboxy-terminus, this phosphorylation is not required for cAMP-dependent activation. The search of sequence databases for possible Rap1 GEFs that might be directly regulated by cAMP led to the discovery of EPAC. In addition to containing homologies to other GEFs for Ras-like proteins, EPAC possesses sequences related to the regulatory subunit of PKA. EPAC is activated both *in vitro* and *in vivo* by direct binding of cAMP. Also, EPAC mutant with deletion of the cAMP-binding domain activates Rap1 *in vitro*. This indicates that the cAMP-binding domain normally inhibits the exchange activity of EPAC until cAMP binds, most likely causing a conformational change that relieves the inhibition.

# VI. SIGNALING BY GPCRs TO MAPK PATHWAY THROUGH OTHER HETEROTRIMERIC G-PROTEIN EFFECTORS

## A. RTK

RTK can be transactivated through an extracellular pathway on GPCR stimulation. It has been shown that a chimeric RTK consisting of the EGFR ectodomain and the transmembrane and intracellular portion of the PDGF receptor (PDGFR) is transactivated by treatment of Rat1 fibroblasts with GPCR ligands but endogenous PDGFR is not. Hence, GPCR-induced transactivation of the artificial RTK does not involve an intracellular pathway and is dependent on the extracellular ligand-binding domain of the EGFR. In the presence of diphtheria toxin mutant CRM197 that specifically blocks heparin-binding EGF (HB-EGF) function or the matrix metalloprotease inhibitor batimastat (BB94), lysophosphatidic acid (LPA)-, carbachol-, or tetradecanoyl-phorbol-13-acetate (TPA)-induced transactivation of the EGFR and tyrosine phosphorylation of SHC are completely abrogated in COS-7 and HEK 293 cells. Flow cytometric analysis directly confirms cell surface ectodomain shedding of proHB-EGF on treatment with GPCR agonists or TPA. Therefore, it has been proposed that activation of heterotrimeric G-proteins by agonist-occupied GPCR induces the extracellular activity of a transmembrane metalloproteinase, which leads to the extracellular processing of a transmembrane growth factor precursor and release of the mature factor, and consequently stimulates RTKs and MAPKs.

### B. PI3K$\gamma$

Overexpression of PI3K$\gamma$ in COS-7 cells activates MAPK in a $G_{\beta\gamma}$-dependent fashion. Wortmannin, an inhibitor of PI3Ks, or expression of a catalytically inactive mutant of PI3K$\gamma$ abolishes the stimulation of MAPK by $G_{\beta\gamma}$ or in response to stimulation of M2 muscarinic G-protein-coupled receptors.

Expression of a mutant Sos protein lacking the domain involved in Ras-specific guanine nucleotide exchange activity, a dominant negative mutant N17-Ras, or a dominant negative mutant of Raf-1 inhibits MAPK stimulation by PI3K$\gamma$ without affecting MAPK stimulation by the activated form of MEK. Therefore, signaling from G-protein-dependent receptors, $G_{\beta\gamma}$, and PI3K$\gamma$ to the MAPK pathway occurs upstream of Sos. Expression of PI3K$\gamma$ in COS-7 cells stimulates tyrosine phosphorylation of Shc and enhances the association of Shc with Grb2, whereas a mutant of Shc lacking the tyrosine phosphorylation site, Y317F, suppresses the stimulation of MAPK induced by LPA, the expression of $G_{\beta\gamma}$ and carbachol in m2-transfected cells, the expression of PI3K$\gamma$, or the expression of the Src-related tyrosine kinase Fyn. The nonspecific tyrosine kinase inhibitor genistein or the Src-like specific inhibitor PP1 potently blocks MAPK activation by PI3K$\gamma$. Thus, stimulation of MAPK by PI3K$\gamma$ requires a tyrosine kinase that, in turn, phosphorylates Shc and induces its association with Grb2. Therefore, receptors coupled to heterotrimeric G-proteins can stimulate the MAPK pathway through $G_{\beta\gamma}$ subunits, PI3K$\gamma$, a tyrosine kinase, and Shc.

## VII. SIGNALING BY HEPTAHELICAL RECEPTORS TO MAPK PATHWAY THROUGH HETEROTRIMERIC G-PROTEIN-INDEPENDENT PATHWAYS

### A. β-Arrestin-Mediated Recruitment of Src Kinase and Activation of MAPK Pathway

Stimulation of $\beta_2$ adrenergic receptors ($\beta_2$ARs) results in the dissociation of heterotrimeric G-proteins into $G_{\alpha s}$-GTP and $G_{\beta\gamma}$ subunits. The release of $G_{\beta\gamma}$ facilitates GRK (G-protein-coupled receptor kinase)-mediated phosphorylation of the agonist-occupied receptor. β-Arrestin-1 functions as an adapter, binding to both GRK-phosphorylated receptor and c-Src. β-Arrestin-1 mutants, disrupted either in c-Src binding or in the ability to target receptors to clathrin-coated pits, block $\beta_2$AR-mediated activation of the MAP kinases Erk1 and Erk2. β-Arrestin-1 binding, which terminates receptor–G-protein coupling, can initiate a second wave of signal transduction in which the "desensitized" receptor functions as a critical structural component of a mitogenic signaling complex.

In HEK-293 cells expressing angiotensin II type 1a receptors (AT1aR), angiotensin stimulation triggers β-arrestin-2 binding to the receptor and internalization of AT1aR-β–arrestin complexes. Within these complexes, β-arrestin-2 acts as a scaffold to assemble component kinases of the MAPK cascade, Raf-1, MEK1, and ERK2.

### B. c-Src

The $\beta_3$AR can directly recruit c-Src. Unlike the other GPCRs, the $\beta_3$AR is not phosphorylated by G-protein receptor kinases and thus does not bind to β-arrestin and undergo internalization. Interestingly, $\beta_3$AR contains proline-rich sequences in both the third intracellular loop and the carboxyl-tail, which appear to mediate the interaction with the SH3 domain of c-Src and activate the MAPK cascade. Disruption of these proline-rich sequences abolishes the ability of the receptor to bind c-Src and to stimulate the MAPK pathway without affecting the signaling by the heterotrimeric G-proteins.

## VIII. MECHANISMS FOR CONTROLLING THE SPECIFICITY OF SIGNALING PATHWAYS

It is apparent that signaling pathways are intertwined with one another to form a large network that is subjected to stimulatory and inhibitory inputs. Such complexity is essential for mediating the pleiotropic biological processes in response to the myriad of extracellular cues. However, it also poses an enormous challenge for the cell to maintain the specificity of the signaling pathways. Several mechanisms have been proposed for the control of specificity in cell signaling.

### A. Cell-Specific Profiling of Signaling Components

As more and more signaling pathways and downstream effectors have been identified, each receptor may activate many potential pathways and effectors. However, the biological outcome of signals generated at the cell surface in response to receptor stimulation is strongly dependent on the developmental stages and cell types. For instance, in early development, FGFR1 plays an important role in the control of cell migration, a process that is crucial for mesodermal patterning and gastrulation, whereas stimulation of FGFR1 in fibroblasts leads to cell proliferation. Moreover, activation of a given membrane receptor by a specific ligand transduces a unique biological response, even though these pathways utilize a common repertoire of proteins. PDGF and EGF, for instance, stimulate unique biological responses in their target tissues, although the intracellular signaling pathways that are activated by PDGF and EGF are very similar indeed.

In addition, activation of the same signaling molecules in different cells leads to distinct responses. For example, stimulation of PI3K by insulin in muscle cells results in the enhancement of metabolic processes, whereas stimulation of PI3K by nerve growth factor (NGF) in neuronal cells leads to an anti-apoptotic signal. Similarly, when cAMP increases on GPCR activation, the MAPK pathway is stimulated in neuronal PC12 cells but inhibited in fibroblast NIH 3T3 cells.

The most plausible explanation for these observations is that there is cell type- and stage-specific expression of signaling components, such as effector proteins and transcriptional factors, in different cell types and at different developmental stages. Therefore, a similar input can lead to a different output in a different cellular context.

## B. Combinatorial Control

Signal specificity can be defined in part by a combinatorial recruitment during signaling processes. For instance, even though every RTK contains a core PTK, there exist some other regulatory elements that recruit and activate a unique set of signaling proteins via their own tyrosine autophosphorylation sites and by means of the tyrosine phosphorylation sites on closely associated docking proteins. The combinatorial recruitment of a particular complement of signaling proteins from a common preexisting pool of signaling cassettes is one mechanism for control of signal specificity. This process is further regulated by differential recruitment of stimulatory and inhibitory proteins by the different receptors and downstream effector proteins, leading to fine-tuning of cellular responses.

## C. Scaffold Proteins

It has been shown that scaffolding proteins that bind simultaneously to several proteins are able to insulate common components of signaling pathways from closely related signaling cascades. In yeast, Sterile 11 (Ste11) is shared by two signaling pathways and functions as the MAPKKK. The scaffolding protein Ste5 has been shown to interact with a pheromone-activated G-protein and with components of the MAP kinase cascade. Ste5 forms a complex with Ste11, Ste7, and Fus3, leading to insulation of the pheromone-induced MAP kinase cascade from the closely related osmolarity response pathway, in which MAPKK Pbs2 acts as both the scaffolding protein and the intermediate kinase that relays signal from MAPKKK Ste11 to downstream MAP kinase Hog1.

Of note, the output of signaling cascades through scaffold protein is determined by a balance of all components. Too much or too little of any component in the scaffolded complexes may decrease the output of the pathway. Therefore, a scaffolded pathway is in principle more sensitive to fluctuations in the concentrations of pathway components, even though scaffolds have the advantage of facilitating efficient signaling with specificity.

## D. Cellular Compartmentalization

In recent years, it has become apparent that the cellular localization of signaling components involved in cell signaling has a profound impact on their biological activity. As mentioned above, AT1aR activation can lead to $G_{\beta\gamma}$ release, which facilitates GRK-mediated phosphorylation of the receptor. The phosphorylated residues on the receptor function as docking sites for β-arrestin translocation from the cytoplasm; the β-arrestin further acts as a scaffolding protein for the assembly of c-Src, Raf-1, MEK1, and ERK2. For RTK signaling, many of the targets of RTKs are located at the cell membrane, and membrane translocation is required for activation of many cellular processes. Binding of SH2, PTB, or SH3 domains to activated receptors or to membrane-linked docking proteins leads to membrane translocation. In addition, membrane translocation is regulated in part by PH or FYVE domains, two protein modules that bind to different phosphoinositides.

The translocation of activated ERK1/2 proteinss from the cytoplasm into the nucleus is another example of the role of protein localization in cell signaling.

## E. Signal Duration and Amplitude

Signal duration and signal strength are essential determinants of signal transmission and biological responses. For instance, RTKs that induce transient stimulation of MAPK (e.g., EGFR) stimulate PC12 cell proliferation, whereas RTKs that stimulate a sustained and robust MAPK response (e.g., NGF receptor, FGFR) promote neuronal differentiation of the same cells. Alternatively, overexpression of EGFR in PC12 cells leads to sustained MAPK response, resulting in cell differentiation, although the same receptors give a proliferative response when expressed at lower levels. These experiments show that the biological outcome (proliferation versus differentiation) is determined by the integrative amplitude of various inputs over time. Signal threshold can be determined by the specific activity of a given RTK and by the balanced action of the various inhibitory or stimulatory signals that are regulated by other pathways. For example, the signal generated by an RTK can be prolonged by increasing the intracellular cAMP level that enhances B-Raf activity by activation of Rap1. Signaling pathways are also subjected to multiple negative feedback mechanisms at the level of the receptor itself by inhibitory protein tyrosine phosphatases and by receptor endocytosis and degradation. In addition, the specific activity of key effector proteins can be negatively regulated by inhibitory signals. The balance between the various stimulatory and inhibitory responses will ultimately determine the strength and duration of the signals that are transmitted through the networks of signaling cascades following their initiation at the cell surface in response to receptor stimulation.

## IX. SUMMARY

It is very common for signaling pathways activated from membrane receptors to be interconnected with one another via complicated intracellular protein networks. As an example, the proliferative response generally controlled by the RTK–MAPK pathway can be regulated by GPCR signaling pathways at multiple levels by different mechanisms. The frequently applied tool of targeted gene disruption used by geneticists for analyzing signaling pathways is complicated by the existence of redundant signaling pathways and multitasking components shared by multiple signaling cascades. Consequently, more sophisticated tools should be developed and applied for the analysis of cellular signaling pathways. To understand the control of the specificity among signaling networks, there is a need for new techniques for determination of protein localization and measurement of kinetics of cellular signaling events in the context of living cells and even in the live animal. In addition, detailed analyses of gene expression patterns by microarray analysis of genes that are expressed in response to growth factor stimulation of cells derived from normal or pathological tissues will reveal new links between signaling pathways. Finally, for the future study of cellular signaling networks, the modern biochemists, geneticists, and transductionists will benefit from the adoption of approaches that have been developed by engineers to describe complicated networks.

## Glossary

**G-protein-coupled receptor** Seven-transmembrane protein that is usually (but not always) coupled to heterotrimeric G-proteins to achieve its biological functions.

**mitogen-activated protein kinase (MAPK) cascade** MAPKs constitute a family of kinases that are activated by phosphorylation in response to extracellular stimuli. MEKs, the kinases that phoshorylate MAPKs, are themselves regulated by phosphorylation. Such sequential chains of kinase phosphorylation and activation are called kinase cascades.

**receptor tyrosine kinase** Transmembrane receptor with a single membrane-spanning region that possesses intrinsic protein tyrosine kinase activity in its cytoplasmic domain and ligand-binding ability in its extracellular domain.

**scaffold protein** A protein capable of binding to multiple signaling components, thereby providing a platform to relay signals and achieve specificity of communication among components.

## See Also the Following Articles

**Activating and Inactivating Receptor Mutations • Coactivators and Corepressors for the Nuclear Receptor Superfamily • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Membrane Receptor Signaling in Health and Disease • Membrane Steroid Receptors • Receptor–Receptor Interactions • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Somatostatin

MALCOLM J. LOW

*Oregon Health and Science University*

I. INTRODUCTION
II. EVOLUTION OF THE SOMATOSTATIN GENE FAMILY
III. SOMATOSTATIN GENE ORGANIZATION AND REGULATION
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VIII. SUMMARY

**Somatostatins are cyclic neuropeptides that exert a broad array of inhibitory and modulatory activities in the endocrine, gastrointestinal, nervous, and immune systems. The archetypal somatostatin, somatotropin release-inhibiting factor, inhibits secretion of pituitary growth hormone. It interacts with five known somatostatin subtypes. The recent development of small-molecule receptor-subtype-specific ligands and new genetic models of somatostatin peptide and receptor mutations promises to provide more definitive answers to the unresolved questions of somatostatin function in extrahypothalamic brain tissue.**

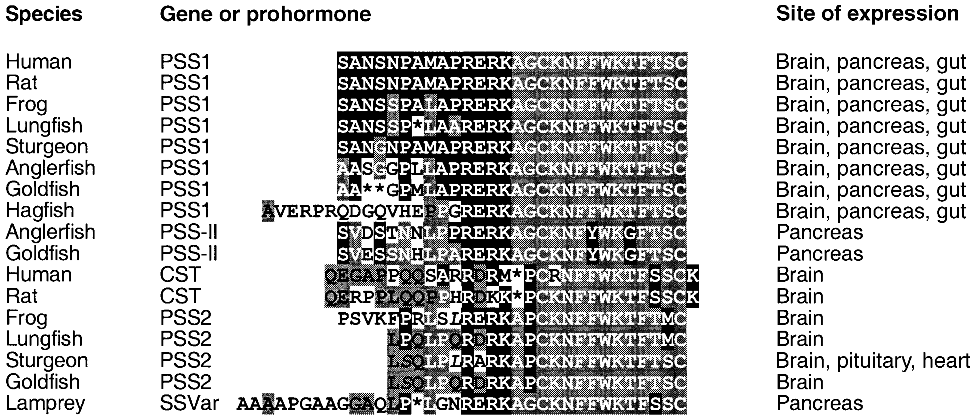
## I. INTRODUCTION

A peptide that potently inhibited growth hormone (GH) release from cultured pituitary cells was unexpectedly identified during the early efforts to isolate a GH-releasing factor from hypothalamic extracts. The factor responsible for both this inhibition of GH secretion and the inhibition of insulin secretion by a pancreatic islet extract was eventually purified from porcine hypothalamus and its amino acid sequence determined by Brazeau and colleagues in 1973. This cyclic peptide containing 14 amino acids was named somatostatin (SS14). Subsequently, a second N-terminal extended form, SS28, was identified as a secretory product from various tissues. Both forms of mammalian somatostatin are derived posttranslationally from a common prohormone by the action of specific prohormone convertases. In addition, the isolation of SS28(1–12) in some tissues suggests that SS14 can also be secondarily processed from SS28. SS14 is the predominant form of somatostatin produced in the brain (including the hypothalamus) and most other tissues, whereas SS28 is found in highest concentrations in the gastrointestinal tract, especially the small intestine.

The biological activities of somatostatin are much wider than the inhibition of GH secretion for which it was named. Somatostatin also inhibits thyrotropin secretion from the pituitary and has nonpituitary roles, including neurotransmitter or neuromodulator activity in the central and peripheral nervous systems and regulatory functions in the gut and pancreas. As a pituitary regulator, somatostatin is a true neurohormone, i.e., a neuronal secretory product that enters the blood (hypophyseal–portal circulation) to affect cell function at remote sites. In the gut, somatostatin is present in both the myenteric plexus (where it acts as a neurotransmitter) and in epithelial cells, where it influences the function of adjacent cells by a paracrine mechanism. Somatostatin can influence its own secretion from pancreatic delta cells (an autocrine function) in addition to acting as a paracrine factor on other endocrine cell types in pancreatic islets. Gut exocrine secretion can be modulated by intralumenal action, so somatostatin can also be considered to be a lumone. Because of its wide distribution, broad spectrum of regulatory effects, and evolutionary history, this peptide can be regarded as an archetypal gut–brain peptide.

## II. EVOLUTION OF THE SOMATOSTATIN GENE FAMILY

The genes that encode somatostatin in humans and a number of other species exhibit striking sequence homology, even in primitive jawless fish (Fig. 1).



| Species | Gene or prohormone | | Site of expression |
|---|---|---|---|
| Human | PSS1 | SANSNPAMAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Rat | PSS1 | SANSNPAMAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Frog | PSS1 | SANSSPALAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Lungfish | PSS1 | SANSSP*LAARERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Sturgeon | PSS1 | SANGNPAMAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Anglerfish | PSS1 | AASCGPLLAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Goldfish | PSS1 | AA**GPMLAPRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Hagfish | PSS1 | AVERPRQDGQVHEPPGRERKAGCKNFFWKTFTSC | Brain, pancreas, gut |
| Anglerfish | PSS-II | SVDSTNNLPPRERKAGCKNFYWKGFTSC | Pancreas |
| Goldfish | PSS-II | SVESSNHLPARERKAGCKNFYWKGFTSC | Pancreas |
| Human | CST | QEGAPPQQSARRDRM*PCRNFFWKTFSSCK | Brain |
| Rat | CST | QERPPLQQPPHRDKK*PCKNFFWKTFSSCK | Brain |
| Frog | PSS2 | PSVKFPRLS*L*RERKAPCKNFFWKTFTMC | Brain |
| Lungfish | PSS2 | LPQLPQRDRKAPCKNFFWKTFTMC | Brain |
| Sturgeon | PSS2 | LSQLP*L*RARKAPCKNFFWKTFTSC | Brain, pituitary, heart |
| Goldfish | PSS2 | LSQLPQRDRKAPCKNFFWKTFTSC | Brain |
| Lamprey | SSVar | AAAAPGAAGGAQLP*LGNRERKAGCKNFFWKTFSSC | Pancreas |

FIGURE 1 Amino acid sequence comparisons of somatostatin-like peptides in species of different vertebrate taxa. The conserved mammalian sequence of SS28 derived from prosomatostatin 1 (PSS1) is shown for humans and rats. The cyclic SS14 peptide (gray shading) at the C-terminus of SS28 has been conserved in all vertebrate PSS1 genes. Teleost fish have a second gene encoding PSS-II that is proteolytically processed to a SS28-like peptide characterized by the C-terminal sequence of [Tyr$^7$, Gly$^{10}$]SS14. A mammalian cortistatin (CST) gene encodes either human CST17 (DRMPCRNFFWKTFSSCK) or rat CST14 (PCKNFFWKTFSSCK), both of which have 11 amino acid homologies with SS14. Frog, lungfish, sturgeon, and goldfish have a PSS2 gene that may be the orthologue of mammalian CST. Lampreys (*Lampetra fluviatilis*) produce a variant of SS14 (SSVar) containing the [Ser$^{12}$] but not the [Pro$^2$] substitutions present in CST. Amino acid identity among sequences is indicated by the different combinations of font shading. An asterisk ( * ) has been inserted in some sequences to maximize alignment.

Furthermore, the amino acid sequence of SS14 is identical in all vertebrates. Until recently, it was accepted that all tetrapods have a single gene encoding both SS14 and SS28 whereas teleost fish have two nonallelic prosomatostatin genes (PSS1 and PSS-II), each of which encodes only one form of the mature somatostatin peptides. Comparative endocrinologists have inferred that a common ancestral gene underwent a gene duplication event after the split of teleosts from the ancestors of tetrapods.

However, both lampreys and amphibians, which pre- and postdate the teleost evolutionary divergence, respectively, have now been shown to contain at least two distinct PSS genes. A more distantly related gene identified in mammals encodes cortistatin (CST), a somatostatin-like peptide that is highly expressed in cortex and hippocampus. Rat CST14 differs from SS14 by three amino acid residues but has high affinity for all known subtypes of somatostatin receptors (see later). The human gene sequence predicts a tripeptide-extended CST17 and a further N-terminally extended CST29 (Fig. 1). A revised evolutionary concept of the somatostatin gene family is that a primordial gene underwent duplication during or before the advent of chordates and the two resulting genes subsequently underwent differing rates of mutation to produce the distinct prosomatostatin and procortistatin genes in mammals. A second gene duplication likely occurred in teleosts to generate PSS1 and PSS-II from the ancestral somatostatin gene.

## III. SOMATOSTATIN GENE ORGANIZATION AND REGULATION

The mammalian gene has a relatively simple organization consisting of two coding exons separated by one intron (Fig. 2). A single promoter directs transcription of the PSS1 gene in all tissues and there are no known alternative mRNA splicing events. Apart from its expression in neurons of the periventricular and arcuate hypothalamic nuclei and involvement in GH secretion, somatostatin is highly expressed in the cortex, lateral septum, extended amygdala, reticular nucleus of the thalamus, hippocampus, and many brain stem nuclei in human and rat brain tissue. Cortistatin, present in the brain at a small fraction of the levels of somatostatin and in a more limited distribution, is primarily confined to the cortex and hippocampus.

The molecular mechanisms underlying the developmental and hormonal regulation of somatostatin gene transcription have been most extensively studied in pancreatic islets and islet-derived cell lines. Less is known concerning the regulation of somatostatin

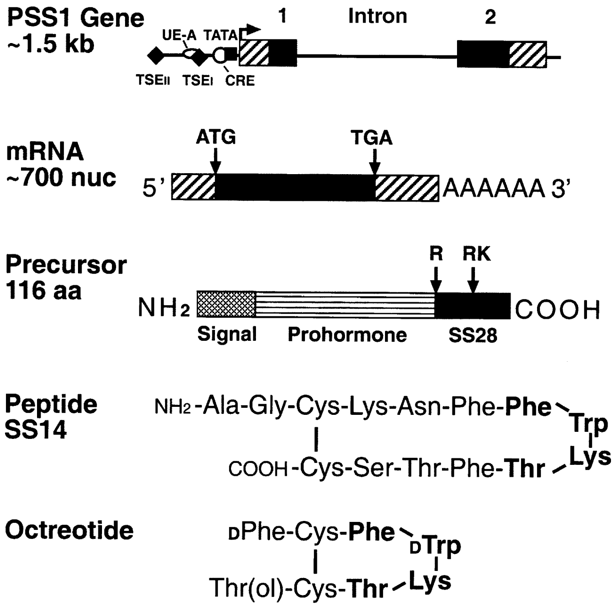


FIGURE 2 Summary of somatostatin biosynthesis in mammals. Structures of the mammalian PSS1 gene, mRNA, precursor protein, mature SS14 peptide, and the synthetic SSTR agonist octreotide are presented schematically. The promoter elements in the PSS1 gene (TSE, UE-A, CRE, and TATA) are discussed in the text. The 5′ and 3′ untranslated regions of exons 1 and 2 and within the corresponding polyadenylated mRNA are indicated by diagonal stripes. ATG, Translation initiation codon; TGA, translation stop codon; R, arginine; K, lysine.

gene expression in neurons, except that activation is strongly controlled by binding of the phosphorylated transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) to its cognate CRE contained in the promoter immediately 5′ to a consensus TATA sequence. Experiments performed in transgenic mice suggest that accurate neural-specific somatostatin gene expression requires distal genomic elements in addition to the proximal 300 nucleotides that are sufficient for islet cell expression. The proximal enhancer elements in the somatostatin gene promoter that bind complexes of homeodomain-containing transcription factors (PAX6, PBX, and PREP1) and up-regulate transcription in pancreatic islets may actually represent gene silencer elements in neurons (promoter elements $\text{TSE}_{\text{II}}$ and UE-A). Conversely, another related cis-element in the somatostatin gene (promoter element $\text{TSE}_{\text{I}}$) apparently binds a homeodomain transcription factor PDX1 (also called STF1/IDX1/IPF1) that is common to developing brain, pancreas, and foregut and regulates gene expression in both the central nervous system (CNS) and gut.

Similar to other genes encoding polypeptide precursors, the posttranslational intracellular trafficking and processing of PSS represent additional control points in the expression of somatostatin (Fig. 2). A peptide motif assuming an amphipathic $\alpha$-helical conformation near the N-terminus of PSS appears to play an essential role in targeting the prohormone through the endoplasmic reticulum and Golgi compartments that constitute part of the regulated secretory pathway. A second highly conserved amino acid motif, NPAMAP (in single-letter code: N, asparagine; P, proline; A, alanine; M, methionine) within the SS28(1–12) sequence is important for directing the endoproteolytic processing of PSS1 to either SS28 at the 5′ single basic amino acid (Arg) residue or to SS14 at the 3′ paired basic amino acid (Arg-Lys) residues by appropriate prohormone convertases.

## IV. SOMATOSTATIN RECEPTORS

Five somatostatin receptor subtypes (SSTR1–SSTR5) have been identified by gene cloning techniques, and one of these (SSTR2) is expressed in two alternatively spliced forms, SSTR2a and SSTR2b. The SSTRs are members of the rhodopsin-like G-protein-coupled receptor clan, and their unique amino acid signature is provided by a seven-element fingerprint of peptide sequences located in conserved regions of the N- and C-termini, extra- and intracellular loops, and transmembrane domains. They are most closely related to the vertebrate opioid receptors and the invertebrate allatostatin receptor family. The five SSTR subtypes are encoded by separate genes located on different chromosomes, are expressed in unique or partially overlapping distributions in multiple target organs, and differ in their coupling to second-messenger signaling molecules, and therefore in their range and mechanism of intracellular actions. The subtypes also differ in their binding affinity to specific somatostatinergic ligands. Some of these differences have important implications for the use of somatostatin analogues in diagnostic imaging and in pharmacotherapy.

All SSTR subtypes are coupled to pertussis-toxin-sensitive G-proteins and bind SS14 and SS28 with high affinity in the low-nanomolar range, although SS28 has a modestly higher affinity for SSTR5. SSTR1 and SSTR2 are the two most abundant subtypes in brain and probably function as both postsynaptic receptors and presynaptic autoreceptors in the hypothalamus and limbic forebrain, respectively. SSTR4 is most highly expressed in the hippocampus. SSTR3 is uniquely localized on nonmotile neuronal cilia, structures that have an unknown role in neuronal signaling. All the subtypes are expressed in the pituitary, but SSTR2 and SSTR5 are the most abundant receptors on somatotrophs. These two subtypes are also the most physiologically important in pancreatic islets. SSTR5 is responsible for inhibition of insulin secretion from beta cells and SSTR2 is essential for inhibition of glucagon from alpha cells (Table 1).

Binding of somatostatin to its receptors leads to activation of one or more inhibitory G-proteins ($G_i/G_o$), which in turn inhibit adenylyl cyclase activity and decrease the concentration of intracellular cAMP. Other G-protein-mediated actions common to all SSTRs are activation of a vanadate-sensitive phosphotyrosine phosphatase (PTP) and modulation of mitogen-activated protein kinases (MAPKs). MAPK activity has been reported to be increased by SSTR1 and SSTR4, decreased by SSTR2 and SSTR5, and modulated in both directions by SSTR3. Different subsets of SSTRs can activate inwardly rectifying $K^+$ channels and/or inhibit voltage-dependent $Ca^{2+}$ channels. The net effects of SSTR coupling to these membrane conductances include hyperpolarization of the resting membrane potential, decreased frequency of action potentials, and decreased probability of vesicle release from nerve terminals. SSTR1 also activates a $Na^+/H^+$ exchanger and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate glutamate receptors, whereas SSTR2 inhibits non-*N*-methyl-D-aspartate (NMDA) glutamate receptors in some cells. SSTR2 and SSTR4 have been reported to couple positively to phospholipase C/inositol 1,4,5-trisphosphate and phospholipase $A_2$ activities, respectively.

The lowering of intracellular cAMP and $Ca^{2+}$ are the most important mechanisms for the inhibition of hormone secretion from endocrine cells, whereas actions on PTP and MAPK are postulated to play a role in antiproliferative effects of somatostatin on tumor cells. Recent provocative data suggests that SSTRs may form oligomeric complexes, including in some cases heterodimers with non-SSTRs, greatly increasing the possible signaling complexity of somatostatin ligands.

## V. REGULATION OF HORMONE SECRETION BY SOMATOSTATIN

### A. Inhibition of GH Secretion and GH Negative Feedback

In the pituitary, somatostatin directly inhibits secretion of GH and thyrotropin and, under limited conditions, of prolactin and adrenocorticotropic hormone (ACTH), from their respective cell types. However, the full role of somatostatin in the regulation of GH secretion is much more complex and involves a constant interplay with hypothalamic growth hormone-releasing hormone (GHRH), circulating hormones, and additional modulatory peptides at the level of both the pituitary and the hypothalamus. The predominant hypothalamic influence on GH release is stimulatory, thus section of the pituitary stalk or lesions of the ventromedial hypothalamus cause reductions of basal and induced GH release. When the somatostatinergic component is inactivated (e.g., by antisomatostatin antibody injection in rats), basal GH levels and GH responses to the usual provocative stimuli are enhanced. Negative feedback

TABLE I Diverse Inhibitory Functions of Somatostatin on Nervous, Endocrine, Gastrointestinal, and Immune System Cell Secretion[a]

| Organ | Cell type | Factor inhibited | SS receptor |
|---|---|---|---|
| Brain (hypothalamus) | SS neuron | Somatostatin | SSTR1 |
| Brain (hypothalamus) | GHRH neuron | GHRH | SSTR2 |
| Brain (hippocampus) | CA1 pyramidal | Glutamate | SSTR4 |
| Pituitary gland | Somatotroph | Growth hormone | SSTR2, SSTR5 |
| Pancreatic islet | β cell | Insulin | SSTR5 |
| Pancreatic islet | α cell | Glucagon | SSTR2 |
| Stomach | Parietal cell | HCl | SSTR2 |
| Immune system | T lymphocyte | Interferon γ | SSTR2 |

[a] Abbreviations: SS, somatostatin; GHRH, growth hormone-releasing hormone; SSTR, somatostatin receptor.

control of GH release is mediated by GH and by insulin-like growth factor-I (IGF-I), which is synthesized in the liver under control of GH. Direct GH effects on the hypothalamus act by short-loop feedback, whereas those involving IGF-I and other circulating factors influenced by GH, including free fatty acids and glucose, are long-loop systems analogous to the pituitary–thyroid and pituitary–adrenal axes. Control of GH secretion thus includes two closed-loop systems (GH and IGF-I) and one open-loop regulatory system (neural) (Fig. 3).

Although most of the evidence for a direct role of GH in its own negative feedback has been derived from animals, an elegant study in normal men demonstrated that GH pretreatment blocks the subsequent GH secretory response to GHRH by a pathway that is dependent on somatostatin. The mechanism responsible for this GH feedback via the hypothalamus has been largely elucidated in rodent models. GH receptors are selectively expressed on somatostatin neurons in the hypothalamic periventricular nucleus and on neuropeptide Y (NPY) neurons in the arcuate nucleus. Expression of the c-*fos* gene in both populations of GH receptor-positive neurons is acutely elevated by GH administration, indicating an activation of hypothalamic circuitry that includes these neurons. Data from many experiments strongly support a model of GH negative feedback regulation that involves the primary activation of periventricular somatostatin neurons by GH. These tuberoinfundibular neurons then inhibit GH secretion directly by release of somatostatin in the median eminence, but in addition they indirectly inhibit GH secretion by way of collateral axonal projections to the arcuate nucleus that synapse on and inhibit GHRH neurons (Fig. 3).

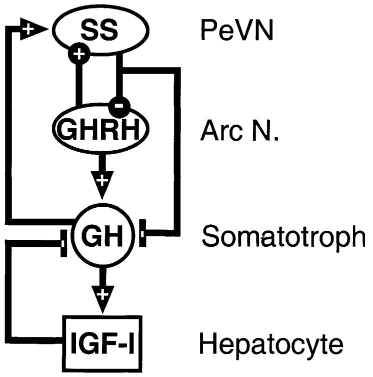


**FIGURE 3** Regulatory feedback loops in the hypothalamic control of pituitary growth hormone secretion. Growth hormone-releasing hormone (GHRH) and somatostatin (SS) are the principal stimulatory and inhibitory factors mediating GH secretion. GH stimulates the synthesis and release of insulin-like growth factor-I (IGF-I) from hepatocytes, which inhibits GH secretion from the pituitary. GH also inhibits its own secretion by a short-loop feedback circuit involving the stimulation of somatostatin neurons in the periventricular nucleus (PeVN) of the hypothalamus. A common neural mechanism leading to the inhibition of GH secretion is the activation of somatostatin neurons. Stimulatory and inhibitory neuronal synapses are represented by circular terminals marked with a plus or minus sign, respectively. Stimulatory and inhibitory hormonal pathways are represented by arrows with a plus sign or bars with a minus sign, respectively. Arc N, Arcuate nucleus.

## B. Neural Regulation of Tuberoinfundibular Somatostatin and GHRH

GHRH-containing nerve fibers that terminate adjacent to portal vessels in the external zone of the median eminence arise principally from within, above, and lateral to the infundibular nucleus in the human hypothalamus, corresponding primarily to the rodent arcuate nucleus. Perikarya of the tuberoinfundibular somatostatin neurons are located almost completely in the medial periventricular nucleus and parvocellular component of the anterior paraventricular nucleus. Neuroanatomical and functional evidence suggests a bidirectional synaptic interaction between the two peptidergic systems.

Multiple extrahypothalamic brain regions provide efferent connections to the hypothalamus and regulate GHRH and somatostatin neuronal activity. Somatosensory and affective information are integrated and filtered through the amygdaloid complex. The basolateral amygdala provides an excitatory input to the hypothalamus whereas the central extended amygdala, which includes the central and medial nuclei of the amygdala together with the bed nucleus of the stria terminalis, provides a $\gamma$-aminobutyric acid (GABA)ergic inhibitory input. Many intrinsic neurons of the hypothalamus also release GABA, often with a peptide cotransmitter. Excitatory cholinergic fibers arise to a small extent from forebrain projection nuclei, but mostly from hypothalamic cholinergic interneurons, which densely innervate the external zone of the median eminence. Similarly, the origin of dopaminergic and histaminergic neurons is local, with their cell bodies located in the hypothalamic arcuate and tuberomammillary bodies, respectively. Two important ascending pathways to the medial basal hypothalamus regulate GH secretion and they originate from serotonergic neurons in the raphe nuclei and adrenergic neurons in the nucleus of the tractus solitarius and ventral lateral nucleus of the medulla.

Both GHRH and somatostatin neurons express pre- and postsynaptic receptors for multiple neurotransmitters and peptides. The α2-adrenoreceptor agonist clonidine reliably stimulates GH release, and for this reason a clonidine test has been a standard diagnostic tool in pediatric endocrinology. The net stimulatory effect on GH secretion appears to involve a dual mechanism of action—inhibition of somatostatin neurons and activation of GHRH neurons—and is blocked by the specific α2 antagonist yohimbine. In addition, a partial attenuation of the effects of clonidine by 5-hydroxytryptamine ($5\text{-HT}_1/5\text{-HT}_2$) antagonists suggests that some of the relevant α2 receptors are located presynaptically on serotonergic nerve terminals and increase serotonin release. Both norepinephrine and epinephrine play physiological roles in the adrenergic stimulation of GH secretion. Adrenergic α1 agonists have no effect on GH secretion in humans but β2 agonists such as the bronchodilator salbutamol inhibit GH secretion by stimulating the release of somatostatin from nerve terminals in the median eminence. These effects are blocked by propranolol, a nonspecific β antagonist. Dopamine generally has a net effect of stimulating GH secretion, but the relative importance of different dopamine receptor subtypes and their localization on specific neuronal structures in the hypothalamus is not known.

The effect of serotonin (5-hydroxytryptamine) on GH release in humans has been difficult to decipher because of the large variety of 5-HT receptor subtypes. However, clinical studies with the receptor-selective agonist sumatriptan clearly implicate the $5\text{-HT}_{1D}$ receptor subtype in the stimulation of basal GH levels. The drug also potentiates the effect of a maximal dose of GHRH, invoking in its mechanism of action the recurring theme of GH disinhibition by inhibition of hypothalamic somatostatin neurons. Histaminergic pathways acting through H1 receptors play only a minor, conditional stimulatory role in GH secretion in man.

Acetylcholine (ACh) appears to be an important physiological regulator of GH secretion. Blockade of muscarinic (m1) ACh receptors reduces or abolishes GH secretory responses to GHRH, glucagon, arginine, morphine, and exercise. In contrast, drugs that potentiate cholinergic transmission increase basal GH levels and enhance GH response to GHRH in normal individuals or in patients with obesity or Cushing's disease. *In vitro*, acetylcholine inhibits somatostatin release from hypothalamic fragments, and acetylcholine can act directly on the pituitary to inhibit GH release. There may even be a paracrine cholinergic control system within the pituitary. However, the sum of evidence suggests that the primary mechanism of action of m1 agonists is to inhibit somatostatin neuronal activity or the release of peptide from somatostatinergic terminals.

Many neuropeptides in addition to GHRH and somatostatin are involved in the modulation of somatotroph activity in the human. GH secretion is stimulated by galanin, opioid peptides, and ghrelin, the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), each of which act at least in part by a GHRH-dependent mechanism. A larger number of neuropeptides are known or suspected to inhibit GH secretion in humans, at least under certain circumstances. The list includes NPY, corticotropin-releasing hormone (CRH), calcitonin, oxytocin, neurotensin, vasoactive intestinal peptide (VIP), and thyrotropin-releasing hormone (TRH). Inhibitory actions of NPY are well established in the rat. Its effect on GH secretion is secondary to stimulation of somatostatin neurons and is of particular interest because of the presumed role in GH autofeedback (discussed earlier) and the integration of GH secretion with regulation of energy intake and expenditure.

## C. Somatostatin and GH Secretory Rhythms

GH secretion in young adults exhibits a true circadian rhythm over a 24-h period, characterized by greater nocturnal secretion that is independent of sleep onset. However, GH release is further facilitated when slow-wave sleep coincides with the normal circadian peak. Under basal conditions, GH levels are low most of the time, with an ultradian rhythm of about 10 (men) or 20 (women) secretory pulses per 24 h, as calculated by deconvolution analysis. Both sexes have an increased pulse frequency during the nighttime hours, but the fraction of total daily GH secretion associated with the nocturnal pulses is much greater in men. Overall, women have more continuous GH secretion and more frequent GH pulses that are of more uniform size compared to men. These sexually dimorphic patterns in the human are actually quite similar to those in the rat, although not as extreme.

The neuroendocrine basis for sex differences in the ultradian rhythm of GH secretion is not fully understood. Gonadal sex steroids play both an organizational role during development of the hypothalamus and an activational role in the adult, in which they regulate gene expression of many of the peptides and receptors central to GH regulation. In the human, unlike the rat, the hypothalamic actions of testosterone appear to be predominantly due to its aromatization to 17β-estradiol and interaction with estrogen receptors. Hypothalamic somatostatin appears to play a more prominent role in men than in women for the regulation of pulsatile GH secretion, and this difference is postulated to be a key factor in producing the sexual dimorphism.

### D. Somatostatin Effects on Gastrointestinal and Immune Systems

Somatostatin exerts inhibitory effects on virtually all endocrine and exocrine secretions of the pancreas, gut, and gallbladder (Table 2). Somatostatin also inhibits secretion by the salivary glands and, under some conditions, the secretion of parathyroid hormone and calcitonin. Somatostatin blocks hormone release in many endocrine-secreting tumors, including insulinomas, glucagonomas, VIPomas, carcinoid tumors, and some gastrinomas. Somatostatin and SSTR expression are coinduced by inflammatory and immune reactions in macrophages, T lymphocytes, splenocytes, and synovial fibroblasts in rheumatoid arthritis, consistent with paracrine or autocrine modulation of proliferative and hormonal responses in these cells.

**TABLE 2 Physiologic Effects of Somatostatin in the Gastrointestinal Tract and Other Tissues**

| Inhibits hormone secretion from | Inhibits other gastrointestinal actions |
|---|---|
| Stomach and intestine | Gastric acid secretion |
| Gastrin | Gastric and jejunal fluid secretion |
| Secretin | Gastric emptying |
| Gastrointestinal polypeptide | Pancreatic bicarbonate secretion |
| Motilin | Pancreatic enzyme secretion |
| Glicentin (enteroglucagon) | (stimulates intestinal absorption of water and electrolytes) |
| Vasoactive intestinal peptide | Gastrointestinal blood flow |
| Pancreatic islets | Vasopressin-stimulated water transport |
| Insulin | Bile flow |
| Glucagon | Extragastrointestinal actions |
| Somatostatin | Inhibits the function of activated immune cells |
| Genitourinary tract | Induction of apoptosis |
| Renin | Inhibition of tumor growth |

## VI. EXTRAHYPOTHALAMIC SOMATOSTATIN AND BRAIN FUNCTION

The physiological actions of somatostatin in extrahypothalamic brain tissue remain the subject of active investigation. In the striatum, somatostatin increases the release of dopamine from nerve terminals by a glutamate-dependent mechanism involving SSTR2. In the pontine reticular formation, somatostatin blocks fear-potentiated acoustic startle responses and attenuates the increase of neuronal activity produced by the application of glutamate. Somatostatin is widely coexpressed with NPY in the limbic cortex and hippocampus GABAergic interneurons that modulate the excitability of pyramidal neurons. Temporal lobe epilepsy is associated with a marked reduction in somatostatin-expressing neurons in the hippocampus, consistent with a putative inhibitory action on seizures. Somatostatin has general arousal properties and induces rapid eye movement (REM) sleep whereas cortistatin, in contrast, has neuronal depressant actions and stimulates slow-wave sleep. A wealth of correlative data has linked reduced forebrain and cerebrospinal fluid concentrations of somatostatin with Alzheimer's disease, major depression, and other neuropsychiatric disorders, raising speculation about the role of somatostatin in modulating neural circuits underlying cognitive and affective behaviors.

## VII. DIAGNOSTIC AND THERAPEUTIC USES OF SOMATOSTATIN

### A. Somatostatin Analogues and Pharmacotherapy

An extensive pharmaceutical discovery program has been ongoing to synthesize somatostatin analogues with receptor subtype selectivity and improved pharmacokinetics and oral bioavailability compared to the native peptide. Initial efforts focused on the rational design of constrained cyclic peptides that incorporated D-amino acid residues and included the [$\mathrm{Trp}^8$-$\mathrm{Lys}^9$] dipeptide of somatostatin, which structure-function studies demonstrated is necessary for high-affinity binding of the peptide to its receptor. Many such analogues have been studied in clinical trials, including octreotide (Fig. 2), lanreotide, vapreotide, and the hexapeptide MK678. Each of these compounds is an agonist with similarly high-affinity binding to SSTR2 and SSTR5, moderate binding to SSTR3, and no (or low) binding to SSTR1 and SSTR4. More recently, a combinatorial chemistry approach has led to a new generation of nonpeptidyl somatostatin agonists that bind selectively and with subnanomolar affinity to each of the five SSTR subtypes. In contrast to the marked success in development of potent and selective somatostatin agonists, there is a relative paucity of useful antagonists.

The actions of octreotide (SMS 201-995, or Sandostatin) illustrate the general potential of somatostatin analogues in therapy. This compound controls excess secretion of GH in acromegaly in most patients and shrinks tumor size in about one-third. Octreotide is also indicated for the treatment of thyrotropin-secreting adenomas that recur after surgery. It is used to treat other functioning, metastatic neuroendocrine tumors, including carcinoid, VIPoma, glucagonoma, and insulinoma, but is seldom of use for the treatment of gastrinoma. It is also useful in the management of many forms of intractable diarrhea (acting on salt and water excretion mechanisms in the gut) and to reduce external secretions in pancreatic fistulas (thus permitting healing). A decrease in blood flow to the gastrointestinal tract is the basis of its use in bleeding esophageal varices associated with hepatic cirrhosis and portal hypertension, but it is not effective in the treatment of bleeding from peptic ulcers.

The only major undesirable side effect of octreotide is reduction of bile production and of gallbladder contractility, leading to "sludging" of bile and an increased incidence of gallstones. Other common adverse effects, including nausea, abdominal cramps, flatulence, and diarrhea secondary to malabsorption of fat, usually subside spontaneously within 2 weeks of continued treatment. Long-term octreotide therapy is not associated with impaired glucose tolerance, despite an inhibitory effect on insulin secretion, presumably because of compensating reductions in carbohydrate absorption and GH and glucagon secretion that are also caused by the drug.

## B. Somatostatin Receptor Imaging and Cytotoxic Therapies

Somatostatin analogues labeled with a radioactive tracer have been used as external imaging agents for a wide range of disorders. A $^{111}$In-labeled analogue of octreotide (Octreoscan) has been approved for clinical use in the United States and several other countries. A majority of the neuroendocrine tumors and many pituitary tumors that express SSTRs are visualized by external imaging techniques after administration of this agent; a variety of nonendocrine tumors and inflammatory lesions are also visualized, all of which have in common the expression of SSTRs. Such tumors include non-small-cell cancer of the lung, meningioma, breast cancer, and astrocytomas. Because activated T cells of the immune system display SSTRs, inflammatory lesions that take up the tracer include sarcoidosis, Wegener's granulomatosis, tuberculosis, and many cases of Hodgkin's disease and non-Hodgkin's lymphoma. Although the tracer lacks specificity in differential diagnosis, its ability to identify the presence of abnormality and the extent of the lesion provides important information for management, including tumor staging. The use of a portable radiation detector in the operating room makes it possible to ensure the completeness of removal of medullary thyroid carcinoma metastases. New developments in the synthesis of positron emission tomography (PET) tracers chelated to octreotide have allowed for the sensitive detection of meningiomas less than 1 cm in diameter and located beneath osseous structures at the base of the skull.

The ability of somatostatin to inhibit the growth of normal and some neoplastic cell lines and to reduce the growth of experimentally induced tumors in animal models has stimulated interest in somatostatin analogues for the treatment of cancer. Somatostatin's tumorostatic effects may be a combination of direct actions on tumor cells due to inhibition of growth factor receptor expression, inhibition of MAPK, and stimulation of PTP. SSTR1, SSTR2, SSTR4, and SSTR5 can all promote cell cycle arrest associated with induction of the tumor suppressor retinoblastoma (Rb) and p21, whereas SSTR3 can trigger apoptosis accompanied by induction of the tumor suppressor p53 and the proapoptotic protein Bax. In addition, somatostatin has indirect effects on tumor growth by its inhibition of circulating, paracrine, and autocrine tumor growth-promoting factors and it can also modulate the activity of immune cells and influence tumor blood supply. Despite this promise, the therapeutic utility of octreotide as an antineoplastic agent remains controversial.

Two new treatment approaches in preclinical trials may yet effectively utilize somatostatin receptors in the arrest of cancer cells. The first is receptor-targeted radionuclide therapy using octreotide chelated to a variety of $\gamma$- or $\beta$-emitting radioisotopes. Theoretical calculations and empirical data suggest that radiolabeled somatostatin analogues can deliver a tumoricidal radiotherapeutic dose to some tumors after receptor-mediated endocytosis. A variation on this theme is the chelation of a cytotoxic chemotherapeutic agent to a somatostatin analogue. A second approach involves somatic-cell gene therapy to transfect SSTR-negative pancreatic cancer cells with an SSTR gene. Therapeutic results could occur from the creation of autocrine or paracrine inhibitory growth effects or with the addition of targeted radionuclide treatments.

## VIII. SUMMARY

Somatostatin is a phylogenetically ancient peptide that occupies numerous regulatory niches in diverse organ systems. Comparative genomic analyses suggest that at least two gene duplications of a primordial somatostatin gene have occurred in the past 500 million years of vertebrate evolution, yielding a family of related peptides that signal through five different subtypes of somatostatin receptors. In the simplest terms, somatostatin can be considered a paninhibitory factor for a large number of hormones, including pituitary growth hormone, cytokines, and exocrine secretions, in addition to its roles in modulating neuronal activity and regulating cell proliferation. A great deal of largely circumstantial evidence points to widespread activity of somatostatin within neural circuits underlying locomotor, cognitive, and emotional processes.

## Glossary

**cAMP response element** Consensus nucleotide sequence 5′-TGACGTCA-3′; found in the promoter of many hormone-regulated genes, including somatostatin; a specific binding site for the transcriptional activator cAMP response element binding protein (CREB).

**hypophyseotropic hormones** Neurotransmitters, including neuropeptides, dopamine, and $\gamma$-aminobutyric acid; secreted from tuberoinfundibular neurons and conveyed as hormones through the long portal vessels, acting at a distance on pituitary cells and regulating their function.

**mitogen-activated protein kinases** Protein serine/threonine kinases that mediate intracellular signaling through three major mitogen-activated protein kinase cascades: ERK1/ERK2, JNK/SAPK, and p38.

**octreotide** A commonly used eight-residue peptidyl analogue of SS14 with a long biological half-life and efficacy as an agonist at SSTR2 and SSTR5.

**phosphotyrosine phosphatase** Enzyme that catalyzes the dephosphorylation of tyrosine residues from signaling molecules and thereby opposes the action of protein tyrosine kinases, which are key mediators of cellular responses such as proliferation and differentiation.

**prohormone** A precursor (either peptide or steroid) to an active hormone; produced in significant amounts as an intermediate in the pathway of production of the active hormone.

**somatostatins** Cyclic neuropeptides, the archetype being SS14, or somatotropin release-inhibiting factor, so named for one of its biological activities, inhibition of the secretion of pituitary growth hormone.

**somatostatin receptors, types 1–5** Five subtypes of G-protein-coupled, seven-transmembrane-domain somatostatin receptors.

**tuberoinfundibular neurons** Located in several nuclei of the medial basal hypothalamus; project axons to the median eminence and infundibulum (pituitary stalk) and release their neurotransmitters adjacent to the fenestrated, tuberohypophyseal portal blood vessels supplying the pituitary gland.

## See Also the Following Articles

**Ghrelin • Glucagonoma Syndrome • Growth Hormone-Releasing Hormone (GHRH) and the GHRH Receptor • Growth Regulation: Clinical Aspects of GHRH • Peptide YY • Peptidomimetics**

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# Spermatogenesis, Hormonal Control of

Sarah Meachem and Robert McLachlan

*Prince Henry's Institute of Medical Research, Australia*

I. INTRODUCTION
II. OVERVIEW OF THE HYPOTHALAMIC–PITUITARY–TESTICULAR AXIS
III. ORGANIZATION OF THE TESTIS
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V. FSH DEPENDENCY
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VII. FSH AND TESTOSTERONE SYNERGY
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IX. CONCLUSION

**The process of spermatogenesis in the adult testis depends on two pituitary hormones, follicle-stimulating hormone and luteinizing hormone; luteinizing hormone, in turn, stimulates the production of androgens, notably testosterone. Specific cellular sites during spermatogenesis are specifically and conjointly regulated by hormonal activities. Production of viable, healthy sperm is a complex process that requires a high degree of cellular, hormonal, and molecular interactions; understanding the precise mechanisms of hormonal interaction is significant in developing a strategy for the design of an effective male hormonal contraceptive.**

## I. INTRODUCTION

Production of fertile sperm, a testicular process under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), is necessary for reproductive viability. LH stimulates the production of androgens, notably testosterone, which is required for the attainment of male secondary sexual characteristics. Results from a wide array of animal and human experiments have identified specific cellular sites during spermatogenesis that are specifically and conjointly regulated by FSH and testosterone. It appears, however, that FSH is not essential for sperm production, because animals congenitally lacking FSH can be fertile, although their testis size and total sperm output are diminished. It seems that FSH plays an important role in establishing the foundations for full adult spermatogenesis by controlling the population of Sertoli cells, which determine adult spermatogenic capacity. In addition, FSH acts to optimize sperm production via involvement in multiple steps of spermatogenesis, most notably the steps involving the earliest germ cell forms, the spermatogonia. Testosterone is essential for spermatogenesis, particularly in supporting spermatid meiosis and maturation. Understanding the basic physiology of sperm production has important ramifications for understanding the common problem of male infertility and, conversely, in the design of a hormonal contraceptive. Thus far, clinical studies of androgen-based contraception have shown that there are two sites of inhibition, both dependent on gonadotropin suppression, at the level of the spermatogonia and sperm release. The following brief review of the basic endocrine control and organization of the testis prefaces a more detailed discussion of the hormonal control of spermatogenesis.

## II. OVERVIEW OF THE HYPOTHALAMIC–PITUITARY–TESTICULAR AXIS

The adult testis has two main functions, to produce sperm for fertility and to produce androgens for maintaining secondary sexual traits. Sperm and androgen production are dependent on stimulation by the gonadotropins LH and FSH. The gonadotropins are produced by and secreted from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) stimulation from the hypothalamus. LH exerts its effects on testicular Leydig cells to stimulate the production and secretion of androgens, most notably testosterone. Testosterone is essential for the initiation and maintenance of spermatogenesis and exerts its effects on the germ cells via receptors on Sertoli, Leydig, and peritubular cells. FSH acts via specific G-protein-coupled surface receptors located exclusively on Sertoli cells and is important for normal testicular growth and sperm production (see Fig. 1).

The process of sperm production, or spermatogenesis, in many mammals is a continuous process that takes place throughout the reproductive life span of the animal. However, in some mammals, spermatogenesis shows marked seasonal variation; in others, a single wave of spermatogenesis is followed by sterility. Spermatogenesis is a complicated process in which stem cells (called spermatogonia) undergo a complex sequence of proliferative and differentiation steps before they give rise to mature sperm (Fig. 1). Spermatogonia must proceed through three main phases before giving rise to mature sperm: (1) spermatogonia first undergo mitosis; (2) cells called spermatocytes then undergo reduction of a diploid chromosome number by the process of meiosis; (3) haploid, round spermatids are finally transformed into highly organized motile spermatozoa, a process termed spermiogenesis. The last event in spermiogenesis is the release of spermatozoa from the seminiferous epithelium into

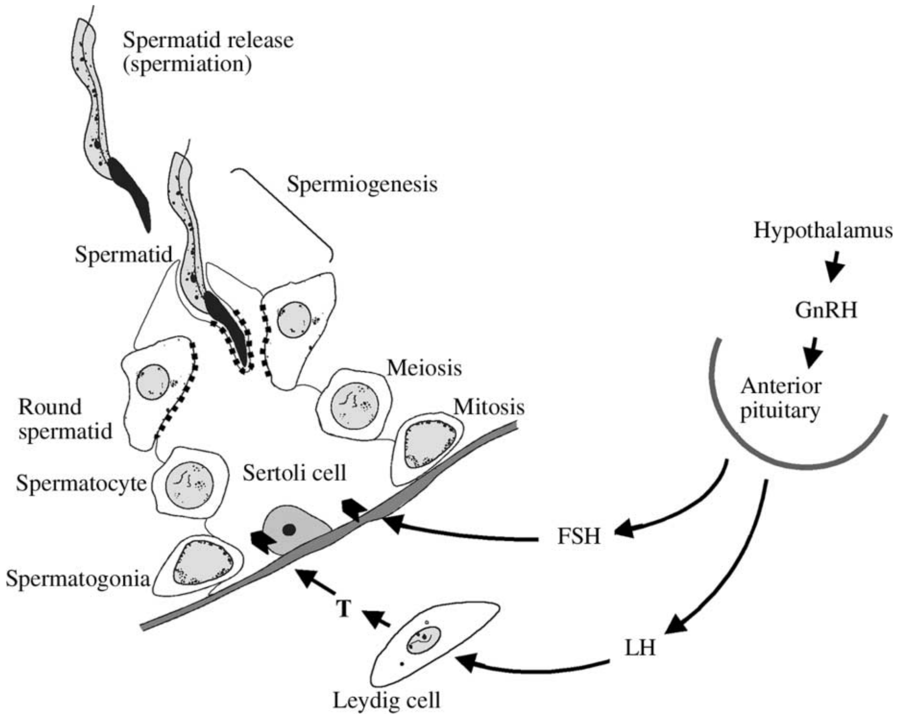

**FIGURE 1** The hormonal control of spermatogenesis and structural organization of the testis. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are secreted from the anterior pituitary under the influence of gonadotropin-releasing hormone (GnRH) from the hypothalamus. FSH targets the developing germ cells via specific G-protein-coupled receptors (⬟) on the Sertoli cell plasma membrane; LH stimulates the Leydig cells to produce and secrete androgens, predominantly testosterone (T), which in turn exerts its effects on the developing germ cells via nuclear receptors (⬟) in the Sertoli cell. Spermatogenesis is an elaborate process by which spermatogonia must proceed through multiple mitotic divisions, then meiosis, to produce haploid, round spermatids; these steps are followed by a complex sequence of morphological transformations that give rise to spermatozoa (spermiogenesis), ending in release of the spermatozoa from the Sertoli cell, into the tubule lumen (spermiation). In humans, the total time for spermatogonia to develop into mature sperm is 65 days; this time varies depending on the species. Extensive animal studies have shown that spermatogenesis depends on both FSH and T, each having both independent and synergistic effects on spermatogenesis. Although FSH is not essential for spermatogenesis, it plays a key role establishing the size of the Sertoli cell population during testicular development and in supporting spermatogonial development in adult animals. In contrast, T is required for the complete maturation of spermatids, and both FSH and T have been shown to play an important role in promoting spermatocyte and spermatid survival and in maintaining specialized junctions (···) involved in the adhesion of spermatids to the Sertoli cell.

the tubule lumen, a process termed spermiation. These phases of spermatogenesis occur along similar lines in all mammals and have been well described.

## III. ORGANIZATION OF THE TESTIS

The mammalian testis is encapsulated by a dense connective tissue called the tunica albuginea. The testis is composed of two major compartments, the seminiferous tubules, containing the nurturing Sertoli cells and the developing germ cells, and the interstitium, where Leydig cells and other interstitial cells reside. The testis contains many seminiferous tubules, which are convoluted loops connected at both ends to the excurrent duct system, also known as the rete testis. The mature spermatozoa pass from the tubules into the rete testis and then pass through the efferent ducts to the epididymis for final maturation before being capable of fertilizing an egg. The seminiferous tubules consist of the seminiferous epithelium and the lumen. Within the epithelium, the Sertoli cell provides structural and nutritional support for the developing germ cells; the lumen allows sperm and secretory products to be transported out of the testis. The interstitium contains the blood and lymphatic vessels responsible for the supply of nutrients into and out of the testis. Leydig cells are responsible for the generation of testosterone and other steroids.

The seminiferous epithelium has three compartments with different functions. Tight junctional complexes connecting the adjacent Sertoli cells form two permanent (basal and adlumenal) compartments and a temporary, intermediate compartment. Spermatogonia and early spermatocytes reside in the basal compartment. These cells have relatively free access to nutrients coming from the lymphatic and vascular systems. The intermediate compartment is formed during transit of spermatocytes from the basal to the adlumenal compartment, which involves the successive breakdown and formation of tight junctions. The adlumenal compartment contains all later stage germ cell types and is stringently controlled by the Sertoli cells via the tight junctional complexes, which limit the passage of macromolecules into the adlumenal environment. The junction is commonly referred to as the blood–testis barrier and becomes functional in early postnatal life. Numerous other Sertoli cell attachments to the developing germ cells assist in maintaining the structural integrity of the epithelium and provide a link for the cells to communicate. The success of spermatogenesis relies on the communication between all testicular cells.

## IV. HORMONAL DEPENDENCY OF SPERMATOGENESIS

The hormonal control of spermatogenesis has been studied extensively for many years, and differing emphasis has been placed on the relative roles of endocrine, paracrine, and autocrine factors. It must be understood that the complexity of germ cell development and the intricate intercellular relationships make it difficult to define clearly the exact control of each step in sperm production. In a wide range of experimental paradigms, different species have been used, different approaches to manipulating hormone levels have been employed, and, most importantly, there have been many different methods for describing or quantifying changes in germ cell production. Broadly speaking, hormone withdrawal and replacement have been used to dissect out the factors that regulate initiation, maintenance, and restoration of the spermatogenic process. This has varied from the complete withdrawal of pituitary factors by hypophysectomy, to active immunization with GnRH, to treatment with GnRH antagonists, and to the use of congenitally GnRH-deficient mice. The relatively selective withdrawal of testosterone has been achieved by sex steroid treatment (which causes gonadotropin suppression and a resultant decrease in testicular testosterone levels to $<5\%$ of normal), treatment with antiandrogens, and immunization against LH. FSH withdrawal has been achieved by disrupting FSH action by genetically modifying mice and by passively immunizing animals against FSH. A diverse range of qualitative and quantitative end points has been used to evaluate the effects of hormone on spermatogenesis. The advent of sophisticated stereological methods for quantitative analysis of cell populations has led to major advances in defining the sites of hormone action.

The precise mechanism by which FSH and testosterone exert their effects on the developing germ cells has not been elucidated but involves a highly orchestrated molecular and genomic response. Studies on the role of FSH and testosterone indicate that both hormones have independent and synergistic effects on germ cell development.

## V. FSH DEPENDENCY

FSH plays a key role in the development of the immature testis, particularly by controlling the size of the Sertoli cell population, which is set early in postnatal life. This is of particular importance for the adult animal because Sertoli cell number dictates sperm output. After debating many conflicting data from animal models, there is agreement that some degree of complete spermatogenesis can be initiated and maintained in the absence of FSH; however, quantitatively normal spermatogenesis depends on FSH. FSH acts at multiple sites in the spermatogenic pathway by promoting spermatogonial proliferation and survival and viability of later germ cell types by presumably maintaining structures and proteins involved in attachment of the germ cell to the Sertoli cell. More specifically, rat and monkey studies have shown that FSH plays a major role in supporting spermatogonial development, with FSH having more pronounced effects on certain subpopulations. FSH has been shown to play a role in later germ cell types, supporting spermatocytes and round spermatid development presumably by supporting cell survival. The specialized Sertoli cell junctional apparatus has been shown to be a hormone-sensitive structure; the structures are found between Sertoli cells and at stages from mature round spermatids to mature sperm before spermiation. This specialized apparatus has been shown to be disorganized in long-term gonadotropin-deplete rats and can be restored by FSH treatment, suggesting that FSH is important for the maintenance of the junctional apparatus. *In vitro* experiments on Sertoli and round spermatid cultures suggest that FSH is important for adherence of the round spermatids to the Sertoli cells. Finally, FSH may be involved in the release of mature sperm from the epithelium, based on reports that more sperm are retained within the seminiferous epithelium following acute FSH withdrawal. Another way that FSH can support germ cell development is via the Leydig cells. FSH has been shown to regulate Leydig cell products that play a role in spermatogenesis and promote maturation of the Leydig cell population.

## VI. TESTOSTERONE DEPENDENCY

Spermatogenesis has an absolute requirement for testosterone, particularly for the maturation of spermiogenic cells. Data from rodent models of testosterone replacement provide no evidence that testosterone supports spermatogonial development, although rodent models expressing high levels of testicular testosterone appear to have a detrimental effect on spermatogonial development. The viability of spermatocytes and spermatids is enhanced in the presence of testosterone and there are marked increases in cell degeneration in the absence of testosterone. The major lesion following selective testosterone withdrawal is during rat spermiogenesis, wherein sperm production is ablated as a consequence, at least in part, of the premature release of round spermatids from the seminiferous epithelium. Hence, round spermatid maturation is dependent on testosterone, which promotes the attachment of round spermatids to the Sertoli cells, possibly by induction of structures and proteins involved in cell adhesion. Finally, sperm release (spermiation) is also partly dependent on testosterone, because when testicular testosterone is reduced, mature sperm are retained and phagocytosed within Sertoli cells.

It has been established that testosterone is the active androgen supporting spermatogenesis in the normal testis; however, within the testis, testosterone can be metabolized by 5α-reductase enzymes to a more potent androgen, dihydrotestosterone (DHT). There is some evidence to support the notion that when testosterone levels are low (such as during LH suppression, when the level falls to $<5\%$ of normal), metabolism of testosterone to DHT may be important in maintaining some degree of spermatogenesis.

## VII. FSH AND TESTOSTERONE SYNERGY

There are many reports suggesting that FSH and testosterone act synergistically and that they exert the same biological effect. For example, it is clear that germ cell apoptosis/viability can be regulated by FSH and testosterone. Acute models of gonadotropin suppression/replacement in rats have revealed that both FSH and testosterone affect spermiation but that both are required for normality. FSH and testosterone can stimulate Sertoli cell products such as androgen-binding protein and transferrin. Synergy has been shown to play a role in maintaining proteins involved in cell adhesion. Sertoli cell N-cadherin, one such protein, has been shown to be maximally produced *in vitro* in the presence of both FSH and testosterone. Furthermore, *in vitro* studies of round spermatids and Sertoli cells show that binding of round spermatids to Sertoli cells is dependent on testosterone only in the presence of FSH. In addition, *in vivo* studies show that spermiogenesis is restored in hypophysectomized testosterone-replaced rats when FSH is administered. Monkey and human studies have demonstrated the relevance of several of these hormonally sensitive steps, notably spermatogonial development and sperm release, which are disrupted following gonadotropin suppression. Whether these are the results of independent or synergistic actions of FSH and testosterone in these species is unclear.

## VIII. RELEVANCE TO MALE CONTRACEPTIVE DEVELOPMENT

Studies in animals and humans have improved our understanding of the physiological basis of male contraception based on testosterone administration to suppress gonadotropins. Two major sites of spermatogenesis in humans have been identified as hormone sensitive, i.e., spermatogonial development and spermiation. Given the data from animal models, it appears that FSH withdrawal is important for spermatogonial development and that both FSH and testosterone are critical for spermiation. Clinical studies have shown that the extent of spermatogenic suppression and time of onset vary among individuals and between racial groups. Although not proved, it is thought that these differences may be attributed to differences in the degree and onset of gonadotropin suppression, and that faster and deeper suppression of both FSH and testosterone will lead to a more profound inhibition of spermatogenesis and thus create an effective contraceptive. Some studies provide evidence indicating that administration of progestins in combination with androgens may provide this desired effect, although further investigation is necessary. Another issue relating to variations in the degree of spermatogenic suppression between racial groups may be differences in androgen metabolism by 5α reduced enzymes; whether these differences are genetic or environmental, such as diet, is unclear.

## IX. CONCLUSION

Control of sperm production is mediated by FSH and LH via testosterone. A wide variety of animal models, most notably rodent models, coupled with quantitative analyses of testicular cell populations, have recognized that the sperm generation pathway contains steps sensitive to the independent action of FSH and testosterone and steps requiring synergistic actions. It is apparent that FSH is particularly important for early testicular growth, particularly in establishing the size of the Sertoli cell population and in supporting spermatogonial development in adulthood. Testosterone, however, is critical for maturation of round spermatids, because FSH is unable to complete this vital step; sperm release seems to require both hormones. Limited monkey and human studies have shown that gonadotropin suppression results in inhibition of spermatogonial development and sperm release. Further elucidation of the precise mechanisms by which these hormones affect sperm production will be significant for the design of the first effective hormonal contraceptive strategy for men.

## Glossary

| | |
|---|---|
| **follicle-stimulating hormone** | Pituitary hormone that induces germ cell production and development of their supporting cells in the ovary and testis. |
| **hormone** | Substance released by an endocrine gland; travels through the bloodstream to exert an effect on remote cells, tissues, and organs. |
| **luteinizing hormone** | Pituitary hormone that acts with follicle-stimulating hormone to stimulate sex hormone release. |
| **Sertoli cells** | The "nurse" cells of the seminiferous epithelium; provide nutritional and structural support for developing germ cells. |
| **spermatids** | Haploid germ cells produced by the second meiotic division in spermatogenesis; differentiate into mature spermatozoa. |
| **spermatocytes** | Tetraploid germ cells that undergo two meiotic divisions, yielding haploid spermatids. |
| **spermatogenesis** | Process whereby spermatogonia divide and differentiate into mature spermatozoa. |
| **spermatogonia** | Diploid germ cells that divide and differentiate; the most immature germ cell type. |
| **stereological** | Methodology that allows structural information (for instance, estimation of cell number) to be derived from sections of a structure. |
| **testosterone** | Male sex hormone (androgen) secreted by interstitial Leydig cells of the testis; responsible for triggering development of sperm and secondary sexual characteristics. |

## See Also the Following Articles

**Anti-Müllerian Hormone • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Estrogen and Spermatogenesis • Follicle Stimulating Hormone (FSH) • Male Hormonal Contraception • Sexual Differentiation, Molecular and Hormone Dependent Events in • Testis Descent, Hormonal Control of**

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# StAR Protein

See ***Steroidogenic Acute Regulatory Protein***

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# Stem Cell Factor[1]

Diana Linnekin, Tanya Jelacic, and Shivakrupa
*National Cancer Institute, Maryland*

I. INTRODUCTION AND HISTORICAL PERSPECTIVE
II. THE SCF LIGAND
III. THE SCF RECEPTOR
IV. BIOLOGY
V. MECHANISMS OF ACTION
VI. PATHOPHYSIOLOGY
VII. SUMMARY

**Stem cell factor (SCF) is a growth factor that binds the receptor tyrosine kinase Kit, leading to the activation of multiple signal transduction components, including members of the Src family. SCF is essential for the survival, growth, and maturation of stem cells involved in gametogenesis, hematopoiesis, and melanogenesis.**

## I. INTRODUCTION AND HISTORICAL PERSPECTIVE

Although stem cell factor (SCF) was cloned in 1990, this growth factor has a remarkably rich history. The receptor for SCF is the receptor tyrosine kinase (RTK) Kit. In mice, the c-Kit gene product maps to the *White Spotting* (*W*) locus on chromosome 5 and SCF maps to the *Steel* (*Sl*) locus on chromosome 10. Mice with mutations in the *W* locus were first identified in 1927. These animals were characterized by alterations in coat pigmentation, abnormalities in reproduction, and macrocytic anemia. In 1956, mice with the identical defects were found to have mutations in the *Sl* locus. The complementary nature of the phenotypes of these animals suggested that the *W* and *Sl* gene products were a receptor and its ligand. The isolation of the v-Kit oncogene from the Hardy–Zuckerman 4 strain of feline sarcoma virus by Peter Besmer and co-workers in 1986 set the stage to test this possibility formally. In 1987, the c-Kit proto-oncogene was cloned and the following year it was mapped to the *W* locus. Multiple groups then cloned the ligand for Kit and demonstrated that it mapped to the *Sl* locus. The cloning of SCF allowed rigorous assessment of its biological activities. Indeed, this growth factor proved to be critical for stem cells involved in gametogenesis, hematopoiesis, and melanogenesis. Although clinical use of SCF may be limited by its capacity to activate mast cells, gain-of-function mutations in Kit are associated with a variety of diseases in humans. Thus, understanding the cellular and molecular biology of the SCF receptor may be useful in designing approaches for treatment of these disorders, as well as harnessing the considerable biological potency of the ligand.

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[1] The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U. S. Government.

## II. THE SCF LIGAND

SCF, also termed mast cell growth factor, Kit ligand, and Steel factor, is the ligand for the receptor tyrosine kinase Kit. The human SCF gene is located on chromosome 12q22–q24, is 50 kb in length, and consists of eight exons. The murine SCF gene has been mapped to chromosome 10 in a region flanked by genes encoding peptidase-2 and phenylhydroxylase. The cDNA for SCF has been cloned and sequenced from a variety of species including human, mouse, rat, pig, and chicken [Accession Nos. (mouse) U44725; (human) M59964; (rat) M59966; (pig) L07786; and (chicken) D13516]. The first exon of the human SCF gene codes for the signal peptide required for membrane anchoring of SCF. Exons 2 through 6 code for the transmembrane region. Exon 7 codes for the extracellular region and exon 8 encodes the cytoplasmic tail. SCF protein is found as both a soluble form (sSCF or $SCF^{165}$) and a membrane-bound form (mSCF or $SCF^{220}$). These forms are generated by alternate splicing of a primary transcript at exon 6. One splice product gives rise to a protein of 275 amino acids containing a cleavable amino-terminal signal peptide. The cleavage of the signal peptide gives rise to $SCF^{248}$, also designated KL-1 or SCF-1. It contains an extracellular domain, a 22-amino-acid transmembrane domain, and a 36-amino-acid cytoplasmic domain. $SCF^{248}$ undergoes proteolytic cleavage at Ala-165 to generate the soluble form of SCF, $SCF^{165}$. The other splice variant of SCF lacks the proteolytic cleavage site and gives rise to a membrane-bound form of SCF, $SCF^{220}$, also termed KL-2 or SCF-2.

Soluble SCF exists as a heavily glycosylated 50 to 60 kDa noncovalent homodimer that can dissociate and reassociate in solution. The recently described crystal structure revealed a large dimerization region and a charged receptor-binding region that includes hydrophobic crevices. The concentration of soluble SCF in human serum is approximately 3 ng/ml. Consequently, it exists primarily as a monomer. In contrast, the close proximity of mSCF in the plasma membrane results in the dimerization of this molecule. Dimerization of membrane-bound SCF facilitates signaling through the Kit receptor. Both forms of SCF are biologically active, with distinct as well as overlapping functions. Membrane-bound SCF induces more persistent tyrosine kinase activation than soluble SCF. This is likely due to the slower internalization of Kit after binding this form of the growth factor.

SCF is expressed by stromal cells, fibroblasts, and endothelial cells and is also present at low levels in the circulation. SCF is also expressed along the migratory pathway of stem cells in embryos and this plays an important role in development. Sertoli cells, ovarian follicular cells, brain, and olfactory bulb also express SCF. The ratio of membrane SCF to soluble SCF varies considerably in different tissues. Fibroblasts, brain, thymus, spleen, and bone marrow express higher levels of $SCF^{248}$, whereas placenta, cerebellum, and testis have higher levels of $SCF^{220}$. The exact mechanism of regulation of the levels of different SCF proteins is unknown.

## III. THE SCF RECEPTOR

The receptor for SCF is Kit, a receptor tyrosine kinase sharing homology with receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1). Kit has also been designated as stem cell factor receptor and CD117. The gene for human c-Kit has been mapped to 4q11–q34, spans approximately 70 kb of DNA, and has 21 exons [GenBank Accession Nos. (human) X06182 and (mouse) Y00864]. Human Kit encodes a 145 kDa glycosylated protein (GenBank Accession No. 1817733) containing an extracellular domain of approximately 500 amino acids, a 30-amino-acid juxtamembrane domain, a cytoplasmic kinase domain divided into two parts by a 77-amino-acid kinase insert region, and a 50-amino-acid carboxy-terminal tail region. The extracellular domain contains five immunoglobulin-like repeat regions, the first three of which constitute the ligand-binding domain. The fourth immunoglobulin-like domain is involved in receptor dimerization and the function of the fifth domain has not been defined. Some of these features are illustrated in Fig. 1. An alternative splice form of Kit, termed KitA, has a four-codon insertion (GNNK) in the extracellular domain just proximal to the membrane-spanning region. Both forms are co-expressed in most tissues. Another variant of Kit that consists of only the second

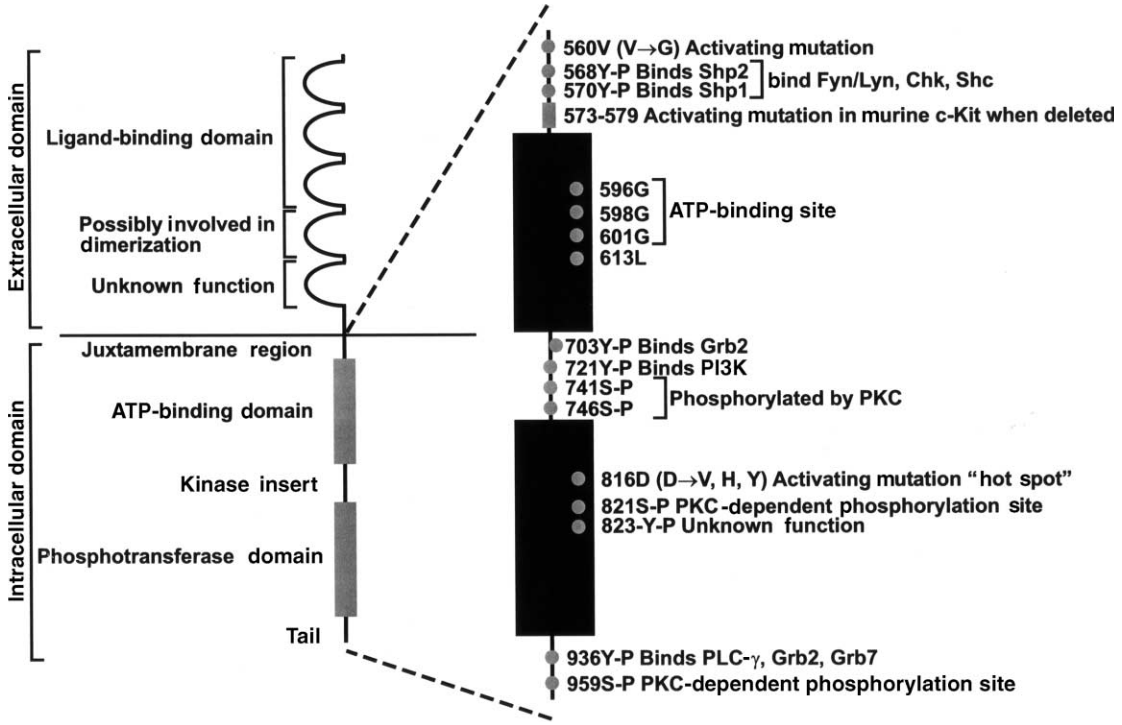
Extracellular domain
Ligand-binding domain
Possibly involved in dimerization
Unknown function
Juxtamembrane region
ATP-binding domain
Kinase insert
Phosphotransferase domain
Tail
Intracellular domain
560V (V→G) Activating mutation
568Y-P Binds Shp2
570Y-P Binds Shp1
bind Fyn/Lyn, Chk, Shc
573-579 Activating mutation in murine c-Kit when deleted
596G
598G
601G
ATP-binding site
613L
703Y-P Binds Grb2
721Y-P Binds PI3K
741S-P
746S-P
Phosphorylated by PKC
816D (D→V, H, Y) Activating mutation "hot spot"
821S-P PKC-dependent phosphorylation site
823-Y-P Unknown function
936Y-P Binds PLC-γ, Grb2, Grb7
959S-P PKC-dependent phosphorylation site

FIGURE 1 Structure–function relationships of the Kit receptor tyrosine kinase. The extracellular domain of Kit is divided into five immunoglobulin-like regions. The intracellular domain is composed of the juxtamembrane region, the catalytic domain, a kinase insert, and a carboxy-tail. Amino acids that contribute to the activation of Kit or that interact with specific signaling molecules are designated by small circles.

catalytic domain and the carboxy-terminal tail is expressed in spermatids. Full-length Kit is proteolytically cleaved in a process dependent on the fifth immunoglobulin-like domain, to give rise to a soluble form of Kit. The function of soluble Kit is not fully defined; however, it binds to SCF and may act as a receptor antagonist *in vivo*.

Kit is expressed in a variety of different cells. In hematopoietic tissue, it is expressed in stem cells, progenitor cells (pluripotent as well as progenitors committed to the myeloid, lymphoid, erythroid, and megakaryocytic lineages), bone marrow mononuclear cells with lymphoid markers, and mast cells. In most hematopoietic lineages, Kit expression decreases during maturation. However, mast cells, activated platelets, and some subsets of NK cells are exceptions. Nonhematopoietic cells expressing Kit include melanocytes, spermatozoa, vascular endothelium, interstitial cells of Cajal (ICCs), breast glandular epithelial cells, sweat glands, astrocytes, oocytes, theca cells, and renal tubules. Kit is also expressed in the human endometrium and placenta during pregnancy.

Surface expression of Kit is regulated by multiple mechanisms. Interaction with SCF induces internalization of the receptor through clathrin-coated pits. Kit is also ubiquitinated and can be degraded in either the lysosome or the proteasome pathway. In addition, certain cytokines regulate surface expression of Kit. Transforming growth factor-β can induce the down-regulation of Kit through reductions in mRNA stability. This is also seen with interleukin-3 (IL-3) stimulation in mast cells. The processing of membrane-bound receptor to generate the soluble form also determines the surface expression levels of Kit in umbilical endothelial cells, mast cells, and human plasma.

## IV. BIOLOGY

Kit and SCF are expressed in many tissues. The correct function of these proteins is necessary for maintenance of gastrointestinal motility, hematopoiesis, melanogenesis, gametogenesis, and some aspects of central nervous system (CNS) function. The following section will summarize the biological role of SCF and Kit in each of these organ systems.

## A. Central Nervous System

The widespread expression of Kit and SCF in the CNS of adult animals was a surprising finding since the *W* and *Sl* mutant mice do not exhibit gross CNS abnormalities. However, a subtle abnormality has recently been demonstrated in *Ws*/*Ws* adult rats, which express a Kit mutant with impaired kinase activity. These rats have reduced long-term potentiation (LTP) in the mossy fiber–CA3 pathway of the hippocampus. Impaired spatial learning and memory in the Morris water maze test were also observed in these animals. $Sl/Sl^{d}$ mice, which express the soluble but not the transmembrane form of SCF, are also known to have impaired hippocampal learning, but have normal LTP.

## B. Gastrointestinal Tract

The ICCs are unusual cells in that they have characteristics of smooth muscle cells, fibroblasts, and neuronal cells. These are the only cells in the gastrointestinal tract (GI) tract that express Kit. The discovery of this exclusive expression of Kit revolutionized the study of these cells. Immunostaining for Kit *in situ* allowed visualization of the complex network that ICCs form throughout the GI tract. Although expression of Kit is universal among the ICCs, expression of other proteins varies, and there are different subpopulations of ICCs within the different regions of the GI tract.

ICCs have characteristics similar to cells of both neural crest and mesenchymal origins. However, studies in the late 1990s revealed that ICCs and the surrounding smooth muscle cells are both derived from mesenchymal precursors that express Kit. Precursors that contact SCF maintain Kit expression and develop into ICCs. Precursors that do not interact with SCF lose Kit expression and develop into smooth muscle cells. Mature smooth muscle cells express SCF, which is necessary for the maintenance of the ICC phenotype of their neighbors.

The ICCs are the pacemaker cells of the GI tract, generating and propagating the slow waves that control the frequency of contraction. Thus, the ICC network is essential to GI motility. These cells also integrate motor signals from the enteric nervous system. Kit signaling is essential to pacemaker function. Chronic exposure to antibodies specific for Kit or to inhibitors of signaling molecules downstream of Kit [i.e., the phosphatidylinositol 3-kinase (PI3K) inhibitors Wortmannin and LY-294002] lead to the loss of slow waves and eventually reductions in the number of ICCs. Interestingly, some subpopulations of ICCs are more resistant to these treatments than others. Similarly, subpopulations of ICCs are altered to differing extents in mice with different *W* or *Sl* mutations.

## C. Hematopoietic Cells

Expression of Kit and SCF is necessary for hematopoiesis in the fetal liver and in adult bone marrow. Kit is expressed primarily on hematopoietic stem cells, pluripotential progenitor cells, and early myeloid, lymphoid, erythroid, and megakaryocytic progenitors, whereas SCF is expressed by supporting stromal cells. SCF supports the survival of the stem cells and pluripotential progenitors, but induces only limited increases in proliferation as a single factor. However, SCF, in combination with certain other growth factors, strongly induces the proliferation of progenitors leading to a variety of lineages. SCF and erythropoietin stimulate the growth of erythroid progenitors. SCF and IL-3 induce the proliferation of progenitors that can differentiate into granulocytes, macrophages, and mast cells. SCF and thrombopoietin stimulate the growth of megakaryocytic progenitors. Expression of Kit decreases as differentiation progresses and is eventually lost altogether on most mature hematopoietic cells. Mast cells, activated platelets, and a subset of natural killer cells are the only fully differentiated hematopoietic cells that express Kit. SCF has a potent effect on mast cells and can induce them to migrate, proliferate, and degranulate.

## D. Melanocytes

Expression of Kit and SCF is necessary for normal pigmentation of skin and hair. The precursors of melanocytes, the pigment-producing cells, arise in the neural crest in the embryo and migrate to the epidermis. The migratory path of the Kit-expressing melanocyte precursors is defined by SCF-expressing mesenchymal cells. Injection of pregnant mice with antibody that blocks binding of SCF during this phase of melanocyte migration leads to offspring that are almost completely lacking in pigmentation. However, injections that do not coincide with melanocyte migration do not alter pigmentation.

After birth, Kit is expressed in melanocytes in the hair follicles and skin, and SCF is expressed in keratinocytes. Hair follicles cycle between states of rest (telogen), active growth (anagen), and regression (catagen). Kit signaling is necessary for the replacement of melanocytes when the follicles progress from telogen to anagen. Injection of antibody specific for Kit into hair follicles at this stage leads to the growth of unpigmented hairs. However, if the injection is not repeated, the follicles produce fully pigmented hairs during their next anagenic phase. In addition to maintaining normal pigmentation in the skin, Kit signaling plays a role in hyperpigmentation in response to ultraviolet B (UVB) light. Both Kit expression and SCF expression increase in response to UVB light. Although the expression of other enzymes involved in melanin production is not dependent on Kit signaling, the expression of tyrosinase, the critical rate-limiting enzyme, is dependent on an increase in cyclic AMP levels mediated by Kit signaling. Thus, injection of anti-Kit antibodies into the skin prevents the hyperpigmentation response to UVB light.

### E. Germ Cells

Expression of Kit and SCF is essential for the formation of primitive gonads in the embryo and for normal fertility in adult animals. Primordial germ cells (PGCs) in both sexes originate outside the genital ridge. PGCs express Kit, whereas cells in and on the way to the genital ridge express SCF. The PGCs follow a "trail" of SCF-expressing cells from their point of origin to the genital ridge. Along the way and at their destination, they proliferate. After the expansion of PGCs in the genital ridge, the primitive gonads differentiate into ovaries or testes, and Kit expression decreases and remains at low levels until the onset of puberty. Although Kit and SCF are crucial for the establishment of primitive gonads in both sexes, their role in adult reproductive function varies with sex. Certain *W* and *Sl* mutations cause infertility in one sex but not in the other.

In adult males, Kit is expressed by spermatogonia and primary spermatocytes, and SCF is expressed by Sertoli cells. Mature spermatozoa express a unique form of Kit that is severely truncated. This form of the protein may be involved in releasing the oocyte from arrest at fertilization. Injection of this protein into oocytes induces parthenogenesis. The necessity of both Kit and SCF is demonstrated by the infertility of male $W^v$ and $Sl^d$ mutant mice, which have reduced Kit function and a lack of transmembrane SCF, respectively.

In adult females, Kit is expressed in oocytes and theca cells, and SCF is expressed by the surrounding granulosa cells. Interaction between Kit and SCF is necessary for follicular development and maturation, but not ovulation and luteinization. There is also evidence that Kit signaling is involved in driving oocytes into meiotic arrest.

## V. MECHANISMS OF ACTION

### A. Receptor Dimerization and Autophosphorylation

As described above, Kit is a RTK. As a monomer, Kit is essentially inactive. Similar to other RTKs, the conformation of the juxtamembrane region imposes structural constraints that reduce spontaneous activation. This may be due to the formation of an α-helical structure by the unoccupied Kit monomer. Consequently, mutations in this region can activate Kit in the absence of ligand. Binding of SCF induces the rapid dimerization of Kit. This is likely promoted by interaction with noncovalently bound ligand dimers. *In vitro*, the first, second, and third Ig-like regions are sufficient to induce the dimerization of soluble forms of Kit. Biochemical data suggest that portions of the fourth immunoglobulin domain contribute to the dimerization of full-length Kit protein in cells. Ligand binding to wild-type Kit induces rapid increases in receptor autophosphorylation. Although the intracellular domain of Kit contains 22 tyrosine residues, autophosphorylation of only tyrosines 568, 570, 703, 721, 823, and 936 of human Kit has been confirmed.

### B. Autophosphorylation of Kit and Initiation of Downstream Signaling Pathways

Multiple signal transduction components are activated after autophosphorylation of Kit. This occurs, in part, through recruitment of SH2-containing proteins to the Kit receptor complex via phosphorylated tyrosine residues. Two important residues in the recruitment of adapter proteins and signaling components to the human Kit receptor complex are tyrosines 568 and 570 in the juxtamembrane region. These are docking sites for Src family members, Shc and Chk (Csk-homologous kinase). In addition, the protein tyrosine phosphatases Shp1 and Shp2 have been reported to interact with tyrosines 569 and 567 on murine Kit, respectively. These correspond to tyrosines 570 and 568 on human Kit. Although most reports indicate that mutation of either of these residues has minimal effects on SCF-mediated growth, the mutation of both residues dramatically impairs growth. Thus, the juxtamembrane region is critical in providing docking sites for a variety of proteins involved in SCF signal transduction as well as maintaining inactive Kit monomers.

Of the signaling components shown to interact with the Kit juxtamembrane region, Src family members have been studied most extensively. Those activated by SCF include Lyn, Fyn, and likely Src and Yes, as well as others. Tyrosine 568 of human Kit is the predominant binding site for Src family members. Interestingly, mutation of this residue results in only subtle alterations in responses to SCF. In contrast, Lyn-deficient mast cells and hematopoietic progenitors have more dramatic abnormalities in SCF-mediated responses. Furthermore, data obtained with Src family inhibitors and dominant inhibitory mutants support an important role for this kinase family in Kit stimulus-response coupling mechanisms. This likely occurs through signaling components activated independent of tyrosine 568. Src family members contribute to the activation of the Jnk family of serine/threonine kinases. Jnks 1 and 2 contribute to the SCF-induced proliferation of mast cells.

Activation of the Ras–Raf–mitogen-activated protein (MAP) kinase cascade may also be initiated through the Kit juxtamembrane region in a Src-dependent manner. In transfected porcine aortic endothelial cells, mutation of tyrosine 570 reduces SCF-induced activation of ERKs and mutation of tyrosine 568 nearly eliminates it. Concomitant with this are decreases in the phosphorylation of Shc that are dependent on Src family members. In contrast, SCF has been reported to activate ERKs in mast cells expressing Y567F murine Kit. Thus, initiation of the ERK signaling pathway likely occurs through multiple sites on Kit. For example, *in vitro* studies have shown that growth factor receptor-binding protein 2 (Grb2) can bind tyrosine 703 or 936 in the kinase insert or carboxy-tail, respectively. Furthermore, ERKs may be activated through different mechanisms in distinct cell lineages

Tyrosine 721 in the kinase insert domain is another important autophosphorylation site on human Kit. Mutation of tyrosine 719, the corresponding residue in murine Kit, does not significantly impair Kit activity, but does eliminate recruitment of PI3K to the Kit receptor complex. Infection of mast cells with this mutant results in a reduction of SCF-induced adhesion of mast cells to fibronectin, as well as minor alterations in survival and growth. Similar defects have been observed in mast cells derived from mice engineered to express this mutant. In addition, significant defects in spermatogenesis occur in transgenic mice expressing Y719F Kit, but no alterations in pigmentation or steady state hematopoiesis were observed. Therefore, direct interaction of Kit and PI3K is essential for maturation of sperm and contributes to some aspects of SCF-mediated responses in mast cells, but is not required for Kit-dependent pigmentation or hematopoiesis. Interestingly, bone marrow mast cells derived from mice deficient for p85α PI3K had dramatic decreases in SCF-induced proliferation *in vitro*. Thus, PI3K also contributes to SCF-mediated responses through mechanisms independent of tyrosine 719.

Two signaling components activated downstream of PI3K are Akt and Jnks. Activation of Akt is dependent on the interaction of PI3K and Kit. Similar to numerous other growth factors, Akt contributes to the capacity of SCF to promote viability. In contrast, activation of Jnk1 and Jnk2 is involved in SCF-induced proliferation.

In the second catalytic domain, phosphorylation of tyrosine 821 of murine Kit (corresponding to tyrosine 823 of human Kit) makes critical contributions to the SCF-induced survival and proliferation of mast cells. Signaling components that interact with this site remain to be identified. Interestingly, tyrosine 821 is not required for SCF-induced activation of PI3K, Ras, or ERKs or for induction of c-myc, c-myb, c-fos, or junB.

Tyrosine 936 is an autophosphorylation site in the carboxy-tail of Kit. *In vitro* studies have shown that it binds to phospholipase Cγ (PLC-γ) and Grb7. The role of the interaction of these proteins with Kit in SCF-mediated responses is not known. Phosphorylation of PLC-γ requires tyrosine 728 in the kinase insert region of murine Kit. This is not critical for proliferation induced by soluble SCF but is required for responses to membrane-bound SCF.

## C. Serine Phosphorylation of Kit

In addition to the important role of autophosphorylated tyrosine residues in Kit signaling, this receptor is also heavily phosphorylated on serine residues. A series of elegant studies by Lars Rönnstrand and coworkers demonstrated that protein kinase C (PKC) isoforms are responsible for serine phosphorylation of Kit at multiple sites. Serines 741 and 746 on human Kit are directly phosphorylated by PKC, and serines 821 and 959 are PKC-dependent sites. Serine phosphorylation reduces interaction of Kit with multiple SH2-containing proteins and is associated with decreased mitogenic responses to SCF as well as increased migration. Increased association with PI3K and increases in Akt activity have been observed in cells expressing Kit with serines 741 and 746 mutated.

## D. Kit Signaling and the Janus Kinase/Signal Transducers and Activators of Transcription Pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is critical for responses mediated by ligands interacting with cytokine receptor superfamily members. Less is known about the activation of this pathway by RTKs such as Kit. We have shown that SCF rapidly and transiently activates JAK2. In addition, reduction in expression of JAK2 using antisense oligonucleotides partially impairs SCF-induced growth. The region of Kit coupling to activation of JAK2 remains to be identified. SCF also activates Stat family members, including Stat1 and Stat5. Stat1 interacts with tyrosine residues in the second catalytic domain of Kit, whereas full activation of Stat5 requires the carboxy-tail of Kit. Stat3 is also phosphorylated on serine residues after stimulation with SCF. Importantly, activation of the JAK/STAT pathway is extremely rapid, transient, and lineage-specific. One means of negatively regulating this pathway could be through Socs1 (suppressor of cytokine signaling). This protein interacts with Kit and constitutive expression reduces SCF-induced growth. Shp1 is also a negative regulator of Kit signaling that may down-regulate JAK2 activity. A summary of the structure–function relationships of the intracellular region of Kit is summarized in Fig. 1. Furthermore, some of the signaling pathways activated by Kit are shown in Fig. 2.

## E. Signaling through Different Receptor and Ligand Isoforms

Two isoforms of human Kit result from alternate splicing of mRNA. This results in the insertion of a 4-amino-acid sequence just proximal to the membrane-spanning region in the extracellular domain. Although the biological significance of this insert remains unclear, recent studies suggest differences in internalization, signaling, and possibly transforming activity of these isoforms. Differences in the signaling of soluble and membrane-bound SCF have also been reported. The kinetics of ligand-induced phosphorylation of Kit are more protracted after stimulation with membrane-bound SCF than with soluble SCF. Thus, this isoform may be more biologically potent. Furthermore, PI3K and PLC-γ may make different contributions to biological responses mediated by soluble SCF versus membrane-bound SCF.

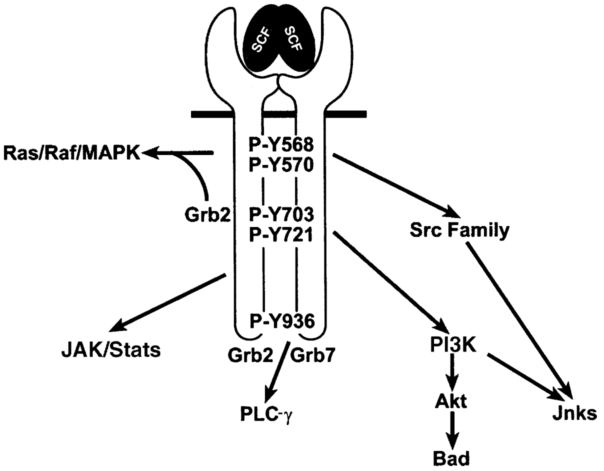

FIGURE 2 Signaling pathways activated by Kit. SCF binds Kit, induces dimerization, and increases autophosphorylation activity of this receptor tyrosine kinase. The indicated autophosphorylation sites recruit signaling components coupled to the biochemical pathways indicated.

## F. Transcription Factors: Activation and/or Induction

As described above, Stat family members phosphorylated after stimulation with SCF include Stat1, Stat3, and Stat5. SCF-induced increases in the DNA-binding activity of both Stat1 and Stat5 have also been reported. Both of these proteins interact with Kit, but it remains to be determined whether Kit directly activates Stat family members or whether nonreceptor kinases such as JAK2 or a Src family member are upstream activators. *In vitro* studies have shown that Kit can phosphorylate Stat1 directly. The role of these events in SCF-mediated responses is yet to be established.

The micropthalmia transcription (Mi) factor is a member of the basic/helix-loop-helix/leucine zipper family. It is highly tissue-specific and expressed in both melanocytes and mast cells. Mice or humans lacking this protein have defects in pigmentation and a reduction in mast cell numbers. Signaling through Kit results in serine phosphorylation of Mi via activation of ERKs. This increases the transcriptional activity of Mi by increasing association with the transcriptional co-activator p300/CREB-binding protein (where CREB is $Ca^{2+}$/cAMP-response element-binding protein).

One family of transcription factors regulated downstream of Akt is the forkhead family. Recent studies indicate that SCF induces the phosphorylation of FOXO1a, 3a, and 4. This did not require the activation of ERKs, p38, or PKC but did require PI3K.

SCF also induces the expression of a variety of immediate-early response genes that are transcription factors. These include c-myc, c-fos, junB, c-myb, and egr-1.

## VI. PATHOPHYSIOLOGY

### A. Preliminary Comments

As discussed above, Kit makes important contributions to the development and function of hematopoietic, germ cell, and pigmented tissues. It is also important in terms of the function of different aspects of the CNS and the GI tract. The critical nature of Kit in normal physiology is highlighted by the aberrations resulting from the absence of functional Kit or its ligand SCF. In addition, a number of diseases are associated with overexpression or gain-of-function mutations.

### B. Gain-of-Function Mutations

The Kit proto-oncogene was identified after the discovery of the v-*Kit* oncogene in the Hardy–Zuckerman strain of feline sarcoma virus. The v-Kit protein contains the catalytic domain and kinase insert region of Kit, but is missing the extracellular domain, as well as portions of the carboxyl-tail and juxtamembrane region. The capacity of Kit to be usurped by a retrovirus as a transforming protein suggested that alterations in the expression or activity of this kinase could be involved in other disease processes. Indeed, in the past decade, gain-of-function mutations in Kit have been identified and associated with a variety of human diseases including gastrointestinal stromal cell tumors (GISTs), mastocytosis, leukemia, lymphoma, germ cell tumors, and small cell carcinoma of the lung.

Two regions of Kit where gain-of-function mutations occur with high frequency are the juxtamembrane region and the second catalytic domain. As described earlier, the secondary structure of the juxtamembrane region maintains the unoccupied receptor in a conformation that inhibits catalytic activity. Mutations in this region alter this configuration and often result in increases in Kit kinase activity in the absence of ligand. The second catalytic domain is the ATP-binding pocket and is another hotspot for activating mutations of Kit. Mutations in the analogous region of Flt3, Met, Ron, and Ret also activate these RTKs.

Gain-of-function mutations in the second catalytic domain of Kit were originally described in mastocytoma cell lines of human, mouse, and rat origin in the early 1990s. More recently, mutations in this region have been found in patients with a variety of different diseases, many of which involve hematopoietic cells. In 1995, mutations in codon 816 of Kit were first described in patients with mastocytosis. Mutations in this region have also been found in some patients with core binding factor leukemias, sinonasal lymphomas, and germ cell tumors. Interestingly, patients with more than one of these conditions have been reported. Mastocytosis can be associated with myeloproliferative disease. Patients with both mastocytosis and germ cell tumors have also been reported.

In 1998, mutations in the juxtamembrane region of Kit were found in patients with GISTs. Interestingly, these mutations occur at higher frequencies in patients with malignant GIST than in those with benign tumors. Malignant GIST is extremely resistant to chemo- and radiotherapy. In 2001, astonishing improvement in a patient with malignant GIST was reported after treatment with STI571, an inhibitor of bcr-abl, Kit, and the PDGF receptor. Subsequent to these findings, the results of several clinical trials indicate that this drug is tremendously beneficial in the treatment of patients with malignant GISTs. *In vitro*, STI571 inhibits the kinase activity of Kit juxtamembrane mutants at lower concentrations than wild-type Kit. Furthermore, cells expressing this form of mutant Kit undergo apoptosis after exposure to this drug. Thus, targeted inhibition of mutant Kit shows great promise in the treatment of GIST.

### C. Alterations in Expression of Kit

In acute myeloid leukemia (AML), large percentages of patients express Kit on leukemic blast cells. This has led to speculation that Kit may be a molecular target for the treatment of AML. Treatment of one AML patient refractory to standard chemotherapy regimens with the Kit inhibitors SU5416 and SU6668 induced remission. However, it remains to be determined whether this approach will be helpful in the management of this disease in the majority of patients.

Inappropriate expression of Kit, SCF, or both has been reported in patients with breast cancer, small cell carcinoma of the lung (SCCL), melanoma, and some tumors of the CNS. Among these diseases, the role of Kit and SCF expression in SCCL and melanoma have been studied most extensively. Therefore, the remainder of this section will focus on studies relating to these two diseases.

Although Kit plays an important role in the development of melanocytes, expression in mature melanocytes may lead to apoptosis. Interestingly, decreases in Kit expression have been observed in tissues from melanoma patients. A strong correlation between reductions in the transcription factor activator protein-2 (AP-2) and Kit expression have been reported. Since the Kit reporter contains multiple AP-2 sites, the loss of AP-2 may lead to a reduction in the expression of Kit and facilitate the escape of melanoma cells from SCF-induced apoptosis. Ectopic expression of Kit in a melanoma cell line dramatically reduced metastatic potential *in vivo*.

In contrast to melanoma, co-expression of Kit and SCF contributes to autocrine growth of SCCL. Seventy percent of SCCL cell lines or primary tumor specimens co-express Kit and its ligand. The role of this putative autocrine loop in the growth of these cells has been illustrated by several approaches. Ectopic expression of a dominant-inhibitory Kit mutant, as well as treatment with drugs that inhibit Kit activity such as AG1296, STI571, SU5416 and SU6597, dramatically inhibits the growth of SCCL cells.

## D. Loss-of-Function Mutations

The phenotypes of the various *W* and *Sl* mutant mice demonstrate the critical role of SCF and Kit in germ cell development, pigmentation, and hematopoiesis, as well as in the functioning of the intestinal tract and portions of the CNS. Reviews extensively describing these animals are listed under Further Reading. This section will briefly summarize the effect of mutations in the Kit or SCF genes in each of these organ systems in mice and indicate whether corresponding defects have been noted in humans.

Mutations in Kit have been associated with defects in pigmentation in mice, rats, pigs, and humans. This may also contribute to roan coloring in horses. In humans, loss-of-function mutations in one allele of Kit are associated with autosomal

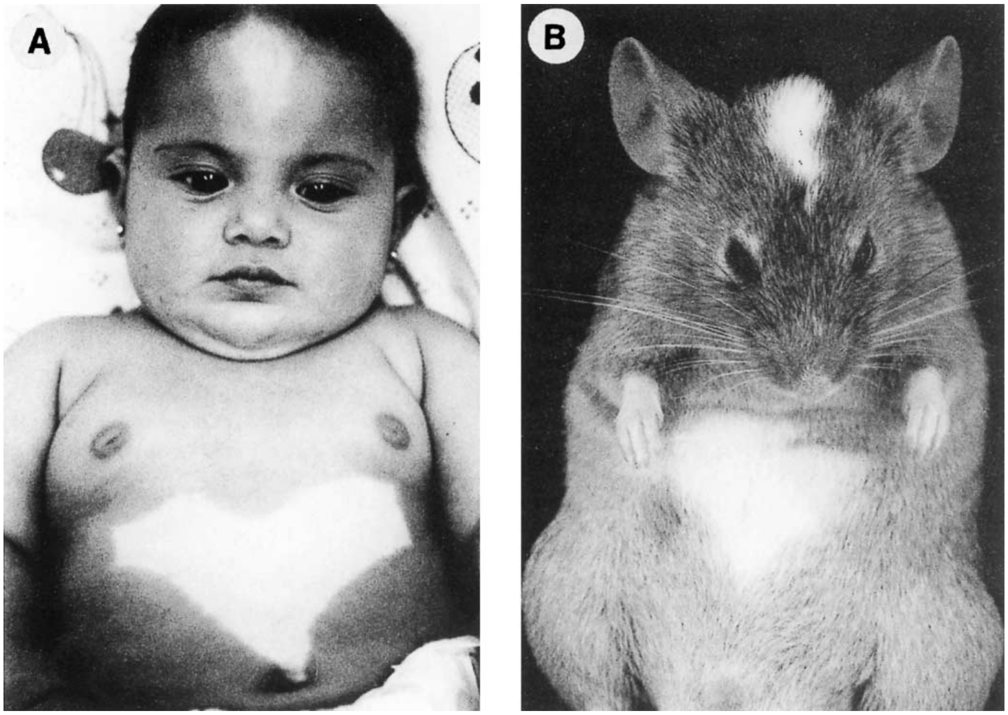

**FIGURE 3** Loss-of-function mutations in one allele of Kit cause alterations in pigmentation in humans and mice. Reprinted from Fleischman *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, p. 10885, with permission.

dominant piebaldism. Mutations in the first catalytic domain of human Kit have been observed in many of these patients. Similar to *W* and *Sl* mice, which are heterozygotes for dominant inhibitory mutations, white spots are observed on the ventral trunk, forehead, and extremities. Shown in Fig. 3 are a child and a mouse that are both heterozygotes for the identical loss-of-function mutation in Kit.

Kit contributes to the development of the interstitial cells of Cajal and these cells are important in the pacemaker activity of the gastrointestinal tract. The importance of Kit in normal intestinal function is highlighted by the abnormality in intestinal pacemaker activity in *W* mice. In humans with autosomal dominant piebaldism, there is an increased frequency of megacolon and other gastrointestinal difficulties. Loss of ICCs is associated with motility disorders in human (e.g., Hirschsprung's disease) and other animals (e.g., grass sickness in horses).

*W* and *Sl* mice also have dramatic alterations in hematopoietic tissue. The more severe variants are mast cell deficient and have macrocytic anemia. The anemia results from decreases in numbers of erythroid progenitors, particularly those corresponding to CFUe (colony forming unit erythroid). No dramatic defects in hematopoiesis have been noted in humans with mutations in Kit. However, the patients examined have had mutations in only one allele of the Kit gene. Similarly, heterozygotic *W* mice have very mild alterations in hematopoietic tissue. Thus, it is unknown whether Kit plays the same nonredundant role in hematopoiesis in humans as it does in mice.

The gene products for both Kit and SCF are expressed at high levels in the murine hippocampus. As discussed previously, mice and rats with mutations in SCF or Kit have deficits in hippocampal-dependent learning. In addition, there are reductions in numbers of sensory nerves in some *W* and *Sl* mice, as well as reports of auditory deficiencies in rats with mutations in Kit. In humans, retardation and sensorineural deafness have been reported in patients with autosomal dominant piebaldism.

The development of both sperm and oocytes requires functional Kit and SCF. Mice with severe defects in either gene product are sterile. Exciting recent studies demonstrate that the interaction of Kit with PI3K is required for spermatogenesis in mice. More subtle alterations in oogenesis may also depend on the association of PI3K and Kit. In humans, reductions in Kit may be linked to defects in sperm development. Kit is also a candidate gene for contributions to ovarian failure.

## VII. SUMMARY

SCF binds the receptor tyrosine kinase Kit. Interaction of this ligand and receptor induces rapid autophosphorylation of Kit, dimerization, and activation of multiple signaling components, including Src family members, the JAK/STAT pathway, the Ras–Raf–MAP kinase cascade, PI3K, and PLC$\gamma$. SCF promotes the survival, growth, and maturation of stem cells from multiple lineages. In addition, when combined with other growth factors, SCF is potently synergistic. Loss of expression of either Kit or SCF is lethal in mice. Mice with mutations resulting in reductions in expression or function of Kit or SCF have defects in pigmentation and hematopoiesis, are mast cell deficient, and have reproductive difficulties. Mutations resulting in the activation of Kit in the absence of ligand are associated with human diseases, including GISTs, mastocytosis, sinonasal lymphomas, germ cell tumors, and some myeloid leukemias. Inappropriate co-expression of Kit and SCF has been implicated in SCCL, and down-regulation of Kit on melanocytes may contribute to metastatic melanoma.

## Glossary

**hematopoiesis** Development of multiple lineages of blood cells from a pluripotential stem cell. These include red blood cells, platelets, lymphocytes (T and B), natural killer cells, monocytes, and granulocytes (neutrophils, eosinophils, and mast cells). This process is regulated by soluble and membrane-bound growth factors, cell contact, and interaction with the extracellular matrix.

**receptor tyrosine kinase** Membrane-spanning protein that binds ligand via the extracellular domain and contains a protein tyrosine kinase in the intracellular domain. Interaction with ligand promotes dimerization and subsequent increases in the catalytic activity of the kinase.

**signal transduction** The process of transferring information from the extracellular milieu to the cellular interior and ultimately into the nucleus. This biochemical process is integral to the control of cellular survival, growth, development, and function.

## See Also the Following Articles

**Angiogenesis • Erythropoietin, Biochemistry of • Protein Kinases**

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# Steroid Hormone Receptor Family: Mechanisms of Action

MILAN K. BAGCHI
*University of Illinois, Urbana-Champaign*

I. INTRODUCTION
II. FUNCTIONAL DOMAINS OF NUCLEAR RECEPTORS
III. HORMONE-INDUCED ACTIVATION
IV. TARGET DNA RECOGNITION
V. TRANSCRIPTIONAL ACTIVATION
VI. TRANSCRIPTIONAL REPRESSION
VII. REGULATION OF CHROMATIN STRUCTURE
VIII. SUMMARY

**The steroid hormones, which include the sex steroids (estrogen, progesterone, and androgens) and adrenal steroids (glucocorticoids and mineralocorticoids), have long been known to control the growth, development, and homeostasis of various mammalian tissues.**

## I. INTRODUCTION

The biological action of a steroid hormone is mediated by its cognate intracellular receptor, which regulates the expression of a specific set of genes in the nucleus. The steroid receptors belong to a large, evolutionarily related family of transcription factors, known as the nuclear receptor (NR) superfamily. This family, which has 48 members, also includes the receptors for thyroid hormones, retinoic acids, and vitamin D as well as receptors for a variety of other metabolic ligands. Many orphan receptors, for which the ligands remain undiscovered, also belong to this family. The signal transduction pathway of the NRs has been studied extensively as a model system to investigate the fundamental mechanisms of eukaryotic gene regulation. These studies have provided a blueprint for the mechanism of action of these important cellular regulatory molecules. The goal of this article is to provide a brief overview of the current mechanistic concepts and emerging models of the NR pathway.

## II. FUNCTIONAL DOMAINS OF NUCLEAR RECEPTORS

Biochemical, molecular genetic, and structure–function analyses during the mid to late 1980s revealed that the NRs possess a modular structure containing discrete functional domains consistent with their role as ligand-inducible transcription factors (Fig. 1). Generally, the receptor structure is composed of three principal modules: (1) an evolutionarily conserved DNA-binding domain (DBD), which anchors the NR to its target DNA, (2) a somewhat less conserved carboxy-terminal ligand-binding domain (LBD), and (3) a poorly conserved amino-terminal activation function region, AF-1. A flexible hinge region (D) joins the DBD and LBD. Mutational analysis of the LBD led to the identification of a highly conserved second activation function region, AF-2, which is

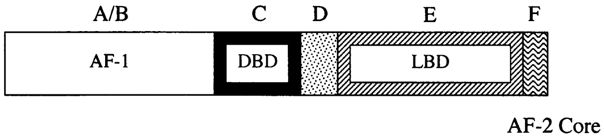

**FIGURE 1** Functional domains (A/B, C, D, E, and F) of a prototype nuclear hormone receptor. The DBD (C) and LBD (EF) represent DNA- and ligand-binding domains, respectively. AF-1 (A/B) and AF-2 (F) indicate constitutive and ligand-inducible transcriptional activation function regions, respectively.

essential for ligand-dependent activation by NRs. In certain receptors, such as thyroid hormone and retinoic acid receptors, the LBD functions as a repression domain in the absence of ligand. In addition, the LBD contains signals for receptor homo- and/or heterodimerization and nuclear localization. The LBD is, therefore, a uniquely important domain involved in many aspects of receptor function.

## III. HORMONE-INDUCED ACTIVATION

Hormones play a pivotal role in activating NRs. A steroid receptor is functionally inactive in cells in the absence of its cognate hormone. Several lines of evidence suggest that the unliganded receptor exists in an oligomeric complex in association with several heat-shock proteins, which are thought to function as chaperone proteins. In this inactive state, the receptor does not interact with DNA. Hormone binding leads to the disaggregation of the receptor from heat-shock proteins and converts it to a form that is able to bind DNA. For certain steroid receptors, such as the glucocorticoid and mineralocorticoid receptors, ligand binding occurs in the cytoplasm of target cells, inducing nuclear translocation of the ligand–receptor complex. Although the hormone-occupied receptors are always localized in the nucleus, many of the NRs are found in the nuclear compartment even in the absence of their cognate ligands. Certain NRs, such as thyroid hormone receptor (TR) and retinoic acid receptor (RAR), bind to DNA in the unliganded state and function as transcriptional repressors of target genes.

Hormone binding triggers a striking conformational change in the LBD of the receptor. Crystal structures of various NR LBDs complexed with agonist or antagonist ligand have thrown considerable light on the molecular nature of this structural change. In the absence of ligand, the LBD structure, which is composed of 12 contiguous α-helices, has a fairly open conformation. The hormone fits into a hydrophobic pocket formed by different layers of helices and directly contacts several amino acid residues of the receptor. On hormone binding, the LBD assumes a rather compact conformation. Comparison of the crystal structures of unliganded and liganded NRs suggested that hormone binding induces a dramatic repositioning of helix 12. Whereas in the unliganded NR, helix 12 extends away from the LBD, in the liganded receptor, this helix folds back tightly against the body of the LBD and makes contacts with the ligand itself. This ligand-induced rearrangement creates a surface consisting of residues contributed by helices 3, 4, and 12 that is utilized by the hormone-bound receptor for interaction with transcriptional co-regulators, such as co-activators (see below), which play critical roles in the hormonal signal transduction pathways.

## IV. TARGET DNA RECOGNITION

A critical step in the hormone-response pathway of NRs is the recognition of the target gene by the receptor. This may occur through direct interaction of the DBD with specific enhancer sequences referred to as hormone-response elements (HREs) or through protein–protein interactions with other classes of DNA-bound transcription factors, such as activating protein 1 (AP-1) or Sp1, at the target promoter. For the classical steroid receptors, estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), and mineralocortocoid receptor (MR), the response elements contain a 15 bp core sequence, composed of two half-sites of 6 bp (AGAACA for GR, PR, AR, and MR; AGGTCA for ER) arranged in a dyad axis of symmetry (palindromic). The half-sites are separated by 3 bp of random composition. These receptors bind to their response elements as head-to-head homodimers. DNA recognition by these NRs involves contacts of amino acids present in two interdependent zinc-finger structures in the DBD with specific base pairs within the major groove of the core HRE motif and the phosphate backbone.

Nonsteroid receptors, such as the TR, RAR, and vitamin D receptor (VDR), bind to response elements that contain direct repeats of a core recognition motif, AGGTCA. These receptors bind mostly as heterodimers with the retinoid X receptor (RXR). A RXR–TR heterodimer can bind to response elements consisting of direct repeats or inverted palindromes of the core AGGTCA motif. The number of base pairs separating the direct repeats of the core recognition motif determines DNA-binding specificities of different heterodimeric pairs. For example, direct repeats separated by 3, 4, and 5 bp represent response elements for VDR, TR, and RAR, respectively.

## V. TRANSCRIPTIONAL ACTIVATION

In a target cell, a DNA- and hormone-bound NR is thought to regulate gene expression by influencing local chromatin structure and enhancing the transcription initiation process at the target promoter. Studies exploiting steroid receptor-regulated *in vitro* gene expression systems indicated that the promoter-bound receptor stimulates transcriptional initiation by facilitating the formation of a stable preinitiation complex containing RNA polymerase II and other basal transcription factors. The precise mechanism by which the receptor achieves this effect remains to be determined. The receptor may promote transcription initiation by facilitating recognition of the promoter by a certain initiation factor(s) or simply by stabilizing the promoter DNA–protein complex once it is formed or perhaps by influencing both reactions. Recent work from a number of laboratories indicates that a class of mediator proteins, termed co-activators, is recruited by the receptor to modify chromatin structure. The co-activators may also function as signaling intermediates between the hormone-occupied receptors and the RNA polymerase II transcription machinery.

A surprisingly large number of nuclear receptor-interacting proteins that may serve as co-activators have been isolated using yeast two-hybrid screening, far Western cloning, and biochemical methods based on NR affinity chromatography. A hallmark of these putative co-activators is that they interact with the nuclear receptors in a ligand-dependent manner and enhance their transcriptional activity. The steroid receptor co-activator-1 (SRC-1), which stimulates transactivation by several nuclear hormone receptors, was the first co-activator reported for the NR superfamily. Additional receptor-interacting proteins, TIF2/GRIP1 and pCIP/ACTR/RAC3/AIB1, which show striking structural similarity to SRC-1, were soon isolated by other laboratories. The remarkable similarity in amino acid sequence among these proteins, all of which exhibit an approximate molecular size of 160 kDa, indicates the existence of a p160 family of nuclear receptor co-activators.

In addition to the p160 family of proteins, several other potential co-activators have been described. Prominent among these is the transcriptional co-activator CREB-binding protein (CBP, where CREB denotes $Ca^{2+}$/cyclic AMP response element-binding protein) and its homologue p300. It is believed that CBP/p300 is recruited as a secondary co-activator of NRs through its direct interaction with primary p160 co-activators. There is strong biochemical evidence that CBP/p300 co-exists with one or more p160 proteins in a large receptor-co-activation complex. These co-activators work together in a synergistic fashion to enhance ligand-dependent transactivation mediated by nuclear receptors. Microinjection of an anti-SRC-1 or anti-CBP antibody into cultured cells partially blocked ligand-dependent gene activation by PR, ER, and other nuclear receptors. Most importantly, gene knockout studies show that loss of SRC-1 function partially impaired the physiological actions of several NRs.

A CBP-associated factor (p/CAF), the mammalian homologue of yeast GCN5, is reported to interact with CBP and the p160 proteins, as well as directly with NR LBDs. p/CAF is also reported to function as a co-activator of several NRs. Interestingly, all three co-activators, p160s, CBP, and p/CAF, possess intrinsic histone acetyltransferase (HAT) activities, suggesting that they might function as chromatin remodelers. Furthermore, CBP has been shown to interact directly with the components of the RNA polymerase II machinery. Collectively, these results indicate that the p160 family of proteins, CBP/p300, and possibly additional co-factors like p/CAF act in unison with the activated nuclear receptors to remodel chromatin structure and then directly recruit the basal transcription apparatus to effect steroid-dependent gene activation.

Another class of co-activator that plays a critical role in NR-mediated transactivation is the thyroid receptor-associated protein/vitamin D receptor-interacting protein (TRAP/DRIP) complex, comprising 13–15 polypeptides. This complex interacts with the LBD of many NRs in a hormone-dependent manner. A single subunit, TRAP220/DRIP205, anchors the entire complex to the LBD and enhances NR-mediated transactivation in an *in vitro* chromatin-free transcription system. Additional protein–protein interactions between the TRAP/DRIP150 subunit and the AF-1 of GR have been documented. Unlike the p160/CBP complex, the TRAP/DRIP complex is devoid of HAT activity. Several subunits of the TRAP/DRIP complex are also present in a mammalian complex corresponding to the yeast mediator complex that associates with the RNA polymerase II holoenzyme. These findings raise the possibility that the TRAP/DRIP complex, once recruited by a NR, may stimulate transcription by directly contacting and influencing the activity of the RNA polymerase II machinery at the core promoter.

The fact that the p160/CBP complex possesses HAT activity but the TRAP/DRIP complex does not has led to the proposal that these co-activator complexes interact with the receptor in a two-step sequential manner to effect transcriptional activation. According to this hypothesis, the NR initially recruits the SRC/CBP complex, which remodels and opens up the chromatin. This is followed by the recruitment of the TRAP/DRIP complex, which interacts with and stimulates the activity of the RNA polymerase II initiation apparatus. Although this is an attractive model, it is not clear how a strict temporal order of recruitment can be maintained or an efficient exchange of co-activators can be achieved. Furthermore, one also needs to consider the possibility that these co-activators function in parallel pathways of receptor-dependent activation, which might be operative in a promoter-specific manner.

Ligand binding to a NR acts as a switch for co-activator recruitment. For steroid receptors such as the GR, ER, and PR, ligand-induced release of receptor-associated heat-shock proteins likely precedes co-activator binding. For certain other receptors such as TR and RAR, ligand-dependent displacement of co-repressors might be a necessary prerequisite for co-activator action. In both cases, the ligand-induced conformational change in the receptor LBD generates a surface to which the co-activator protein docks. Recent studies in several laboratories indicated that multiple conserved leucine-rich sequences, termed NR boxes, exist in the p160 family of co-activators. These NR boxes contain the signature LXXLL motif, where L is leucine and X is any amino acid. An amphipathic helical peptide containing a LXXLL motif mediates the interaction of the co-activator with the LBDs of various nuclear receptors. The leucine residues 1 and 5 form intimate contacts with well-conserved amino acids within the core hydrophobic pocket of the LBD. The residues immediately surrounding the LXXLL motif determine receptor specificity. Crystal structure data support the view that two LXXLL motifs of a single SRC-1 molecule may interact with the individual AF2 domains of both subunits of a NR homo- or heterodimer. Recent studies also indicate that the AF-1 domains of several steroid receptors have the ability to interact with and recruit p160 co-activators, although the molecular basis of this interaction is unclear. One can envision that the co-activator molecule, once docked via interaction with AF-1 or AF-2, may then function as a physical bridge between the transactivation domain of the receptor and the RNA polymerase II transcription machinery during gene activation. Further studies are clearly necessary to test this plausible mechanism.

## VI. TRANSCRIPTIONAL REPRESSION

Transcriptional repression by ligand-bound NRs is well documented. Typically, overexpression of a given nuclear receptor ("interfering receptor") in a transient transfection system often results in the repression of ligand-induced transcriptional activation of target genes by another member of the superfamily ("activating receptor"). Such mutually antagonistic interactions have been described between various pairwise combinations of the steroid receptors ER, GR, PR, and TR and also between NRs and AP-1. No DNA binding by the interfering receptor is required for this effect. Conceptually, transcriptional interference or "squelching" occurs due to the interaction of the activation domains of nuclear receptors with a common but limiting target protein, such as a co-activator, in their signaling pathways. Consistent with this hypothesis, overexpression of SRC-1 appears to alleviate the antagonistic competition between liganded ER and PR in a cell. Similar overexpression of CBP/p300 was also noted to partially overcome the antagonistic interplay between GR and AP1, indicating that this co-activator could be a limiting component common to both signal transduction pathways.

Another mode of transcriptional repression, termed silencing, is displayed by certain nuclear receptors, such as TR and RAR. These receptors actively repress the transcription of cellular genes bearing the cognate hormone-response elements. In the absence of hormone, TR or RAR binds to its response element in a ligand-independent manner and functions as a silencer of basal level transcription from the target promoter. Ligand binding to the receptor releases transcriptional silencing and leads to the activation of target gene expression. The mechanism of this ligand-induced switch from repression to activation of gene expression is a topic of intense investigation in many laboratories.

Recent studies indicated that transcriptional silencing by TR or RAR is dictated, in part, by the association of the receptor with cellular co-repressors. A number of candidate co-repressors have been described among which two distinct but structurally related co-repressors, NR co-repressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT), have been studied most extensively. NCoR/SMRT harbors multiple independent repression domains, which contribute to its overall repression function. The co-repressor uses its carboxy-terminal receptor interaction domain to interact with unliganded TR or RAR but fails to interact with the ligand-occupied receptors. Within the receptor interaction domain of the co-repressor, two CoRNR boxes containing I/LXXI/VI signature motifs mediate interactions with the unliganded LBD. These amphipathic helical sequences are reminiscent of the LXXLL motifs found in the co-activator. It is likely that one NCoR/SMRT molecule is bound per DNA-bound heterodimer, with each CoRNR box contacting a single NR LBD.

The critical determinants of receptor–co-repressor interaction appear to be the presence of the I/LXXI/VI motifs in the co-repressor and the structure of the NR LBD. Although the co-repressor uses the same hydrophobic pocket in the NR LBD that is used by the co-activator, there are important differences. The AF-2 helix is clearly inhibitory to co-repressor binding. In the unliganded receptor, this helix is displaced outside the ligand-binding pocket, allowing the co-repressor to bind. Ligand binding triggers a dramatic change in the position of helix 12, resulting in the displacement of the co-repressor and formation of the co-activator-binding surface. Ligand-induced exchange of co-repressors with co-activators can conceivably take place without the receptor coming off the DNA.

Recent biochemical studies indicate that NCoR/SMRT exists in a large multiprotein complex containing histone deacetylase 3 (HDAC3) and additional polypeptides such as WD40 repeat protein TBL1 and G-protein suppressor 2. Although HDAC3 is the most prevalent histone deacetylase that was found biochemically to be associated with the co-repressor complex, there are reports that NCoR/SMRT associates with Sin 3, HDAC1, and HDAC2. Once anchored to the DNA-bound NR, the co-repressor complex apparently utilizes the histone deacetylase to maintain a repressive chromatin state. In addition, one can envisage that one or more repression domains of the recruited co-repressor may directly contact critical components of the basal transcription machinery and negatively impact the assembly of a functional initiation complex.

NCoR and SMRT are also involved in the transcriptional repression pathway of nuclear receptors other than TR and RAR. Most interestingly, estrogen or progesterone receptor complexed with certain antagonist ligands recruits these co-repressors to the target promoter to block transcription. In tamoxifen- or raloxifen-bound estrogen receptor, due to the presence of an additional side chain in the antagonist, helix 12 is positioned improperly. Helix 12, instead of packing normally as in the hormone-bound LBD, overlaps with the surface that docks the co-activator. This ligand-specified variation prevents co-activator interaction and facilitates co-repressor binding. The recruited co-repressor then negatively modulates the transcriptional activity of the target promoter.

## VII. REGULATION OF CHROMATIN STRUCTURE

In the cell, the transcription units are packaged into nucleosomes and remain in a repressed state. An essential first step in the NR-dependent gene activation pathway is, therefore, chromatin remodeling. Several multisubunit ATP-dependent chromatin-remodeling complexes, such as yeast SWI/SNF or *Drosophila* ISWI, which use the energy of ATP to alter nucleosome structure, have been characterized. Brg1, a subunit of human SWI/SNF, interacts with the GR and is required for efficient receptor-dependent activation of a MMTV (mouse mammary tumor virus) promoter stably integrated into chromosomal DNA. Brg1 is also recruited to an ER-regulated promoter in response to the hormone and critically regulates the transcriptional activity of the receptor. Furthermore, the addition of purified Brg1 to an *in vitro* transcription system reconstituted from chromatinized templates facilitated RAR-dependent transactivation. Taken together, these results point to an important role of ATP-dependent chromatin remodelers in NR-mediated gene activation.

NR-associated factors can also remodel chromatin by modulating acetylation of histones in nucleosomes. Whereas certain co-activators are found to possess intrinsic histone acetyltransferase activity, the co-repressors are associated with histone deacetylases. It is known that hyperacetylation of the lysine-rich tails of histones H3 and H4 on a chromatin DNA can destabilize nucleosomes and facilitate the binding of transcription factors to the promoter regulatory elements, leading to gene activation. Hypoacetylation of acetylated H3 and H4 by deacetylases, on the other hand, creates a repressive chromatin conformation, leading to gene repression. It is therefore postulated that targeted recruitment of acetyltransferase or deacetylase to a particular gene may modulate its transcriptional activity. The recruitment of a co-repressor by a promoter-bound receptor is thought to induce local hypoacetylation of histones to create a repressive chromatin conformation, leading to gene repression. The recruitment of a co-activator, in contrast, would lead to local hyperacetylation of histones, which may destabilize nucleosomes on a chromatin DNA. This, in turn, is likely to allow the binding of a RNA polymerase II transcription initiation complex at the core promoter, leading to gene activation. Consistent with this hypothesis, many of the candidate nuclear receptor co-activators, such as CBP, p/CAF, and SRC-1/p160, are known to possess intrinsic histone acetyltransferase activity. Additionally, recent reports suggested that CBP/p300 or SRC-1 enhanced steroid receptor-dependent transactivation from a chromatinized hormone-responsive template. It has been shown that this effect involves the acetylation of histones and is critically dependent on HAT activity of CBP/p300.

Unliganded TR functions as a repressor of a target promoter by recruiting a co-repressor complex containing histone deacetylase to the promoter. In the presence of thyroid hormone, the co-repressor complex containing the deacetylase is released from the promoter-bound receptor, thereby relieving the transcriptional repression. The receptor then recruits a co-activator complex, which consists of one or more histone acetyltransferases, to promote gene activation. The co-activators and co-repressors appear to act by regulating histone acetylation at the target promoter in an opposing fashion (Fig. 2).

In addition to acetylation, other covalent modifications, such as phosphorylation, methylation, and ubiquitination of histones and nonhistone proteins, have been known to modulate NR function at the target promoter. There is ample evidence that phosphorylation via various kinase cascades influences the transactivation activity of several members of the steroid receptor superfamily such as the GR, PR, and ER. There is now evidence that specific phosphorylation events may modulate co-activator recruitment and function. For example, mitogen-activated protein kinase (MAPK)-induced phosphorylation of specific serine residues in the AF-1 of ER-β facilitates SRC-1 recruitment by the tamoxifen-complexed receptor. In contrast, MAPK-induced phosphorylation in the amino-terminus of peroxisome proliferator-activated receptor-γ leads to inhibition of receptor function.

Most striking among the hormone- and NR-induced chemical modifications at the target promoter is the methylation of nucleosome and transcriptional co-factors by a family of arginine-specific methyltransferases. The p160 co-activators, anchored to promoter-bound NRs, recruit the co-activator-associated methyltransferase 1 (CARM1). Interestingly, the carboxy-terminal region of p160 proteins contains distinct binding sites for both CARM1 and CBP/p300. Consistent with this scenario, CARM1 and p300 synergistically stimulate transactivation by ER and this synergism is dependent on the presence of a p160 co-activator. Whereas CARM1 methylates specific arginine residues in histone H3, CBP/p300 possesses intrinsic HAT activity. It is therefore conceivable that CARM1 and p300 cooperate to induce multiple concurrent histone modifications to induce efficient chromatin remodeling, which allows subsequent recruitment of the transcription machinery. It is also interesting to note that CARM1 and CBP/p300 can undergo direct protein–protein interaction with each other. A functional consequence of this interaction is methylation of CBP/p300 by CARM1. Although the methylated CBP/p300 retains HAT activity and can still act as a co-activator for NR-mediated

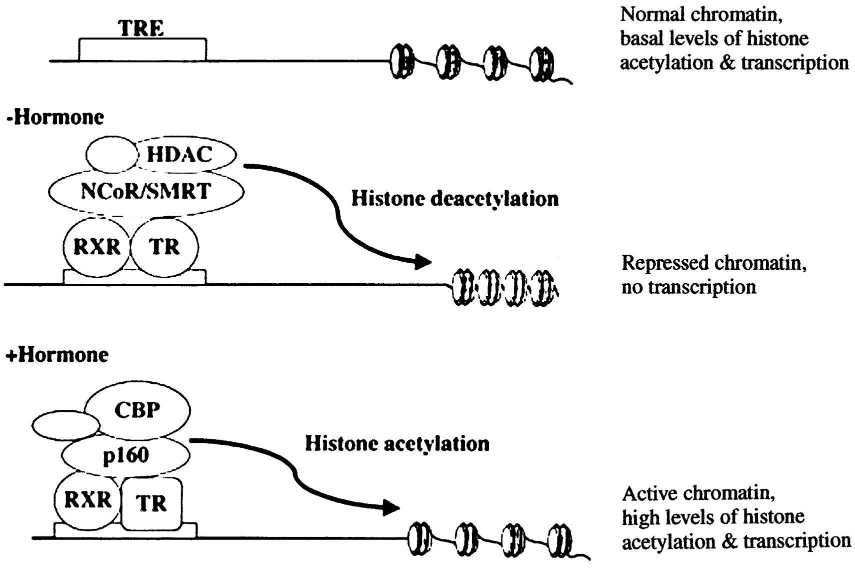
TRE
Normal chromatin, basal levels of histone acetylation & transcription
-Hormone
HDAC
NCoR/SMRT
RXR
TR
Histone deacetylation
Repressed chromatin, no transcription
+Hormone
CBP
p160
RXR
TR
Histone acetylation
Active chromatin, high levels of histone acetylation & transcription

FIGURE 2 A model of nucleosomal remodeling of a TR-responsive promoter by co-repressor and co-activator complexes containing HDAC and HAT activities, respectively. TRE indicates thyroid hormone-response element.

transcription, it fails to interact with CREB, resulting in a block in CREB activation. These results unveil a new regulatory mechanism involving co-factor methylation and stress the point that a combinatorial network of various NRs, their ligands, and co-regulatory proteins underlie the complexity of hormonal signaling.

## VIII. SUMMARY

The research on the NR pathway, which was initiated with the discovery of the concept of steroid receptors by Elwood Jensen and colleagues more than 40 years ago, has made tremendous progress through the years and led to the development of the best-understood model of eukaryotic gene regulation to date. The recent discovery of the co-activators and co-repressors has added additional layers of complexity to this regulatory pathway. Many details of the biochemistry, structure, function, and biology of these new molecules remain to be explored. A better mechanistic understanding of how the interplay of these co-regulatory molecules regulates the expression of specific NR-regulated genes during development and homeostasis in living cells will likely emerge from future studies.

## Glossary

**AF-1 and AF-2** Amino-terminal and carboxyl-terminal activation function regions of a nuclear receptor.

**chromatin** DNA packaged into nucleosomes or histone octamers.

**co-activator** A cellular co-regulatory protein that facilitates gene activation by a transcription factor.

**co-repressor** A cellular co-regulatory protein that facilitates gene repression by a transcription factor.

**histone acetyltransferase** An enzymatic activity that transfers an acetyl group from acetyl coenzyme A to histones.

**histone deacetylase** An enzymatic activity that removes an acetyl group from histones.

**hormone antagonist** A drug that counteracts the action of a hormone by binding to a nuclear receptor.

**hormone-response element** Short DNA sequences bound by nuclear receptors.

***in vitro* transcription** Study of RNA synthesis from a DNA template in cell extracts.

**methyltransferases** Enzymes that promote methylation of substrates.

**nuclear receptors** A family of ligand-inducible transcription factors.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Crosstalk of Nuclear Receptors with STAT Factors • Effectors • Orphan Receptors, New Receptors, and New Hormones • Steroid Nomenclature • Steroid Receptor Crosstalk with Cellular Signaling Pathways**

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# Steroid Nomenclature

Anthony W. Norman and Helen L. Henry
*University of California, Riverside*

I. BASIC RING STRUCTURE
II. CLASSES OF STEROIDS
III. STRUCTURAL MODIFICATION
IV. ASYMMETRIC CARBONS

**Steroids are relatively complex organic molecules with approximately 18–27 carbon atoms. To accurately describe the nature and position of the functional groups attached to steroids (and other organic molecules), chemists have devised a formal system of nomenclature. This article provides an introduction to steroid nomenclature and provides the systematic names of some common steroids as well as the structures of several steroid hormones.**

## I. BASIC RING STRUCTURE

Steroids are derived from a phenanthrene ring structure (**1**) to which a pentano ring has been attached; this yields in the completely hydrogenated form cyclopentanoperhydrophenanthrene or the sterane ring structure (**2**).

Steroid structures are not normally written with all the carbon and hydrogen atoms as illustrated in 2 of Fig. 1; instead, the shorthand notation as presented for sterane (**3**, Fig. 1) is usually employed. In this representation, the hydrogen atoms are not indicated and unless specified otherwise it is assumed that the cyclohexane (A, B, C) or cyclopentane (D) rings are fully reduced; that is, each carbon has its full complement of carbon and/or hydrogen bonds. Also indicated in sterane (**3**) is the standard numbering system for all the carbon atoms in the four rings as well as the letter designation of each ring of a steroid.

## II. CLASSES OF STEROIDS

In vertebrate systems, there are six families of steroid hormones that can be classified both on a

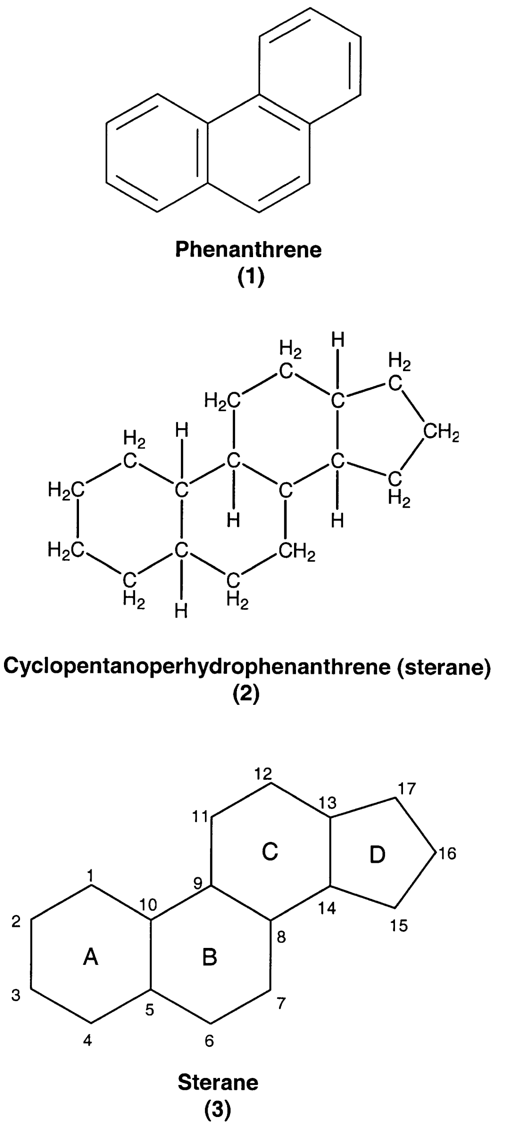

FIGURE 1 Parent ring structures of steroids. Phenanthrene (**1**) is the building block to generate the two 4-ring structures **2** and **3**. The structures for cyclopentanoperhydrophenanthrene (**2** and **3**) represent the same molecule; in **2**, all the hydrogen atoms are indicated, whereas in **3** the tetrahedral bonding of all carbons is assumed (no double bonds are present) and for the sake of convenience the hydrogen atoms are not shown. In (3), the standard numbering system for the carbons of the A, B, C, and D rings of the steroid nucleus is indicated.

structural basis and on a biological (hormonal) basis. They are the estrogens (female sex steroids), the androgens (the male sex steroids), the progestins, the mineralocorticoids, the glucocorticoids, and vitamin D along with its daughter metabolites. The bile acids, structurally related to cholesterol, constitute a seventh class of steroids. All of these steroids are biologically derived from cholesterol (Fig. 2).

## III. STRUCTURAL MODIFICATION

The basic steroid ring structures illustrated in sterane (**3**) can undergo an array of modifications by introduction of hydroxyl or carbonyl substituents and by introduction of unsaturation (double or triple bonds). In addition, heteroatoms such as nitrogen or sulfur can replace the ring carbons, and halogens and or amino groups may replace steroid hydroxyl moieties. Ring size can be expanded or contracted by addition or removal of carbon atoms. The consequences of these structural modifications are designated by application of the standard organic nomenclature conventions of steroids. The pertinent aspects of this system are summarized in Table 1. Prefixes and suffixes are used to indicate the type of structural modification. Any number of prefixes may be employed (each with its own appropriate carbon number and specified in order of decreasing preference of acid, lactone, ester, aldehyde, ketone, alcohol, amine, and ether); however, only one suffix is permitted.

TABLE 1 **Steroid Nomenclature Conventions**

| Modification | Prefix | Suffix |
|---|---|---|
| Hydroxyl group (–HO) | Hydroxy | -ol |
| Hydroxyl above plane of ring | β-OH | — |
| Hydroxyl below plane of ring | α-OH | — |
| Keto or carbonyl group (C=O) | Oxo- | -one |
| Aldehyde (–CHO) | — | -al |
| Carboxylic acid (COOH) | Carboxy | -oic acid |
| Double bond (–C=C–) | — | -ene |
| Triple bond (–C≡C–) | — | -yne |
| Saturated ring system | — | -an |
| One less carbon atom | -Nor | — |
| One additional carbon atom | -Homo | — |
| One additional oxygen atom | -Oxo | — |
| One less oxygen atom | -Deoxy | — |
| Two additional hydrogen atoms | -Dihydro | — |
| Two less hydrogen atoms | -Dehydro | — |
| Two groups on same sides of plane | *Cis* | — |
| Two groups on opposite sides of plane | *Trans* | — |
| Other ring forms (rings A and B *trans*, as in allopregnane) | Allo | — |
| Opening of a ring (as in vitamin D) | Seco- | — |
| Conversion at a numbered carbon from conventional orientation (as in epicholesterol or 3α-cholesterol) | -Epi | — |

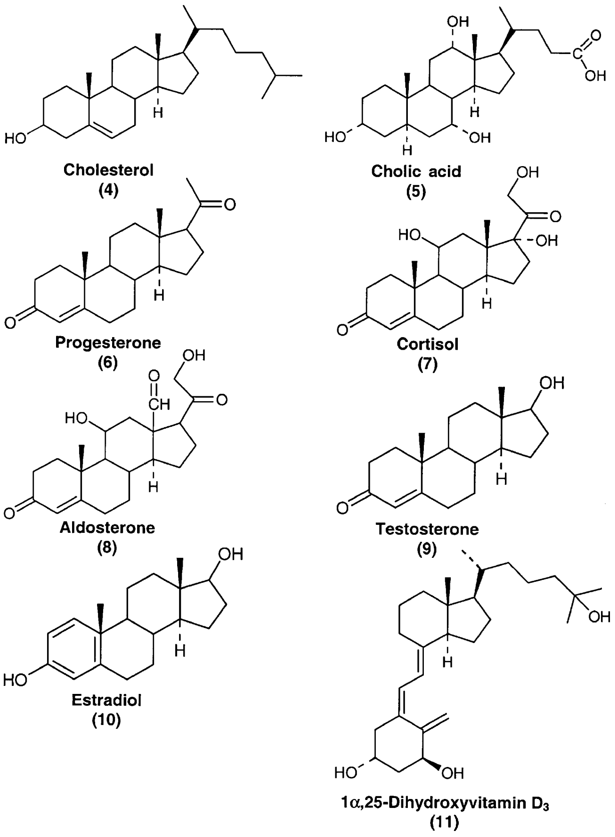
**Cholesterol (4)**
**Cholic acid (5)**
**Progesterone (6)**
**Cortisol (7)**
**Aldosterone (8)**
**Testosterone (9)**
**Estradiol (10)**
**1α,25-Dihydroxyvitamin D$_3$ (11)**

FIGURE 2 Structure of cholesterol (**4**) and cholic acid (a bile acid) (**5**) and representative structures of a steroid hormone from each steroid family (structures **6–10**). The hormone form of vitamin D$_3$ is $1\alpha,25(OH)_2$ vitamin D$_3$ (**11**).

The formal names of steroids are devised in accordance with the official nomenclature rules for steroids laid down by the International Union of Pure and Applied Chemistry. Table 2 lists the trivial and systematic names of a number of common steroids.

## IV. ASYMMETRIC CARBONS

An important structural feature of any steroid is the presence of asymmetric carbon atoms and designation in the formal nomenclature of the structural isomer that is present. For example, reduction of pregnane-3-one (**12**) to the corresponding 3-alcohol (**13**) will produce two epimeric steroids (**14**) and (**15**) (see Fig. 3). The resulting hydroxyl may be above the plane of the A ring and is so designated on the structure (**14**) by a solid line; it is referred to as a -3β-ol. The epimer or -3α-ol (**15**) has the hydroxyl below the plane of the A ring and is so designated by a dotted line for the –C···OH bond. If the α or β orientation of a substituent group is not known, it is designated with a wavy –C–OH line.

Another locus where asymmetric carbon atoms play an important role in steroid structure determination is the junction between each of the A, B, C, and D rings. Figure 4 illustrates these relationships for cholestanol and coprostanol. In the 5α form, the 19-methyl and the α-hydrogen on carbon 5 are on opposite sides of the plane of the A:B ring; this is referred to as a *trans* fusion. When the l9-methyl and β-hydrogen on carbon 5 are on the same side of the A:B ring fusion, this is denoted *cis* fusion. In this case, the steroid structure can no longer be drawn in one plane (as in **24**). Thus, in all 5β steroid structures that have *cis* fusion between rings A and B, the A ring is bent into a second plane that is approximately at right angles to the B:C:D rings (see **24** of Fig. 4). Thus, each of the ring junction carbons is potentially asymmetric and the naturally occurring steroid will have only one of the two possible orientations at each ring junction. Although there are two families of naturally occurring steroids with either *cis* or *trans* fusion of the A:B rings, it is known that the ring fusions of B:C and C:D in virtually all naturally occurring steroids are *trans*.

**TABLE 2 Trivial and Systematic Names of some Common Steroids**

| Trivial name | Systematic name |
|---|---|
| Aldosterone | 18,11-Hemiacetal of 11β,21 -dihydroxy-3,20-dioxopregn -4-ene-18-al |
| Androstenedione | Androst-4-ene-3, 17-dione |
| Androsterone | 3α-Hydroxy-5a-androstan-17-one |
| Cholecalciferol (vitamin $D_3$) | 9,10-Secocholesta-5,7,10(19) -trien-3β-ol |
| Cholesterol | Cholest-5-ene-3β-ol |
| Cholic acid | 3α,7α,12α,-Trihydrozy-5b-cholan -24-oic acid |
| Corticosterone | 11β,21-Dihydroxypregn-4-ene-3, 20-dione |
| Cortisol | 11β,17,21-Trihydroxypregn-4-ene -3,20-dione |
| Cortisone | 17,21-Dihydroxypregn-4-ene-3, 20-dione |
| Dehydroepiandrosterone | 3β-Hydroxy-5-androstene-17-one |
| Deoxycorticosterone | 21-Hydroxypregn-4-ene-3,20-dione |
| Ergocalciferol (vitamin $D_2$) | 9,10-5-eco-5,7,10(19), 22-ergostatetraen-3β-ol |
| Ergosterol | 5,7,22-Ergostatrien-3-β-ol |
| Estriol | Estra-1,3,5(10)-triene-3,16α, 17β-triol |
| Estrone | 3-Hydroxyestra-1,3,5(10) -triene-17-one |
| Etiocholanolone | 3α,-Hydroxy-5b-androstane-17-one |
| Lanosterol | 8,24-Lanostadiene-3β-ol |
| Lithocholic acid | 3α,-Hydroxy-5β-cholan-24-oic acid |
| Progesterone | Pregn-4-ene-3,20-dione |
| Testosterone | 17β-Hydroxyandrost-4-ene-3-one |

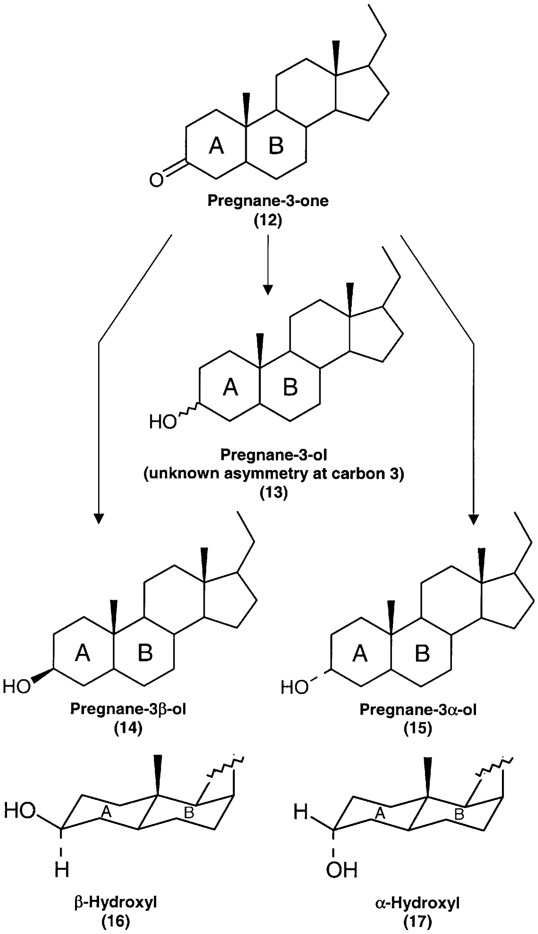

FIGURE 3 Structural consequences resulting from reduction of pregnane-3-one (**12**). The orientations of the α- and β-hydroxyls of compounds pregnane-3β-ol and pregnane-3α-ol as equatorial (**16**) or axial (**17**) substituents, respectively, on the chair version of the A rings are shown in the bottom row.

The chemical determination and designation of the absolute configuration of asymmetric carbon atoms on the side chain according to formal rules of nomenclature are complex. The "sequence rules" of Cahn *et al.* must be applied. These rules describe operational procedures to generate an unambiguous nomenclature specification of the absolute configu-

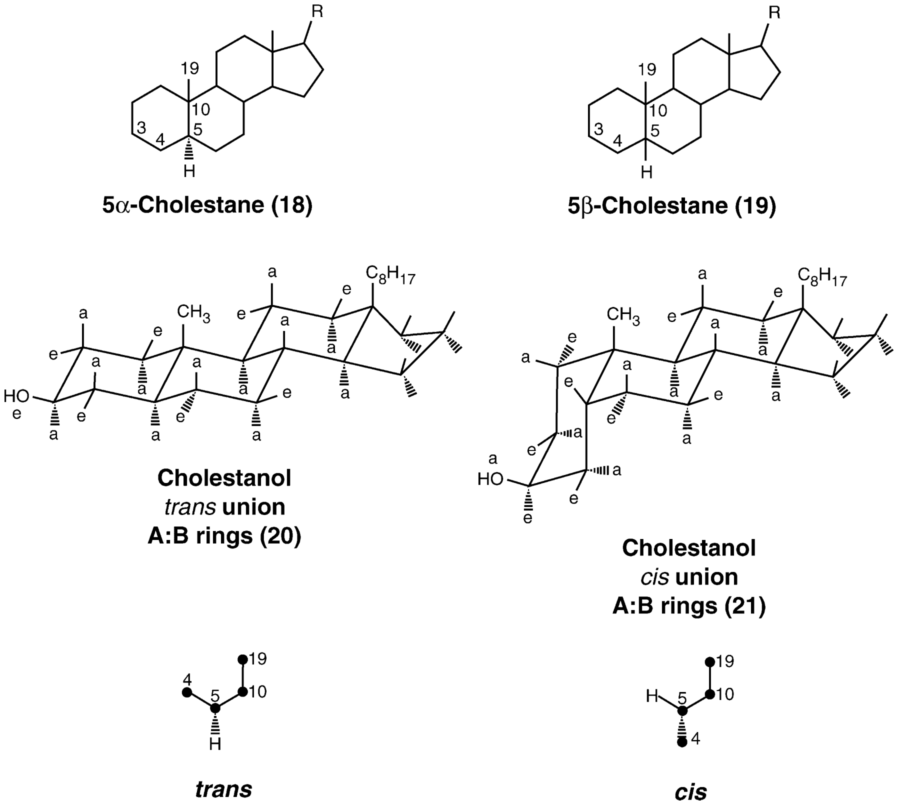

FIGURE 4 Structural relationships resulting from *cis* or *trans* A:B ring fusion in two typical steroids. In 5α-cholestane and cholestanol, the A:B ring fusion is *trans*, whereas in 5β-cholestane and coprostanol, the A:B ring fusion is *cis*. The orientation of substituents around carbon 5 for the *cis* and *trans* circumstances is illustrated in the bottom row. a, axial orientation; e, equatorial orientation; ●–●, carbon–carbon bonds.

ration of all chemical compounds whether they be steroids, sugars, amino acids, thiopolymers, or other compounds.

## Glossary

**asymmetric carbon** A carbon atom in a complex molecule that is chemically bonded to four different substituents.

**chirality** The right- or left-handedness of an asymmetric carbon of organic molecules.

**steroid** A member of the lipid class of compounds composed of the four-ring cyclopentanoperhydrophenanthrene nucleus, it is the basic structural component of steroid hormone families such as estrogens, progestogens, androgens, mineralocorticoids, glucocorticoids, and vitamin D and its metabolites.

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# Steroidogenic Acute Regulatory (StAR) Protein, Cholesterol, and Control of Steroidogenesis

Jerome F. Strauss, III
*University of Pennsylvania Medical Center*

I. INTRODUCTION
II. THE CRITICAL ROLE OF StAR
III. MOLECULAR GENETICS OF CONGENITAL LIPOID ADRENAL HYPERPLASIA

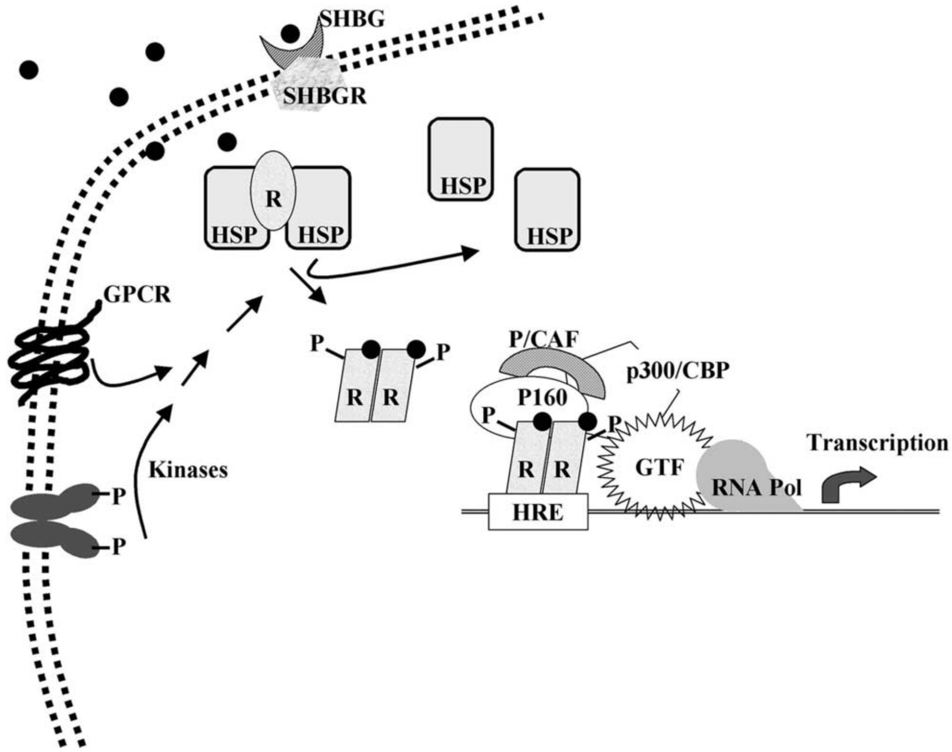

**FIGURE 1** In the absence of hormone, steroid receptors (R) associate with heat-shock proteins (HSP). Binding of steroid (●) results in dissociation from heat-shock proteins, dimerization, and binding to specific hormone response elements (HRE) in the promoters of target genes. The receptor recruits co-activators, including the p160 (also called steroid receptor co-activator) family of proteins, P/CAF, and p300/CBP. These proteins perform a variety of functions, including acetylation of histones, and interact with general transcription factors (GTF) to stimulate transcription of target genes. Activation of a variety of membrane receptors, including G-protein-coupled receptors (GPCR) and growth factor receptors, stimulates a cascade of phosphorylations; this results in enhanced phosphorylation (P) of steroid receptors and their associated proteins, altering the resulting transcriptional activity. Among the signaling pathways that can be induced is steroid-mediated activation of steroid hormone-binding globulin (SHBG) and its receptor, steroid hormone-binding globulin receptor (SHBGR), which elevates cAMP levels, activating protein kinase A. In some cases, altered cell signaling is sufficient to activate a steroid receptor in the absence of any hormone, a pathway termed ligand-independent activation.

containing sequences (hormone response elements) specifically recognized by the receptor; these sequences are in the promoters of target genes. In addition, the receptors recruit complexes of proteins, termed co-activators, which serve a variety of functions to enhance the transcriptional activity of the receptor. Among the best characterized activities of the co-activators is the ability to acetylate histones at target genes, opening up chromatin and allowing access for RNA polymerase and other factors needed for transcription. The receptors and their co-activators are phosphoproteins. Signals emanating from membrane receptors, including growth factor receptors and G-protein-coupled receptors, regulate the phosphorylation and activity of the steroid receptors. Sometimes these changes in cell signaling are sufficient to activate a steroid receptor in the absence of hormone, a process termed ligand-independent activation.

The general structures of the steroid receptors are very conserved; the structure of the estrogen receptor-α is depicted in Fig. 2. Steroid receptors contain highly conserved DNA-binding domains, which consist of two $Zn^{2+}$ finger motifs (region C) and less well-conserved hormone-binding domains (region E) linked to the DNA-binding domain by the hinge region (D). The hormone-binding domains also contain a region important for transcriptional activity, activation function-2, (AF-2), which binds co-activators, facilitating transcription. The hinge region contains a nuclear localization signal. Some receptors contain an additional carboxyl-terminal

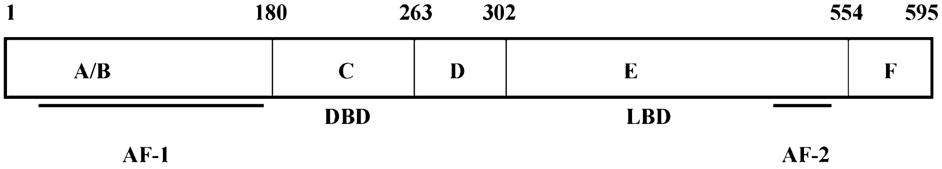

FIGURE 2 The general structural organization of a steroid receptor, depicting the structure of the human estrogen receptor-α. All receptors contain regions A–E. The numbering indicates the amino acid positions bordering the functional domains in estrogen receptor-α. Regions C and E of steroid receptors contain the DNA-binding domain (DBD) and the ligand-binding domain (LBD), respectively. Within the A/B domain and the E domain, each steroid receptor contains a region important for transcriptional activity (AF-1 and AF-2). Region D, termed the hinge region, contains a nuclear localization signal. Although estrogen receptor-α contains an F domain, which is not required for hormone binding, some receptors lack this region.

segment (F), which is not required for hormone binding. The amino-terminal regions of the receptors (A/B) are the least conserved and are the most variable in length, ranging from less than 200 amino acids in the estrogen receptor to more than 500 in the progesterone receptor. This region contains an additional activation function (AF-1). The relative importance of AF-1 and AF-2 in inducing transcription is dependent on receptor, promoter, cell type, and activation signal.

## III. REGULATION OF STEROID RECEPTOR FUNCTION BY CELLULAR SIGNALING PATHWAYS

Two major approaches have been utilized to assess the role of cellular signaling in the activity of steroid receptors. The first method is to modulate the activity of cellular signaling pathways and to determine the effect on receptor activity. The advantage of this scheme is that one can rapidly identify cellular signaling pathways that contribute to the regulation of steroid receptors. However, it is frequently difficult to identify the means by which the alterations in cell signaling modulate the receptor activity. The target of the kinases may be the receptor, associated proteins, or both. The second technique is to mutate phosphorylation sites in the steroid receptors and to determine the roles of the individual phosphorylation sites. This approach is limited by the necessity of first identifying the phosphorylation sites in the steroid receptors, a difficult undertaking that has not been completed for the majority of the steroid receptors.

### A. Activation of Steroid Receptors by Cellular Signaling Pathways

Investigators have known for a long time that some steroid-regulated genes are also regulated by signal transduction pathways. Initially, it was assumed that the cell signaling pathways were acting independently of steroid receptors through proteins such as cAMP response element binding protein (CREB), the activation of which is dependent on phosphorylation. However, it is now evident that alterations in kinase activity can activate some steroid receptors in the absence of hormone. This was first shown by introducing into cells lacking the progesterone receptor a plasmid encoding the chicken progesterone receptor and a reporter plasmid containing progesterone response elements linked to the coding region of chloramphenicol acetyltransferase (CAT). CAT activity, induced in response to progesterone or 8-Br cyclic adenosine monophosphate (cAMP) (an activator of protein kinase A), was then measured. Surprisingly, both treatments induced CAT activity and both were dependent on the progesterone receptor. Subsequent studies revealed that activators of diverse signal transduction pathways cause ligand-independent activation of chicken progesterone receptor. These include epidermal growth factor (EGF), which acts through a membrane-bound receptor that induces its tyrosine kinase activity, and dopamine, which acts through a serpentine membrane receptor that causes activation of G-protein-coupled pathways. Responses of steroid receptors to signal transduction pathways are receptor, activator, and cell type specific. The estrogen receptor appears to be the most responsive of the receptors. Although there are unique genes for most of the steroid receptors, the discovery of a second gene encoding an estrogen receptor has altered the nomenclature in the field. The estrogen receptor that was cloned originally and is the best studied is now termed ER-α; the more recently discovered receptor is termed ER-β. Discussions of the estrogen receptor in literature prior to 1996 refer to ER-α. Many stimuli, including dopamine, 8-Br cAMP, EGF, and other growth factors, activate ER-α, and there is evidence that ER-β can also be activated by cell signaling pathways in the absence of hormone. Most studies of ligand-independent activation have been done in cells transfected with expression plasmids for receptors and with artificial reporters, raising the question of whether such activation can occur *in vivo*. Two types of studies support the belief that these are pathways that contribute to biological activity *in vivo*. First, EGF can induce responses (such as DNA synthesis) that are also induced by estrogen in the uteri of ovariectomized mice; the EGF action is blocked by the estrogen receptor antagonist, ICI 164384, indicating that EGF is acting through the estrogen receptor. Moreover, EGF fails to induce DNA synthesis in the uteri of mice that lack ER-α. Second, dopamine can induce a sexual receptivity response in rats (lordosis) that is normally progesterone dependent. Administration of a progesterone receptor antagonist, mifepristone, inhibits this response, confirming that dopamine is acting through the progesterone receptor.

Other steroid receptors are less responsive to changes in cell signaling. Although the human androgen receptor can be activated under specific circumstances by treatments that elevate cAMP levels or by growth factors, in many cases the changes in cell signaling are insufficient to activate the receptor, although they do increase the response to hormone. Although avian and rodent progesterone receptors can be activated in the absence of ligand, comparable conditions fail to activate the human progesterone receptor. However, treatment with growth factors or activation of protein kinase A does enhance hormone-dependent activity. Moreover, treatment with 8-Br cAMP causes the antagonist mifepristone to act as an agonist. The glucocorticoid receptor responds similarly. It is resistant to ligand-independent activation, but altered cell signaling enhances activity either in combination with agonists or with the antagonist mifepristone.

## B. Role of Phosphorylation in Steroid Receptor Action

All of the steroid receptors, as well as some, if not all, of the co-activators, are phosphoproteins. Although the identification of the phosphorylation sites is incomplete, most have been identified. The number of sites in each receptor ranges from as few as four in chicken progesterone receptor to more than a dozen in the human progesterone receptor. The sites are predominantly serines, although phosphothreonine has been identified in the glucocorticoid receptor and in the human progesterone receptor. Tyrosine phosphorylation in these proteins occurs rarely. Under some conditions, phosphorylation of Tyr-537 in the hormone-binding domain of the estrogen receptor is detected. A majority of the phosphoserines and phosphothreonines are followed by prolines, implicating proline-directed kinases, such as the cyclin-dependent kinases and the mitogen-activated kinases, in the regulation of their phosphorylation. Almost all the phosphorylation occurs in the amino termini of the receptors. The major exception is a Ser-Pro motif in the hinge region of the steroid receptors. In contrast to the other sites, this site appears to be conserved among all receptors, and phosphorylation has been demonstrated in chicken and human progesterone receptors, mouse estrogen receptor, and human androgen receptor. Typically, the receptors exhibit some phosphorylation in the absence of hormone, but the degree of phosphorylation is enhanced upon hormone treatment. The phosphorylation of some sites such as Ser-118 in the estrogen receptor and several of the sites in the progesterone receptor is almost exclusively dependent on hormone or an activating signal such as EGF.

The roles of the phosphorylation sites are diverse and have not been fully elucidated. Reported functions range from increasing response to low levels of hormone, to altering affinity for DNA, regulating transcriptional activity, and playing a role in the stability of the receptors. One of the best characterized sites is Ser-118 in human ER-α. This site is phosphorylated either in response to hormone or to treatment with EGF. Mutation of this site to an alanine modestly reduces the transcriptional activity of the receptor in response to hormone. However, a negative charge (provided either by a phosphate or an artificially substituted glutamic acid) at this position is absolutely required for EGF-induced activation of ER-α in some cell lines. Substitution of a glutamic acid for the serine is insufficient to produce a constitutively active receptor. Thus, EGF must induce phosphorylation either of other sites in ER-α or, more likely, on associated proteins to induce ligand-independent activation.

The receptors are substrates for a variety of kinases. Although most of the sites are proline-directed kinase sites, the human progesterone receptor contains a casein kinase II site, and Ser-167 in the human estrogen receptor is a potential target of multiple kinases, including casein kinase II, Rsk, and Akt. Estrogen, progesterone, and glucocorticoid receptors are all substrates for the cyclin-dependent kinase 2 (Cdk2); activation of Cdk2 enhances the transcriptional activity of estrogen and glucocorticoid receptors. On treatment with EGF, Ser-118 in the estrogen receptor is phosphorylated by mitogen-activated protein kinase (MAPK; also called extracellular signal-regulated kinase, or ERK), contributing to ligand-independent activation. However, hormone-induced phosphorylation of Ser-118 is not dependent on MAPK; instead, cyclin H Cdk7 plays a role in hormone-dependent phosphorylation.

There are receptor-specific effects on steroid receptors of the MAPK family of proline-directed kinases, which includes the ERK, Jun N-terminal kinase (JNK) or stress-activated protein kinase, and p38/HOG signaling cascades. Activation of JNK stimulates the activity of ER-α whereas it inhibits the activity of the glucocorticoid receptor through phosphorylation of Ser-246 in the glucocorticoid receptor. Activation of ERK stimulates the activity of estrogen and progesterone receptors, but inhibits glucocorticoid receptor activity. Thus, the activities of the steroid receptors are regulated by many kinases and the consequences are receptor specific.

In addition to altering the activities of the steroid receptors through phosphorylation of the receptors, signal transduction pathways alter phosphorylation and activity of co-activators. This aspect of receptor function is less well studied, but there is good evidence that the steroid receptor co-activator (SRC) family of nuclear receptor co-activators is phosphorylated by ERK and that these phosphorylations modulate the ability of the SRC proteins to serve as co-activators.

## IV. STEROID RECEPTOR INTERACTIONS WITH REGULATED TRANSCRIPTION FACTORS

Although steroid receptor activities, which result from binding directly to specific DNA sequences, are the best characterized of steroid receptor actions, there is ample evidence that steroid receptors also regulate transcription of target genes through interactions with other transcription factors, independently of a direct interaction of the receptor with the DNA. These activities may be stimulatory or inhibitory, depending on the target gene, receptor, and interacting transcription factor. Conversely, these transcription factors influence the activities of the steroid receptors. In some cases, the cross talk involves DNA-independent protein/protein interactions, and in others, the response is dependent on the sequence including and surrounding the element to which the transcription factor binds in its target gene. Steroid receptors influence several of the transcription factors for which expression, subcellular localization, and activity are highly dependent on cellular signaling pathways.

### A. NF-κB

Steroid receptors modulate the function of nuclear factor κB (NF-κB), a ubiquitously expressed transcription factor. Five members of the family have been identified, with the most well-characterized active complex being a heterodimer of the p50 and p65 (RelA) subunits. Prior to activation, p50 and p65 are located in the cytoplasm and are bound to an inhibitory κB subunit, IκB. Activating signals such as growth factors or inflammatory cytokines such as tumor necrosis factor α (TNFα) induce phosphorylation of IκB, leading to its degradation by the proteasome pathway. This frees the p50/p65 complex, permitting nuclear translocation, binding to DNA, and activation of target genes. Steroid receptors interact with NF-κB through the p65 subunit and can reciprocally modulate transcriptional activity. Both ER-α and androgen receptor-dependent mutual repression of NF-κB activity have been reported. Repression may occur either through direct physical interaction or through competition for a limited pool of co-activators. The best studied example of mutual repression between a steroid receptor and NF-κB is the functional interaction between glucocorticoid receptor and NF-κB. Studies of glucocorticoid receptor repression of NF-κB-induced activation of intercellular adhesion molecule or interleukin (ICAM-1 or IL-8) genes, for example, reveal that the glucocorticoid receptor interacts with RelA at the promoter, but does not block binding of RelA to DNA or formation of the preinitiation complex. However, the presence of the glucocorticoid receptor blocks phosphorylation of Ser-2 in the carboxyl-terminal domain (CTD) of RNA polymerase, a step required for transcription of target genes.

### B. AP-1

The proteins that bind to AP-1 response elements are diverse, consisting of complexes containing either a heterodimer of Fos and Jun transcription factors or homodimers of Jun proteins alone. These proteins are regulated both at the level of transcription and by posttranslational modification. Originally characterized as proteins that are activated by protein kinase C, these proteins are regulated by numerous cell signaling pathways. Among these is the JNK pathway. Functional interactions between AP-1 proteins and steroid receptors are numerous and the response is

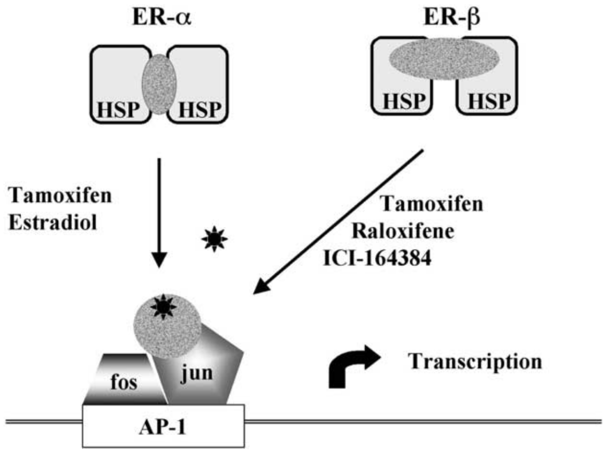

FIGURE 3 Estrogen receptor-mediated activation of transcription through an activator protein (AP-1) site. Estrogen receptors can promote transcription of a target gene through binding to the Jun partner of a Fos/Jun heterodimer bound to an AP-1 site. Although both estrogen receptor-α (ER-α) and ER-β are capable of activating transcription through AP-1 sites, the hormone specificity of the activation differs. HSP, Heat-shock protein complex.

receptor and promoter specific. Both ER-α and ER-β interact with AP-1 complexes. ER-α stimulates AP-1 activity on treatment with either an agonist such as estradiol or an antagonist such as tamoxifen (Fig. 3). Surprisingly, ER-β stimulates AP-1 activity only on binding antagonists. ER interacts with the Jun partner of the heterodimer. The finding that antagonists of conventional hormone response element-dependent transcription can stimulate estrogen receptor-dependent transcription through AP-1 elements complicates the simple model of antagonists as compounds that block the actions of steroid receptors. The ability of a specific compound to antagonize receptor activity is clearly not universal, but is dependent on the function measured.

In contrast to the estrogen receptor, the glucocorticoid receptor frequently inhibits the activity of AP-1 on activation by agonists, although this is, again, dependent on the promoter. The glucocorticoid receptor inhibits the activity of the AP-1 element in the proliferin gene when a Fos/Jun heterodimer binds to the site, but stimulates the activity of Jun homodimers. Similar to the ER, the glucocorticoid receptor binds to Jun; mutations in the glucocorticoid receptor that abrogate binding to conventional hormone response elements do not eliminate regulation of AP-1 proteins either *in vitro* or in a transgenic mouse expressing only the mutant receptor. The striking difference in phenotype between glucocorticoid receptor null mice (embryonic lethal) and mice expressing only a glucocorticoid receptor that lacks the ability to bind to a hormone response element provides strong evidence that many glucocorticoid receptor actions do not require binding to a conventional response element. For example, the glucocorticoid effects on the immune system are retained in mice with the mutant receptor.

Although studies of the androgen receptor have not been as extensive, there is evidence that AP-1 complexes acting through the Jun partner can inhibit the activity of the androgen receptor and that the androgen receptor inhibits the activity of the AP-1 complex.

### C. Interactions with Other Transcription Factors

The functional interaction of steroid receptors with other transcription factors is an active area of research. Among the other transcription factors that interact with steroid receptors are a member of the signal transducer and activator of transcription (STAT) family of transcription factors, STAT5. STAT proteins are cytoplasmic prior to activation. Activation of membrane-bound receptors, including cytokine receptors, causes activation of the Janus family tyrosine kinases (Jak kinases), which, in turn, phosphorylate cytoplasmic STAT proteins, promoting dimerization of the STAT proteins, translocation to the nucleus, and transcriptional activation. Both STAT proteins and glucocorticoid receptors are cytoplasmic in the absence of activating signals. Activation of either can promote nuclear localization of the other. These interactions can stimulate or inhibit the activities of the transcription factors. Activation of the glucocorticoid receptor stimulates STAT-dependent transcription of the β-casein gene, but STAT can also inhibit glucocorticoid receptor activity. Another example of a steroid receptor influencing transcription of target genes through other transcription factors is the activation of the cathepsin D gene by estradiol through interaction of estrogen receptor with the transcription factor Sp-1.

## V. STEROID-INDUCED ACTIVATION OF CELLULAR SIGNALING PATHWAYS

Steroids, acting through classical steroid receptors as well as through other means, induce the activation of a variety of signal transduction pathways. Although there has been evidence for a role of steroids in the activation of cellular signaling pathways for many years, elucidation of the precise mechanisms by which steroids transmit signals to activate kinases is a relatively new and active field of research. The actions of steroids are transmitted by diverse means, including through classical steroid receptors associated with membranes or acting in the cytoplasm, through steroid hormone-binding globulin, and through G-protein-coupled receptors. In addition, there is evidence for membrane receptors, which are structurally unrelated to classical steroid receptors, but these have not yet been cloned and little is known about these proteins.

A variety of studies have provided evidence that either the classical steroid receptor proteins or closely related proteins function as activators of cell signaling pathways. Membrane-bound glucocorticoid and estrogen receptors contain regions on the extracellular surface that cross-react with antibodies to the well-characterized estrogen and glucocorticoid receptors. Binding of estradiol by the membrane estrogen receptor in GH3 pituitary cells induces release of prolactin and subsequent activation of its signaling cascade. Interestingly, one of the ER-α antibodies blocks estradiol-induced prolactin response whereas another induces prolactin release in the absence of estradiol. Finally, reduction in ER-α expression in these cells using antisense oligonucleotides inhibits membrane estrogen receptor expression.

Steroid hormones acting through estrogen, androgen, or progesterone receptors can activate ERK through interaction with and activation of Src kinase, a tyrosine kinase. Although the result, i.e. activation of Src kinase, is similar, estrogen receptors and progesterone receptors interact with different regions of Src. Downstream actions of estradiol, such as activation of endothelial nitric oxide synthase (NOS), are blocked by inhibitors of ERK activation, suggesting a role for ERK in many estradiol-dependent nongenomic actions. In many instances steroids induce rapid alterations in calcium flux and/or induce the activities of a variety of kinases. Some of these actions appear to be independent of classical steroid receptors, although the mechanisms that induce the changes in signaling molecules have not been identified. In one case, a G-protein-coupled receptor (GPCR) is activated.

One steroid pathway that has been partially elucidated is the sex hormone-binding globulin (SHBG) pathway. SHBG serves as a carrier for steroids in the blood. In addition, it has the capacity to bind to a specific membrane receptor. When receptor-bound SHBG binds to an appropriate steroid, such as estradiol or dihydrotestosterone (DHT), the levels of cAMP are increased (Fig. 4). The receptor for SHBG has not been cloned, but there is indirect evidence that it is a G-protein-coupled-receptor because compounds or proteins that inhibit G-protein signaling block SHBG-mediated induction of cAMP.

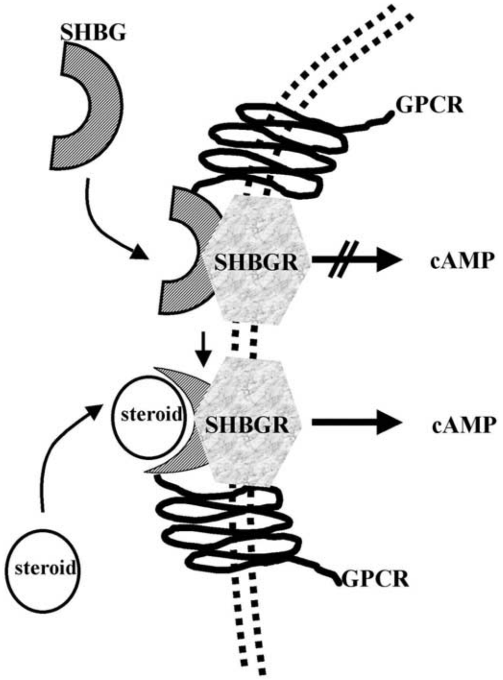

**FIGURE 4** Steroids increase cAMP levels through activation of steroid hormone-binding globulin receptor (SHBGR). In the absence of an activating steroid, the steroid hormone-binding globulin (SHBG) can bind to SHBGR, but does not induce production of cAMP. Binding of steroid activates the receptor, which may be a G-protein-coupled receptor (GPCR), or associates with a GPCR to stimulate synthesis of cAMP.

## VI. SUMMARY

Steroid hormone action and cellular signaling pathways intersect at many levels of the signaling pathways, providing many opportunities for mutual regulation and feedback between steroid receptors, kinases, and downstream transcription factors regulated by signal transduction pathways. For example, when an estrogen receptor-containing cell is exposed to estradiol, the result is a plethora of signaling events, including classical gene regulation through estrogen response elements, regulation of a subset of genes regulated by other transcription factors, such as AP-1 and Sp-1, and direct induction of a variety of cell signaling pathways leading to kinase activation.

## Glossary

**agonist** Compound that binds to a cellular receptor, stimulating its biological activity.

**antagonist** Compound that binds to a cellular receptor and blocks agonist-dependent induction of biological activity.

**co-activator** Molecule that binds to a transcription factor and stimulates the transcription of the target gene to which the factor is bound.

**G-protein-coupled receptors** Seven-transmembrane-spanning regions (also termed serpentine regions) that interact with heterotrimeric GTP-binding proteins, resulting in alterations in downstream signaling.

**proteasome** Large protein complex responsible for degrading proteins that have typically been modified by ubiquitination.

## See Also the Following Articles

**Androgen Receptor Crosstalk with Cellular Signaling Pathways • Co-activators and Corepressors for the Nuclear Receptor Superfamily • Crosstalk of Nuclear Receptors with STAT Factors • Estrogen Receptor Crosstalk with Cellular Signaling Pathways • GPCR (G-Protein-Coupled Receptor) Structure • Progesterone Receptor Structure/Function and Crosstalk with Cellular Signaling Pathways • Steroid Hormone Receptor Family: Mechanisms of Action**

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# Stress

Paul J. Rosch

*New York Medical College, Valhalla, and American Institute of Stress*

I. ORIGINS OF THE STRESS CONCEPT
II. THE GENERAL ADAPTATION SYNDROME AND DISEASES OF ADAPTATION
III. STRESS-RELATED DISORDERS AND MECHANISMS OF ACTION

**Stress, a term used interchangeably to refer to stimuli and to the psychophysiologic responses and pathologic consequences when such stimuli are severe or prolonged, is a highly personalized phenomenon. It is thus difficult to define, much less measure. Our current concepts of stress derive from early studies of physiologic responses to stressors, but technological advances have provided new insights into the mechanisms that may be involved in mediating the role of stress in different disorders.**

IV. STRUCTURE AND ACTIVITY OF StAR
V. THE ROLE OF PHOSPHORYLATION
VI. THE TISSUE-SPECIFIC EXPRESSION OF StAR
VII. THE StAR GENE AND ITS REGULATION
VIII. HOW DOES StAR WORK?
IX. StAR PARALOGUES

**Steroidogenic acute regulatory protein (StAR) plays an essential role in controlling the rate-determining step of steroid hormone synthesis. StAR is a 30 kDa protein and its mRNA is found at high levels in tissues that are involved in steroidogenesis: human adrenal cortex, ovary, and testis. It is also found at lower levels in other tissues (e.g., kidney). This article discusses the role of StAR, its structure and activity, the regulation of the *StAR* gene, and possible models for the mechanism of action of the protein.**

## I. INTRODUCTION

Cholesterol is the substrate for steroid hormone synthesis, and in humans, circulating lipoproteins are the major source of cholesterol used for steroidogenesis. Low-density lipoproteins (LDLs) are a primary reservoir of steroidogenic cholesterol, although other lipoproteins including very-low-density lipoproteins and high-density lipoproteins (HDLs) also contribute to the steroidogenic pool. The LDL receptor family and HDL (SR-BI) receptors are highly expressed on cells that produce large amounts of steroids, and tropic hormones up-regulate the expression of these receptors, ensuring that an adequate supply of substrate is available for steroid biosynthesis. *De novo* synthesis provides cholesterol for steroidogenesis when lipoprotein-derived substrate is insufficient. Excess cholesterol accumulated by steroidogenic cells is esterified and stored in cytoplasmic lipid droplets ready for mobilization by cholesterol ester hydrolase, which is activated by protein kinase A in response to tropic hormone stimulation. Thus, in contrast to protein hormone-secreting glands, which store preformed hormone, steroidogenic glands store hormone precursors.

The initial step in steroid hormone synthesis is the cleavage of the cholesterol side chain to form pregnenolone. This reaction is catalyzed by an enzyme system located on the matrix side of the inner mitochondrial membranes consisting of cytochrome P450 side chain cleavage ($P450_{scc}$); adrenodoxin, an iron sulfur protein electron shuttle; and adrenodoxin reductase, a flavoprotein that transfers electrons from NADPH to adrenodoxin. Cholesterol must be translocated from the relatively cholesterol-rich outer mitochondrial membrane to the inner mitochondrial membrane to come into contact with $P450_{scc}$, requiring it to traverse the aqueous space separating the two membranes. The acute regulation of steroidogenesis is due largely to the increased delivery of the hydrophobic cholesterol to the inner mitochondrial membrane. This acute mode of regulation of steroidogenesis is superimposed on the longer-term control that includes changes in the transcription of genes involved in steroid biosynthesis, cellular cholesterol uptake, and intracellular sterol trafficking.

*In vivo* and *in vitro* studies from more than 30 years ago demonstrated that cycloheximide and other inhibitors of translation block tropic hormone-stimulated pregnenolone production and cause cholesterol to accumulate in the outer mitochondrial membranes. These observations led to the concept that the acute increase in steroidogenesis requires a short-lived, cycloheximide-sensitive protein that is activated or synthesized in response to tropic hormone stimulation. Orme-Johnson and colleagues and Stocco and colleagues reported the induction of an ~30 kDa phosphoprotein in cells stimulated by tropic hormones or cyclic AMP (cAMP) analogues. The 30 kDa protein was localized to mitochondria and shown to be derived from a larger precursor molecule. The 30 kDa protein was subsequently isolated from MA-10 Leydig tumor cells and partial amino acid sequences were obtained, allowing the cloning of the cDNA for the murine protein and later the human protein. The protein was named steroidogenic acute regulatory protein (StAR) and it was proposed to be the cycloheximide-sensitive factor controlling the rate-determining step in steroidogenesis.

## II. THE CRITICAL ROLE OF StAR

The following evidence supports the notion that StAR has a critical role in steroid hormone synthesis: (1) Expression of StAR in MA-10 Leydig tumor cells correlates with the enhanced steroidogenesis. (2) In monkey kidney COS-1 cells, which are not steroidogenic, co-transfection of StAR and the cholesterol side-chain cleavage system results in enhanced steroidogenesis. In the absence of StAR, COS-1 cells expressing the cholesterol side-chain cleavage system produce substantial amounts of pregnenolone only when a polar hydroxysterol precursor that readily enters into mitochondria is provided as an exogenous substrate. However, COS-1 cells expressing both StAR and the cholesterol side-chain cleavage enzyme are capable of producing large amounts of pregnenolone from endogenous cholesterol. StAR also increases cholesterol flux through other mitochondrial P450 enzymes that oxidize cholesterol including P450c27, the enzyme that synthesizes 27-hydroxycholesterol, a bile acid precursor and potent regulator of cellular sterol homeostasis. (3) Mutations that inactivate StAR cause congenital lipoid adrenal hyperplasia, a rare autosomal recessive disorder in which the synthesis of all adrenal and gonadal steroid hormones is severely impaired at the cholesterol side-chain cleavage step, resulting in massive accumulation of cholesterol in the adrenal cortex and testicular Leydig cells. (4) Targeted deletion of the murine StAR gene results in a phenotype in nullizygous mice that mimics human congenital lipoid adrenal hyperplasia.

## III. MOLECULAR GENETICS OF CONGENITAL LIPOID ADRENAL HYPERPLASIA

The clinical phenotype of congenital lipoid adrenal hyperplasia includes the onset of profound adrenocortical insufficiency shortly after birth, hyperpigmentation reflecting increased production of proopiomelancortin, elevated plasma renin activity as a consequence of reduced aldosterone synthesis, and male pseudo-hermaphroditism resulting from deficient fetal testicular testosterone synthesis. The affected offspring are the products of uneventful pregnancies, delivered at term. Early in the disease, the steroidogenic cells of the enlarged adrenal cortices in affected individuals are engorged with lipid droplets containing cholesterol esters, giving rise to the condition’s name. Administration of adrenocorticotropic hormone or human chorionic gonadotropin to subjects with congenital lipoid adrenal hyperplasia does not elicit the normal acute increase in serum levels of adrenal or gonadal steroid hormones.

Congenital lipoid adrenal hyperplasia is a rare disease, except in Japan and Korea, where it accounts for 5% or more of all cases of congenital adrenal hyperplasia. Mutations in the *StAR* gene have been identified in more than 60 unrelated patients with congenital lipoid adrenal hyperplasia. Analysis of DNA from the parents of several patients confirmed that this disease is inherited in an autosomal recessive pattern. Mutations found in the *StAR* gene, which is composed of seven exons and is located on chromosome 8p11.2, include frameshifts caused by deletions/insertions, splicing errors, and nonsense and missense mutations, all of which lead to the absence of StAR protein or the production of functionally inactive protein. Several nonsense mutations were shown to result in C-terminal truncations of StAR. One of these mutations, Q258X, which results in the deletion of the final 28 amino acids of the StAR protein, accounts for 80% of the known mutant alleles in the affected Japanese population. All of the known point mutations that produce amino acid substitutions occur in exons 5–7 of the gene, the exons that encode the C-terminus. The metabolic defect in congenital lipoid adrenal hyperplasia is progressive, with adrenal and gonadal steroidogenesis becoming increasingly impaired with time after birth. A model has been proposed to explain the disease process that postulates the existence of some StAR-independent steroidogenesis prior to the severe cellular damage resulting from cholesterol accumulation and cholesterol oxidation, which ultimately results in a nonfunctional steroidogenic cell (Fig. 1). Comparison of the clinical course of 46,XX females with congenital lipoid adrenal hyperplasia to that of 46,XY subjects reinforced the proposed pathophysiological mechanism. 46,XX females underwent spontaneous puberty and secondary sexual development, whereas 46,XY patients were unable to undergo spontaneous puberty and had insufficient testicular androgen production *in utero* to masculinize the external genitalia. The sparing of some ovarian function in the face of mutations that inactivate StAR reflects the presence of modest StAR-independent steroidogenesis in follicles that is sufficient to sponsor estradiol synthesis. Because the ovary is essentially steroidogenically quiescent during fetal life and because follicles produce steroids only when they are recruited to mature, most follicles are spared from the ravages of cholesterol engorgement, leaving a source of viable, albeit impaired, steroid-producing cells at the time of puberty.

## IV. STRUCTURE AND ACTIVITY OF StAR

Human StAR is synthesized as a 285-amino-acid protein in the cytoplasm. The N-terminus of StAR is characteristic of proteins imported into mitochondria: the first 26 amino acid residues are predicted to form an amphipathic helix. Pulse–chase studies reveal that newly synthesized StAR pre-protein (37 kDa) is rapidly imported into mitochondria and processed to the mature 30 kDa form. The pre-protein has a very short half-life (minutes) but the mature form is longer-lived (hours). Drugs that collapse the mitochondrial proton gradient inhibit StAR import, and agents that block mitochondrial matrix metalloendoproteinases prevent the cleavage of the StAR N-terminal mitochondrial targeting sequence from imported protein.

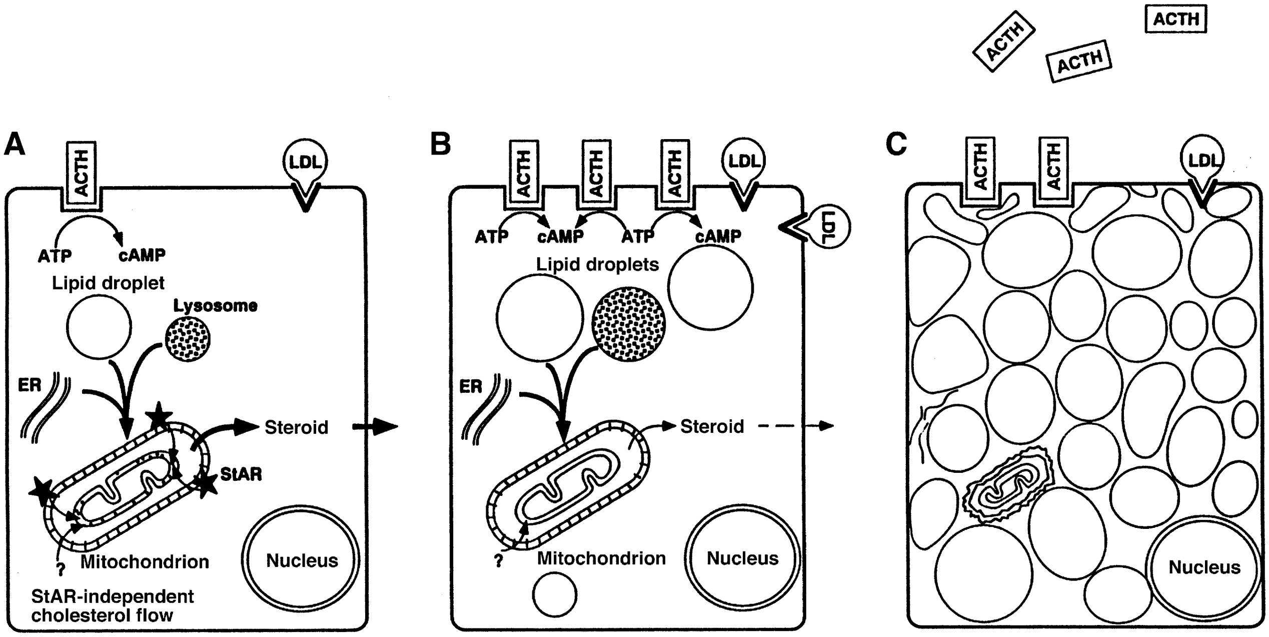

FIGURE 1 Model of the pathophysiology of congenital lipoid adrenal hyperplasia. (A) Normal adrenal cell. (B) Adrenal cell deficient in StAR at an early stage of the disease. The efficient flow of cholesterol to the inner mitochondrial membranes is impaired, leaving only a modest StAR-independent steroidogenesis. Consequently, corticotropin secretion is increased, resulting in adrenal cortical hyperplasia and increased uptake of LDL cholesterol, with accumulation of internalized cholesterol in lipid droplets due to inefficient movement of the substrate to P450scc. (C) Late stage of the disease, with massive accumulation of cholesterol in cytoplasmic lipid droplets, leading to organelle compression and auto-oxidation of cholesterol with subsequent peroxidative damage to proteins and organelles and a severe impairment of steroidogenesis.

To identify the domains of StAR critical for steroidogenesis, Arakane *et al.* generated StAR mutants and examined their activity in COS-1 cells transfected with the cholesterol side-chain cleavage enzyme. Deletion of the C-terminal 28 amino acids ablated StAR's steroidogenic activity, whereas removal of the last 10 amino acids reduced steroidogenic activity by 50%. These experiments indicated that residues in the C-terminus are critical for StAR's activity. The location of critical domains in the C-terminus of StAR is consistent with the analysis of gene mutations causing congenital lipoid adrenal hyperplasia in which mutations that inactivate StAR result from amino acid replacements located in exons encoding the C-terminal half of the protein.

It has been suggested that StAR takes on a molten globule configuration at pH 3.5–4.0, a pH that may be generated in the immediate vicinity of mitochondria. The molten globule configuration may facilitate the unfolding of StAR in preparation for action on the mitochondria and the subsequent movement through the import pore into the mitochondrial matrix. As discussed below, the crystal structure of a protein with a C-terminal domain similar to that of StAR has been determined. The crystal structure reveals that the StAR C-terminus contains a hydrophobic tunnel that can bind cholesterol.

## V. THE ROLE OF PHOSPHORYLATION

The mature StAR protein contains two consensus sequences for cAMP-dependent protein kinase phosphorylation at serine 57 and serine 195. Tropic hormones act via cAMP-mediated signaling cascades to rapidly increase the production of steroids. The cAMP second-messenger system activates protein kinase A, which phosphorylates proteins on either threonine or serine residues in a specific sequence context. Phosphorylation of StAR is a plausible mechanism by which preexisting or newly synthesized StAR can be rapidly activated.

Serine 195 of human StAR must be phosphorylated for maximal steroidogenic activity. cAMP promotes the incorporation of phosphorus into this residue within minutes of stimulation. The other consensus protein kinase phosphorylation site at serine 57 does not appear to be essential for StAR's steroidogenic activity. In contrast, mutation of serine 195 to an aspartic acid residue, which mimics the charge effect of phosphorylation, modestly increased the steroidogenic activity of the protein. These observations suggest that post- or co-translational modification of StAR can increase the activity of existing or newly made StAR protein, providing a mechanism by which tropic hormones acting through the intermediacy of cAMP can rapidly increase steroidogenesis.

## VI. THE TISSUE-SPECIFIC EXPRESSION OF StAR

Tissues that express StAR at high levels carry out tropic hormone-regulated mitochondrial sterol hydroxylations through the intermediacy of cAMP. StAR mRNA is abundant in human adrenal cortex, ovary, and testis. It is also found in lower abundance in kidney and monocytes, findings consistent with the fact that 1α-hydroxylation of vitamin D takes place in the kidney as well as monocyte/macrophages, a reaction catalyzed by a mitochondrial P450 enzyme. The brain also expresses StAR in some species, which may reflect a role in neurosteroid production. StAR mRNA was not detected in the human placenta, an observation that is consistent with the fact that pregnancies hosting a fetus affected with congenital lipoid adrenal hyperplasia go to term. Although estrogen production is impaired in these pregnancies as a result of diminished fetal adrenal androgen production, placental progesterone synthesis is not significantly affected, indicating that the trophoblast cholesterol side-chain cleavage reaction is independent of StAR.

StAR protein is detectable in thecal cells of human ovarian follicles in the adult ovary, but only in granulosa cells of luteinized follicles. Theca and granulosa cells of the fetal ovaries do not stain for StAR. StAR is prominent in fetal and adult testicular Leydig cells and present at low levels in Sertoli cells of adult testis. In the kidney, StAR is localized to the distal convoluted tubules. These observations are consistent with the clinical phenotype of congenital lipoid adrenal hyperplasia in which fetal adrenal and testicular steroidogenesis is markedly affected, whereas ovarian steroidogenic activity is spared to some extent because the ovaries are relatively quiescent until puberty.

## VII. THE *StAR* GENE AND ITS REGULATION

The abundance of StAR protein in steroidogenic cells is determined primarily by the rate of *StAR* gene transcription. In differentiated cells, *StAR* transcription is rapidly (within 15 to 30 min) activated by the cAMP signal transduction cascade. In differentiating cells (e.g., luteinizing granulosa cells), the induction of *StAR* transcription takes a longer time (hours) and requires on-going protein synthesis. *StAR* gene transcription is controlled, in part, by steroidogenic factor-1 (SF-1), also known as Ad4BP, an orphan nuclear receptor. The human *StAR* promoter contains three cooperative *cis* elements that bind SF-1 and are required for cAMP stimulation of *StAR*transcription. In addition to SF-1, CCAAT enhancer-binding protein-β, Sp1, GATA-4, and SREBP-1a (a member of the transcription factor family that governs the expression of many genes involved in lipid metabolism) contribute to the transcriptional control of StAR expression in a positive fashion, whereas DAX-1, another orphan nuclear receptor, inhibits *StAR* transcription, probably through interactions with SF-1.

## VIII. HOW DOES StAR WORK?

StAR was originally thought to stimulate cholesterol movement from the outer to the inner mitochondrial membrane as it was imported into the mitochondria. The importation process was proposed to create contact sites between the two membranes, allowing cholesterol to flow down a chemical gradient. However, a StAR mutant lacking the N-terminal 62 amino acids (N-62 mutant) that contain the mitochondrial targeting sequence was as effective as wild-type StAR in stimulating steroidogenesis. The overexpressed N-62 StAR protein was distributed throughout the cytoplasm of the transfected cells, whereas wild-type StAR was almost exclusively located inside mitochondria. Recombinant human N-62 StAR stimulated pregnenolone production by isolated ovarian mitochondria in a dose- and time-dependent fashion, with significant increases in steroid production observed within minutes with nanomolar concentrations. A mutant recombinant protein in which the A218V mutation, which is found in subjects with congenital lipoid adrenal hyperplasia, was completely inactive. The recombinant N-62 StAR also stimulated the transfer of cholesterol but not phosphatidylcholine from cholesterol-rich phophatidylcholine vesicles to sterol-poor acceptors. The transfer of sterols in these assays could not be ascribed to fusion of the donor vesicles and acceptor membranes. These experiments are most consistent with the idea that StAR enhances the desorption of cholesterol from sterol-rich donor membranes (Fig. 2).The desorption process may involve the

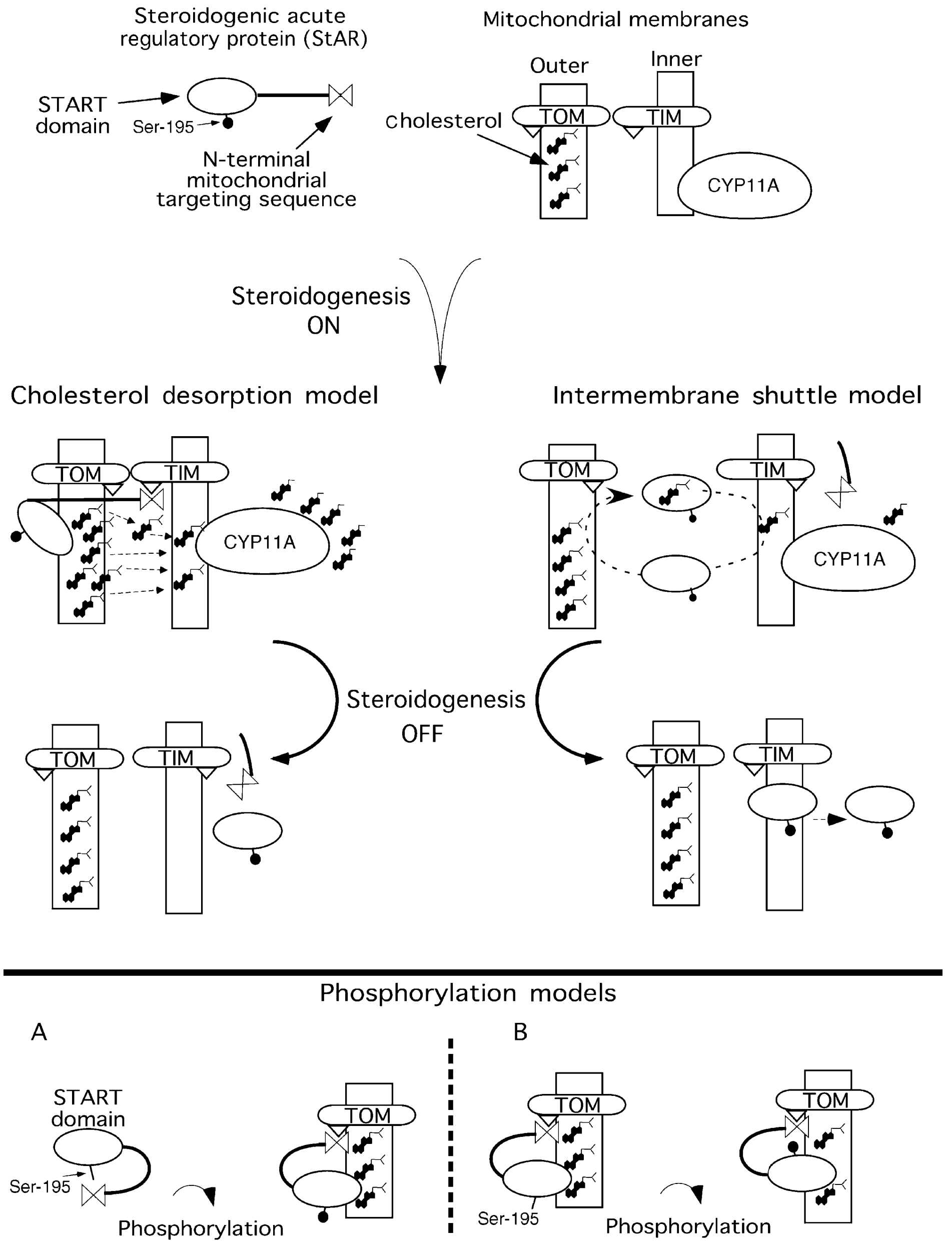
Steroidogenic acute regulatory protein (StAR)
START domain
Ser-195
N-terminal mitochondrial targeting sequence
Mitochondrial membranes
Outer
Inner
Cholesterol
TOM
TIM
CYP11A
Steroidogenesis ON
Cholesterol desorption model
Intermembrane shuttle model
TOM
TIM
CYP11A
TOM
TIM
CYP11A
Steroidogenesis OFF
TOM
TIM
TOM
TIM
Phosphorylation models
A
START domain
Ser-195
Phosphorylation
TOM
B
TOM
Ser-195
Phosphorylation
TOM

FIGURE 2 Models of StAR action. The StAR N-terminal mitochondrial targeting sequence binds to one or more of the known transmembrane outer mitochondrial transporters (TOM) and transmembrane inner mitochondrial transporters (TIM), linking it to the importation machinery. Cholesterol desorption model: The C-terminus of the protein (START domain) stimulates cholesterol desorption. The importation of the START domain removes the protein from its site of action, terminating sterol movement to the inner membrane. During or following importation, the StAR pre-protein is processed to yield the mature StAR form. Intermembrane shuttle model: The StAR protein is imported into the intermembrane space [the StAR-related lipid transfer (START) domain predicted model], and the targeting sequence is proteolytically cleaved, leaving the START domain in the intermembrane space, where it rapidly oscillates from the outer to the inner mitochondrial membranes, delivering cholesterol one molecule at a time. StAR is removed from the intermitochondrial space by an undetermined mechanism, thereby terminating sterol movement. Phosphorylation models: (A) Cyclic AMP-mediated phosphorylation of serine 195 located in the START domain of StAR reduces an inhibitory action of the N-terminal targeting sequence on StAR function, allowing more efficient interaction with the mitochondria. Alternatively (B), cyclic AMP-mediated phosphorylation of serine 195 might reduce an inhibitory action of the N-terminal targeting sequence on the START domain, allowing greater activity of this sterol transporter domain.

binding of cholesterol by the StAR C-terminus, which forms a hydrophobic tunnel, as predicted from the crystal structure of a StAR-like protein (MLN64), that can accommodate cholesterol molecules. Although the ability of StAR to bind cholesterol might suggest that it functions as shuttle, the relatively small amount of StAR in cells and its short functional life cannot account for the needed flux of cholesterol to P450scc if StAR can move only one or two moles of sterol per mole of StAR. Consequently, a "catalytic" role for the protein is required.

The observations reviewed above suggest a mechanism of StAR action that entails the efficient targeting of newly synthesized StAR pre-protein to the mitochondria by the protein's N-terminus. On reaching the mitochondria, the StAR C-terminus interacts with the relatively sterol-rich outer membrane, causing cholesterol to desorb from the outer membrane and transit to the relatively sterol-poor inner membrane, perhaps through preexisting contact sites. The import of StAR into the mitochondria and its subsequent processing remove the protein from its locus of action, effectively terminating sterol movement from the outer to the inner mitochondrial membranes. Thus, the mature protein represents a record of the past action of StAR.

It has been attractive to postulate that a "receptor" for StAR on the mitochondrial outer membrane is involved in the cholesterol translocation process. The hypothetical receptor, which could be a protein or a lipid, is evidently not specific for mitochondria of steroidogenic cells, since StAR works in the context of COS-1 cells, which are not normally steroid hormone-producing cells. The fact that immunohistochemical and electron microscope studies suggest that N-62 StAR mutants are not selectively accumulated by mitochondria argues against an abundance of high-affinity receptors for the StAR C-terminus on the mitochondria. Hence, either very few high-affinity sites or transient interactions are sufficient to promote cholesterol movement.

## IX. StAR PARALOGUES

When StAR was first identified, it was thought to be a unique molecule. It is now evident that a family of proteins sharing a domain that is similar to the C-terminus of StAR exist, the so-called StAR-related lipid transfer (START) domain proteins. The absence of StAR from the human placenta, an organ that produces a significant amount of pregnenolone, documented the existence of StAR-independent steroidogenesis but raised the possibility that another protein might subserve StAR's function in the placenta. MLN64, a gene discovered to be amplified in breast and ovarian cancer cells, shares significant structural homology with StAR. MLN64 was shown to increase pregnenolone production in COS-1 cells expressing the cholesterol side-chain cleavage enzyme system. MLN64 is anchored to late endosomes through N-terminal membrane-spanning domains; freeing the C-terminus of MLN64, which is homologous to StAR, from the membrane-spanning domains increases steroidogenic activity. Cleavage of the MLN64 protein releasing its C-terminus apparently occurs in various tissues, including the human placenta. Thus, the MLN64 C-terminus may replace StAR's function in the placenta. The roles of other START domain proteins in intracellular sterol trafficking and in steroidogenesis remain to be explored.

## Acknowledgments

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## Glossary

**congenital lipoid adrenal hyperplasia** Inherited metabolic disease characterized by markedly impaired adrenal and gonadal steroid hormone synthesis due to mutations in the *StAR* gene.

**cytochrome P450scc (CYP11A)** The catalytic component of the cholesterol side-chain cleavage system that performs three cycles of oxidation and reduction on the cholesterol side chain, resulting in the formation of pregnenolone and isocapraldehyde.

**steroidogenic factor 1** An orphan nuclear receptor that plays a key role in controlling the transcription of the *StAR* gene.

## See Also the Following Articles

**Glucocorticoid Biosynthesis: Role of StAR Protein • Lipoprotein Receptor Signaling • Luteinizing Hormone (LH) • Neuroactive Steroids**

## Further Reading

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# Steroid Receptor Crosstalk with Cellular Signaling Pathways

Nancy L. Weigel and Irina U. Agoulnik
*Baylor College of Medicine*

I. INTRODUCTION
II. STEROID RECEPTOR STRUCTURE AND FUNCTION
III. REGULATION OF STEROID RECEPTOR FUNCTION BY CELLULAR SIGNALING PATHWAYS
IV. STEROID RECEPTOR INTERACTIONS WITH REGULATED TRANSCRIPTION FACTORS
V. STEROID-INDUCED ACTIVATION OF CELLULAR SIGNALING PATHWAYS
VI. SUMMARY

**Steroid hormone receptors are hormone-activated transcription factors. Regulated by a wide variety of cell signaling pathways, steroid hormone receptors alter phosphorylation of target proteins. Conversely, steroids and their receptors act at many levels to regulate diverse cell signaling pathways and their target transcription factors. Thus, the actions of steroids and other cellular regulators are intricately intertwined.**

## I. INTRODUCTION

As hormone-activated transcription factors, the steroid hormone receptors and the cell signaling-regulated transcription factors such as activator protein 1 (AP-1) can regulate components of each other's pathways at the transcriptional level. The focus here, however, is on the cross-regulation of the activities of the signaling pathway components. Three major mechanisms are of importance: (1) regulation of steroid receptor phosphorylation and function by cellular signaling pathways, (2) interactions between steroid receptors and transcription factors that have activities regulated by cellular signaling pathways, and (3) activation of cellular signaling pathways by steroids.

## II. STEROID RECEPTOR STRUCTURE AND FUNCTION

The steroid hormone receptors belong to a large family of ligand-activated transcription factors; the family includes steroid receptors such as the estrogen and androgen receptors, receptors for thyroid hormone and retinoic acid, and a large number of receptors for other small, hydrophobic molecules. In the absence of hormone, steroid receptors associate with heat-shock protein complexes, which hold the receptor in a conformation capable of binding hormone and protect the receptor from degradation (Fig. 1). The location of the receptor in the absence of hormone is specific to the receptor and sometimes to the cell type. Glucocorticoid and androgen receptors are predominantly cytoplasmic in the absence of hormone, whereas the estrogen and progesterone receptors are predominantly nuclear. The steroid hormones are hydrophobic cholesterol derivatives and can freely pass through the cell membrane. Binding of hormone causes conformational changes in the hormone-binding domain that favor dissociation from the heat-shock protein complexes. The receptors form dimers and associate with DNA

# I. ORIGINS OF THE STRESS CONCEPT

## A. Claude Bernard's Milieu Intérieur and Walter Cannon's "Homeostasis"

Although the word "stress" has been used for over five centuries as a synonym for distress, its current meaning dates back only six decades, when the term was essentially coined by the brilliant Canadian investigator Hans Selye. Selye's concept of stress had its roots in the research of Claude Bernard, who first demonstrated the mechanisms responsible for maintaining blood sugar levels and body temperature within a physiologic range whenever normalcy was threatened. In his 1865 "Introduction to the Study of Experimental Medicine", Bernard wrote that "all the vital mechanisms, however buried they may be, have only one object: that of preserving constant the conditions of life in the milieu intérieur (internal environment). ...It is a fixity of the *milieu intérieur* which is the condition of free and independent life." Bernard also identified the existence of ductless (endocrine) glands, as opposed to those with ducts (exocrine), and originated the term "internal secretion."

A half-century later, Walter Cannon referred to this "fixity of the milieu intérieur" as the "steady state" and coined the term "homeostasis" to describe the numerous balancing mechanisms necessary to maintain the steady state. In studying the motor activities responsible for progressively propelling food through the gut, he noted that when his experimental animals were hungry, peristaltic waves increased in frequency and amplitude; however, if they became frightened, no such increases were seen and there was a diminution or even transient cessation of peristalsis. His further investigations revealed that this and other responses during fright were due to an outpouring of adrenaline and stimulation of the sympathetic nervous system. By 1915, Cannon had concluded that the greatest stimulation occurred under conditions of extreme hunger, thirst, fear, or rage, and during sexual activity. All of these situations automatically and immediately precipitated a cascade of coordinated nervous system and metabolic responses throughout the body. Cannon theorized that these integrated activities had been exquisitely honed over the lengthy course of evolution to facilitate "fight or flight" life-saving measures. He summarized the mechanisms responsible for maintaining heart rate, blood pressure, temperature, and blood concentrations of sugar, protein, fat, calcium, and oxygen during normal and emergency conditions in "The Wisdom of the Body," first published in 1932.

## B. Hans Selye and "They Just Looked Sick"

As a medical student, Selye had noted that patients who subsequently developed very different diseases often exhibited identical signs and symptoms during the first few days of their illness. They all had low-grade fevers, feelings of malaise, fatigue, generalized aches, and "they just looked sick." He was 18 and excited about the possibility of using his weekends to study the mechanisms that might be responsible for these common nonspecific signs and symptoms, but was advised that this would be fruitless and that he should devote any spare time to his studies. Selye graduated first in his class; he later earned a doctorate in organic chemistry and received a Rockefeller scholarship in 1931, which he used to study under the renowned biochemist, J. B. Collip, at McGill Medical School. At the time, only two ovarian hormones had been identified, but Collip thought there was a third and Selye was sent to the Montreal slaughterhouses to retrieve as many cow ovaries as possible. Collip processed these into an extract for Selye to inject into female rats; the rats were subsequently examined for any changes in tissues that might be attributed to a new ovarian hormone. Not only were no such effects detected, but most of the rats became very sick and several died.

Although Selye found no gross or microscopic changes in the ovaries or breasts of the injected rats, he observed that all of the animals showed ulcerations in the stomach, enlargement of the adrenals, and shrinkage of the thymus and lymphoid tissues. The most plausible cause seemed to be some contaminant in Collip's chemical concoction. There was a bottle of formaldehyde used to fix tissues for microscopic study in front of him; on a whim, he injected liberal amounts of this toxic substance into several rats and was amazed to find that it produced results identical to those observed with Collip's new extract. Injecting other toxic chemicals produced these same changes; he subsequently demonstrated (in experiments that would be impossible to perform today) that noxious physical and/or emotional stimuli could produce these same pathologic changes. Selye viewed these common pathologic findings, as well as the very early similar symptoms in sick patients he had observed as a medical student, as a nonspecific response to what he chose to call "biologic stress."

In 1936, Selye submitted his findings to the editor of *Nature*, in the form of a 74-line letter; the editor agreed to publish the letter if Selye omitted the word "stress," because at the time, in colloquial speech, the word had the connotation of "nervous strain." Also, Selye was unaware that stress had been used for centuries by engineers and physicists to describe something quite different from what Selye was proposing. Hooke's Law (1658) stated that the magnitude of stress (an external force) would produce a proportional amount of strain (deformation) in a metal, depending on its degree of malleability. Therefore, "strain" would have been preferable, and Selye later complained on several occasions that, had he been aware of this, he would have gone down in history as the father of the "strain" concept. Considerable confusion was created when his research had to be translated into other languages. In 1946, when invited to explain his theories at the prestigious Collège de France, where 100 years previously, the great Claude Bernard had presented his concept of *the milieu intérieur*, the academicians responsible for maintaining the purity of the French language were unable to find a suitable word or phrase to convey Selye's concept of biologic stress. After several days of debate, they decided that a new word would have to be created and *le stress* was born, quickly followed by *el stress*, *il stress*, *lo stress*, *der stress* in other European languages, with similar neologisms in Russian, Japanese, Chinese, and Arabic. Stress is one of the few words still preserved extant in languages that do not use the Latin alphabet.

Other difficulties arose as "stress" was increasingly incorporated into vernacular speech and eventually became a popular buzzword; it was used interchangeably to refer to both physical and emotional challenges, the body's response to these stimuli, as well as to the pathological consequences of such interactions. Even Selye had difficulties when he tried to extrapolate his laboratory research to humans. In helping him to prepare his *First Annual Report on Stress* 1951, I included a critical letter that had appeared in the *British Medical Journal*. Using verbatim citations from Selye's own writings, one physician had concluded that "stress, in addition to being itself, was also the cause of itself, and the result of itself." Because it was apparent that most people viewed stress as some unpleasant threat, Selye had to create a new word, "stressor," to differentiate stimulus from response. Another problem was that all of his experiments had been conducted in laboratory animals and had focused only on the damaging effects of stress. It was apparent that stress was not always necessarily harmful for humans. Increased stress results in increased productivity—up to a certain level that differs for each of us. It is only when this level is exceeded that damage is apt to occur. Nor is stress always a synonym for something bad. Winning a race or election may be just as stressful as losing, or more so. A passionate kiss and contemplating what might follow could be described as stressful but hardly comparable to the feeling experienced during a root canal procedure. Were the physiological changes associated with "good" stress different? Could good stress negate the effects of bad stress? Selye subsequently created "eustress" to refer to good stress that might promote health.

## II. THE GENERAL ADAPTATION SYNDROME AND DISEASES OF ADAPTATION

Selye viewed the initial stereotyped response to stress described in his 1936 *Nature* report as a "call to arms" of the body's defense mechanisms and referred to it as an "alarm reaction." If a state of stress persisted, a "stage of resistance" ensued, during which there was increased adaptation to the noxious stimulus. When prolonged further, resistance/adaptation became inadequate or disappeared in a third and final stage of exhaustion. He termed this three-phased response the General Adaptation Syndrome, the initial component being the alarm reaction, when adaptation had not yet been acquired, a subsequent stage of resistance, during which adaptation to the offending stimulus was maximal, and a final stage of exhaustion, when all resistance was lost.

Autopsy findings during various phases of the General Adaptation Syndrome revealed pathologic changes in the cardiovascular system, kidneys, gastrointestinal tract, soft tissues, and other structures reminiscent and often indistinguishable from those seen in patients with rheumatoid arthritis, disseminated lupus, gastrointestinal ulcers, and cardiovascular and kidney disorders. Selye reasoned that if stress could produce this type of pathology in his laboratory animals, perhaps it could contribute to these and other diseases in humans; he referred to these as "diseases of adaptation." These pathologic changes were influenced by various factors, including the nature and severity of the stressor, the duration of exposure, and prior sensitization through dietary alterations or the administration of certain steroids. He subsequently discovered how to produce consistently different disorders such as myocardial necrosis or nephrosclerosis by placing the animals on a high-sodium diet and administering desoxycorticosterone acetate (DOCA). Conversely, a high-potassium diet and strategies that conserved potassium minimized myocardial damage.

## III. STRESS-RELATED DISORDERS AND MECHANISMS OF ACTION

Selye identified over two dozen different steroids in the adrenal venous effluent, but it was difficult to distinguish between those with physiologic hormonal activities and others that represented metabolites or precursors having little clinical significance. After numerous experiments, he concluded that there were three main types of adrenal cortical hormones, which he classified as glucocorticoids, mineralocorticoids, and testoids. Glucocorticoids, or "sugar hormones," produced in the zona fasciculata had a pronounced influence on carbohydrate metabolism because they promoted gluconeogenesis by breaking down protein to provide glucose. Other catabolic or antianabolic effects included lympholysis, eosinopenia, and inhibition of inflammation. Whereas these anti-inflammatory effects delayed wound healing and increased susceptibility to infection, glucocorticoids such as cortisone were later found to provide clinical benefits, especially in patients with rheumatoid arthritis. Mineralocorticoids, or "salt hormones," such as DOCA, manufactured in the zona glomerulosa, had their major effects on electrolytes by promoting the reabsorption of sodium and the excretion of potassium. The testoids, or sex steroids, also known as "protein" hormones, originated in the zona reticularis and appeared to have similar but much weaker effects than testosterone on secondary sex characteristics and protein anabolism. Selye's categorizations were somewhat deceptive because the close structural similarity of all adrenal cortical steroids resulted in overlapping effects. Glucocorticoids had some sodium retention properties and mineralocorticoids could mimic certain glucocorticoid carbohydrate responses. When 9a-halogenated and 2-methylated analogues of cortisone were subsequently synthesized, it was found that they had very potent glucocorticoid and mineralocorticoid actions. Conversely, compounds with a double bond in the 1 and 2 positions separated these effects.

In 1937, Selye tried to determine what stimulated the adrenal cortex during stress. Until the late nineteenth century, the human pituitary was thought to be a vestigial organ and no pituitary hormone had yet been isolated. After many complicated surgical interventions, he discovered that only removal of the anterior pituitary prevented the adrenal cortical response to stress. Experiments designed to elucidate the role of the pituitary showed that administering crude lyophilized anterior pituitary extract (LAP), the only hypophyseal product that was available at the time, definitely caused enlargement of the adrenal cortex. Of equal interest was the observation that LAP tended to simulate the damaging effects of DOCA with respect to the production of nephrosclerosis and myocardial damage during stress. When subsequent advances led to the isolation and purification of pituitary hormones, it became obvious that adrenocorticotropic hormone (ACTH) was not responsible for these LAP effects. On the other hand, somatotropic hormone (STH) or growth hormone could, under certain conditions, produce changes similar to those observed with DOCA and LAP. Further investigations revealed that STH could antagonize or reverse the catabolic effects of ACTH and tended to prevent the weight loss and susceptibility to infection seen following the administration of ACTH and glucocorticoids. For example, the rat's normal resistance to tuberculosis could be temporarily lost by pretreatment with ACTH but was restored when STH was administered concomitantly. ACTH clearly stimulated the production of glucocorticoids and STH augmented mineralocorticoid activities either by increasing their production or by exaggerating their effects. This suggested that there were checks and balances between ACTH and STH in the pituitary and between glucocorticoids and mineralocorticoids in the adrenal. ACTH and glucocorticoids had strong anti-inflammatory effects whereas STH and mineralocorticoids stimulated inflammatory and proliferative connective tissue responses. How these activities were influenced by emotional stress in humans or could contribute to "diseases of adaptation" was not clear.

When DOCA was used to treat patients with adrenal insufficiency due to adrenalectomy or Addison's disease, there were reports of focal areas of necrosis in the heart and skeletal muscle, evidence of periarteritis nodosa, nephrosclerosis, worsening of joint distress, and even the development of incapacitating arthritis, as had been seen in experimental animals. The tendency to develop hypertension even with very small doses of DOCA was also a frequent problem encountered during the treatment of adrenal insufficiency. DOCA is not manufactured in any appreciable amount in humans. When aldosterone, the naturally occurring human mineralocorticoid, became available, attempts to demonstrate its ability to counter the anti-inflammatory effects of cortisone or produce the pathology seen with DOCA in laboratory studies were disappointing. It seemed unlikely that aldosterone caused significant pathology in humans but subsequent evidence supports Selye's theory. Researchers have now confirmed that aldosterone can contribute to cardiovascular and renal pathology as well as to fibrosis and collagen formation by promoting sodium influx and hypertrophy in vascular smooth muscle cells, generation of oxygen free radicals, and stimulation of growth factors and by potentiating the pressor effects of angiotensin II.

Extrapolating the results of animal studies to humans can be hazardous. This is especially true when dealing with stress-related research. Contemporary stress is most often due to a variety of emotional challenges that can occur several times a day, rather than to the physical threats primitive man was subjected to on a sporadic basis. Although "flight or fight" responses to severe stress can occur, we are much more apt to experience other signs and symptoms that are subtler and cover a wide range of emotional and physical responses, as illustrated in Table 1.

Although there is a great deal of anecdotal evidence that stress can contribute to emotional and physical disorders, attempting to prove this by delineating the mechanisms of actions that may be involved is difficult. There are numerous confounding influences and no animal models that reflect the wide range of emotions that humans experience. The consensus of opinion is that when secreted in response to acute stress, hormones such as adrenaline and cortisol stimulate protective body defense mechanisms and help the body adapt, but when repeatedly invoked or produced in excess for sustained periods, the resultant physiologic responses become damaging. The most relevant research has focused on the cardiovascular system, showing that stress due to lack of control on the job, for example, results in hypertension and coronary heart disease. Other factors, such as social instability, can also cause changes in clotting factors that contribute to heart attacks and strokes. One report has revealed that the 40% spike in mortality rates seen in Russian men following the fall of communism was largely due to cardiovascular disease.

Chronic stress has been shown to reduce immune system resistance to the common cold and has been implicated in other viral-linked disorders, ranging from herpes and autoimmune disease syndrome (AIDS) to certain malignancies. Repeated and/or long-term stress can also affect how we age, especially with respect to neuronal brain damage. Memory loss for recent events, one of the hallmarks of aging, is due to progressive atrophy of the hippocampus, which is responsible for memory retrieval and learning. As the old adage goes, "You can't teach an old dog new tricks." Stress hormones such as cortisol cause the same type of hippocampal shrinkage seen in the elderly. Studies show that patients suffering from depression or posttraumatic stress disorder often

**TABLE 1 Common Signs and Symptoms of Stress**

1. Frequent headaches, jaw clenching, or pain
2. Gritting and grinding teeth
3. Stuttering or stammering
4. Tremors; trembling of lips or hands
5. Neck ache, back pain, or muscle spasms
6. Light headedness, faintness, or dizziness
7. Ringing, buzzing or "popping" sounds
8. Frequent blushing or sweating
9. Cold or sweaty hands or feet
10. Dry mouth or problems swallowing
11. Frequent colds, infections, or herpes sores
12. Rashes, itching, hives, or "goose bumps"
13. Unexplained or frequent "allergy" attacks
14. Heartburn, stomach pain, or nausea
15. Excess belching or flatulence
16. Constipation, diarrhea, or loss of bowel control
17. Difficulty breathing or frequent sighing
18. Sudden attacks of life-threatening panic
19. Chest pain, palpitations, or rapid pulse
20. Frequent urination
21. Diminished sexual desire or performance
22. Excess anxiety, worry, guilt, or nervousness
23. Increased anger, frustration, or hostility
24. Depression or frequent or wild mood swings
25. Increased or decreased appetite
26. Insomnia, nightmares, or disturbing dreams
27. Difficulty concentrating; racing thoughts
28. Trouble learning new information
29. Forgetfulness, disorganization, or confusion
30. Difficulty in making decisions
31. Feeling overloaded or overwhelmed
32. Frequent crying spells or suicidal thoughts
33. Feelings of loneliness or worthlessness
34. Little interest in appearance or punctuality
35. Nervous habits; fidgething or feet tapping
36. Increased frustration, irritability, or edginess
37. Overreaction to petty annoyances
38. Increased number of minor accidents
39. Obsessive or compulsive behavior
40. Reduced work efficiency or productivity
41. Lies or excuses to cover up poor work
42. Rapid or mumbled speech
43. Excessive defensiveness or suspiciousness
44. Problems in communication or sharing
45. Social withdrawal and isolation
46. Constant tiredness, weakness, or fatigue
47. Frequent use of over-the-counter drugs
48. Weight gain or loss without dietary changes
49. Increased smoking, alcohol, or drug use
50. Excessive gambling or impulse buying

complain of memory loss for recent events and difficulties in learning and concentration that correlate with increased cortisol levels. An increase in blood sugar, insulin, and cholesterol levels and a decrease in bone density and muscle mass have also been linked to chronic stress. These and other adverse effects can be aggravated by poor lifestyle habits, such as alcohol, drug, and tobacco abuse, faulty diet, and lack of exercise, all of which are also often stress related.

To test the hypothesis that allostatic load reflected the effects of stress on the body and contributed to disease, a group of healthy elderly people were studied; their blood pressure, blood glucose, and cholesterol levels were correlated with cortisol measurements. Follow-up 3 years later confirmed that those with the highest allostatic loads were the ones most apt to develop newly diagnosed cardiovascular disease and were significantly more likely to show declines in mental as well as physical functioning.

Advances in our understanding of how the body responds to stress or to any attempt to change the status quo have always depended on the information available about biochemical and physiologic functions. Although Claude Bernard first described the existence of endocrine glands, he had scant knowledge of their activities. Walter Cannon knew little about the functions of the adrenal cortex, nor was Hans Selye aware of the host of neurotransmitters (such as endorphins, dopamine, and serotonin) that play an important part in the response to stress. The endorphins, which have powerful effects on mood and pain perception, are secreted simultaneously with ACTH in amounts that are proportional to the magnitude of the stressful stimulus. Measurements following the removal of the pituitary or adrenal, or following the administration of dexamethasone, confirm that the production and release of endorphins are regulated by the same adrenal steroid feedback mechanisms that control the secretion of ACTH, the premier stress hormone.

There are far more questions than answers about how the body responds to stress or how emotions can contribute to disease. As René Dubos noted, "what happens in the mind of man is always reflected in the diseases of his body." Selye's proposal that stress-related hormones caused different diseases was criticized as being simplistic because the available data supported other interpretations, such as a "permissive" or "conditioning" role. It was pointed out that interactions between stress and the adrenal could include the following effects:

- Nonspecific effects due to overproduction of corticoids.
- Nonspecific effects due to decreased elimination of corticoids.
- Nonspecific effects due to conditioning of the target by corticoids, causing increased sensitivity.
- Nonspecific effects due to the stressor, but requiring the presence of corticoids to maintain tissue reactivity.
- Specific effects due to the stressor, and not influenced by adrenal factors.
- Specific effects due to the stressor, and requiring adrenal activity.
- Any combination of the above.

The stress saga is far from over. Many of Selye's theories may be incorrect or incomplete based on subsequent observations. However, as he often reminded me, "Theories don't have to be correct, only facts do. Some theories are valuable for their heuristic merit, in that they encourage others to discover new facts that lead to better theories." Selye's concepts of stress and its effects on health are a good example of this and are likely to be his greatest legacy.

## Glossary

**alarm reaction** First stage of the General Adaptation Syndrome in which gastric ulcerations, adrenal hypertrophy, and shrinkage of the thymus and lymphatic tissues are consistently seen within 48 h following exposure to noxious stimuli.

**desoxycorticosterone acetate** Adrenal cortical steroid with predominant effects on electrolytes such as sodium and potassium.

**diseases of adaptation** Maladies observed during the course of the General Adaptation Syndrome in experimental animals; Selye believed the maladies were the counterpart of diseases in humans with similar pathologic changes.

**eustress** Selye's term for "good" stress that promotes health.

**General Adaptation Syndrome** Selye's term for the manifestations of stress in the whole body following prolonged stress exposure; the syndrome develops over time in three phases: the alarm reaction, the stage of resistance, and the stage of exhaustion.

**homeostasis** Walter Cannon's term for the body's tendency to maintain physiological stability in response to influences that threaten to disturb the steady state.

**milieu intérieur** Claude Bernard's term for the internal environment of the body, or the "soil" in which all biologic reactions occur.

**stage of exhaustion** Third and final stage of the General Adaptation Syndrome, during which the ability to adapt to stress is exhausted and death often ensues.

**stage of resistance** Second stage of the General Adaptation Syndrome, during which the body's resistance to stress is maximized.

**stressor** Selye's term for any stimulus that produces a state of stress.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Corticotropin-Releasing Hormone Receptor Signaling • Corticotropin-Releasing Hormone, Stress, and the Immune System • Endocrine Rhythms: Generation, Regulation, and Integration • Glucocorticoid Biosynthesis: Role of StAR Protein • Mineralocorticoids and Hypertension • Stress and Reproduction**

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# Stress and Reproduction

Judy L. Cameron

*Oregon National Primate Research Center, Oregon Health and Science University*

I. INTRODUCTION
II. REPRODUCTIVE CONSEQUENCES OF STRESS
III. MULTIPLE FORMS OF STRESS
IV. MECHANISMS OF STRESS ACTION

**Many forms of stress, including energetic stresses (e.g., undernutrition, exercise, temperature stress, and lactation), stresses associated with injury and illness (e.g., pain and infection), and psychosocial stresses (e.g., fear, anxiety, and discomfort), can lead to a suppression of reproductive hormone secretion and, if sustained, a suppression of fertility. Exposure to stress in the adolescent period can delay the onset of puberty. Stress-induced reproductive dysfunction can occur in both females and males.**

## I. INTRODUCTION

All forms of stress act primarily at the level of the central nervous system (CNS) to lead to a suppression of the activity of the hypothalamic neurons that provide the central neural drive to the reproductive axis [i.e., gonadotropin-releasing hormone (GnRH) neurons]. Not all stresses cause a suppression of reproductive axis activity in all animals. Multiple factors, including dominance rank, magnitude of the stress, perception of the stress, aggressiveness of the animal, and level of activity of the reproductive axis prior to stress exposure, play important roles in modulating the response of the reproductive axis to both acute and chronic stresses. The neural mechanisms by which stress signals modulate the function of GnRH neurons are in the early stages of being mapped. Although some neural systems, such as the hypothalamic–pituitary–adrenal axis, are activated by many stresses, they do not play a causal role in all forms of stress-induced reproductive dysfunction.

## II. REPRODUCTIVE CONSEQUENCES OF STRESS

Stress exposure can be acute, chronic, or intermittent and vary from mild to severe. The reproductive consequences of stress exposure depend on both the severity of the stress and the duration of the stress. Individual differences in stress sensitivity also play an important role in determining the outcome of stress exposure. In females, mild or acute forms of stress can lead to a suppression of circulating gonadotropin secretion, which in turn can lead to a lengthening of the menstrual cycle (primarily due to a lengthening of the follicular phase due to slow follicular development), a suppression of ovulation, or impaired luteal function (characterized by low progesterone secretion and in more severe cases a shortening of the luteal phase). Exposure to more severe or chronic forms of stress can result in impaired ovarian cyclicity with the occurrence of irregular menstrual cycles (oligomenorrhea) or the complete loss of menstrual cyclicity (amenorrhea). As ovarian steroid production is decreased, there is also a decline in secondary sexual characteristics, including breast size and amount of subcutaneous fat. In males, stress-induced reproductive impairment is characterized by a decrease in testosterone secretion and thus a loss of libido and a decrease in spermatogenesis and hormonal support for secondary sexual characteristics. Chronic stress occurring during the process of pubertal development can impair the progression of puberty, leading in some cases to a very marked delay in the pubertal development of reproductive capacity and the accompanying development of secondary sexual characteristics.

The primary site of stress-induced disruption of the reproductive axis appears to be at the level of the GnRH neurons, which provide the central neural drive to the reproductive axis (Fig. 1). Using animal models of various stresses, it has been shown for at least some stresses that GnRH secretion is impaired. However, more typically, it is inferred that GnRH secretion is impaired by measuring a suppression in pituitary gonadotropin secretion. This conclusion is further supported by the finding that in all conditions of stress-induced reproductive dysfunction studied to date, administration of exogenous GnRH can stimulate the function of the reproductive axis, indicating that stress is not acting to directly suppress pituitary or gonadal activity. In some forms of acute stress, a fall in gonadotropin secretion can be noted within minutes to hours. With more subtle stresses, impairment of gonadotropin secretion is generally noted when the stress is present on a chronic basis.

## III. MULTIPLE FORMS OF STRESS

### A. Energetic Stresses

#### 1. *Undernutrition*

Much of what is known about mechanisms by which nutritional status modulates the activity of the reproductive axis comes from the clinical study of patients with the psychiatric disorder anorexia nervosa. This syndrome involves an obsessive fear of being fat and leads to a profound decrease in food intake and extreme weight loss that can become life-threatening. Nearly all women who develop anorexia nervosa show a loss of ovarian cyclicity and amenorrhea. Gonadotropin secretion during the weight loss phase of anorexia nervosa is very low and often nonpulsatile in nature or pulsatile only during the nighttime period, as it is in the early pubertal period. Anorexia can often start in the teenage years, and if it starts prior to menarche, it can delay menarche for many years, holding the girl in a prepubertal state long beyond the normal time of puberty. This delay in activation of adult-like gonadotropin secretion and ovarian function is accompanied by minimal secretion of the ovarian steroid hormones, and thus development of secondary sexual characteristics is delayed. Such a prolongation of childhood and maintenance of a girlish body habitus can be advantageous in certain sports, such as ballet, and the prevalence of anorexia in girls participating rigorously in such sports is much higher than in the normal population. Although over 90% of anorexic patients are female, the incidence of this disease is increasing in males, and they too show a profound suppression of reproductive hormone secretion and a loss of fertility and sexual function when low body weight is sustained.

A number of studies with experimental animals show that brief periods of severe undernutrition (such as fasting) can also lead to a suppression of reproductive hormone secretion. In addition, decreased reproductive hormone secretion has been documented in humans after several days of fasting. However, in general, fertility would not be expected to be compromised by brief periods of undernutrition because sperm production and development of oocytes take place over a prolonged period (weeks to months). There is evidence in rodents that acute undernutrition in the late follicular phase can block ovulation, but this has not been demonstrated in larger species.

The relationship between chronic mild-to-moderate undernutrition and activity of the reproductive axis is more controversial. There is generally little animal research addressing this question, probably because maintaining animals on diets for a prolonged period of time would be a rather inefficient method of determining the mechanisms by which energy availability modulates reproductive function, in terms of both time and money. Livestock that are maintained with suboptimal nutritional support have compromised fertility, but again, most of the experimental work examining the mechanisms by which nutrition impairs fertility in these species has utilized rather severe forms of undernutrition. In human populations in which mild to moderate undernutrition is relatively common, minimal impact on adult fertility has been reported. However, there are stronger correlations between moderate nutritional compromise and later timing of puberty onset, as well as poorer pregnancy outcome. Overall, it would appear that although prolonged periods of mild to moderate undernutrition do have some impact on function of the reproductive axis and fertility, the impact is relatively weak compared to either acute or chronic forms of severe undernutrition.

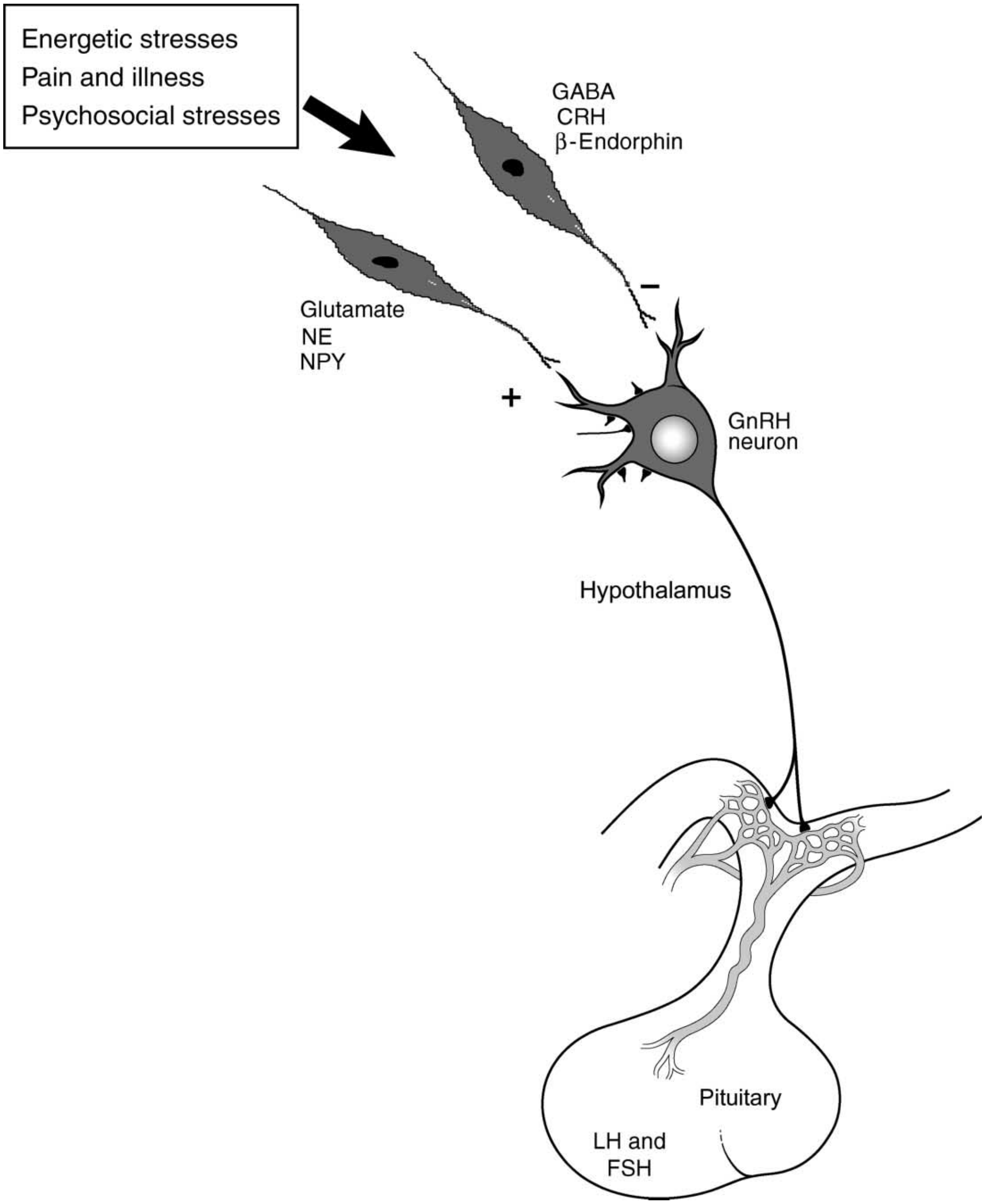
Energetic stresses
Pain and illness
Psychosocial stresses
GABA
CRH
β-Endorphin
−
Glutamate
NE
NPY
+
GnRH neuron
Hypothalamus
Pituitary
LH and FSH

**FIGURE 1** Schematic diagram showing that stresses act to suppress the activity of the reproductive axis by affecting neural systems that modulate the activity of hypothalamic GnRH-containing neurons.

### 2. *Exercise*

Women participating in vigorous exercise training programs have an increased incidence of menstrual disorders. These range from irregular cyclicity to a complete loss of menstrual cycles for prolonged periods of time. The more marked forms of exercise-induced reproductive dysfunction are associated with low circulating levels of luteinizing hormone (LH), follicle-stimulating hormone, and estrogen. If exercise regimens are very heavy in the adolescent years, individuals can experience a delay in puberty, which can be quite extensive in length. Exercise-induced reproductive dysfunction can be very rapidly reversed by a brief hiatus in the exercise regimen, as often occurs in athletes when they experience an injury or take a vacation. Exercise-induced reproductive dysfunction can also be rapidly reversed by increasing food intake, indicating that the mechanism by which exercise impairs reproductive function involves decreases in energy availability. Moreover, reproductive dysfunction in athletic women is often associated with the presence or history of eating disorders or an abnormal preoccupation with weight and diet. Exercise-induced reproductive dysfunction is more prevalent in females participating in sports where emphasis is put on a lean body image, such as ballet, compared to sports where increased body mass in encouraged. Exercise-induced reproductive dysfunction is less commonly reported in men, perhaps in part because of the lower emphasis on leanness and in part because changes in reproductive hormones in men are less readily detected compared to loss of menstrual cycles in women.

### 3. *Lactation*

Energetic stress occurring during lactation is also an important regulator of fertility and has the potential to profoundly suppress the activity of the reproductive axis. Suppression of the hypothalamic–pituitary–gonadal axis stems both from the energetic drain associated with milk production and from other neuroendocrine signals that occur with lactation, including increased prolactin and oxytocin release. As with other energetic stresses, the degree of reproductive suppression is directly related to the energy stress; thus, in animals there is good evidence that nursing a greater number of offspring has more of a suppressive effect on reproductive function than nursing one or two offspring. In humans, provision of breast milk as the sole source of nutrition for an infant, with no bottle supplementation, generally leads to a longer period of postpartum amenorrhea. Other important variables that play a role in determining the effects of lactation on reproduction include the frequency of nursing and the amount of food available to the mother.

### 4. *Pain, Illness, and Immunological Stresses*

Chronic illness and exposure to pain, such as in patients with fibromyalgia, can be associated with an increased incidence of reproductive dysfunction. Inflammation and other conditions associated with activation of the immune system can also lead to a suppression of reproductive function, particularly when CNS levels of cytokines are elevated. Much of the experimental work examining the interactions between immune activation and the activity of the reproductive axis has utilized acute administration of endotoxin. This inflammatory-like stress is accompanied by a suppression of LH secretion, a delay in folliculogenesis, and decreases in luteal function. However, the response of the reproductive axis is influenced by the endocrine environment at the time of the inflammatory challenge. For example, an elevation in CNS interleukin-1α levels can rapidly suppress LH secretion in nonhuman primates when estrogen levels are low, but leads to an elevation of LH secretion in the presence of significant estrogen concentrations.

### 5. *Psychosocial Stress*

The effects of behaviorally induced stresses, that is, psychological and social stresses involving elicitation of anxiety, fear, and discomfort, on reproductive function have been studied extensively in animal models. Both restraint stress and the expectation of mild footshock (as a conditioned stimulus) can lead to rapid suppression of reproductive hormone secretion in rodent models. In human populations, it has been more difficult to selectively study the impact of psychosocial stress on reproductive function because these stresses rarely occur in isolation from other stresses or in a timely fashion so that they can be easily studied. One of the best characterized forms of psychosocial stress-induced reproductive dysfunction comes from studies of women who present to infertility clinics with functional hypothalamic amenorrhea (FHA). By definition FHA is a state of subfertility that is not associated with substantial undernutrition or exercise, does not involve lactation, and is not associated with any organic or structural causes of decreased fertility. Studies of women with FHA show that they experience more psychological stress than other women, although they do not experience more stressful life events, but rather react more profoundly to the stressful events they do experience. They also show increased activation of physiological systems that respond to stress, including increased activation of the hypothalamic–pituitary–adrenal axis. Treatment of these patients with cognitive behavior therapy or with drugs that reduce the activity of some central neural systems that are activated by stress can restore fertility, although not in all cases. For example, both opiate antagonists and metaclopromide, a dopamine agonist, have been shown to stimulate reproductive hormone secretion and restore menstrual cyclicity in some, but not all women with FHA.

Although the majority of studies examining the effects of psychosocial stress on reproduction have documented stress-induced suppression of reproductive function, there are a handful of human studies that have reported that girls who have grown up under conditions of family stress, such as in homes where the father is absent, in homes where there has been family conflict, or in homes where the parents have divorced, enter puberty at a significantly earlier age. However, mechanisms by which such stress exposure would advance the onset of puberty have not been established. Moreover, there is the possibility that early stress exposure does not cause advancement of puberty, but rather, that the likelihood of early puberty and exposure to early life family stresses may simply be correlated because they are both influenced by a common factor(s). For example, one of the factors governing the age of menarche in a girl is the age of her mother at menarche. Thus, it is possible that mothers who experienced early menarche are more likely to have family conflict or divorce when their children are young and to have daughters who exhibit early menarche.

A more detailed understanding of how psychosocial stress can impact on reproduction comes from animal studies, with investigations in nonhuman primates having particular relevance to understanding this human condition. Nonhuman primates live in complex social groups and have higher cortical brain areas similar to those of humans; moreover, the anatomical and functional organization of their reproductive axis is very similar to that of humans. A number of studies have shown that acute stresses (i.e., restraint, receipt of aggressive attacks, and placement in an unfamiliar social situation) can rapidly suppress reproductive hormone secretion in a variety of nonhuman primates. However, not all acute stresses have this effect, particularly those that are associated with less direct threat to the individual. Thus, the perception of severity of stress appears to be an important factor in determining whether a stress will lead to a suppression of reproductive hormone secretion. Chronic exposure to social stresses (such as troop reorganization or being placed in a new social environment) can lead to a marked and sustained suppression of reproductive hormone secretion. However, not all individuals respond to chronic stresses with suppressed reproductive function. Studies have shown a high degree of correlation between aggressiveness and testosterone titers, with the more aggressive males showing higher circulating levels of testosterone. Dominance rank, particularly in times of social instability, is also often correlated with circulating testosterone levels in males. It has also been hypothesized that social status (i.e., dominance rank) plays an important role in determining lifetime reproductive success in primates, with subordinate animals experiencing a greater degree of social stress and having a lesser degree of reproductive success. However, the support for this hypothesis is not uniform and this remains a controversial notion. It would appear that multiple factors, including dominance rank, magnitude of stress, perception of the stress, aggressiveness of the animal, and level of activity of the reproductive axis prior to stress exposure, can play important roles in modulating the response of the reproductive axis to both acute and chronic stresses (Fig. 2).

## IV. MECHANISMS OF STRESS ACTION

There are two general ways in which stress exposure is thought to be able to suppress GnRH neuronal activity, by increasing the activity of neural systems that are inhibitory to GnRH neurons or decreasing the activity of neural systems that are stimulatory to GnRH neurons (Fig. 1). To date, most attention has been focused on the activation of inhibitory systems; however, the complete neural mechanisms by which stress modulates the reproductive axis for any of the stresses discussed in this article are far from being understood. The mechanisms by which various forms

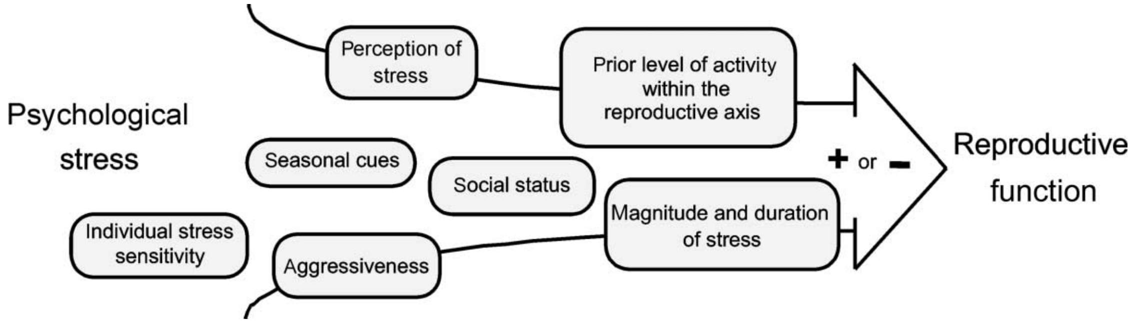
Psychological stress
Perception of stress
Prior level of activity within the reproductive axis
Seasonal cues
Social status
Individual stress sensitivity
Aggressiveness
Magnitude and duration of stress
+ or −
Reproductive function

**FIGURE 2** Schematic diagram showing modulatory factors that play an important role in determining whether stress exposure will lead to reproductive dysfunction within an individual.

of stress impair reproductive axis activity appear to have some common elements, but there also appear to be mechanisms that are specific to each type of stress. For example, many forms of stress can activate the hypothalamic–pituitary–adrenal axis, and experimental studies have shown that CRH can suppress the activity of GnRH neurons and that several forms of stress-induced impairment of reproductive function can be reversed by administering an antibody to CRH (i.e., footshock stress and inflammatory stresses). However, transgenic mice without expression of the CRH gene still show stress-induced suppression of reproductive hormone secretion in response to restraint stress and undernutrition. Thus, the activation of a particular neural system in response to stress does not necessarily indicate a causal role for that neurotransmitter in the etiology of stress-induced reproductive dysfunction. For many forms of stress, neural systems that are activated and inhibited are beginning to be mapped, but the role of these systems in mediating the suppression of GnRH neuronal activity, and how information regarding various modulatory factors (e.g., dominance status, perception of stress, and aggressiveness) interacts with these systems, awaits future research.

## Glossary

**amenorrhea** A loss of ovarian cyclicity.

**corticotropin-releasing hormone** The hypothalamic neuropeptide that stimulates pituitary production and secretion of adrenocorticotropin, a hormone that regulates adrenal function.

**functional hypothalamic amenorrhea** A clinical state of subfertility in women resulting from stress exposure.

**gonadotropin-releasing hormone** The hypothalamic neuropeptide that stimulates pituitary production and secretion of gonadotropins.

**gonadotropins** Luteinizing hormone and follicle-stimulating hormone; hormones produced by the anterior pituitary gland that regulate ovarian and testicular function.

**menarche** The time of first menstrual bleeding.

**stress** An internal or external stimulus that causes a physiological challenge or evokes a state of anxiety.

## See Also the Following Articles

**Corticotropin-Releasing Hormone, Stress, and the Immune System • Eating Disorders • Endocrine Rhythms: Generation, Regulation, and Integration • Gonadotropin-Releasing Hormone (GnRH) • Sexual Differentiation, Molecular and Hormone Dependent Events in • Stress • Thyroid and Reproduction**

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# Systemins

Clarence A. Ryan and Gregory Pearce
*Washington State University*

I. INTRODUCTION
II. TOMATO SYSTEMIN
III. TOMATO SYSTEMIN HOMOLOGUES
IV. TOBACCO SYSTEMIN
V. MODE OF ACTION
VI. SUMMARY

**Systemin is an 18-amino-acid polypeptide hormone that is released in *Solanaceae* species from sites where there is tissue damage. Systemin activates genes throughout plant tissues for defense against predators and pathogens. The wound hormone is cleaved from a 200-amino-acid precursor called prosystemin that is synthesized in vascular bundle cells.**

## I. INTRODUCTION

Polypeptide hormones are common regulators of numerous physiological processes in eukaryotes. In 1922, the first polypeptide hormone discovered, insulin, was initially identified in the pancreatic secretions of dogs. Following the discovery of insulin, hundreds of polypeptide hormones that regulate a wide variety of physiological processes were isolated and characterized.

Polypeptide hormones in animals are nearly always produced as precursors that are proteolytically processed to their active forms. Many of the polypeptide hormones are derived from precursors that contain one copy of the hormone, but in many cases, the precursor contains more than one copy, and in other cases more than one type of hormone is produced from a single precursor.

Before 1991, polypeptides were not recognized as signaling molecules in plants; small organic molecules were thought to regulate the numerous physiological processes that govern growth and development. Evidence that supported a role for polypeptides as signaling molecules was not obvious. In 1991, an 18-amino-acid polypeptide called systemin was isolated and was found to regulate the expression of defensive genes in tomato leaves in response to insect attacks or severe mechanical wounding. Since then, five families and subfamilies of polypeptide signals that fulfill the definition of hormones have been identified in plants as regulators of various processes of defense and development.

## II. TOMATO SYSTEMIN

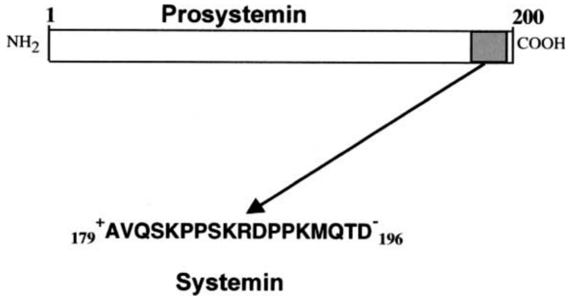

FIGURE 1 The location of the systemin sequence within its precursor, prosystemin.

Systemin, like animal polypeptide hormones, is derived proteolytically from the C-terminal region of a 200-amino-acid precursor protein called prosystemin (Fig. 1). The systemin precursor does not exhibit a signal sequence at its N-terminus, is not glycosylated or posttranslationally modified, is found in the cytoplasm, and is not known to be associated with any organelles. Alanine substitutions at each amino acid residue in systemin have variable effects on activity, with only a substitution of Thr to Ala at position 17 totally eliminating activity. This analogue is a powerful antagonist of native systemin. Removal of the C-terminal Asp totally abolishes activity, and it too is a potent antagonist of native systemin. The N-terminal 14 amino acids are likely involved in receptor binding, whereas the C-terminal sequence [MQTD (using the one-letter amino acid code): M, methionine; Q, glutamine; T, threonine; and D, aspartic acid] appears to have a direct role in signaling.

The tertiary structure of systemin in solution has been analyzed by nuclear magnetic resonance (NMR) and circular dichroism spectroscopy; systemin exists as a mixture of random coils with β-sheet and β-turn motifs. The most interesting features of the systemin sequence are the prolines, at positions 6, 7, 12, and 13, which suggests that a poly(l-proline) II $3_1$ helix secondary structure is present in the central region of the polypeptide. The polyproline II $3_1$ helix may provide a signature kink in the systemin structure that is likely important to its recognition by the systemin receptor.

Prosystemin, generated in *Escherichia coli* or in a baculovirus/insect cell system, has been found to be as active as systemin. It has not been determined whether the intact prosystemin protein is active without being processed, or whether it is processed after being supplied to the plants. A prosystemin lacking the systemin sequence is totally inactive when supplied to excised tomato plants, indicating that the systemin sequence must be present for signaling to occur.

## III. TOMATO SYSTEMIN HOMOLOGUES

Homologues of tomato prosystemin genes are found in other Solanaceae species, including potato, black nightshade, and bell pepper. The homologues are highly conserved among the species, with identities ranging from 73–88% and each encoding a systemin sequence that contains at most two to three amino acid replacements compared to tomato systemin

| | |
|---|---|
| **Tomato** | **A**VQ**S**K**PP**S**KR**D**PPKMQTD** |
| **Potato-1** | **A**VH**S**T**PP**S**KR**D**PPKMQTD** |
| **Potato-2** | **A**AH**S**T**PP**S**KR**D**PPKMQTD** |
| **Nightshade** | **A**VR**S**T**PP**P**KR**D**PPKMQTD** |
| **Pepper** | **A**VH**S**T**PP**S**KR**P**PPKMQTD** |

FIGURE 2 Amino acid sequences of systemins from potato, nightshade, and pepper, compared to the sequence of tomato systemin. Conserved amino acids are in bold type.

(Fig. 2). No substitutions have been found among the seven residues at the C-terminus, and all prolines are conserved. Each systemin actively induces defense genes in tomato plants, with the exception that the nightshade systemin is about 10-fold less effective than the others.

## IV. TOBACCO SYSTEMIN

A homologue of the tomato prosystemin gene has not been found in tobacco, a more distant relative of the tomato than nightshade, pepper, and potato. Additionally, tomato systemin does not induce defense gene expression in leaves of tobacco plants. A search for tobacco systemin in leaves of tobacco plants has resulted in the isolation of two tobacco 18-amino-acid hydroxyproline-rich, glycosylated polypeptides that are structurally dissimilar from tomato systemin, but that possess a strong defense gene induction in tobacco plants. All of the plant-derived polypeptides that activate defense genes are included under the general name of systemins. The two tobacco systemins, called Tob Sys I and Tob Sys II, are produced from a single gene that encodes a 150-amino-acid polyprotein precursor from which both systemins are processed, one systemin from the N-terminal region and one from the C-terminal region (Fig. 3).

Tobacco prosystemin bears no homology to tomato systemin, but the central regions of both of the tobacco systemins contain hydroxyproline-rich regions, which are similar to the proline-rich regions found in tomato systemin. These regions would likewise cause a kinking of the secondary structure and, like tomato systemin, are likely to be important in recognition of the polypeptides by their respective receptors.

## V. MODE OF ACTION

A synthetic $^{14}$C-labeled tomato systemin derivative has been shown to be mobile when applied to wounds, and its movement in the phloem correlates with the movement of the systemic signal that is produced in response to wounding. Prosystemin synthesis is localized in the vascular bundles of the plants, and systemin signaling may be amplified in parenchyma cells as it moves through the phloem. This scenario is temporally compatible with the known rate of movement of the wound signal from a severe wound site to cells throughout the plants.

The functional role of systemin has been demonstrated by transforming tomato plants with an antisense prosystemin gene, driven by the constitutive 35S promoter. The transgenic antisense plants exhibit a severely reduced systemic induction of defense genes in response to wounding and are rapidly consumed by *Manduca sexta* larvae. However, supplying the plants with systemin through their cut stems results in a normal response. Additionally, tomato plants that are transformed with prosystemin cDNA in the sense orientation, driven by the 35S promoter, act as if they are in a permanently wounded state, synthesizing defense proteins constitutively. This phenotype is apparently caused by expression of the gene in cells in which it is not normally expressed, resulting in the release of systemin at a low but constitutive level, causing the plants to behave as if they are under attack by chewing insects. Grafting wild-type tomato scions to rootstocks overexpressing prosystemin also causes the wild-type scions to accumulate defense proteins as if they are wounded. This appears to be the result of the constitutive release of systemin in the rootstock. Wild-type plants grafted onto wild-type rootstocks do not exhibit this response. These experiments support the role of systemin as a mobile signal.

Systemin interacts with a 160-kDa membrane-bound receptor with a $K_D$ of about $10^{-13}$ $M$ and initiates a signal transduction cascade that activates over 20 defensive genes that help protect the plant from insect and pathogen attacks.

Two sets of genes are activated in response to wounding and systemin—signal pathway genes and defensive genes directly involved in deterring predators. Signal pathway genes are activated within 1–4 h after wounding and include the prosystemin gene and genes that code for some members of the octadecanoid pathway. The octadecanoid pathway is part of the signaling pathway that leads to synthesis of jasmonic acid, a second messenger for up-regulating the pathway as well as for signaling downstream activation of the defense response. The defensive genes are activated at about 4–8 h after wounding and they code for several proteinase inhibitor proteins that have specificities against all four classes of proteolytic

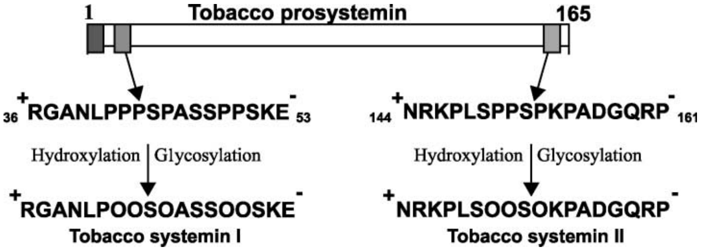
1
Tobacco prosystemin
165
$_{36}{}^{+}$RGANLPPPSPASSPPSKE$^{-}_{53}$
$_{144}{}^{+}$NRKPLSPPSPKPADGQRP$^{-}_{161}$
Hydroxylation
Glycosylation
Hydroxylation
Glycosylation
$^{+}$RGANLPOOSOASSOOSKE$^{-}$
Tobacco systemin I
$^{+}$NRKPLSOOSOKPADGQRP$^{-}$
Tobacco systemin II

FIGURE 3 The origin of Tob Sys I and Tob Sys II from a common precursor. The precursor consists of 165 amino acids that contain a 16-amino-acid signal sequence (in black) that is lost during processing through the secretory pathway. Within the secretory pathway, the prolines are modified to hydroxyprolines and carbohydrate units are attached. The specific hydroxyprolines that are glycosylated have not been identified.

enzymes, and also for polyphenol oxidase. The proteinase inhibitor proteins can inhibit proteolytic digestive enzymes of attacking organisms, whereas the polyphenolase cross-links proteins in the digestive tracts of the predators, reducing the nutritional value of the proteins. The overall effect of the defense response is to facilitate systemic signaling and to reduce the ability of the predators to obtain nutrition from plant proteins, resulting in protein starvation.

## VI. SUMMARY

Homologues of tomato systemin with a high percentage of amino acid similarities are found in potato, black nightshade, and pepper. Systemins have also been found in tobacco but are structurally different than tomato systemin and are cleaved from a polyprotein precursor. However, all systemins contain a central motif containing prolines or hydroxyprolines; this motif is likely involved in providing a structure that is recognized by cellular receptors. Although systemins have been isolated only from Solanaceae species, systemic signaling for defense is known to occur in over 100 plant families in which systemins may also play a hormonal role in signaling.

## Glossary

**octadecanoid pathway** Signaling pathway in many plants; regulates a variety of cell-specific responses and is initiated by release of linolenic acid from membranes, in response to specific signals. Free linolenic acid is converted to jasmonic acid, a second messenger that activates genes, via several enzymes that cumulatively comprise this pathway.

**polypeptide hormones** Found ubiquitously in animals as two major classes: those synthesized in the endoplasmic reticulum, processed through the secretory pathway, stored in vesicles, and released in response to an appropriate signal, and those synthesized in the secretory pathway, anchored to membranes with the hormone domain in the extracellular space, and released by proteolysis in response to an appropriate signal. Polypeptide hormones have been identified only recently in plants, and their mode of synthesis, storage, and release is poorly understood.

**prohormone** Precursor of an active hormone.

**prohormone processing enzyme** Enzyme that processes polypeptide prohormones to their active form.

**prosystemin** Precursor of a systemin.

**systemins** Polypeptide plant hormones that are released following attack (wounding) by insects and pathogens; regulate activation of defense-related genes.

## See Also the Following Articles

**Abscisic Acid • Auxin • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Jasmonates • Salicylic Acid**

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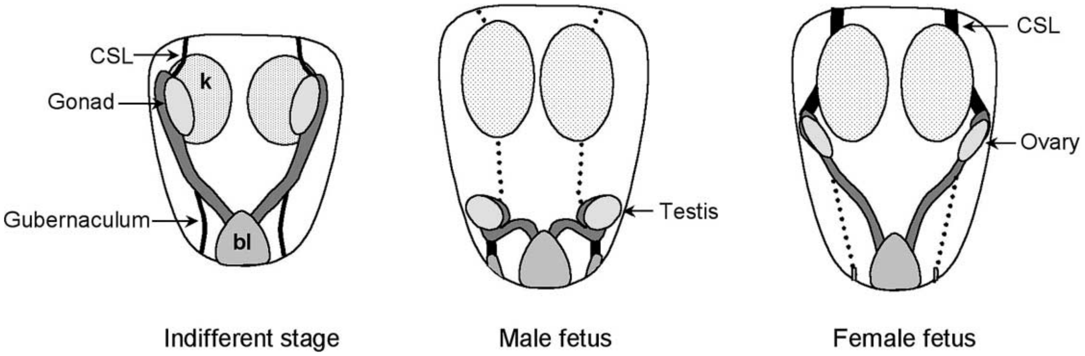

**FIGURE 1** Sexually dimorphic development of the cranial suspensory ligament (CSL) and the gubernaculum. At the undifferentiated stage, before gonadal sex determination and differentiation have started, each gonad is located next to a kidney (k). The gonad and its associated duct system are attached to the abdominal body wall by two ligaments: the cranial suspensory ligament (CSL) and the gubernaculum. In the male fetus, the first phase of testis descent results the testis being positioned lateral to the bladder (bl), connected to the inguinal region via a developing gubernaculum; development of the CSL is lacking. In the female fetus, the ovary is found in a position lateral to the kidney, attached to a well-developed CSL; there is no further development of the gubernaculum. Based on Emmen, J. M., McLuskey, A., Adham, I. M., Engel, W., Grootegoed, J. A., and Brinkman, A. O. (2000). Hormonal control of gubernaculum development during testis descent: Gubernaculum outgrowth in vitro requires both insulin-like factor 3 and androgen. *Endocrinology* **141**, 4720–4727. Copyright owner, The Endocrine Society.

are related to, and include, transforming growth factor-$\beta$. The term hormone in the name AMH refers to its signaling role, rather than giving information about its position in the context of the endocrine and growth factor classification system. The action of AMH is mediated by a multimeric complex containing two related serine/threonine kinase receptors, a type I receptor and a type II receptor. The type II receptor for AMH (AMHR-II) has been identified, whereas ALK2 and ALK6 (BMPR-1B) are considered as candidate AMH type I receptors.

AMH is the first hormonal factor known to be produced by the testis, in the precursor Sertoli cells following embryonic testis determination. Later in life, these cells will develop into mature Sertoli cells, providing support to the germ cells during spermatogenesis. In addition to being the first testicular secreted hormonal factor, another hallmark of AMH production involves its testis specificity. The *AMH* gene is silent in embryonic ovaries and is also not transcribed in any other tissue.

## B. AMH Action

Much is known about the role of AMH in embryonic/fetal sex differentiation. Evidence from studies on both humans and laboratory animals shows that AMH induces the regression of the Müllerian ducts in the male embryo. In the female, the Müllerian ducts develop into oviducts and uterus (and also the upper part of the vagina). Male *AMH* and *AMHRII* knockout mice, which are deficient for either AMH or AMHRII, retain Müllerian ducts that will develop. Also, female transgenic mice expressing AMH during fetal life lack oviducts and a uterus. There exists a rare syndrome in humans, called persistent Müllerian duct syndrome (PMDS), in which 46,XY individuals have Müllerian duct derivatives. PMDS in humans has been associated with mutations in either the *AMH* gene or the *AMHRII* gene. Patients with PMDS often have undescended testes. Additional research demonstrated that boys born with undescended testes often show a relatively low AMH level. Taken together, the available evidence indicates that incomplete regression of the Müllerian duct system in boys can lead to impairment of testis descent. However, this impairment is caused mainly by mechanical interference, due to the presence of female genital duct structures. Typically, the undescended testes are not seen in the mouse models. Male *AMH* knockout mice retain Müllerian ducts but their testes descend normally. In contrast to human females, which have a simple uterus, the uterus in rodents is bicornuate and highly movable, allowing the testes to reach the scrotum in a PMDS situation. Furthermore, female transgenic mice overexpressing AMH do not demonstrate ovarian descent or any difference in the position of the ovaries. The current view is that AMH is not a hormonal factor that is directly involved in the control of testis descent.

## III. ANDROGENS

### A. Androgens and the Androgen Receptor

Following the onset of AMH production by Sertoli cells, the next step in testis differentiation involves the formation of an interstitial population of Leydig cells situated in between the testicular tubules. Whereas the tubules are the spermatogenic compartment of the testis, the interstitial Leydig cells are responsible for steroid hormone production, or steroidogenesis, during fetal and postnatal life. The main steroid hormone to be produced is testosterone, formed by a series of enzymatic conversions from cholesterol. An important metabolite of testosterone is 5α-dihydrotestosterone. Each androgen has a quite specific role during male sexual differentiation; testosterone is directly involved in differentiation and development of the Wolffian duct-derived structures, including the epididymides, whereas 5α-dihydrotestosterone is the main active ligand in the differentiation and development of the prostate and male external genitalia.

The actions of testosterone and 5α-dihydrotestosterone are mediated by the androgen receptor, which is a member of the family of nuclear receptors. The available evidence indicates that there is only one gene encoding one type of androgen receptor. The *androgen receptor* (*AR*) gene is located on the X chromosome, in the human at Xq11–q12, and consists of eight coding exons, encoding a protein with a molecular mass of approximately 110 kDa. A polyglutamine stretch, encoded by a polymorphic $(CAG)_nCAA$ repeat, is present in the $NH_2$-terminal domain. Variation in length (9–33 glutamine residues) is observed in the human population, and the length of the repeat has been suggested to be negatively associated with a very mild modulation of androgen receptor activity. Whether subtle differences in $(CAG)_nCAA$ repeat lengths are important for modulation *in vivo* of androgen receptor activity is still a matter of debate, but could possibly play a role in later life, in relation to aging.

### B. Androgen Insensitivity Syndrome

It is generally accepted that mutational defects in the androgen receptor can lead to impairment of the normal development of both internal and external male structures in 46,XY individuals. Detailed information about the DNA sequence of the human *AR* gene and the molecular structure of the AR protein has facilitated the study of mutational defects associated with androgen insensitivity. The end-organ resistance to androgens has been designated as androgen insensitivity syndrome (AIS). The main phenotypic characteristics of 46,XY individuals with the complete form of AIS are as follows: female external genitalia, a short and blind-ending vagina, the absence of Wolffian duct-derived structures (including epididymides) and the prostate, development of gynecomastia, the absence of pubic and axillary hair, and cryptorchidism. This cryptorchidism is not only related to the absence of male external genitalia, including a scrotum, but also is caused by marked disturbance of the early steps in the process of testis descent.

Mutations of the *AR* gene in AIS individuals are mostly single base mutations resulting in amino acid substitutions or premature stop codons, although complete or large deletions of the *AR* gene, and also intronic mutations in splice donor or acceptor sites, have been found.

Androgen insensitivity has also been noted in other mammalian species including the rat and the mouse. In the mouse, this condition also appeared to be X-linked and the locus was designated *Tfm* (testicular feminization). *Tfm* mice completely lack Wolffian duct-derived structures and show female external genitalia and cryptorchidism.

### C. Androgens and Testis Descent

In male androgen-resistant *Tfm* mutant mice, development of the cranial suspensory ligament (CSL) is observed. This is of much interest, because outgrowth of this ligament is lacking in normal male mice, as opposed to its further development in normal female mice. The CSL is considered to be one of the main structures involved in determination of the gonadal position.

Several studies have supported the concept that development of the CSL is sex-dimorphic, which is probably relevant for the male-specific transabdominal phase of testis descent. When pregnant rats are treated with androgens to expose the fetuses to an increased level of androgens, this prevents the outgrowth of the CSL in the female fetuses, whereas males prenatally exposed to synthetic anti-androgens display CSL development. Furthermore, the AR is clearly expressed in primordial cells of the CSL, shortly before the onset of the first phase of testis descent, when this structure is highly sensitive to androgens. Interestingly, the sensitivity to androgens occurs within a small developmental time window and is lost after embryonic day 17 in the mouse, despite AR expression. The molecular mechanisms underlying the suppressive action of androgens on CSL development are largely unknown and remain to be elucidated. Thus far, no evidence that cell proliferation and cell death parameters of the CSL are different in female and male mouse fetuses has been obtained. The interactions between cells and their extracellular matrix, and the controlling actions of androgens and local signaling factors in the development of the CSL primordium, need to be studied in more detail.

A second important structure, which certainly plays a major role in testis descent and also is under androgenic control, is the mesenchymal layer of the gubernaculum. Initially, in both female and male fetuses, the gubernaculum expresses AR protein. Subsequently, when the sex differentiation mechanisms have started to come into play, AR expression decreases dramatically in the female gubernaculum, compared to maintenance of AR expression in the male gubernaculum. Most likely, this is an androgen-dependent process. *In vivo* and organ culture experiments have demonstrated that rat gubernaculum tissue shows cell proliferation under the influence of androgens. Given the fact that expression of the AR, which is a prerequisite for androgen action, is detected in the mesenchymal core of rodent gubernaculum, it is suggested that androgens act directly on the mesenchymal cells. These mesenchymal cells, in turn, may elicit a growth response of the myogenic cells through paracrine mechanisms. In several androgen-responsive tissues, including the prostate, androgens act via the AR expressed in the mesenchymal compartment.

It is concluded that androgens have a clear and unmistakable role in testis descent during the first phase, as demonstrated in mice and rats. This role involves the inhibition of CSL development and the stimulation of growth of the gubernaculum, probably through an indirect action, mediated by mesenchymal cells on the muscular component of the gubernaculum.

## IV. INSULIN-LIKE GROWTH FACTOR 3

### A. Insl3

For many years, it has been quite clear that testicular factors, in particular androgens, are involved in the control of testis descent. Yet, it also was clear that a piece of the puzzle was missing, and as discussed herein, this missing piece does not seem to be AMH. Several investigators have put much effort in trying to identify the factor, which was believed to be produced by the testis in addition to AMH and androgens. Descendin, a candidate protein, was not characterized in molecular detail. A very strong candidate for the unknown testicular factor was recently identified, by analysis of the phenotype of *Insl3* knockout mice. The *Insl3* gene is expressed in embryonic testis, by the early Leydig cell population. Like the expression of the *AMH* gene, this expression is specifically found in testis and not in ovaries or other embryonic tissues. The phenotype of the *Insl3* knockout mice showed a complete lack of testis descent.

Insl3 is a member of the insulin-like hormone family, which includes insulin, relaxin, and insulin-like growth factors I and II. Insl3, also called relaxin-like factor, is a single gene product, and is synthesized as a prepropeptide consisting of a signal peptide, a B-chain, a connecting C-peptide, and an A-chain. It is expected that bioactive Insl3 is formed after enzymatic removal of the C-peptide, as for insulin and relaxin. Insl3 probably mediates its action through binding to a specific receptor. However, no such receptor has been identified thus far.

With regard to transcriptional control of the *Insl3* gene, it is of interest to note that the transcriptional regulator steroidogenic factor 1 (SF1) might be involved. This factor plays a prominent role in the development of the gonads and steroidogenic tissues. Based on the identification of functional SF1 sites in the *Insl3* promoter, it can be suggested that SF1 may act as a co-mediator of *Insl3* gene expression during male sex differentiation. The production of Insl3 by the fetal Leydig cells is independent of gonadotropin stimulation. However, gonadotropins become essential for the maintenance of *Insl3* expression in the postnatal and adult testis, as supported by the observation that *hpg* mice, carrying a deletion in the *GnRH* gene, lack Insl3 expression in adulthood.

### B. Insl3 Deficiency

Mice with a targeted deletion of the *Insl3* gene have been generated. Since no gross abnormalities are found in female Insl3-deficient mice, which are fertile and have normal-sized litters, it was initially assumed that Insl3 plays a redundant role in ovarian function. Recently, however, careful examination of female mutant mice showed that their estrous cycle was twice as long as that of wild-type littermates. In addition, the *Insl3* knockout ovary shows an acceleration of follicular atresia and luteolysis, indicating that Insl3 plays a role in normal ovarian function during reproductive life. Male mice deficient for Insl3 have a very clear phenotype: all have undescended testes in adulthood. This phenotype was unexpected but confirmed the previous proposed involvement of an additional testicular factor in testis descent. Analysis of male *Insl3* knockout mice revealed that gubernaculum development is severely affected. The action of both androgen and AMH appears not to be affected in these mutant mice, since they are completely virilized and no Müllerian duct remnants could be identified. The testis-specific expression of Insl3 during embryonic life is consistent with a role in gubernaculum development. It was demonstrated that impairment of spermatogenesis seen in Insl3-deficient mice is caused by the intra-abdominal position of the testis, rather than by a direct role of Insl3 in control of spermatogenesis. The molecular and cellular mechanism of Insl3 action on the gubernaculum remains to be studied.

### C. Human INSL3 Gene

The human gene encoding INSL3 has been characterized and mapped to region p13.2–p12 of chromosome 19. Human serum contains a detectable level of INSL3, and the circulating INSL3 level in boys increases around puberty, resulting in a significantly higher level in postpubertal males than in females and prepubertal children. Similarly, the concentration of testosterone in plasma increases at puberty in normal boys. Since both testosterone and INSL3 are produced by testicular Leydig cells, such a correlation between testosterone and INSL3 levels might reflect coordinated control of their production.

Based on observations in mice, genetic analysis of cryptorchidism has recently been performed, by screening for *INSL3* gene mutations in cryptorchid boys. Several studies have been published, resulting in information about 350 individuals with (a history of) (one- or two-sided) cryptorchidism. Three mutations and several polymorphisms were found. Two heterozygous mutations are located in the connecting C-peptide and one mutation is found in the B-chain. No functional studies have been performed yet, so it remains to be determined whether these mutations are the underlying cause of the observed phenotypes.

It can be concluded that mutations in the *INSL3* gene appear not to be a frequent cause of cryptorchidism in human. One possible explanation might be that such a mutation will be effectively eliminated from the genetic pool, due to its adverse effect on fertility. There might also be a difference in the role of INSL3 among species. In human, mutations in the *INSL3* gene may lead to a phenotype possibly related to gonadal function but not resulting in a disturbance of testis descent.

It is to be expected that INSL3 acts through binding to a cell plasma membrane receptor. Recently, high-affinity INSL3-binding sites have been reported, although the binding molecules have not yet been identified. It would be of great interest to study the possible occurrence of INSL3 receptor mutations in the human population, in relation to the incidence of cryptorchidism.

## V. ESTROGENS

Genes playing a role in the synthesis and biological action of estrogens have been identified, and mouse gene knockout models have been generated for the two genes encoding the two different estrogen receptors (ER-α and ER-β) and for the gene encoding aromatase, the enzyme that converts testosterone into estradiol. These mouse models indicate that there is no prominent direct role for estrogens in the development of the male reproductive tract, including the process of testis descent. In ER-α-deficient mice, quite excessive development of the cremaster muscle was observed, but there was no direct effect on the process of testis descent.

In contrast, exposure to an exogenous estrogenic agent before birth has been found to exert a clear adverse effect on male reproductive tract development. Urogenital tract abnormalities (including epididymal cysts, microphallus, testicular hypoplasia, and cryptorchidism) were found in male offspring of mothers taking the synthetic estrogenic compound DES (diethylstilbestrol) during pregnancy. The incidence of hypospadias, cryptorchidism, and testicular cancer in the general population has been reported to have increased over the past several decades. There is a general concern that chemicals with estrogenic activity that are present in the environment might act as endocrine disruptors and thereby contribute to the increasing incidence of male reproductive tract abnormalities, similar to that seen in DES-exposed males.

A possible mechanistic explanation for disruption of testis descent after fetal exposure to an exogenous estrogenic compound has become apparent during analysis of Insl3-deficient mice. The reproductive phenotype of Insl3-deficient mice is strikingly similar to a phenotype that can be induced by prenatal exposure of male mice to DES: an intra-abdominal position of the testes and a lack of gubernaculum development. It was hypothesized that this effect of DES might involve down-regulation of *Insl3* gene expression. Indeed, the expression of *Insl3* mRNA is markedly reduced in DES-exposed testes, compared to testes of control mice. Thus, the undescended testes

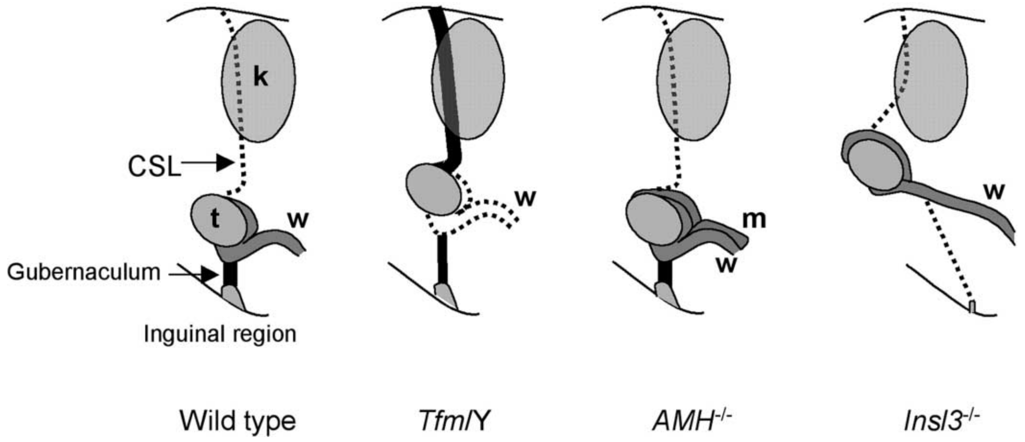

**FIGURE 2** The first phase of testis descent in different genetic mouse models. In the normal (wild type) male fetus, the gubernaculum develops, whereas CSL development is lacking. When a functional androgen receptor is absent (*Tfm*/Y mouse), both the CSL and the gubernaculum develop, although shortening of the gubernaculum is less pronounced. The Wolffian duct system is not stabilized. In AMH-deficient male mice ($AMH^{-/-}$), both the Müllerian and Wolffian duct systems are maintained. CSL development is lacking, whereas the gubernaculum shows normal development. Finally, in Insl3-deficient male mice ($Insl3^{-/-}$), CSL development does not occur, and gubernaculum development is severely impaired. CSL, cranial suspensory ligament; k, kidney; m, Müllerian duct system; w, Wolffian duct system; t, testis.

observed in DES-exposed male mouse fetuses might be related to decreased Insl3 expression.

## VI. CONCLUDING REMARKS

A clear picture has emerged with regard to the control of testis descent in the mouse, with androgens and Insl3 acting as principal factors (Fig. 2). However, the control of testis descent in the human male seems to involve additional factors, perhaps still unknown. It is of great importance to study this in more detail. The incidence of disturbed testis descent in the human male is very high—some type of problem is observed in at least 1% of all newborn males—and may be increasing due to environmental factors. Disturbed testis descent, resulting in unilateral or bilateral cryptorchidism, often can be completely repaired by surgical intervention. The surgical intervention might minimize the loss of fertility, as the scrotal location of the testis is needed to obtain normal spermatogenesis. Also, there is a correlation between testis maldescent and testicular cancer, which warrants the prevention and treatment of cryptorchidism, although it is not clear whether this correlation reflects a cause and effect relationship or whether a shared etiological factor is involved. By learning more about the mechanisms involved in testis descent and the factors that control this process, an understanding of other aspects of male reproductive health will be acquired.

## Glossary

**cranial suspensory ligament** A ligament that connects the undifferentiated gonad to the upper wall of the abdominal cavity, in the early fetus. This ligament does not develop further in the male fetus, which is a prerequisite for testis descent. In adult females of most mammalian species, the cranial suspensory ligament (CSL) is a muscular cordlike structure that keeps the ovary and uterus in place, especially during pregnancy. The development and function of the human CSL are not completely understood.

**cryptorchidism** A disorder in which one testis or both testes do not descend into the scrotum, as detected at birth. This condition can originate from impairment at an early step or at later steps in testis descent. When cryptorchidism is not reversed spontaneously, surgical placement of the undescended testis into the scrotum is required to obtain normal spermatogenesis and fertility with the onset of adulthood.

**gubernaculum** A paired ligamentous cord present in both sexes that attaches to the gonad at the superior end and attaches to the bottom of the abdomen at the expanded inferior end (gubernacular bulb). Development and relative shortening of the gubernaculum, which occur only in the male, are essential for the first abdominal phase of testis descent. Later changes in the configuration of the gubernaculum are involved in passage of the testes through the inguinal canal into the scrotum.

**insulin-like factor 3** A protein hormone that may be involved in the control of testis descent. During fetal development, insulin-like factor 3 (Insl3) is produced by the testis, in the interstitial Leydig cells, but not by the ovary. Inactivation of the gene encoding Insl3 in the mouse (*Insl3* knockout) results in complete blockade of testis descent.

## See Also the Following Articles

**Anti-Müllerian Hormone • Dihydrotestosterone, Active Androgen Metabolites and Related Pathology • Estrogen and Spermatogenesis • Estrogen in the Male • Insulin-like Growth Factor Signaling • Male Hormonal Contraception • Sexual Differentiation, Molecular and Hormone Dependent Events in • Spermatogenesis, Hormonal Control of**

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# Thyroglobulin

Geraldo Medeiros-Neto

*Hospital das Clinicas, University of São Paulo*
*Medical School*

I. INTRODUCTION
II. THE STRUCTURE OF THYROGLOBULIN AND SYNTHESIS OF THYROID HORMONE
III. THE Tg GENE: STRUCTURE, EXPRESSION, AND REGULATION
IV. ANIMAL MODELS OF DEFECTIVE Tg SYNTHESIS
V. HUMAN CONGENITAL GOITER WITH DEFECTIVE Tg SYNTHESIS
VI. SERUM THYROGLOBULIN MEASUREMENTS IN THE MANAGEMENT OF THYROID CANCER
VII. SUMMARY

**Thyroglobulin (Tg) is a glycoprotein that is synthesized and secreted by thyroid cells; it is essential for the synthesis of thyroid hormone. In addition, large reserves of iodine and thyroid hormone are stored in the Tg molecule and are available for secretion when needed. Mutations in the Tg gene cause a structurally defective protein with a severely impaired ability to function. Defects in the synthesis of Tg have been described in both animals and humans, resulting in congenital goiter and reduced thyroid function.**

## I. INTRODUCTION

Thyroid hormone synthesis is intimately tied with thyroglobulin (Tg). Indeed, after the active transport of iodide into the thyroid cell, every subsequent step of triiodothyronine ($T_3$) and thyroxine ($T_4$) formation occurs within the Tg molecule. Thus, synthesis of $T_3$ and $T_4$ follows a metabolic pathway that depends on the integrity of the Tg structure. This large glycoprotein, a dimer of 660,000 Da, is synthesized and secreted by the thyroid cells into the lumen of the thyroid follicle (Fig. 1). Thyroglobulin serves two main purposes in the function of the thyroid gland. The first is related to the process of hormone production. Thus, Tg provides for the efficient coupling of the hormone precursors mono- and diiodotyrosine to form $T_3$ and $T_4$. The second function is that of a repository within the gland of a large supply of iodine and of hormone for secretion at a steady rate or upon demand. These two properties of Tg seem to permit the organism to operate in an

TSLPR. However, the primary sequence of the TSLPR differed from that of other members of the cytokine receptor family in several important ways. First, the second of four conserved cysteine residues in the extracellular domain was missing. Second, the hallmark WSXWS sequence found in all other members of the family was missing, with the sequences PSEWT or WTAVT present instead. The significance of these changes remains unclear. In the cytoplasmic domain, the TSLPR had a sequence that corresponded to the "Box 1" motif found in members of the cytokine receptor family, but it lacked the linked "Box 2" motif. These sequences have been shown to be important in initiating signal transduction cascades following receptor engagement through binding of members of the Janus family of protein tyrosine kinases. The final structural feature of note in the TSLPR cytoplasmic domain is a single tyrosine residue, 4 amino acids from the carboxy-terminus.

As discussed above, only cells of the hematopoietic lineage were found to bind TSLP. However, using RNA analyses, Pandey *et al.* found TSLPR mRNA in a wide variety of tissues, with the highest levels in liver, lung, and testis. Message was also found in thymus, spleen, brain, and heart. Fujio *et al.* found a similar expression profile. One possible explanation for the widespread expression of TSLPR mRNA comes from Hiroyama *et al.*, who found that myeloid cells displayed the highest levels of TSLPR expression. The expression of TSLPR mRNA in nonhematopoietic tissue may reflect the presence of resident cells of the myeloid lineage in those tissues. Support for this hypothesis comes from expression studies of the human TSLPR. Like the mouse gene, the human TSLPR gene was found to be expressed predominantly in myeloid cells, with the highest levels seen in myeloid-derived dendritic cells. The fact that these cells are known to be present throughout the body may help to explain the broad expression profile of the TSLPR gene.

As described above, two affinity classes of TSLP-binding sites were found, suggesting the possibility of multiple subunits in a functional TSLP receptor complex. In support of this model, when the TSLP-binding properties of the TSLPR were examined by Scatchard analysis, only the low-affinity binding site was seen. Clues as to the identity of this second receptor subunit were suggested by the similarities between the biological responses generated by TSLP and those generated by IL-7 (see below) and by the observation that anti-IL-7 receptor α (IL-7Rα) chain antibodies inhibited responses to TSLP. Hence, experiments were performed to test whether the IL-7Rα chain contributed to TSLP-binding affinity. Binding studies using cells transiently transfected with cDNAs encoding TSLPR, IL-7Rα, or both showed that only those cells expressing both chains displayed high-affinity TSLP binding and only cell lines expressing both receptors responded to TSLP. The absolute requirement for IL-7Rα in delivering TSLP signals was further supported by the observation that chimeric receptors containing the TSLPR cytoplasmic domain and an extracellular domain capable of homodimerization failed to generate intracellular signals when expressed in cell lines. However, responses could be stimulated when chimeric molecules including the cytoplasmic domains of the TSLPR and IL-7Rα were induced to heterodimerize. Taken as a whole, these data demonstrate that the low-affinity binding of TSLP to the TSLPR does not generate responses and that a functional TSLP receptor complex consists of the TSLPR and IL-7Rα (Fig. 1).

## III. SIGNAL TRANSDUCTION THROUGH THE TSLP RECEPTOR

The inclusion of IL-7Rα in both the TSLP and IL-7 receptor complexes suggested that TSLP and IL-7 may generate similar biochemical signals. Generally speaking, cytokine binding by members of the cytokine receptor family activates a similar signaling pathway, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Following receptor engagement, one or more members of the Janus family of protein tyrosine kinases are phosphorylated, associate with the receptor at the Box 1 and Box 2 motifs, and become activated. In turn, these activated kinases phosphorylate a member of STAT family of transcription factors, leading to their dimerization, localization to the nucleus, and induced transcription of specific target genes. For example, engagement of the IL-7 receptor activates JAK1 and JAK3, leading to the tyrosine phosphorylation and subsequent activation of STAT5 (Fig. 1).

TSLP signaling studies have taken advantage of the ability of the fetal liver-derived pre-B-cell line NAG8/7 to respond to either TSLP or IL-7, allowing a comparison of the signaling properties of each receptor complex. As expected, treatment of NAG8/7 cells with IL-7 led to the tyrosine phosphorylation of JAK1, JAK3, and STAT5. Likewise, TSLP treatment of these cells led to the tyrosine phosphorylation of STAT5, but not of JAK1 or JAK3 (Fig. 1). In fact, TSLP was unable to activate any of the known JAK family members. This is in contrast to data from Fujio

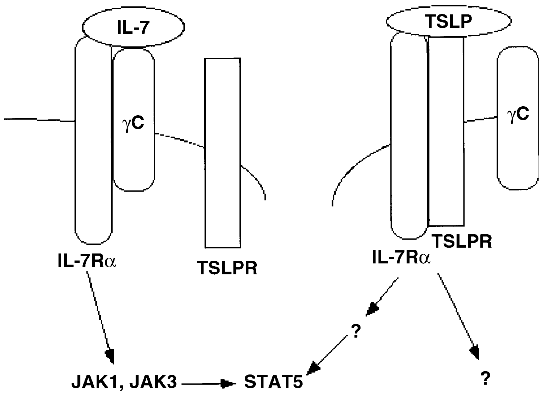

FIGURE 1 The TSLP–receptor complex utilizes the IL-7Rα chain and the TSLPR chain, but not γc. Whereas the IL-7–receptor complex utilizes the IL-7Rα chain and γc, the TSLP–receptor complex uses the IL-7Rα chain and the TSLPR complex. This leads to a number of downstream signaling differences, the most notable of which is JAK-independent activation of the STAT5 transcription factor in response to TSLP. IL-7 also activates STAT5, but does so through the more conventional pathway of activating JAKs (specifically, JAK1 and JAK3).

*et al.*, who found that substituting the membrane-proximal region of the erythropoietin receptor with that of the TSLPR did lead to tyrosine phosphorylation of JAK2. Similarly, Hiroyama and colleagues found JAK2 constitutively associated with a chimeric receptor consisting of the extracellular domain of CD8 and the TSLPR cytoplasmic domain. One possible explanation for the different results may be that the chimeric receptors, by virtue of being homodimers, may adopt a confirmation that allows JAK2 to associate. In addition, it now appears that there is a fundamental difference in the signaling properties of the mouse and human TSLP receptors. The human receptor can recruit and activate JAK2, whereas the mouse receptor cannot. This difference may in part account for some of the unique biological properties of human and mouse TSLP (see below).

Support for the lack of JAK activation by engagement of at least the mouse TSLP receptor comes from receptor reconstitution experiments. Isaksen *et al.* used transient transfection of the hepatoma cell line HepG2 to demonstrate that TSLP signaling required STAT5 uniquely and that activation of STAT5 was independent of JAK. Treatment of cells that were co-transfected with cDNAs encoding the TSLPR, IL-7Rα, and a STAT-responsive reporter plasmid did not lead to reporter activation. However, addition of a STAT5 cDNA did lead to activation. As these cells express STAT1 and STAT3, these data showed that STAT5, but not STAT1 or STAT3, could be activated by TSLP. In addition, dominant-negative forms of JAK1 and JAK2 did not affect reporter activity driven by TSLP treatment, although responses to IL-7 were inhibited by dominant-negative JAK1. Other experiments have demonstrated that JAK3 also has no effect on TSLP-mediated responses. Collectively, these data support the notion of JAK-independent STAT5 activation. Notably however, a cDNA encoding dominant-negative Tec partially inhibited TSLP-mediated STAT5 activation, suggesting a role for Tec family kinases in TSLP signal transduction.

## IV. FUNCTION OF TSLP *IN VITRO*

Three cell types have been shown to respond to TSLP *in vitro*. First, thymocytes and peripheral T cells exposed to suboptimal antigen receptor stimulation can be co-stimulated by the addition of TSLP. The magnitude of the augmentation of proliferation mediated by TSLP supplementation is similar to the co-stimulatory effect of IL-7 on the same populations. However, IL-7 alone will induce a low level of proliferation in these cells as well, whereas TSLP by itself does not promote DNA replication at all. Consistent with what is known about the TSLP receptor complex, antibody blocking experiments have shown that TSLP co-stimulation is dependent on the IL-7Rα chain and the TSLPR but is independent of γc.

Second, TSLP has been shown to influence the development of B lymphocytes in *in vitro* culture systems. B-cell development from both bone marrow and fetal liver cultures is enhanced in the presence of TSLP. However, although IL-7 will also promote B lymphopoiesis in the same culture systems, there are several differences in the effects of IL-7 and TSLP. First, the vast majority of cells that develop in IL-7-supplemented bone marrow or fetal liver cultures fail to express surface immunoglobulin M (IgM). This is due to the fact that although many of these cells have rearranged heavy chain alleles, they have not yet rearranged their light chain loci. On the other hand, most of the cells that develop in cultures supplemented with TSLP express surface IgM, indicating that these cells have successfully rearranged one or more light chain loci. However, it should also be noted that TSLP imparts a less dramatic proliferative effect than IL-7, although the absolute number of IgM$^{+}$ cells produced in TSLP-treated cultures is still generally greater than what is obtained from IL-7-treated cultures.

Similar results have been obtained evaluating the contribution of TSLP to human B-cell development using an *in vitro* culture system. Specifically, human TSLP (huTSLP) promoted the development of CD19$^+$/VpreB$^+$ pre-B cells from CD34$^+$ cord blood progenitors. However, TSLP induced only a modest increase in total cell numbers with a concomitant increase in the percentage of CD19$^+$/Vpre-B$^+$ cells. In contrast, IL-7 induced a prominent expansion in cell numbers, but a proportional decrease in the CD19$^+$/Vpre-B$^+$ cells. Moreover, addition of TSLP to CD19$^+$ cells derived from established cultures resulted in a marked increase in the number of CD19$^+$/IgM$^+$ cells in the absence of stroma. These events were associated with an increase in kappa light chain transcripts, suggesting that TSLP may promote light chain rearrangement, perhaps by altering locus accessibility or the expression of germ-line transcripts. These effects were not observed in cultures treated with other B-cell lineage cytokines, including IL-7. The similar influence of TSLP on B-cell development in both the mouse and human systems strongly suggests that a unique function of TSLP may be to promote the development of IgM$^+$ B cells.

Finally, it has recently been established that human TSLP will stimulate the maturation and survival of CD11c$^+$ dendritic cells (DCs) and induce the production of a number of chemokines that promote the recruitment of T lymphocytes. This information collectively suggests the possibility that the enhanced proliferation of T cells and thymocytes induced by TSLP after suboptimal antigen receptor stimulation could be the consequence of the maturation and up-regulation of co-stimulatory molecules on antigen-presenting cells in these cultures. However, despite these clear effects of huTSLP on human DCs, mouse TSLP does not have similar effects on mouse DCs. This species-specific difference is somewhat surprising given that, as discussed above, the mouse receptor complex is expressed at high levels on cells from the myeloid lineage.

## V. BIOLOGY OF TSLP *IN VIVO*

An interesting aspect of the biology of TSLP centers around the observations that mice that are genetically deficient in the gene encoding IL-7R$\alpha$ have more severe defects in the development of B and T lymphocytes than those lacking the IL-7 gene. When this observation was initially made, it was suggested that perhaps the ability of another IL-7R$\alpha$-chain-utilizing cytokine accounted for the differences. Hence, it is possible that the action of TSLP ameliorates some of the effects of IL-7 deficiency but that this is also eliminated in IL-7R$\alpha$-deficient mice. The specific differences in the developmental defects support this possibility. For example, B-cell precursors in IL-7R$\alpha$-chain knockout mice generally progress poorly beyond the pro-B-cell Hardy fraction A/B stage, which leads to dramatic reductions in mature B cells. B-cell development in IL-7-deficient mice generally proceeds to the pre-B-cell or Hardy fraction C stage, but the cells subsequently fail to expand, leading to reduced numbers of mature B cells. This is consistent with the *in vitro* activity of these two cytokines since IL-7 treatment of bone marrow or fetal liver precursors *in vitro* supports considerable expansion. TSLP, on the other hand, is less able to support expansion, but does promote maturation to later stages and some pre-B-cell expansion. Hence, it seems likely that either TSLP is responsible for the transition from the pro-B- to pre-B-cell stage normally or it can adequately serve the function of IL-7 in its absence at this stage. In any case, in the mouse, it is clear that TSLP cannot completely replace the need for IL-7 in expanding the numbers of pre-B cells *in vivo* or *in vitro*.

A possible role for TSLP in T-cell development has been suggested by the fact that the thymus was one of the areas of highest expression and by the observation that TSLP co-stimulates thymocytes and mature T cells. Moreover, observed differences in thymocyte maturation in IL-7R$\alpha$-chain- and IL-7-deficient mice also suggest this possibility. However, results regarding this hypothesis have been inconsistent. Specifically, IL-7-deficient mice have smaller thymi (approximately 10% of the normal cell numbers) and reduced numbers of T lymphocytes, although subset distributions appear grossly normal. Mice lacking IL-7R$\alpha$ had an interesting dichotomy in their thymic phenotype, with some mice grossly resembling the IL-7-deficient mice and others showing more dramatic perturbations. This second group had thymocyte numbers approximately 1% of normal and they failed to develop CD4$^+$/CD8$^+$ double-positive thymocytes. Again, a potential role for TSLP in mediating these different phenotypes was suggested, although it is less clear why TSLP might partially rescue thymocyte development in some mice and not others. One possibility might be a difference in TSLP expression in these two subsets of mice in the thymus, with more severely affected individuals expressing less TSLP than the IL-7 knockout “look-a-likes.” However, this is only speculation and has not been investigated.

A knockout of the *Tslp* gene has not yet been reported, but the gene encoding the receptor has recently been disrupted. However, no discernible phenotype involving the development of either B or T lymphocytes has been noted in mice that lack the receptor. It is possible that this reflects an ability of IL-7 to completely substitute for TSLP or that the subtlety of the defect has thus far escaped detection.

Perhaps the most intriguing clues as to the function of TSLP come from the study of mice that overexpress TSLP as a transgene. Notably, mice that express the TSLP protein in immature thymocytes develop an autoimmune disease that is characterized by the development of skin lesions, splenomegaly, lymphadenopathy, and mixed leukocytic infiltrates in multiple tissues including the skin, lungs, and liver. This autoimmune disease exhibited an earlier onset with greater severity in female mice, which intriguingly is also observed in many human autoimmune diseases as well. In addition, there was considerable evidence for an accumulation of polyclonal immunoglobulins precipitating from serum samples at low temperature. Accumulation of such "cryoglobulins" is characteristic of a type III cryoglobulinonemia, which is observed in certain types of infections (e.g., hepatitis C) and in various human autoimmune diseases. Apart from the skin lesions, the nature of this autoimmune disease is considerably different than what is observed when other cytokines including IL-7 are overexpressed in transgenic mice. In addition, the phenotype of mice overexpressing TSLP under the control of the Lck proximal promoter was very similar to that of mice expressing the cytokine under control of the keratin-14 promoter. The similarity in phenotype of transgenic mice expressing the same cytokine under a different type of transcriptional regulation suggests that these soluble proteins are systemically distributed and produce similar effects regardless of their point of origin. The same was true of IL-7 transgenic mice in which different promoters drove expression of the cytokine. Hence, it seems likely that the abnormalities observed in mice overexpressing these cytokines reflect perturbations of cell populations that they normally affect and that the development and character of this autoimmune disease in TSLP-transgenic mice reveals something important about its function in the regulation of the mouse immune system.

As discussed above, there is some evidence that both IL-7 and TSLP serve different functions in mice and humans. Most notably, as discussed above, deficiencies in the mouse IL-7 or IL-7 receptor gene result in severe defects in both B- and T-cell development. However, humans deficient in the IL-7 receptor have defects in T-cell development, but normal B-cell compartments. This indicates that neither IL-7 nor TSLP signaling is an obligate requirement for B-cell development in humans. Moreover, expression of the human TSLP receptor is highest in cells of the myeloid lineage and human TSLP triggers the release of T-cell-attracting chemokines in both monocytes and dendritic cells. In addition, TSLP treatment triggers the maturation of $CD11c^+$ dendritic cells, which enhances their ability to co-stimulate T cells. This suggests a possible mechanism whereby TSLP overexpression could mediate the lymphocytic infiltrates and autoimmune disease discussed above in transgenic mice. However, it has been reported that mouse TSLP does not have the same effects on mouse monocytes and dendritic cells despite the fact that monocytes at least express both the IL-7 and the TSLP receptors.

These effects on myeloid cells have now been shown to have profound consequences on the development of allergic inflammatory responses in humans. Soumelis and co-workers have recently demonstrated that TSLP triggers the maturation of $CD11c^+$ DCs. These mature DCs promote $CD4^+$ T-cell expansion, recruit $T_H2$-phenotype T cells, and promote the development of $T_H2$ cells from naive T cells. This turns out to be physiologically important because it was also shown that epithelial cells isolated from sites of acute atopic dermatitis expressed high levels of TSLP, whereas expression was undetectable in normal skin. Collectively, these data suggest a model whereby affected skin produces TSLP and promotes the recruitment, maturation, and survival of DCs to the site and the subsequent recruitment and development of allergy-promoting $T_H2$ T cells.

## VI. CONCLUSIONS

The initial characterization of TSLP and its biological effects suggested that it may be simply a "lesser" version of IL-7—it does everything IL-7 does, but simply not as well. The study of TSLP is still in its infancy and the availability of reagents and systems to facilitate understanding of this unique cytokine suggest that the next few years will produce a vast expansion in information regarding TSLP. At this point, it is known that TSLP can influence the development of B lymphocytes in both mice and humans. In addition, findings in TSLP transgenic mice strongly suggest that this cytokine influences immune system homeostasis at some level and can contribute to the development of autoimmune disease. Such a role is also suggested by the recent demonstration that TSLP plays a major role in allergic inflammatory responses in humans. Collectively, available information suggests that TSLP is much more than just a "poor man's IL-7."

## Glossary

**common cytokine receptor γ-chain (γc)** A transmembrane protein that was first identified for its contribution to interleukin-2 (IL-2) binding and signaling. It has subsequently been shown to be involved in binding and signaling from a number of other cytokines, which are collectively known as the γc-utilizing cytokines. These include IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.

**interleukin-7** A cytokine that promotes the growth of pre-B cells and co-stimulates thymocytes and mature T cells.

**interleukin-7 receptor α-chain** A transmembrane protein that was first identified for its capacity to bind interleukin-7 (IL-7). Subsequent research revealed that it binds IL-7 in conjunction with another transmembrane protein termed the common cytokine receptor γ-chain.

**Janus family kinases (Jaks)** A family of protein tyrosine kinases that are activated by cytokines binding to their receptors. Their best-characterized substrates are members of the signal transducers and activators of transcription family.

**signal transducers and activators of transcription (Stats)** A family of transcription factors that are induced to dimerize and translocate to the nucleus after being tyrosine phosphorylated in response to cytokine stimulation. This phosphorylation is most commonly carried out by members of the Janus kinase family.

## See Also the Following Article

**Interleukin-7**

## Further Reading

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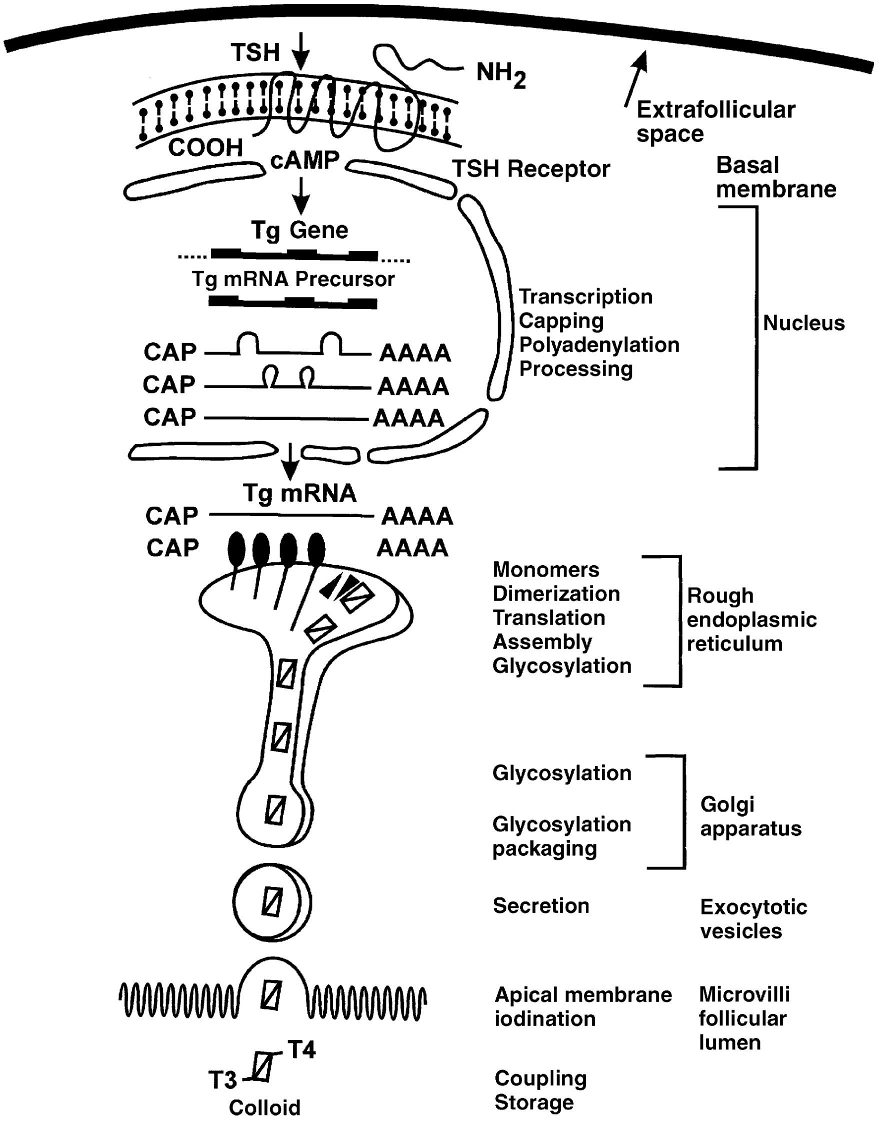
TSH
NH2
Extrafollicular space
COOH
cAMP
TSH Receptor
Basal membrane
Tg Gene
Tg mRNA Precursor
Transcription
Capping
Polyadenylation
Processing
Nucleus
CAP AAAA
CAP AAAA
CAP AAAA
Tg mRNA
CAP AAAA
CAP AAAA
Monomers
Dimerization
Translation
Assembly
Glycosylation
Rough endoplasmic reticulum
Glycosylation
Glycosylation packaging
Golgi apparatus
Secretion
Exocytotic vesicles
Apical membrane iodination
Microvilli follicular lumen
T4
T3
Colloid
Coupling
Storage

FIGURE 1 Tg gene expression, product synthesis, and Tg gene transcription are dependent on TSH stimulation through the interaction of TSH within the TSH receptor, via the cAMP pathway. The Tg gene (300 kb long) is transcribed into a Tg mRNA of 8448 bp and translated into a 2748-amino-acid (12S) Tg subunit. The mature protein is formed by joining two 12S subunits. After carbohydrate incorporation, the Tg is secreted into the follicular lumen via the Golgi apparatus. Modified from Medeiros-Neto, G., Targovnik, H. M., and Vassart, G. (1993). Defective thyroglobulin synthesis and secretion causing goiter and hypothyroidism. *Endocr. Rev.* **14**(2), 165–183, with permission from The Endocrine Society.

environment that is usually deficient in iodine and to accommodate wide variations in iodine supply. The efficiency of hormone synthesis of Tg depends on structural factors intrinsic to the protein matrix that favors the coupling reaction. It may be assumed that genetic mutations that would result in a structurally defective protein would severely impair the functional ability of Tg to serve as a matrix for the generation of $T_3$ and $T_4$.

## II. THE STRUCTURE OF THYROGLOBULIN AND SYNTHESIS OF THYROID HORMONE

Thyroglobulin is synthesized on the endoplasmic reticulum as single polypeptide chains of approximately 300,000 Da. The nascent protein is transported to the Golgi, where the carbohydrate chains are completed. It then migrates to the apical membrane of the thyroid cell. Meanwhile, the iodide that has been trapped by the thyroid also accumulates at the apical cell border. Here, a complex series of reactions occurs, in which the iodide is oxidized through the action of a thyroidal peroxidase and hydrogen peroxide and is attached to tyrosyl residues within the Tg peptide chain. At this point, the Tg molecule contains iodinated tyrosines, monoiodotyrosine (MIT), and diiodotyrosine (DIT), but no thyroid hormone.

Through a further action of the peroxidase, two DIT residues, both in peptide linkage within Tg, couple to form $T_4$. This occurs by the formation of a diphenyl ether from the two DITs, leaving dehydroalanine at the site formerly occupied by the DIT donating the outer ring of $T_4$. The other thyroid hormone ($T_3$) is formed by coupling one molecule of MIT with one molecule of DIT. Normally, approximately one-third of the iodine of Tg will be in the thyroid hormones $T_4$ and $T_3$, and the remainder will be in the inactive precursors DIT and MIT. By the time hormone synthesis is completed, two chains of 330,000 Da will associate to form the mature 660,000 Da Tg molecule. The two associating chains are probably identical. Thyroglobulin is stored extracellularly in the lumen of the thyroid follicle, where it is virtually the sole occupant (Fig. 1).

When the stored hormone is needed, Tg is retrieved by pinocytosis and ingested by the cell, where it fuses with lysosomes and is digested, and the hormone is released into the circulation. DIT and MIT are deiodinated and the iodine is returned to the intracellular iodide pool, where it is recycled. This is an important mechanism for iodine conservation.

The main metabolic steps of the biosynthesis of Tg appear in Fig. 1. Despite its huge size, Tg is not rich in tyrosine. Of more than 5000 amino acids in the 660,000 Da protein, only 4 to 8 are actually incorporated into hormone molecules. The synthesis of such an enormous molecule for this relatively small yield has perplexed many observers. The apparent wastefulness of this process may be explained in part by the storage function of Tg, through which large amounts of iodine are retained for long periods of time. This provides the thyroid with a constant reserve of iodine for hormone synthesis, even during periods when iodine is not immediately available from the environment.

Structural changes in the Tg molecule or its precursors, inability to couple iodotyrosines, defective glycosylation, or abnormal transport through the membrane system of the cell could impair or substantially alter the synthesis of $T_4$ and $T_3$ and result in congenital goiter and various degrees of thyroid hypofunction.

## III. THE TG GENE: STRUCTURE, EXPRESSION, AND REGULATION

The Tg gene, mapped to human chromosome 8q24.2–q24.3, covers at least 300 kb of genomic DNA and contains 8.5 kb of coding sequence divided over 48 exons separated by introns varying in size up to 64 kb. In 1987, the primary structure of human Tg was deduced from the sequence of its 8448-base messenger RNA and corresponding coding DNA (cDNA) sequence of 8304 bp. More recently, it was shown that the open reading frame of human Tg consists of 8307 bp, due to an extra nucleotide triplet (CAG) after position 2952. The frame encodes 2768 amino acid residues, including the signal peptide. The revised nucleotide positions resulted in the change of 12 amino acid residues and reduced the original number of tyrosine residues in the Tg monomer from 67 to 66.

The complete coding sequence of the 8307 nucleotides of the human Tg has been published with the encoded amino acid sequence (19-amino-acid signal peptide plus 2749 amino acids). To indicate the complexity of the Tg protein, other characteristics have been included such as the acceptor and donor tyrosine residues, the sites where thyroid hormones are synthesized, the N-glycosylation sites for the addition of carbohydrate molecules, the cysteine-rich repeated domains, the acetylcholinesterase homologous domain, and the most prominent antigenic epitopes. This work also described previously identified mutations and deletions found in human thyroid pathology and homologous positions in animals with hereditary thyroid disorders linked to a Tg defect (such as goats, Afrikander cattle, and *cog/cog* mice).

Four hormonogenic acceptor sites lie at positions 5, 1291, 2554, and 2747 in human Tg. Iodothyronine donor sites are at positions 130 and 1448. The acceptor sites map to the nonrepeated amino- and carboxy-terminal segments. The central portion of the molecule does not contain hormonogenic sites, yet it probably plays an important structural role in allowing the precise positioning of the hormonogenic tyrosines.

Two mechanisms for coupling are possible, one involving the free interaction of the donor and acceptor and the other assuming rigid juxtaposition of donor and acceptor. For this latter mechanism, a specific three-dimensional Tg structure is necessary.

The expression of the Tg gene is controlled at the level of transcription by TSH, the intracellular effects of which are mediated via cAMP. It is mimicked by forskolin, a universal activator of adenyl cyclase. No significant increase of Tg gene transcription was observed with chronic hyperstimulation of the rat thyroid gland with endogenous TSH. This indicates that the gene is close to being maximally expressed (at least in relative terms) under normal physiological conditions. In contrast, a dramatic decrease in Tg gene transcription was observed when endogenous TSH levels were suppressed. Injection of exogenous TSH in experimental animals restores transcriptional activity of the gene.

## IV. ANIMAL MODELS OF DEFECTIVE Tg SYNTHESIS

Defects in Tg synthesis have been described in detail in sheep, cattle, bongo antelope, goats, and mice. Some of the animal studies are very informative. They serve as models for defective Tg synthesis in humans and provide important information about the molecular mechanisms of Tg defects.

### A. Hereditary Goiter of the Afrikander Cattle

Congenital goiter with Tg deficiency has been described in a South African breed of cattle. Affected homozygotes were euthyroid despite huge goiters. The tissue contained iodoproteins, immunologically related to Tg but of abnormally low molecular weight. Iodoproteins with sedimentation coefficients of 4S, 9S, 12S, and 18S were isolated and purified into homogenous forms by gel chromatography and sucrose gradient ultracentrifugation.

In the Afrikander cattle thyroid, iodoalbumin constituted only 2.6% of the total soluble protein in the supernatant of the thyroid homogenate. In other examples of defective Tg biosynthesis, iodinated albumin frequently replaces Tg. The goiter of the homozygous animal contained a 7.3 kb Tg mRNA species in addition to the normal-sized 8.4 kb Tg mRNA. The mutation responsible for the disease is a cytosine to thymine transition, creating a stop codon at position 697 in exon 9. As a consequence, the goiter Tg mRNA encodes the shorter peptide of 75 kDa. The normal reading frame is conserved in the defective message, which is translatable into a potentially functional protein of approximately 2400 residues instead of the normal 2769 amino acids. The euthyroid state of the affected animals is compatible with the fact that the major $T_4$ hormonogenic region of Tg near the amino-terminus is present on both the short and the long Tg-related peptides found in the goiter.

### B. Hereditary Goiter of the Dutch Goats

A strain of hypothyroid goats with congenital goiter due to a defect in Tg synthesis has been extensively studied. The disease is inherited as an autosomal recessive. Only minute amounts of Tg were detected by radioimmunoassay (RIA). Ultracentrifugation, immunodiffuson, and immunoelectrophoresis demonstrated an absence of a normal 19S Tg in the goiter tissue.

Sequencing studies on goat complementary DNA were reported. A cytosine to guanine point mutation that caused a change from TAC (Tyr) to TAG (termination signal) at amino acid position 296 was found in exon 8. Chain termination in exon 8 will result in a Tg polypeptide chain with an estimated molecular weight of 39,000 that is similar in size to the *in vitro* translation product. The Tg fragment found in the goat's goiter containing the N-terminal hormonogenic site was able to produce $T_4$ *in vivo*. Administration of 1 mg iodide per day to a goitrous goat resulted in iodinated Tg with an iodotyrosine:iodothyronine ratio of 2.0:2.4. This is probably why the goitrous goats could be kept euthyroid by the administration of extra iodide.

### C. Hereditary Goiter of the *cog/cog* Mice

A mouse mutation that in the homozygous state (*cog/cog*) causes primary hypothyroidism with goiter has been found. Young adult mutant mice are hypothyroid, as evidenced by significantly lower total serum concentrations of $T_4$ and $T_3$ and elevated serum levels of TSH. Studies of goitrous tissue showed marked deficiency in immunoreactive Tg. Further studies demonstrated linkage of goiter to the Tg gene on chromosome 15. A single amino acid change at position 2265 (leucine to proline) causes abnormal storage of the mutant thyroglobulin in the endoplasmic reticulum.

Ultracentrifugation procedures followed by RIA were used to determine the protein sedimentation properties. A small peak in the 3S to 8S area (13.7% of the total apparent immunologic activity) contained iodinated albumin. There were also broad, overlapping peaks at 12S, 19S, and 27S (86.3%). The sedimentation properties indicate that the formation of 19S Tg was not normal. There was an increase in unassociated 12S subunits and aggregated species larger than 19S.

## V. HUMAN CONGENITAL GOITER WITH DEFECTIVE Tg SYNTHESIS

### A. Clinical Features

Characteristically, the majority of patients have congenital goiter, with hypothyroidism or goiter appearing shortly after birth. Goiters are usually large and have a soft, elastic consistency. Many of these patients present as adults with nodular hyperplasia (Fig. 2). Symptoms of compression of the surrounding neck structures are quite common. Hypothyroidism may be partially compensated for by preferential $T_3$ secretion and normal serum $T_3$ levels. Therefore, many of these patients may attain near-normal stature and some are able to perform simple tasks and even to learn how to read and write. There is no impairment in hearing and the structure of the cochlea is normal in the few cases that have been examined by computerized tomography.

### B. Genetics

A positive family history of goiter has been obtained in more than 50% of these patients. Both sexes are affected, but males are more frequently affected (38 females, 54 males). The parents were considered heterozygous and unaffected; frequently more than one sib was affected in a generation and the pattern of inheritance is autosomal recessive.

### C. Laboratory Investigation and Diagnosis

In the early reports of defective Tg synthesis, it was found that the serum protein-bound iodine concentration (PBI) was higher than thyroxine iodine measured by RIA. This suggested the presence in serum of abnormal iodoproteins. These may be non-19S Tg subunits, iodoalbumin, iodohistidines, or iodogammaglobulin. The presence of abnormal iodoproteins is also frequently found in endemic goiter, sporadic goiter, and goitrous Hashimoto

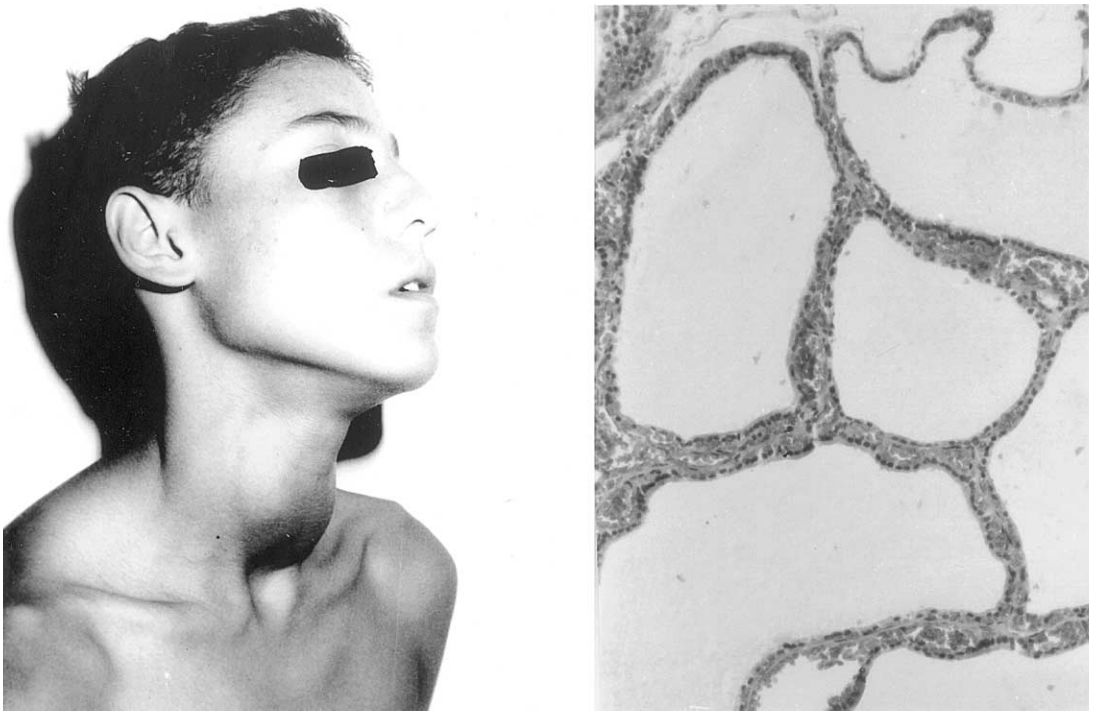

**FIGURE 2** A 16-year-old male with congenital goiter with few signs of hypothyroidism treated with L-T4 since early childhood (left). Serum Tg was very low (1.8 ng/ml) and did not rise after 0.45 mg of human recombinant TSH (2.1 ng/ml) despite a large goiter (78 g). The pathological specimen was typical of a thyroglobulin defect with absent colloid (right). Immunochemistry was able to demonstrate immunoreactive Tg inside the follicular cells.

thyroiditis and should be considered as only indicative of a possible Tg defect.

With the increasing use of serum Tg determination by RIA, the presence of an abnormally low or borderline low serum Tg level in a goitrous individual became a good indicator of a Tg defect. The TSH stimulation test is used to differentiate the group of Tg defects from other possible types of dishormonogenesis. Subjects with Tg defects have relatively low or undetectable serum Tg values that do not increase 48 h after a single injection of bovine or, more recently, human recombinant TSH. By contrast, a fivefold increase in serum Tg values was obtained in goitrous patients with organification defects (low or absent thyroid peroxidase active). Therefore, goitrous patients with a suspected hereditary Tg defect may be differentiated from other congenital goitrous patients through the TSH stimulation test.

The radioiodide uptake is invariably elevated, indicating an activation of the iodine-concentrating mechanism, probably due to chronic TSH stimulation. As the thyroperoxidase system is also activated, there is an increased incorporation of iodine into protein. Since thyroglobulin is not available, albumin and other proteins are iodinated and secreted. Incorporation of iodine into proteins other than Tg generates iodotyrosines and iodohistidines. In situations in which the patient lives in an area where the supply of iodine is low, the loss of iodide may aggravate the functional deficiency of the dishormonogenetic gland. On the other hand, an adequate supply of iodide can lead to compensation for the relative lack of thyroidal Tg, as demonstrated in experimental animals.

The perchlorate discharge test is usually negative (less than 15% of the trapped iodide is released after an oral dose of 2.0 g of potassium perchlorate).

Thyroid images obtained by isotope scanning or by ultrasonography are not different from those seen in multinodular or hyperplastic goiters. Thus, although these procedures are routinely employed to estimate the thyroid volume and thyroid anatomy, they add little to the differential diagnosis.

## D. Microscopic Examination

A number of reports include the histological examination of the thyroid, by both light microscopy and electron microscopy. The absence or pronounced scarcity of colloid and large follicular spaces lined by predominantly cuboidal cells with frequent atypical nuclei are indicative of a lack of Tg synthesis and chronic TSH stimulation (Fig. 2). In patients with impaired Tg export, electron microscopy has shown fragmented endoplasmic reticulum cisternae, with irregular contours that appeared overdistended. The Golgi complexes were numerous and had a large surface area. There were no colloid droplets inside the follicular cell and no immunofluorescence in the lumina after exposure to both anti-human Tg and anti-human serum albumin. Similarly, immunostaining with anti-human Tg using the immunoperoxidase method in two patients with a quantitative defect have shown Tg-related protein only inside the cytoplasm.

## E. Biochemical Studies on Tissues with Defective Tg Synthesis

In patients with a defective synthesis of Tg, the normal Tg peak is usually absent in the filtration elution pattern and an abnormally large albumin peak preceding the hemoglobin peak is observed.

This pattern of absence of Tg can be confirmed by Tg radioimmunoassay. Normally, Tg is measurable in the thyroid-soluble protein fraction, ranging from 50 to 90 mg/g of tissue. In all patients with the quantitative Tg defect, the Tg content was less than 0.5 mg/g of tissue. Other methods for evaluating the presence of Tg in the thyroid extract are immunoelectrophoresis (using rabbit anti-human Tg) and sodium dodecyl sulfate-agarose gel electrophoresis. In both methods, the absence of Tg bands confirms the quantitative Tg defect.

## F. Identified Defects in Human

Five different mutations in the Tg gene have been reported. All patients are homozygous for the mutations and fit the clinical description of a Tg synthesis defect, although the locations of the mutations and the corresponding defects at the protein level are strikingly dissimilar. The Tg disorders can be divided into three groups:

1. Structurally defective Tg that may not be functional or will yield thyroid hormones only at a very high intake of iodine.
2. The defective Tg molecule is not exported to the colloid (endoplasmic reticulum storage disease) as indicated in Fig. 3.
3. Defective glycosylation of Tg (low sialyc acid incorporation).

Approximately 113 patients from several families have been described in detail and molecular mechanisms have been determined in many of these patients. Two important deletions, of 68 amino acids from the

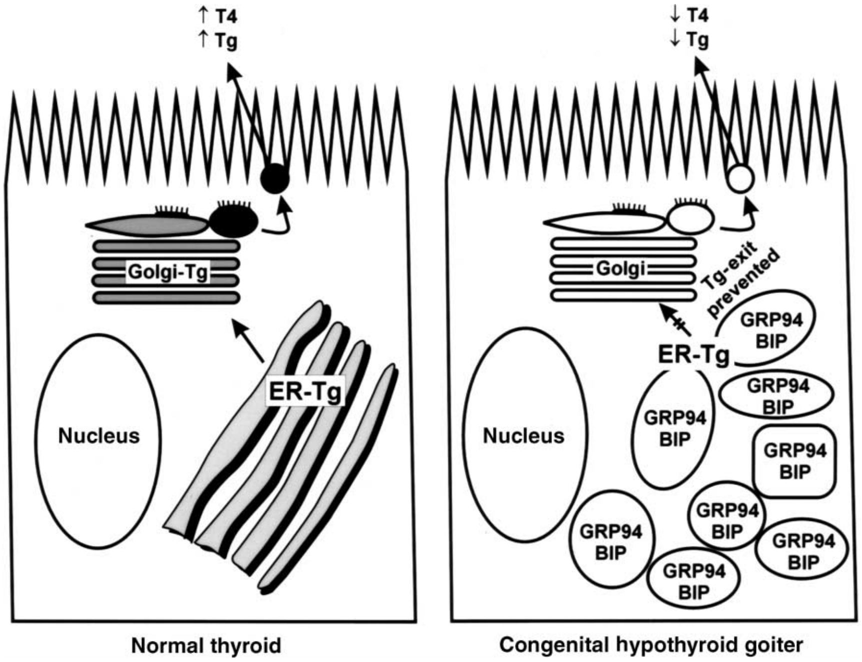

FIGURE 3 Schematic representation of the consequences of defective Tg that leads to an endoplasmic reticulum storage disease causing congenital hypothyroid goiter. In affected patients, the arrival of Tg in the Golgi complex and the subsequent secretion of Tg to form T4 are drastically inhibited. Instead, Tg accumulates in the ER, with accumulation of ER chaperones (GRP94, BIP) that assist the mutant Tg in migrating to the Golgi apparatus. The diminished synthesis of T4 triggers pituitary secretion of TSH, further stimulating the thyroid gland and contributing to goiter growth. Modified from Arvan *et al.* (1997). Intracellular protein transport to the thyrocyte plasma membrane: Potential implications for thyroid physiology. *Thyroid* 7(1), 89–105, with permission from Mary Ann Liebert Publishers.

N-terminal part of the Tg proteins and 46 amino acids of the middle of the protein, have been attributed to the in-frame deletion of exons 4 and 30, respectively.

A truncated Tg protein has been reported in a patient in whom exon 22 contains a premature stop codon that results in a very small Tg protein. The thyroid tissue also contains an alternative splice variant from which exon 22 was deleted.

In many patients, similar to the findings in *cog/cog* mice, the mutant Tg may not leave the endoplasmic reticulum, due to the quality control system of the cell. Therefore, very little Tg protein is actually stored in the colloid (Fig. 3).

## VI. SERUM THYROGLOBULIN MEASUREMENTS IN THE MANAGEMENT OF THYROID CANCER

Thyroglobulin is a glycoprotein that is produced only by normal or neoplastic thyroid follicular cells. Most differentiated thyroid carcinomas secrete Tg. Patients with lung and bone metastases have the highest serum Tg concentrations, whereas those with metastases in lymph nodes may have only modest elevations of serum Tg. Medullary and anaplastic carcinomas do not secrete Tg, whereas Hürthle cell carcinoma, a variant of follicular carcinoma, secretes Tg but does not concentrate radioiodine.

Serum Tg should not be detectable in patients who have undergone total thyroidectomy followed by radioiodine ablation and its detection in such patients signifies the presence of persistent or recurrent disease.

Good thyroglobulin assays can detect concentrations as low as 1 ng/ml. The results, however, can be artifactually altered by the presence of serum anti-thyroglobulin antibodies, which are found in approximately 15% of patients with thyroid carcinoma. Tests for these antibodies should always be performed when serum thyroglobulin is measured, but the extent to which the presence of the antibodies alters the results of serum Tg assays depends on whether a radioimmunoassay (less affected) or an immunoradiometric assay (more affected) is performed.

The production of thyroglobulin in both normal and neoplastic thyroid tissue is in part dependent on TSH. Interpretation of the serum Tg concentrations should take into account the serum TSH value, as well as the presence or absence of thyroid remnants.

If the serum Tg level is detectable during L-T4 suppressive treatment, it will increase after the treatment is withdrawn, indicating that thyroid remnant or neoplastic tissue is actively producing and releasing thyroglobulin under the stimulation of endogenous TSH.

The serum thyroglobulin concentration is an excellent prognostic indicator. Patients with undetectable serum Tg concentrations after total thyroidectomy and radioiodine ablation and under L-T4 suppressive therapy are free of the disease (Fig. 4). Conversely, 80% of patients with serum thyroglobulin concentrations that are higher than 10 ng/ml during L-T4 treatment and higher than 40 ng/ml after withdrawal of L-T4 treatment have detectable foci of radioiodine uptake in the neck or bone/lung metastases (Fig. 4).

Patient 1, as shown in Fig. 4, developed a serum Tg that was close to 1 ng/ml by the end of the first postoperative year and subsequently had no recurrences during 15 years of follow-up. In contrast, patient 2 never had a serum Tg below 1 ng/ml despite a total thyroidectomy and radioiodine ablation. After approximately 5 years, this patient developed a clinically evident recurrence despite negative radioiodine scans and died of the disease 10 years later.

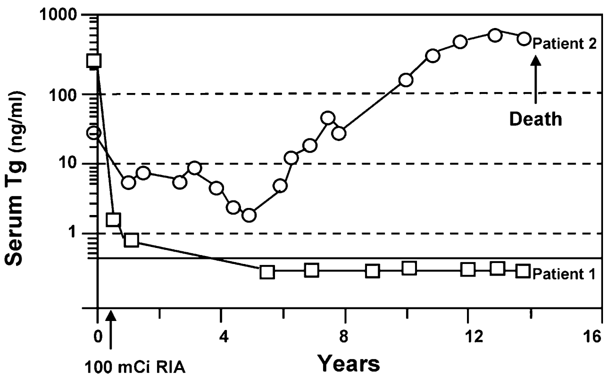

**FIGURE 4** Serum Tg concentrations during L-T4 suppressive therapy in two patients. Patient 1 consistently had serum Tg levels below 1 ng/ml, whereas Patient 2 had elevated serum Tg values despite negative RAI scans. This patient developed clinically evident distant metastases. Modified from Spencer, C. A. (1998). Detection of residual and recurrent thyroid cancer by serum thyroglobulin measurement. American Thyroid Association 70th Annual Meeting, with permission.

In conclusion, it should be remembered that measurement of Tg in serum is technically challenging and it is difficult to compare serum Tg obtained by different methods. The clinical interpretation of serum Tg results should be made relative to two main factors that influence serum Tg concentrations: the mass of differentiated thyroid tissue present and the level of serum TSH and TSH receptor stimulation. Therefore, changes in the serum Tg levels during TSH suppression by L-T4 are a good indicative sign of the TSH sensitivity of the tumor and may indicate the potential efficiency of TSH suppression therapy.

Finally, the ability to stimulate serum Tg with recombinant human TSH during thyroxine suppression and to measure Tg mRNA from peripheral blood holds promise for improvement in the management of thyroid cancer patients over the next decade.

## VII. SUMMARY

The integrity of the Tg protein structure is essential for adequate synthesis of thyroid hormone. Also, a large supply of iodine and of thyroid hormone is stored in the Tg molecule and is available for secretion on demand.

Mutations in the Tg gene or hyposialylated Tg due to defective sialyltransferase activity cause a structurally defective protein and severely impair the functional ability of Tg. Abnormalities in the synthesis of Tg have been described in both animals and human. Hereditary congenital goiter with or without hypothyroidism is the phenotypic major clinical finding in these species. Affected animals include sheep, cattle, bongo antelope, goats, and mice. The inheritance mode is autosomal recessive. In most animal studies, structurally abnormal Tg is present. The molecular basis for the defective Tg synthesis is attributable to a nonsense mutation in exon 9 (Afrikander cattle) and in exon 8 (Dutch goats).

In human, defective Tg synthesis has been reported in 113 patients and frequently more than one sibling is affected in a given generation. Characteristically, these patients exhibit hereditary congenital goiter with relatively low Tg levels that do not increase after stimulation with bovine TSH. High PBI concentrations with low serum $T_4$ values indicate the presence in serum of iodinated proteins (mainly iodoalbumin). Also, iodinated peptides are frequently excreted into the urine. Tissue studies confirm that there is no normal Tg peak on gel filtration and virtually no immunoassayable Tg in the tissue extracts.

The described mutations in the Tg gene include deletions of entire exons (exon 4 and exon 30) and point mutations with a premature stop codon generating a very truncated Tg protein. The mutant Tg protein may be functional (generating $T_3$ and $T_4$) in the constant presence of excess iodine. Many of the mutant Tg proteins are retained in the endoplasmic reticulum system of the cell and do not reach the follicular lumen (colloid).

Serum Tg measurements have greatly facilitated the clinical management of patients with differentiated thyroid cancer. The interpretation of any given Tg value requires the careful synthesis of all pertinent clinical and laboratory data available to the clinician. For instance, Tg autoantibodies remain a significant obstacle to the clinical use of Tg assays, although their presence may be indicative of persistent or residual metastatic disease. Finally, the ability to stimulate serum Tg with recombinant human TSH during L-T4 suppression and to measure Tg mRNA from peripheral blood holds promise for the management of thyroid cancer patients.

## Glossary

**allele** Variant form of a gene at a specific locus.

**autosomes** A term for any chromosome other than the X or Y sex chromosome.

**base pair** Two nucleotides located on complementary strands of DNA and paired by hydrogen bonds.

**cDNA (complementary DNA)** DNA strand created *in vitro* by reverse transcription of a messenger RNA molecule.

**chaperone** A protein that assists in the correct folding (and assembly) of another protein.

**codon** Sequence of three nucleotides that encodes a particular amino acid or signals the initiation or termination of protein synthesis.

**deletion** Mutation that results in the removal of a sequence of DNA from a chromosome.

**endocytosis** Process by which extracellular material is transported into a cell, forming membrane-bound vesicles within the cytoplasm.

**endonuclease** Enzyme that cleaves a nucleotide chain.

**endoplasmic reticulum** Cellular organelle consisting of sheets of membranes; it functions in the synthesis of lipids and membrane proteins.

**exon** Portion of a gene that encodes a protein sequence or a specific part of a protein.

**frameshift mutation** Change in the DNA sequence that occurs by insertion or deletion of some number of nucleotides that is not a multiple of 3, thus altering the reading frame for protein translation.

**gene** Specific sequence of DNA at a particular chromosomal location; it codes for a specific protein.

**heterozygote** Organism with two different alleles at a particular locus.

**homozygote** Organism with the same alleles at a particular locus.

**intron** Noncoding segment of a gene that is transcribed into RNA but is spliced out before translation occurs.

**linkage** The association between two genes that are located near each other on the same chromosome and that as a result tend to be inherited together.

**missense mutation** Change in the DNA sequence that alters a codon so that it codes for a different amino acid.

**mutation** Change in the DNA sequence of a gene.

**nonsense mutation** Change in the DNA sequence that results in a codon that specifies an amino acid being replaced by a stop codon (codon that terminates protein synthesis).

**oncogene** Gene that can cause or contribute to cancerous growth or other unregulated cell proliferation.

**PCR (polymerase chain reaction)** Laboratory technique for exponentially amplifying the number of copies of a short segment of DNA.

**point mutation** Change in one nucleotide in a DNA molecule.

**recessive allele** Allele that is not expressed in the phenotype of a heterozygote, due to the presence of the dominant allele.

**somatic mutation** Change in the DNA sequence of any cell in an organism other than a germ cell; it cannot be inherited by the organism's offspring.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Iodine: Symporter and Oxidation, Thyroid Hormone Biosynthesis • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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# Thyroid and Reproduction

Christopher Longcope
*University of Massachusetts Medical School*

I. THYROID HORMONE ACTION ON THE GONADS
II. CLINICAL ASPECTS OF THYROID DYSFUNCTION
III. PREGNANCY

**Thyroid hormones appear to have some effects on male and female reproductive capacities. Thyroid hormone receptors found on cells of reproductive tissues in both sexes are involved in activation of steroid hormones. Disorders of the thyroid gland are common in patient populations, and thus it is important to understand the impact thyroid dysfunction my have on sexual development and function.**

## I. THYROID HORMONE ACTION ON THE GONADS

Although much of the work on the effects of thyroid hormone at the tissue level has been carried out in animals, the results appear to be applicable to humans. The following discussions present the known effects of thyroid hormones on the reproductive axis in men and women.

### A. Testis

Because the testis is stimulated by both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thyroid hormone effects on the pituitary, the source of LH and FSH, can result in changes in testicular function. In rats, both thyroid hormone administration and thyroidectomy result in decreases in concentrations of circulating LH and FSH. In the testis, thyroid hormone has been reported to increase LH receptors on Leydig cells initially, but with continued exposure there is a decrease in receptor number. Thyroid hormone appears to be active in the developing testis but to play a lesser role on the adult testis. Receptors for thyroid hormones, primarily TR$\alpha$1 and TR$\alpha$2, have been identified in prepubertal testes; primarily in the Sertoli and germ cells. Acting mainly through TR$\alpha$1, thyroid hormone increases differentiation of the Sertoli cells and inhibits their proliferation. Similarly, thyroid hormone increases differentiation of Leydig cells from mesenchymal precursor cells. In adult testis, thyroid hormone has an acute effect, increasing steroid synthesis and enhancing steroidogenic acute regulatory protein (StAR) function. However, continued exposure to thyroid hormone results in decreases in both end points. Thyroid hormone also decreases aromatization in Sertoli cells. With maturation through puberty, both TR$\alpha$1 and TR$\alpha$2 decline in number; however, the decline appears to be much greater for TR$\alpha$1 than TR$\alpha$2, thus the ratio of TR$\alpha$2/TR$\alpha$1 increases. Because TR$\alpha$2 may inhibit the action of TR$\alpha$1, the fact that it is at a higher level than TR$\alpha$1 may explain why thyroid hormone has less effect on the adult testis, compared to the prepubertal testis. However, male mice lacking both TR$\alpha$1 and TR$\beta$2 are fertile. Although some individuals with thyroid dysfunction may have problems related to reproduction, the presence of thyroid hormone does not appear to be absolutely necessary for male reproductive function.

### B. Ovary

Thyroid hormone acts to decrease circulating levels of LH and to synergize with FSH to increase differentiation of granulosa cells. It is not clear that ovarian development is critically dependent on normal thyroid hormone levels. Thyroid hormone receptors TR$\alpha$1 and TR$\beta$1 are present in human and animal granulosa and stromal cells. Female mice lacking either TRα1 or TRβ are fertile, but those lacking both TRα1 and TRβ1 have markedly reduced fertility. Thus, it would appear that thyroid hormones are more important for reproduction processes in females, as opposed to males. A lack of thyroid hormone has been associated with the development of polycystic ovaries in rodents. Granulosa cell aromatase activity is inhibited by excess thyroid hormone, although progesterone synthesis is increased. Thyroid hormone not only has a direct effect on ovarian steroidogenesis but also augments action of gonadotropins in steroid synthesis. Thyroid hormones are important for normal ovulation and normal steroidogenesis.

Studies have indicated that triiodothyronine (T3) decreases estradiol secretion in both medium and large follicles but increases progesterone synthesis in medium follicles and decreases progesterone synthesis in large follicles. T3 also stimulates 3β-hydroxysteroid dehydrogenase activity in the pig corpus luteum. The development of hypothyroidism in prepubertal rats results in increased proliferation of granulosa cells but inhibition of differentiation. No copora lutea are noted in the ovaries of hypothyroid rats. Receptors for thyroid hormone have been noted in the endometrium and excess thyroid hormone in mice may cause a thickened endometrium. Thyroxine (T4) has been noted to decrease estradiol uptake by the rat uterus, but thyrotoxicosis in rats results in a decreased response to estrogen in the uterus.

## II. CLINICAL ASPECTS OF THYROID DYSFUNCTION

### A. Hypothyroidism

#### *1. Males*

Hypothyroidism during pregnancy may lead to the condition known as cretinism. Hypothyroidism does not appear to interfere with gonadal development in the male fetus, however, and cretins usually have a normal reproductive tract. As a reflection of the role of thyroid hormone in stimulating the differentiation of Sertoli cells, hypothyroidism in early life results in an increased number of Sertoli cells and an accordingly enlarged testis. Leydig cells may also be increased in number. Thus, hypothyroidism occurring in prepubertal boys may be associated with enlarged testis and precocious pseudopuberty. True puberty will occur in these individuals, however, and they will be capable of normal reproduction. Prepubertal hypothyroidism is more commonly associated with delayed sexual maturation.

Hypothyroidism in adults may be associated with defects in spermatogenesis and fertility, but this is not a universal phenomenon and many hypothyroid men will be fertile. In part, this is a manifestation of the degree of hypothyroidism. Hypothyroidism may alter the pituitary responsiveness to gonadotropin-releasing hormone (GnRH), resulting in lower levels of LH and a decrease in levels of circulating testosterone. In addition, hypothyroidism can be associated with hyperprolactinemia, which can be associated with hypogonadism.

#### *2. Females*

Perhaps because thyroid dysfunction is more common in women than in men, there have been more studies of hypothyroidism in females. Female cretins generally have normally developed reproductive tracts at birth. Hypothyroidism in prepubertal girls may cause precocious pseudopuberty with galactorrhea, caused by an increase in prolactin, and vaginal bleeding as a result of increased ovarian estrogen secretion, which stimulates the endometrium. The exact mechanisms are uncertain, but these conditions may be due to excess pituitary secretion of thyroid-stimulating hormone (TSH) and gonadotropins. A more common result of hypothyroidism in girls is delayed menarche and sexual maturation.

In postpubertal women, hypothyroidism is often associated with menstrual dysfunction, menometrorrhagia (irregular menstrual cycle) being the commonest. However, the incidence of menstrual irregularities appears to be less common now than previously. Earlier studies reported that 60–80% of hypothyroid women had menstrual abnormalities as compared to only 23% in more recent studies. The commonest symptom is menometrorrhagia in most studies. Anovulation is frequently present and endometrial biopsies often are out of phase. Paradoxically, hypothyroid women taking oral contraceptives may report an absence of withdrawal bleeding. LH and FSH levels are often low and, although this may be a direct result of low thyroid hormone levels, prolactin levels are often elevated and may result in hypogonadism.

### B. Hyperthyroidism

#### *1. Males*

Excess thyroid hormone in prepubertal males may result in early maturation; a marked excess of thyroid hormone may cause a decrease in testis volume and an impairment of sexual development associated with low levels of LH.

In adults, increased levels of thyroid hormone result in an increase in circulating levels of sex hormone-binding globulin (SHBG) and an increase in circulating levels of testosterone and estradiol. Free testosterone levels generally remain within the normal range but bioavailability may be decreased. The increase in estrogen levels can be sufficient to cause gynecomastia. An increase in LH levels may occur; but this is not a universal finding. In males with thyrotoxicosis, fertility may be decreased because of low sperm counts and alterations in sperm motility. Receptors for T3 have been found in rat epididymis and these may play a role in the sperm abnormalities.

### 2. *Females*

In prepubertal women, thyrotoxicosis can result in delayed sexual maturity, but women with polycystic fibrous dysplasia (McCurre–Albright syndrome) may have associated precocious puberty and thyrotoxicosis. In adult women, menstrual abnormalities are frequently noted, with oligo- or amenorrhea being common. Gonadotropin levels are increased but the ovulatory peak of LH may be absent.

## III. PREGNANCY

It has been proposed that hypothyroid women rarely became pregnant, but, despite the frequency of anovulation, pregnancy in untreated hypothyroid women is not uncommon. The course of pregnancy and its eventual outcome are influenced by thyroid function, and, in turn, pregnancy has an effect on thyroid function in euthyroid women.

Pregnancy in an untreated hypothyroid woman is associated with an increased risk of spontaneous abortion, gestational hypertension, low-birth-weight infants, and congenital fetal malformations. Subclinical hypothyroidism, i.e., elevated TSH levels with borderline low T4 and T3 levels, has been reported to be associated with an increased risk for these abnormalities. The frequency of these disorders is difficult to determine because of the scattered nature of the reports, many of which involve a questionable diagnosis of hypothyroidism or the institution of appropriate treatment at the time of diagnosis.

In a normal pregnancy, the high levels of human chorionic gonadotropin (hCG) stimulate the thyroid gland and there is a small increase in thyroxine and free T4 and T3. Elevations of thyroid hormone levels in turn suppress TSH levels. As hCG levels fall, the stimulus to the thyroid decreases and TSH levels start to rise.

Thyroid hormone has been shown to increase the concentration of SHBG, but the marked increase in thyroxine-binding globulin (TBG) and SHBG that occurs in pregnancy is secondary to the increase in estrogen levels. As TBG levels rise, the levels of T4 and T3 also increase and in most normal pregnancies will be above the upper limits for normal nonpregnant women. The free T4 and free T3 levels remain within the normal limits. However, in pregnant women being treated with replacement doses of thyroid hormone, the dose will need to be increased because of the increase in TBG.

Thyrotoxicosis in pregnant women is also associated with abnormalities, and there is an increased risk of low-birth-weight infants, congenital fetal malformations, pre-eclampsia, and premature delivery. Pregnant women with thyrotoxicosis are usually treated with an antithyroid drug, although in extreme cases surgery may be indicated. Antithyroid drugs cross the placenta and can act on the fetal thyroid, resulting in a fetal goiter. Thus, the dose of the antithyroid drug should be kept at the lowest dose needed to ameliorate the thyrotoxicosis, and in many instances can be discontinued in the third trimester.

In the postpartum period, thyroiditis may develop in association with hypothyroidism or hyperthyroidism. Either condition may be transient or prolonged.

## Glossary

**galactorrhea** Production and secretion of milk from breast tissue.

**granulosa cells** Line the follicle in the ovary; take up and aromatize androgens to estrogens, primarily estradiol.

**gynecomastia** Enlargement of the breast; usually used in reference to the male breast.

**Leydig cells** Present in the interstitial space of the testes; stimulated by luteinizing hormone to synthesize steroids, primarily the androgen testosterone.

**Sertoli cells** Line the seminiferous tubules of the testes; necessary for spermatogenesis; respond to follicle-stimulating hormone.

**sex hormone-binding globulin** Protein made in the liver that binds certain steroids with high affinity, making them less available for uptake by tissues compared to albumin-bound or free (bioavailable) steroids. The binding affinity of SHBG is highest for dihydrotestosterone and testosterone but less for estradiol.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Follicle Stimulating Hormone (FSH) • Luteinizing Hormone (LH) • Sex Hormone-Binding Globulin (SHBG) • Stress and Reproduction • Thyroid Hormone Action on**

**the Heart and Cardiovascular System • Thyroid Hormone Action on the Skeleton and Growth • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH)**

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# Thyroid Hormone Action on the Heart and Cardiovascular System

BERND R. GLOSS
*University of California, San Diego*

I. INTRODUCTION
II. T3 TARGET GENES IN THE HEART
III. T3 EFFECTS ON MYOCYTE PHYSIOLOGY
IV. TARGET GENE PROMOTERS
V. T3 RECEPTOR EXPRESSION IN THE HEART
VI. SUMMARY

**Both hyperthyroidism and hypothyroidism are associated with changes in cardiac function. A number of genes expressed in the heart are known to be regulated by thyroid hormone. This article examines the changes in cardiac performance that are associated with changes in thyroid hormone levels and discusses thyroid hormone regulation of target gene expression in the heart.**

## I. INTRODUCTION

In the late 1700s, a physician named Caleb Hillier Parry described an interesting correlation that he had observed during his medical career. He noted that an enlargement of the thyroid gland coincided with an enlargement of the heart.

Since these early descriptions of the correlation between thyroid disorders and cardiac function, many detailed studies have crystallized the concept that there must be genes in the heart that are regulated by thyroid hormone and that up- or down-regulation of such genes brings about the physiological effects in the heart that are observed in a particular thyroid condition. What are the changes in cardiac physiology that are associated with elevated or lowered thyroid hormone levels?

Hyperthyroidism is associated with an elevated heart rate (tachycardia) and to a significant degree also with cardiac arrhythmias. In addition, the speed and force of cardiac contraction are both elevated and there are hemodynamic changes largely caused by a decreased arterial tone and increased venous tone. Because most of the biological actions of thyroxine are mediated by its conversion to triiodothyronine (T3), in the hyperthyroid state T3 increases heart rate (chronotropic effects), increases the force and speed

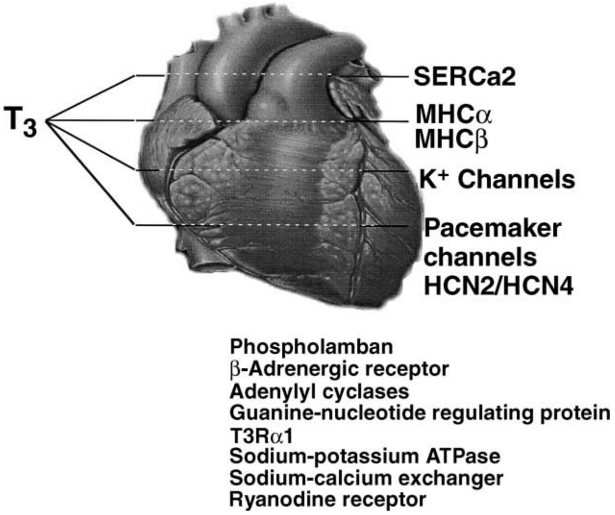

FIGURE 1 Thyroid hormone in the form of T3 regulates the cardiac genes listed in this figure. The regulatory regions of the genes on the right have been studied extensively and in some the T3-response elements have been identified and characterized.

of systolic contraction (inotropic effect), and accelerates diastolic relaxation (lusitropic effect).

Hypothyroidism, in the long run, causes a lowering in heart rate (bradycardia) and often leads to an elevated blood pressure. It is the hemodynamic changes combined with the altered metabolic functions, for example, hypercholesterolemia, that eventually manifest in a failing heart, often in conjunction with coronary artery disease.

The T3-induced changes in hyper- and hypothyroidism in cardiac target genes are best studied in rodents and will be discussed in detail in Section II. Figure 1 illustrates schematically which target genes are described in this article and lists other genes known to be influenced by thyroid hormone but that are less well studied thus far.

## II. T3 TARGET GENES IN THE HEART

### A. The SERCa2 Gene

Thyroid hormone regulates the expression of sarcoplasmic–endoplasmic calcium ATPase type 2a (SERCa2) in myocytes. When rodents are injected with thyroxine at a dose of 1 mg per kilogram of body weight to make them hyperthyroid, both the mRNA for the SERCa2 gene and the protein levels are elevated. Figure 2 shows a Northern blot with total ventricular RNA of mice with different thyroid status. Conversely, when rodents are made hypothyroid by treatment with 5-propyl-2-thiouracil and a low-iodine diet, the mRNA for SERCa2a as well as the protein levels are reduced by 20–30% (see Fig. 2). Interestingly, it has been noted that in heart failure patients and in rodents with failing hearts, the SERCa2 gene is down-regulated. Aortic constriction (banding) in mice and rats that leads to cardiac hypertrophy also reduces the transcription of the SERCa2 gene. Very recent findings indicated that in both the failing heart and the hypertrophied heart, the levels of thyroid hormone receptors, especially the T3Rα1 receptor, are reduced, which in turn could be the reason for the diminished transcription of the SERCa2 gene.

### B. The Myosin Heavy Chain Genes

The gene for myosin heavy chain α (MHCα) encodes a large protein that is part of the thick filament in myocytes, facilitating contraction and relaxation. Transcription of the α isoform is regulated by thyroid hormone, as shown in Fig. 2, especially in mice, where hyperthyroidism leads to an elevation of

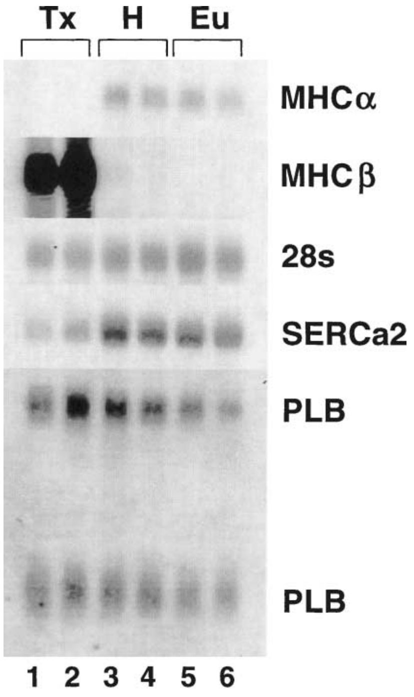

FIGURE 2 Northern blot with mouse heart total RNA. The mRNA levels in ventricles of hypothyroid (Tx, lanes 1 and 2), hyperthyroid (H, lanes 3 and 4), and euthyroid (Eu, lanes 5 and 6) animals were determined by probing the blot with radiolabeled oligonucleotides for myosin heavy chain α (MHCα), myosin heavy chain β (MHCβ), and 28S rRNA or with cDNA probes random-labeled for sarcoplasmic–endoplasmic reticulum calcium ATPase (SERCa2) and phospholamban (PLB).

transcripts and protein in cardiac myocytes. Particularly in the mouse heart, the up-regulation of MHCα expression is linked to the development of the neonatal myocyte into an adult myocyte and the onset of MHCα isoform expression coincides with an increase in circulating T3 and tetraiodothyronine levels during this developmental process.

The gene for the MHCβ isoform encodes a high-molecular-weight protein similar to MHCα and also localizes to the thick myosin filament. Transcription of the MHCβ gene is also regulated by thyroid hormone, and in mouse myocytes this T3 regulation is reversed compared with MHCα, which is nicely demonstrated in Fig. 2. In the adult mouse heart, there is very little MHCβ transcription when the animals are euthyroid; however, in hypothyroidism, the transcription of MHCβ is strongly induced, which points to a T3-dependent negative regulation of the promoter of this gene. This ligand-dependent repression of the MHCβ gene can be reestablished when the euthyroid status is restored.

It should be noted here that the expression of the myosins in general is very species specific although in mammals all members of the MHC gene family are regulated by T3. Surprisingly, however, whether T3 induces or represses the expression of a given MHC gene depends not only on the gene itself, but also on the muscle in which it is expressed. Indeed, the same gene can be induced by T3 in one muscle and repressed in another.

## C. The $K^+$ Channel Genes

Some genes for the cardiac delayed inward rectifier potassium channels have been cloned in recent years. These channels are pores in the plasma membrane that are not always homogenous structures but are composed of two or more different proteins encoded by different genes. This makes it difficult to study the regulation of one particular gene and make a conclusion regarding the effect on a particular current that flows across the membrane using the channel pore that contains the gene product studied.

Nevertheless, a strong correlation was found between the prolonged relaxation times in hypothyroid papillary muscles of the mouse heart and reduced expression of genes that code for delayed inward rectifier channels, namely, Kv1.4, Kv4.2, and Kv4.3. Patch-clamping cardiac myocytes from hypothyroid hearts have indeed shown a reduced net current flow across the plasma membrane, which could explain the prolonged repolarization time and relaxation period observed in hypothyroid mouse papillary muscles. Conversely, in hyperthyroid animals, the Kv4.3 gene was moderately up-regulated and the Kv1.4 gene was strongly up-regulated. This could lead to a faster repolarization of the myocyte to accommodate the more rapid action-potential cycles needed for the faster heartbeat that is observed in hyperthyroidism.

The Kv1.5 gene was also investigated for its regulation by thyroid hormone. In hypothyroid rat hearts, this gene was markedly down-regulated in contrast to the hyperthyroid status when the gene was expressed at a higher level. Interestingly, these thyroid hormone-induced changes in gene expression were not found in the mouse heart, which points toward a possible species-specific action of the thyroid hormone signaling pathway on this gene, similar to the species-specific regulation of the myosin genes by thyroid hormone.

The Long QT syndrome is a dominant autosomal disease, in some cases linked to congenital deafness. It can be linked to mutations in various ion channel genes, among them the KvLQT1 gene. In hypothyroid animals, the QT interval of the electrocardiogram (ECG) is significantly prolonged and the question of whether the KvLQT1 gene was down-regulated in hypothyroidism was examined. This was not the case, however, it was found that an associated protein encoded by the minK gene was actually up-regulated in hypothyroidism and down-regulated in hyperthyroidism. The increased expression of minK could potentially interfere with the gating properties of the pore formed by KvLQT1 and thereby contribute to the prolongation of the QT interval in the ECG.

## D. Pacemaker Channels

Rapid rhythmic changes in membrane potentials are most prevalent in the cells of the brain and the heart. Rhythmic pacing of neuronal networks has been implicated with the encoding and controlling of information flow in the CNS. The involvement of a membrane current that was called $I_h$ in the brain and $I_f$ in the heart has been fairly well characterized. Recently, the HCN gene family whose gene products constitute the $I_h$ or $I_f$ channel was discovered. This article focuses on the two family members that are expressed in the heart, namely, HCN2 and HCN4. Both genes are highly homologous and their encoded proteins form the entire or at least the majority of the pore-forming units that presumably carry the $I_f$ current. The $I_f$ current acts at the very end of the action potential in cardiac myocytes just before the membrane rapidly depolarizes and the cell contracts. It is believed that the activation of the

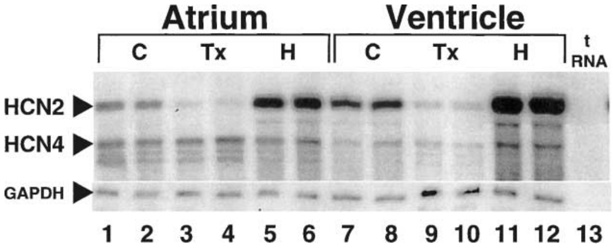

FIGURE 3 RNase protection, showing the mRNA levels for the pacemaker channels HCN2 and HCN4 in atria (lanes 1–6) and ventricles (lanes 7–12) of euthyroid (C), hypothyroid (Tx), and hyperthyroid (H) mice. In lane 13, a tRNA control was hybridized to all probes used and no specific protected signal was detected. A probe for the glyceraldehyde3-phosphate dehydrogenase (GAPDH) was used to confirm equal RNA input into each hybridization.

channel serves as a trigger for a new depolarization/repolarization cycle. These cycles are slower at a reduced heart rate and faster at an elevated heart rate. Because thyroid hormone modulates heart rate, the T3 responsiveness of the HCN2 and HCN4 genes has been investigated. Indeed, it could be demonstrated that mRNA levels for both genes are elevated in hyperthyroidism and diminished in hypothyroidism in mouse, rat, and rabbit hearts. Data from RNase protection experiments in mouse hearts are shown in Fig. 3. Because heart rate is controlled from the sinus node in the right atrium, the expression of HCN2 and HCN4 in atria and ventricles was studied and it was found that HCN2 is regulated by T3 in atria and ventricles; however, HCN4 responds to T3 only in ventricles and not in atria. The reason for this differential regulation of the pacemaker channels is currently unknown.

## III. T3 EFFECTS ON MYOCYTE PHYSIOLOGY

When myocytes are isolated from adult or neonatal rat hearts and put in tissue culture, a spontaneous contraction can be observed. Addition of T3 to the culture medium increases this spontaneous beating, indicating an effect of T3 on the contractile physiology of these cells. Prolonged treatment of myocytes with T3 also increases the protein content of these cells, similar to what is seen in cardiac hypertrophy. Although not all of these T3 effects can be explained on the molecular level, the transcriptional stimulation of the SERCa2 gene by T3 and the concomitant decrease of phospholamban mRNA, as shown in Fig. 2, facilitate a more efficient shift of calcium from the cytoplasm into the sarcoplasm. The relocation of calcium to the cytoplasm to bring about a contraction via the myosin/actin apparatus works through the ryanodine channel in the sarcoplasm; interestingly, the gene for this channel is also up-regulated by T3. In this way, T3 increases the calcium transients between the sarcoplasm and the cytoplasm, which provides for more rapid contraction/relaxation cycles. To coordinate the increased calcium cycling in the myocyte with an increased contractile capability, depolarizing and repolarizing must be accelerated as well. The depolarizing trigger could potentially be shifted toward a more positive voltage by modulating the $I_f$ current that works just before the depolarization phase of the myocyte action potential. This is schematically indicated in Fig. 4, showing a ventricular myocyte action potential diagram. An increase of mRNA for HCN2 and HCN4 by T3 could potentially bring about the voltage shift in the $I_f$ current. In the repolarization phase of the action potential, several delayed inward rectifier potassium channels control the flow of $K^+$ ions into the cytoplasm. Most of the genes that encode these potassium channels have been identified and it could be shown that Kv1.4, Kv1.5, Kv4.2, Kv4.3, and the minK gene are regulated by T3. This provides a basis for accelerated and decelerated depolarization and repolarization, indirectly evident in ECGs of hyper- and hypothyroid animals.

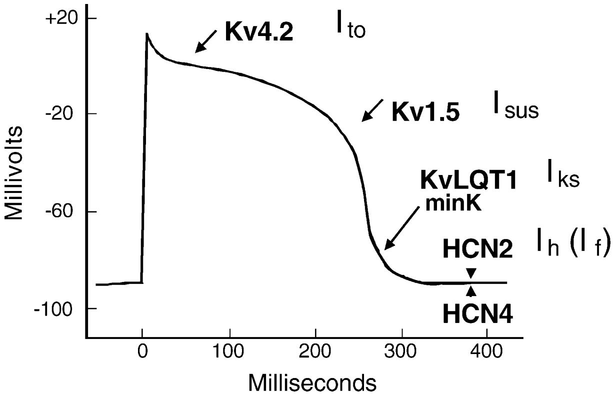

FIGURE 4 Ventricular action potential curve. During a single contraction of a ventricular myocyte, its membrane potential changes within 300 to 400 ms from a resting negative potential of 80–90 mV to a positive potential of approximately 20 mV. This is largely due to a rapid influx of sodium and calcium ions. The slow repolarization phase is characterized mainly by potassium efflux through channels that carry the $I_{to}$, $I_{sus}$, and $I_{ks}$ currents. Pacemaker channels carry the $I_h$ or $I_f$ current with both sodium and potassium ions, triggering the rapid depolarization phase. $\mathrm{I}_{to}$, transient outward potassium current; $\mathrm{I}_{sus}$, sustained outward potassium current; $\mathrm{I}_{ks}$, slow delayed rectifier potassium current.

Finally, the mechanical contraction apparatus within the cytoskeleton of the myocyte can be modified by T3. Especially in rodents, the myosin heavy chains (MHCα and MHCβ) that make up the thick filament of the contractile apparatus are thyroid hormone responsive. Transcription of MHCα is activated by T3, whereas transcription of MHCβ is repressed. In the mouse, this leads to an almost complete myosin isoform switch in hypo- and hyperthyroidism. Myosin V3 contains only MHCβ and myosin V1 is composed of two molecules of MHCα. In hypothyroidism, the V3 form, which hydrolyzes ATP slowly and therefore causes a slower contraction, dominates. In contrast, euthyroid and hyperthyroid mouse hearts contain the fast ATP-hydrolyzing V1 myosin that facilitates a rapid contraction.

Taken together, these findings indicate that thyroid hormone regulation of gene expression links the calcium pumps, ion channels, and myosins with one another for a coordinated faster performance or slower performance in the hyperthyroid heart and hypothyroid heart, respectively.

## IV. TARGET GENE PROMOTERS

The most extensively studied promoters are those of the myosin heavy chain genes and the promoter of the rat SERCa2 gene. The MHCα promoter contains a complex arrangement of transcription factor-binding sites; these include the classical TATA-box and CAAT-box, a muscle enhancer factor 2 (MEF2)-binding site, a brain factor 2 (BF2) site, and a PNR (pannier gene encoded GATA transcription factor) element that binds two Ets factors as well as a thyroid hormone response element (TRE) with the consensus sequence 5′-AGGTGAcaggAGGACA-3′. Further upstream of the transcription start site at approximately −1.9 kb, a DNase I hypersensitive site that contained a conserved GATA-binding factor motif was found.

The rat and mouse MHCβ promoter is very strongly repressed by T3; a transferable negative regulatory element has not yet been found but several hexamer half-sites close to the transcription start site have been identified. It has also been hypothesized that the promoter could be T3 inhibited by a mechanism proposed for negative regulation by the glucocorticoid and retinoic acid receptors. In these models, the ligand-bound receptor molecules interact with positive-acting transcription factors (presumably off DNA), preventing them from transactivating their target gene. Among the factors proposed to bind to the MHCβ promoter within 300 bp upstream of the transcription start site are TFIID (TATA box binding factor IID)-binding TATA-box, MyoD (muscle gene-transactivating helix-loop-helix factor), NFe (tissue specific nuclear factor), SP1 (Simian-virus 40 protein 1), M-CAT (muscle specific TEF), and AP5/GT-II (TEF-I binding element), as well as other binding sites that are shared between many muscle specific genes. Which of these factors could be inhibited by the ligand-bound T3 receptor is currently not known.

The rat SERCa2 promoter has been examined also. T3 responsiveness lies within 560 bp upstream of the transcription start site, and three TREs have been identified. All three TREs are needed for full activation or repression by non—ligand-bound T3R, but each TRE has a different half-site configuration. TRE1 is a direct repeat spaced by 4 nucleotides (DR + 4), TRE2 is an inverted palindrome spaced by 4 nucleotides (IP + 4), and TRE3 is configured as an IP + 6. Interestingly, a novel thyromimetic (GC-1) stimulates TRE1 but represses TRE2 and TRE3 when each TRE is isolated by pairwise mutation of the other two in the natural promoter context. The TREs of the rat SERCa2 promoter can potentially interact with the other transcription factors that bind to the promoter. One such interaction has been demonstrated with the MEF2a factor potentiating the T3 stimulation in a T3Rα isoform-specific manner. Other factors that could bind to the region within 560 bp upstream of the transcription start site are the TFIID-binding TATA-box, C/EBP (CCAAT/enhancer binding protein), SP1, and an E-box-binding helix-loop-helix protein.

Very recently, the promoters of the T3-regulated delayed inward rectifier potassium channel Kv4.2 and the pacemaker channels HCN2 and HCN4 were cloned. The Kv4.2 region linked to a CAT reporter gene responded significantly to T3 stimulation in neonatal rat myocyte transfections. The promoter regions of HCN2 and HCN4 have not been characterized fully and the HCN2 region failed to respond to T3 when linked to a reporter gene, which raises the possibility that other mechanisms besides transcriptional regulation may operate on this gene to elevate and diminish mRNA levels in hyper- and hypothyroidism, respectively.

## V. T3 RECEPTOR EXPRESSION IN THE HEART

The above-described changes in nuclear gene regulation are likely to be mediated by the nuclear hormone receptors that bind T3. The ligand-binding isoforms that are found in the heart are the T3Rα1 and T3Rβ1 isoforms. Expression of T3Rα1 dominates in the heart with the mRNA levels of T3Rβ1 approximately 30% of those for T3Rα1. This is shown in an RNase protection experiment with total RNA from mouse hearts in Fig. 5. In the liver, for example, this ratio in inverted with the T3Rα1 mRNA levels approximately 10% of the T3Rβ1 mRNA levels. Which factors promote enhanced expression of the T3Rα gene in the heart and a lower ratio of expression of the T3Rβ gene is currently unknown.

Much has been learned from the knockout of the α or β isoforms of the thyroid hormone receptors in the mouse. Lack of T3Rα expression in the heart led to bradycardia and a "hypothyroid-like" gene expression profile. In contrast, lack of T3Rβ expression in the heart had virtually no phenotype when the animals were brought to a euthyroid status, except for the notion that T3Rα expression was reduced by nearly 40% in T3Rβ knockout animals (see Fig. 5). Interestingly, homozygous mutation of the ligand-binding domain of T3Rβ in the mouse to generate a dominant negative receptor significantly disturbed T3Rα action. This shows that T3Rβ is obviously expressed in the same cardiac cells as T3Rα. The question arises as to why T3Rβ cannot compensate for the lack of T3Rα expression. Do certain promoters of cardiac genes respond preferentially to T3Rα, and if so, what are the discriminating elements in such promoters? Further studies are necessary to understand the contribution of each receptor isoform in the heart, for example, analyzing the cardiac phenotype and gene expression in receptor double-knockout mice. Rescue experiments of knockout mice with specific T3R isoforms in a cardiac gene therapy experiment will also reveal what each isoform can contribute to cardiac function. Finally, the temporal and spatial disruption of either T3R isoform in the heart will enable a refined analysis of the cardiac phenotype in inherently euthyroid animals.

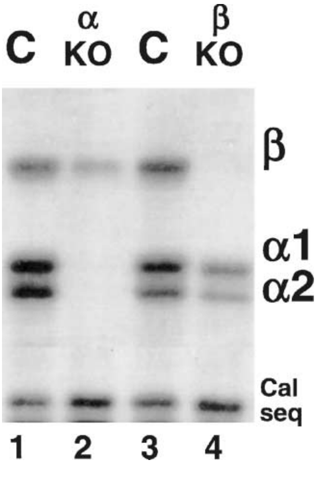

FIGURE 5 RNase protection, showing the mRNA levels for thyroid hormone receptors β (β1 and β2), α1 (α1), and α2 (α2) in mouse ventricles. Controls (C, lanes 1 and 3) were hybridized with RNA from isogenic wild-type animals of the respective mouse strain that was lacking either thyroid hormone receptor α (lane 2) or thyroid hormone receptor β (lane 4), designated αKO and βKO, respectively. To control for equal RNA input in each lane, a probe for calsequestrin (Calseq) was used.

## VI. SUMMARY

Thyroid status in humans and in animals influences cardiac output. This article has attempted to describe the known molecular targets in the heart that T3 reaches to alter cardiac performance. The genes that are regulated by T3 contribute to the lusitropic effect, for example, the potassium channels Kv1.4, Kv1.5, Kv4.2, Kv4.3, and minK. Genes that contribute to the inotropic effect are, on the one hand, calcium pumps and channels like SERCa2a, phospholamban, ryanodine channel and the sodium–calcium exchanger and, on the other hand, the myosin heavy chains. The chronotropic effect of T3 probably has several components including the pacemaker channels that are up-regulated in hyperthyroid tachycardia and down-regulated in hypothyroid bradycardia. But there are also reports that the β-adrenergic receptor, adenylyl cyclase, and the T3Rα gene itself are regulated by T3, which may contribute to the chronotropic effect. Some of the target gene promoters have been characterized very well and contain distinct thyroid hormone-response elements, as well as muscle-specific elements. Other promoters still lack TREs and there may be other mechanisms by which T3 regulates the expression of those genes. Two isoforms of the thyroid hormone-binding nuclear receptors are expressed in the heart, T3Rα1 and T3Rβ1. Both seem to be present in the same myocytes but subserve distinct functions that are currently under intense investigation.

## Glossary

**$K^+$ channels** Multisubunit structures located in the plasma membrane that gate the flux of potassium into the cell. These include the so-called delayed inward rectifier channels that open to repolarize the cell after a contraction. Some of the genes for individual subunits are regulated by thyroid hormone.

**myosins** Proteins that are part of the contractile fibers found in myocytes. The genes for myosin heavy chain α

(MHCα) and myosin heavy chain β (MHCβ) are regulated by thyroid hormone in some species. In rodents, MHCα is predominant in the adult heart and MHCβ can be strongly induced by hypothyroidism.

**pacemaker channels** The genes for these channels were only recently cloned and this article focuses on those that are expressed in the heart and regulated by thyroid hormone, namely, the HCN2 and HCN4 genes. A characteristic of the $I_f$ conductance constituted by the gene products of HCN2 and HCN4 is that both sodium and potassium ions are conducted through the pore. Furthermore, gating is regulated by cyclic AMP and hyperpolarization of the cell membrane.

**phospholamban** A small 6 kDa protein that associates with SERCa2a and regulates the calcium pump activity dependent on the phospholamban phosphorylation status.

**SERCa2a** Sarcoplasmic-endoplasmic calcium ATPase type 2a is found primarily in the myocytes of the heart. The approximately 100 kDa protein is embedded in the sarcoplasmic membrane and transports calcium from the cytoplasm into the sarcoplasm while hydrolyzing ATP to ADP. The expression of the gene coding for SERCa2a is regulated by thyroid hormone.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action**
**• Thyroid Hormone Action on the Skeleton and Growth**
**• Thyroid Stimulating Hormone (TSH)**

## Further Reading

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# Thyroid Hormone Action on the Skeleton and Growth

GRAHAM R. WILLIAMS
*Imperial College London, Hammersmith Campus, London*

I. CLINICAL AND EPIDEMIOLOGICAL OBSERVATIONS
II. THYROID HORMONE RECEPTORS
III. THE EPIPHYSEAL GROWTH PLATE
IV. THYROID HORMONE EFFECTS ON CHONDROCYTES AND GROWTH
V. GROWTH ABNORMALITIES IN TR NULL MICE
VI. THYROID HORMONE EFFECTS ON OSTEOBLASTS, OSTEOCLASTS, AND BONE TURNOVER

**The skeleton is a specialized and complex organ that remodels its structure in response to systemic, local, and physical signals. It is highly vascularized, contains the hematopoietic bone marrow, and provides structural support and a reserve of calcium and phosphate ions for the maintenance of mineral homeostasis. Bone is formed either by direct transformation and ossification of condensed mesenchyme (intramembranous ossification) or by replacement of a cartilage scaffold with calcified bone (endochondral ossification). Development and growth of the skull and closure of its sutures result from intramembranous ossification. Linear growth, fracture repair, and bone remodeling, together with development of the remaining skeleton, result from endochondral ossification. A considerable number of clinical, epidemiological, and basic scientific studies indicate that thyroid hormones play a key role in skeletal development. They are essential for normal linear growth and are important regulators of bone turnover and the maintenance of bone mass.**

## I. CLINICAL AND EPIDEMIOLOGICAL OBSERVATIONS

Hypothyroidism in childhood causes growth arrest together with delayed bone age, epiphyseal dysgenesis, and immature body proportion. Furthermore, up to 50% of patients with the phenotypically variable syndrome of resistance to thyroid hormone (RTH), caused by mutant thyroid hormone receptor (TR) β proteins, suffer from growth retardation and developmental abnormalities of bone that may reflect tissue hypothyroidism. Thyroxine (T4) replacement therapy in hypothyroid children induces rapid catch-up growth that may be incomplete because bone age advances faster than height, leading to early epiphyseal growth plate fusion and speculation that skeletal responses to thyroid hormones are exaggerated in hypothyroidism. Accordingly, childhood thyrotoxicosis causes accelerated growth and advanced bone age, which may lead to premature growth plate closure and short stature. Advanced intramembranous ossification may also result in craniosynostosis in thyrotoxic children due to premature closure of the skull sutures. Indeed, stimulation of mineral apposition and osteogenesis, resulting in premature narrowing of the skull sutures, has been documented in a hyperthyroid rat model.

Thyrotoxicosis in adults results in increased bone turnover and net bone loss and causes osteopenia and fracture in patients with established disease. Thus, a series of studies has shown that thyrotoxicosis is associated with reduced bone density and an increased risk of osteoporotic hip fracture. Postmenopausal women are particularly at risk when confounding factors including age and sex are accounted for. Such findings have led to the contentious issue regarding whether treatment of hypothyroid or thyroid cancer patients with supraphysiological doses of T4 is also associated with osteoporosis and increased fracture risk. The current consensus suggests that overzealous treatment with T4, leading to suppression of circulating thyrotropin (TSH) concentrations, is probably associated with increased fracture risk, particularly in postmenopausal women and in those with a previous history of thyrotoxicosis.

## II. THYROID HORMONE RECEPTORS

The complex actions of thyroid hormone (3,5,3′-L-triiodothyronine, T3) are mediated by TR proteins, which function as hormone-inducible transcription factors that regulate the expression of target genes and are members of the superfamily of hormone and orphan nuclear receptors. The TRα and TRβ genes are conserved in vertebrates. TRα encodes the widely expressed α1 and α2 C-terminal variants: TRα1 is a functional receptor that binds T3 and DNA, whereas TRα2 fails to bind T3 and is a weak antagonist *in vitro*, although its physiological role is unknown. A novel promoter in intron 7 of TRα generates the truncated Δα1 and Δα2 variants that act as repressors *in vitro* and have been implicated in intestinal development. TRβ encodes the functional β1, β2 isoforms and a new β3 N-terminal variant was recently identified in rat. TRβ2 expression is restricted to pituitary and hypothalamus, where it primarily regulates the activity of the hypothalamo–pituitary–thyroid feedback loop, but β1 and β3 are expressed widely. A truncated rat TRΔβ3 isoform is also expressed widely and acts as a potent antagonist in vitro, but its physiological significance is not yet known.

TRs are located at sites of intramembranous and endochondral bone formation in a variety of species; TRα1, TRα2, and TRβ1 mRNAs and proteins are expressed in osteoblasts and osteocytes and in reserve and proliferative zone epiphyseal growth plate chondrocytes. Functional TRs have also been identified in primary cultured osteoblasts and growth plate chondrocytes and in immortalized osteoblastic and chondrogenic cells from several species. Recent reports suggest that skeletal osteoblastic responses to T3 may vary according to anatomical site, although the mechanisms responsible for these findings are unknown. Thyroid hormones also stimulate osteoclastic bone resorption, but this effect is likely to be mediated by T3-responsive osteoblasts, as osteoclasts do not express functional TRs.

## III. THE EPIPHYSEAL GROWTH PLATE

The epiphyses and metaphyses of long bones originate from independent ossification centers and are separated by a growth plate, which becomes ossified after puberty when epiphyseal fusion occurs (Fig. 1). In the normal growth plate, reserve zone progenitor cells lie immediately below the epiphysis and mature chondrocytes are located above the primary spongiosum, which communicates with the bone marrow. Immature reserve zone cells undergo clonal expansion to form organized columns in the proliferative zone, where they secrete a cartilage matrix containing type II collagen and proteoglycans. The largest proliferative cells differentiate to form hypertrophic chondrocytes, which secrete type X collagen, enlarge by five times their volume, and eventually undergo apoptosis to leave a cartilage scaffold. New blood

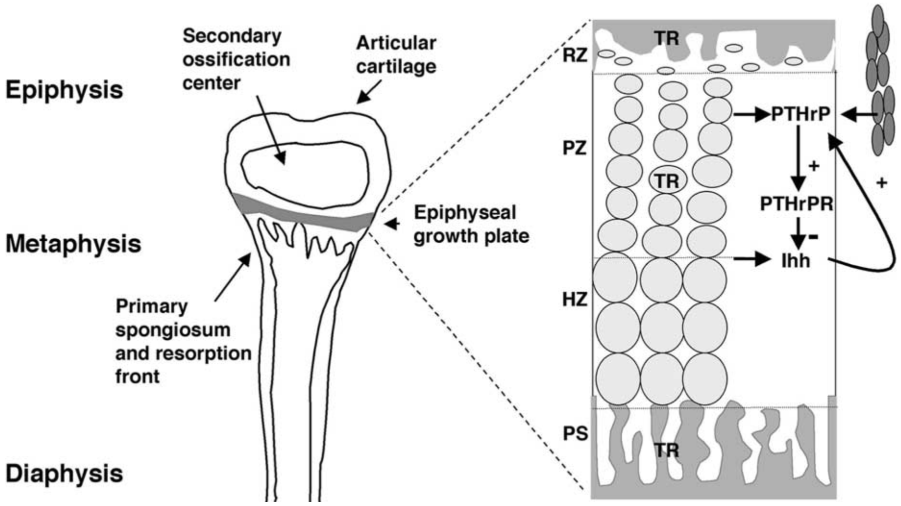

**FIGURE 1** Long bone showing the location of the epiphyseal growth plate and an enlarged view to show the organization of chondrocytes, the localized expression of thyroid hormone receptors, and the feedback loop that controls the pace of chondrocyte proliferation. RZ, reserve zone; PZ, proliferative zone; HZ, hypertrophic zone; PS, primary spongiosum; TR, thyroid receptor; Ihh, Indian hedgehog; PTHrP, parathyroid hormone-related peptide; PTHrPR, PTHrP receptor.

vessels enter from the primary spongiosum and osteoblasts invade the growth plate to lay down trabecular bone on this cartilage template and complete the endochondral ossification process. Recent experiments have established that the pace of chondrocyte differentiation and bone formation is regulated by a negative feedback loop involving the paracrine factors Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP). Ihh is secreted by pre-hypertrophic chondrocytes and stimulates PTHrP production from the peri-articular region of the epiphysis during bone development. PTHrP acts on PTHrP-receptor-expressing pre-hypertrophic chondrocytes to maintain cell proliferation, reduce Ihh production, and complete a feedback loop in which PTHrP exerts a negative signal that inhibits hypertrophic chondrocyte differentiation and delays bone formation (Fig. 1).

## IV. THYROID HORMONE EFFECTS ON CHONDROCYTES AND GROWTH

Hypothyroidism results in gross abnormalities of growth plate structure with disorganized proliferating chondrocyte columns, abnormal cartilage matrix, reduced hypertrophic chondrocyte differentiation, and impaired vascular invasion of the growth plate at the primary spongiosum (Fig. 2). Alterations in PTHrP and PTHrP receptor mRNA expression in growth plate chondrocytes were identified in hypothyroid and thyrotoxic animals, suggesting that the setpoint of the critical Ihh/PTHrP feedback loop is sensitive to thyroid status. Whereas the majority of these effects probably result from direct actions of T3 in TR-expressing chondrocytes, other studies also indicate that the hypothyroid growth plate is relatively insensitive to the actions of growth hormone (GH) because of reduced local expression of insulin-like growth factor-I (IGF-I). Similar findings of delayed hypertrophic chondrocyte differentiation and impaired neovascularization of newly formed cartilage in fracture callus in hypothyroid rats reinforce the view that endochondral ossification during skeletal growth and repair requires thyroid hormones.

Until recently, the locations of T3 target cells within the growth plate were unknown. It has been shown that TRα1, TRα2, and TRβ1 proteins are expressed in reserve and proliferating zone chondrocytes but not in hypertrophic cells, suggesting that progenitor cells and proliferating chondrocytes are primary T3-target cells but that differentiated chondrocytes may lose the ability to express TRs and become unresponsive. A large body of data, in a variety of *in vitro* model systems using chondrocytes derived from several species, indicates that T3 regulates chondrocyte proliferation and the organization of proliferating chondrocyte columns, is required for terminal hypertrophic differentiation,

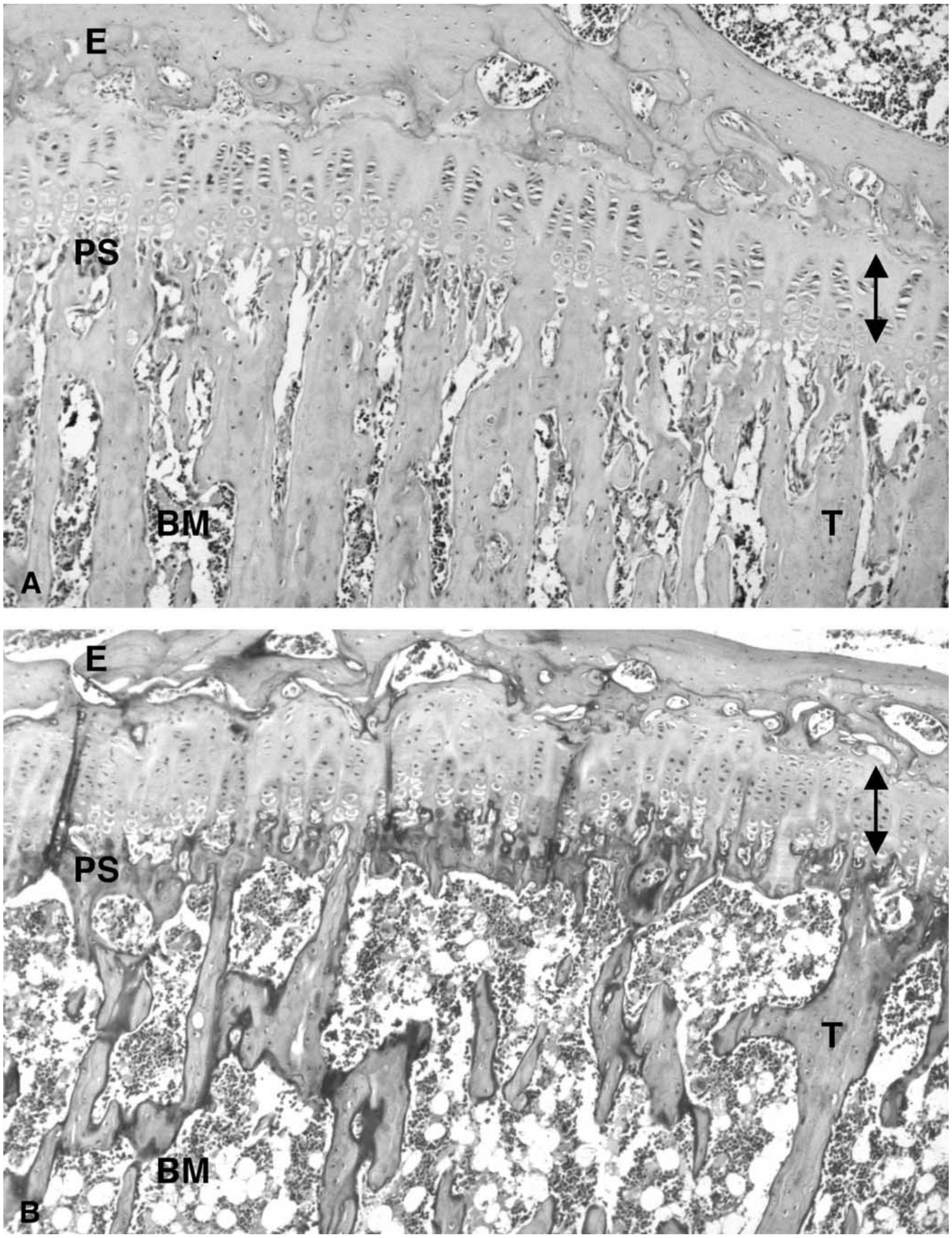

**FIGURE 2** Sections of proximal tibial growth plate from 12-week-old euthyroid (A) and hypothyroid (B) rats stained with hematoxylin and eosin. The euthyroid growth plate contains organized columns of chondrocytes with neovascularization at the primary spongiosum. Longitudinal trabecular bone lies in continuity with proliferating and hypertrophic chondrocyte columns. The hypothyroid growth plate is disorganized and neovascularization is absent. Trabecular bone formation is diminished and disorganized relative to normal. E, epiphysis; PS, primary spongiosum; BM, bone marrow; T, trabecular bone; arrow delineates the growth plate.

and induces calcification of cartilage matrix. In other studies, rat tibial growth plate chondrocytes grown in primary suspension culture express TRs and maintain their differentiation potential. In this system, T3 directly inhibits clonal expansion and cell proliferation, while simultaneously promoting hypertrophic chondrocyte differentiation. Recent studies indicate that T3 might achieve this by induction of cyclin-dependent kinase inhibitors that may arrest cell cycle progression in proliferating chondrocytes and enable terminal hypertrophic differentiation to progress.

## V. GROWTH ABNORMALITIES IN TR NULL MICE

The skeletal phenotypes of TR knockout mice reinforce the view that T3 acts directly in growth plate cartilage but reveal the complexity of thyroid hormone action in bone (Table 1). Deletion of the TRβ gene does not result in growth retardation or evidence of developmental abnormalities in bone and cartilage, indicating that TRβ is not essential for skeletal development. Nevertheless, the creation of distinct mouse models with different RTH mutations in TRβ has highlighted the phenotypic variability of the human RTH syndrome as only one mouse model, harboring the severe TR$\beta^{PV}$ mutation, suffers from impaired growth.

Several TRα knockouts have been produced, in which differing products of the TRα locus were deleted, with varying effects on the skeleton and on thyroid and GH/IGF-I status. TR$\alpha1^{-/-}$ mice lack expression of TRα1 and Δα1, but TRα2, Δα2, and β are retained and skeletal development is normal. Combination of the TR$\alpha1^{-/-}$ and $\beta^{-/-}$ mutations to produce a TR$\alpha1^{-/-}$ $\beta^{-/-}$ double knockout results in growth retardation and delayed bone maturation. TR$\alpha1^{-/-}$ $\beta^{-/-}$ mice are growth hormone and IGF-I deficient; GH replacement reverses some of the growth retardation but does not influence the defective ossification, indicating that T3 exerts important direct effects on growth plate chondrocytes but mediates some of its effects on growth via GH and IGF-I.

In contrast, in TR$\alpha^{-/-}$ mice there is growth arrest after weaning and disorganization of growth plate chondrocytes with delayed cartilage mineralization and bone formation. The TR$\alpha^{-/-}$ mutation results in deletion of TRα1 and α2 but preservation of Δα1, Δα2, and TRβ. These animals become progressively hypothyroid in the postnatal period due to impaired thyroid hormone production and the skeletal phenotype can be rescued by T4 replacement, suggesting that TRβ can compensate for TRα in the growth plate in appropriate circumstances. Nevertheless, the delayed bone maturation seen in TR$\alpha^{-/-}$ mice is not further modified in mice harboring a TR$\alpha^{-/-}$ $\beta^{-/-}$ double gene deletion, suggesting that TRβ, in contrast to TRα, is dispensable in bone.

These studies have been interpreted to suggest that the non-T3-binding variants TRα2, Δα1, and Δα2 may play important roles in bone development. Thus, mice devoid of all TRα isoforms were generated. These TR$\alpha^{0/0}$ mice display growth delay and retarded endochondral ossification with evidence of impaired hypertrophic chondrocyte differentiation and disorganized growth plate architecture. TR$\alpha^{0/0}$ mice express TRβ and maintain normal TSH concentrations with slightly reduced T4 levels, indicating mildly increased thyroid hormone sensitivity and further suggesting that TRα is functionally predominant in bone. TR$\alpha^{0/0}\beta^{-/-}$ double-mutant

**TABLE 1 Genotypes and Growth Characteristics of Thyroid Hormone Receptor (TR) Null Mice**

| TR Knockout | Deleted TR mRNAs | Expressed TR mRNAs | Thyroid status | GH Status | Growth Retardation |
|---|---|---|---|---|---|
| $\alpha1^{-/-}$ | α1, Δα1 | α2, Δα2, all β isoforms | Mildly hypothyroid | Normal | − |
| $\alpha^{-/-}$ | α1, α2 | Δα1, Δα2, all β isoforms | Grossly hypothyroid | Normal | ++ |
| $\alpha^{0/0}$ | All α isoforms | All β isoforms | Euthyroid[a] | Normal | + |
| $\beta^{-/-}$ | All β isoforms | All α isoforms | RTH | Mildly deficient | − |
| $\beta2^{-/-}$ | β2 | All α isoforms, β1, β3, Δβ3 | RTH | Mildly deficient | − |
| $\alpha1^{-/-}\beta^{-/-}$ | α1, Δα1, all β isoforms | α2, Δα2 | Severe RTH | GH/IGF-I[b] deficient | ++ |
| $\alpha^{-/-}\beta^{-/-}$ | α1, α2, all β isoforms | Δα1, Δα2 | Severe RTH | Not determined | ++ |
| $\alpha^{0/0}\beta^{-/-}$ | All α isoforms, all β isoforms | None | Severe RTH | GH deficient | ++ |

*Note*. GH, growth hormone; RTH, resistance to thyroid hormone; IGF-I, insulin-like growth factor-I, GH status was determined by comparison of pituitary GH mRNA expression in wild-type and mutant animals.

[a] Dynamic testing demonstrated mild increased pituitary sensitivity to thyroid hormones.

[b] GH and IFG-I serum concentrations and GH mRNA.

mice are viable and more severely growth retarded than their TR$\alpha^{0/0}$ counterparts but less so than Pax8$^{-/-}$ mice in which congenital hypothyroidism results from thyroid follicular cell agenesis. Taken together, these findings indicate that differing consequences result from thyroid hormone deficiency, in which unoccupied receptors are present, compared to TR deficiency, and suggest a physiological role for unliganded TR or non-T3-binding TR variants in bone development. They also indicate that the TRα gene is essential for skeletal development and the timing of bone mineralization.

## VI. THYROID HORMONE EFFECTS ON OSTEOBLASTS, OSTEOCLASTS, AND BONE TURNOVER

Thyroid hormones activate bone-forming osteoblasts and bone-resorbing osteoclasts, but in hyperthyroidism the normally tightly coupled activities of these two cell types are dissociated, resulting in bone loss. The bone remodeling cycle begins with activation of osteoclast lineage cells, which begin to resorb bone. Osteoblasts invade the area once a certain resorption depth is reached and lay down new matrix, which is mineralized to form new bone in areas of previous resorption. The activation–resorption–formation cycle normally lasts up to 200 days and occurs at discrete sites called bone-remodeling units. Bone remodeling occurs at differing rates in trabecular and cortical bone and in differing anatomical locations. The rate at which each site undergoes remodeling is known as the activation frequency and this is a major determinant of total bone turnover. Bone resorption, matrix deposition, and mineralization times are shortened in hyperthyroidism and the activation frequency is increased. These disproportionate changes uncouple osteoblasts and osteoclasts and lead to a net bone loss of approximately 10% per remodeling cycle in hyperthyroidism.

It is clear that osteoblasts express functional TRs, which have been identified at the mRNA and protein levels, by nuclear T3-binding activity and in transient transfection studies using T3-inducible reporter genes. Although osteoblasts in primary culture and several osteoblastic cell lines respond directly to T3 *in vitro*, the specific consequences of T3 stimulation vary considerably between studies. Such differences may occur between species and depend on the degree of cellular confluence, the stage of osteoblast differentiation, the cell type, passage number, and origin, and the dose and duration of T3 treatment. Furthermore, the response of osteoblastic cells to T3 has been reported to vary according to the anatomical site from which the cultured cells originate. Thus, T3 has been shown to stimulate, inhibit, or have no effect on osteoblastic cell proliferation. A general consensus, however, indicates that osteoblast activity is stimulated by T3. T3 increases production of osteocalcin and collagen type I matrix proteins, collagenase 3, gelatinase B, tissue inhibitor of metalloproteinase-1, alkaline phosphatase, IGF-I, IGF-binding proteins 2 and 4, fibroblast growth factor receptor 1, interleukin-6, and interleukin-8 in various osteoblastic cell culture systems. Furthermore, T3 may potentiate some of the osteoblast responses to PTH by modulating the expression of PTH/PTHrP receptor. Despite the many potential T3 target genes identified in osteoblasts, little information is available regarding mechanisms by which their expression is modulated and T3 regulation in many cases may be indirect and involve other signaling pathways. In contrast to effects on the activity of mature osteoblasts, osteoblast progenitor cell differentiation is inhibited by thyroid hormones, although the effects in vitro are complex and dose-related and involve interactions with other factors including IGF-I, steroid hormones, vitamin D, cytokines, and other growth factors.

Bone resorption is stimulated by T3 in bone organ cultures by mechanisms that are unclear but certainly complex and likely to involve other systemic hormones and locally acting cytokines. There is controversy regarding whether osteoclasts express TRs, although most studies indicate they do not and co-culture experiments have revealed that osteoclastic bone resorption is stimulated by T3 only in the presence of osteoblastic cells. These findings suggest that the resorptive effects of T3 in bone are mediated via direct stimulation of TR-expressing osteoblasts, which may induce the recruitment and differentiation of osteoclast progenitor cells or increase the activity of mature osteoclasts.

## Glossary

**cortical bone** Dense surface layer of mature compact bone.

**endochondral ossification** Bone formation by replacement of a preformed cartilage scaffold that occurs during skeletal development and fracture repair.

**epiphyseal growth plate** Specialized strip of cartilage located at the ends of long bones, in which programmed proliferation and maturation of chondrocytes result in the production of cartilage to form a template for new bone formation during linear growth.

**intramembranous ossification** Bone formation by direct transformation and ossification of condensed mesenchyme that occurs during development of flat bones, including the skull, mandible, scapula, and ileum.

**osteoporosis** A progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture.

**primary spongiosum** Region of trabecular bone forming the interface between the growth plate and bone marrow and the site for neovascularization and osteoblast invasion during endochondral ossification.

**trabecular bone** Interior network of organized calcified spongy bone, the space between which contains bone marrow and communicates with the marrow cavity.

## See Also the Following Articles

**Bone Morphogenetic Proteins • Environmental Disruptors of Thyroid Hormone Action • Osteogenic Proteins • Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Thyroid Hormone Action on the Heart and Cardiovascular System • Thyroid Hormone Receptor Isoforms • Thyroid Stimulating Hormone (TSH)**

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# Thyroid Hormone Receptor Isoforms

Paul M. Yen

*National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Maryland*

I. TR ISOFORMS AND RELATED GENE PRODUCTS
II. TISSUE EXPRESSION OF TR ISOFORMS
III. HORMONAL REGULATION OF TR ISOFORMS
IV. ISOFORM-SPECIFIC REGULATION OF TARGET GENES
V. TR KNOCKOUT MODELS OF TR ISOFORMS
VI. CONCLUSION

**Multiple isoforms of thyroid hormone receptors are expressed in a variety of tissues. Serving as transcription factors, these receptors play isoform-specific roles in regulation of hormonal activity and target gene expression. Genetic studies of the isoforms as well as their tissue expression, hormone regulation, and isoform-specific functions with respect to phenotype and transcription of target genes provide insights into mechanisms of disorders related to thyroid hormones.**

## I. TR ISOFORMS AND RELATED GENE PRODUCTS

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that belong to the nuclear hormone receptor superfamily. TRs and other nuclear hormone receptor family members share a similar domain organization in that they contain a central DNA-binding domain with two zinc fingers, a carboxy-terminal ligand-binding domain, and multiple transactivation domains (Fig. 1). They also bind to thyroid hormone response elements (TREs) composed of two or more hexamer sequences arranged as direct repeats or inverted repeats (separated by four and six nucleotides, respectively), typically located in the promoter region of target genes.

There are two major TR isoforms, TRα and TRβ, encoded on separate genes located on human chromosomes 17 and 3, respectively. The multiple TR isoforms have been found in tissues of amphibia, chicks, mice, rats, and humans. In mammalian species, TRα and TRβ vary from 400 to slightly over 500 amino acids in size and contain highly homologous DNA-binding and ligand-binding domains (Fig. 2). Both TR isoforms bind triiodothyronine (T3) with high affinity and mediate thyroid hormone-regulated gene expression. In positively regulated target genes, liganded TR recruits co-activators such as the steroid-related co-activator (SRC) family members and other cofactors that lead to increased histone acetylation of chromatin in the promoter of target genes (Fig. 3). This may then be followed by recruitment of another complex that contains components of the RNA polymerase II complex, which, in turn, leads to recruitment and stabilization of RNA polymerase II and enhanced transcription. In contrast, unliganded TRs bind to TREs and recruit corepressors, including the silencing mediator of retinoic acid and thyroid hormone (SMRT) receptors and the nuclear receptor corepressor (N-CoR), with histones deacetylases, which cause decreased acetylation of local chromatin and repression of transcription.

In addition to the TRs encoded by the two TR genes, TR isoforms can be produced by alternative splicing. Alternative splicing of the initial RNA transcript of the TRα gene generates two mature mRNAs, each encoding two proteins, TRα-1 and c-erbAα-2. In the rat, these proteins are identical from amino acid residue 1 to residue 370, but their respective sequences diverge markedly afterward. As a consequence of the replacement of the carboxy terminus with a 122-amino-acid sequence, c-erbAα-2 does not bind T3. Additionally, c-erbAα-2 binds TREs weakly but cannot transactivate thyroid hormone-responsive genes. It also may block TH action on certain target genes by binding to TREs and preventing TRs from regulating transcription.

The TRα gene also encodes another gene product, rev-erbA, on the opposite strand. The rev-erbA mRNA contains a 269-nucleotide stretch that is complementary to the c-erbAα-2 mRNA due to its transcription from the DNA strand opposite of that used to generate TRα-1 and c-erbAα-2. The protein product also belongs to the nuclear hormone receptor superfamily, is expressed in adipocytes and muscle cells, and can bind to TREs and retinoic acid response elements (RAREs) and repress gene transcription. However, rev-erbA should be considered an orphan receptor because its cognate ligand and function are not known. The rev-erbA receptor may be involved in regulating the splicing that generates c-erbAα-2 because increased levels of rev-erbA mRNA correlate with increased TRα-1 mRNA relative to c-erbAα-2. The rev-erbA receptor also may be involved in adipocyte differentiation.

The TRβ gene also generates two isoforms, TRβ-1 and TRβ-2. This gene contains two promoter regions that regulate the transcription of an mRNA encoding TRβ-1 or TRβ-2. One or both of the coding mRNAs are generated by selective promoter choice. The amino acid sequences of the DNA-binding, hinge region, and ligand-binding domains of these two TRβ isoforms are identical; however, the amino-terminal regions share no sequence homology. Both are bona

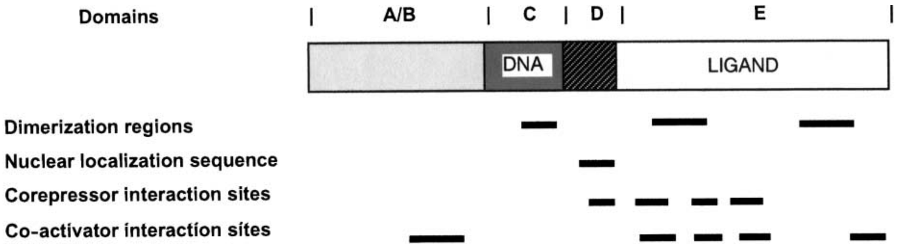
Domains
A/B
C
D
E
DNA
LIGAND
Dimerization regions
Nuclear localization sequence
Corepressor interaction sites
Co-activator interaction sites

FIGURE 1 General organization of major thyroid hormone receptor domains and functional subregions.

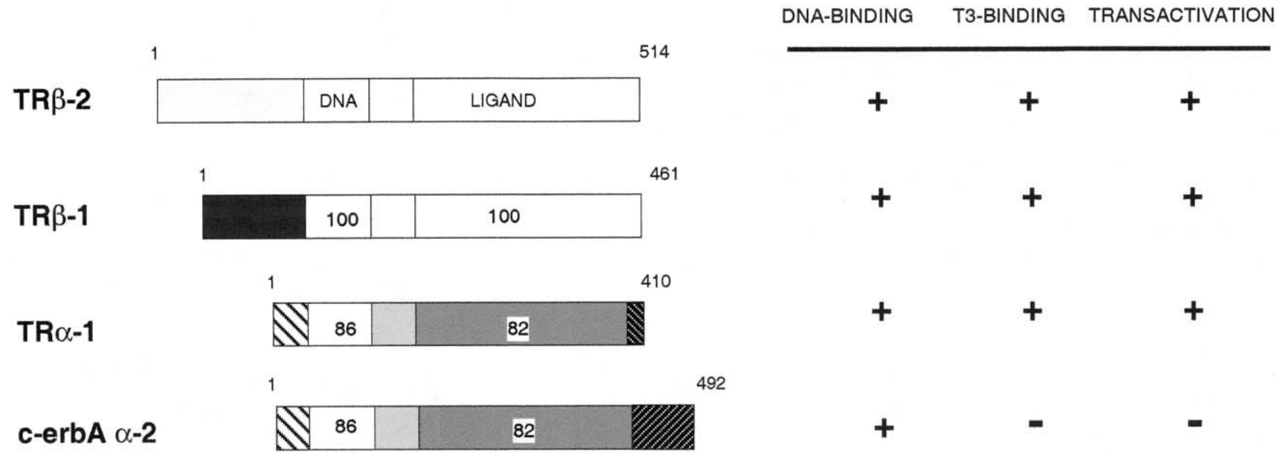

FIGURE 2 Comparison of amino acid homologies and their functional properties among thyroid hormone receptor isoforms; length of each receptor is indicated by numbers just above receptor diagrams; amino acid homology (%) with TRβ-2 is indicated by numbers in the receptor diagrams.

fide receptors in that they bind TREs and TH with high affinity and specificity and mediate TH-dependent transcription. Expression of the two TRβ isoforms may be regulated by pituitary-specific transcription factors such as Pit-1.

## II. TISSUE EXPRESSION OF TR ISOFORMS

TRα-1 and TRβ-1 mRNAs and proteins are ubiquitously expressed in rat and human tissues. However, TRα-1 mRNA has highest expression in rat skeletal muscle and brown fat whereas TRβ-1 mRNA has highest expression in brain, liver, and kidney. TRβ-2 mRNA and protein have a restricted tissue expression in the anterior pituitary, hypothalamus, and developing brain and inner ear. In chicks and mice, TRβ-2 mRNA also is expressed in the developing retina. Last, a number of short forms of TRα and TRβ generated by alternative splicing of mRNA or by use of intrinsic promoters have been found in embryonic stem cells and in fetal bone cells, and may have biological significance. In particular, some of these short forms have dominant negative activity on TH-mediated transcription and may also affect intestine and bone development.

## III. HORMONAL REGULATION OF TR ISOFORMS

TR mRNA regulation varies among the different isoforms. In the intact rat anterior pituitary, T3 decreases TRβ-2 mRNA, modestly decreases TRα-1 mRNA, and slightly increases rat TRβ-1 mRNA. Despite these countervailing effects, the total T3 binding decreases by 30% in the T3-treated rat pituitary. In other tissues, T3 slightly decreases TRα-1 and c-erbAα-2 mRNA, with the exception of the brain, where c-erbAα-2 levels are not changed. TRβ-1 mRNA is minimally affected in nonpituitary tissues. The hypothalamic tripeptide, thyrotropin-releasing hormone (TRH), also regulates TR mRNA expression because it decreases TRβ-2 mRNA, slightly decreases TRα-1 mRNA, and minimally affects TRβ-1 mRNA in cultured rat pituitary cells. Retinoic acid blunts the negative regulation by T3 in these cells. Additionally, in patients with nonthyroidal illness who have decreased circulating serum free T3 and tetraiodothyronine (T4) levels, TRα and TRβ mRNAs are increased in peripheral mononuclear cells and liver biopsy specimens. Thus, increased TR expression may potentially compensate for decreased circulating thyroid hormone levels in these patients.

## IV. ISOFORM-SPECIFIC REGULATION OF TARGET GENES

The amino acids of each of the TR isoforms are highly homologous across mammalian species. This conservation suggests that there may be important specialized functions for each TR isoform. Recent studies have suggested that TRβ-1 may exhibit isoform-specific regulation of the TRH and myelin basic protein genes, and TRβ-2 may play important roles in the regulation of the growth hormone and thyroid-stimulating hormone β (TSHβ) gene expression in the pituitary. The differential expression of TR isoforms in various tissues (e.g., TRβ-1 is the

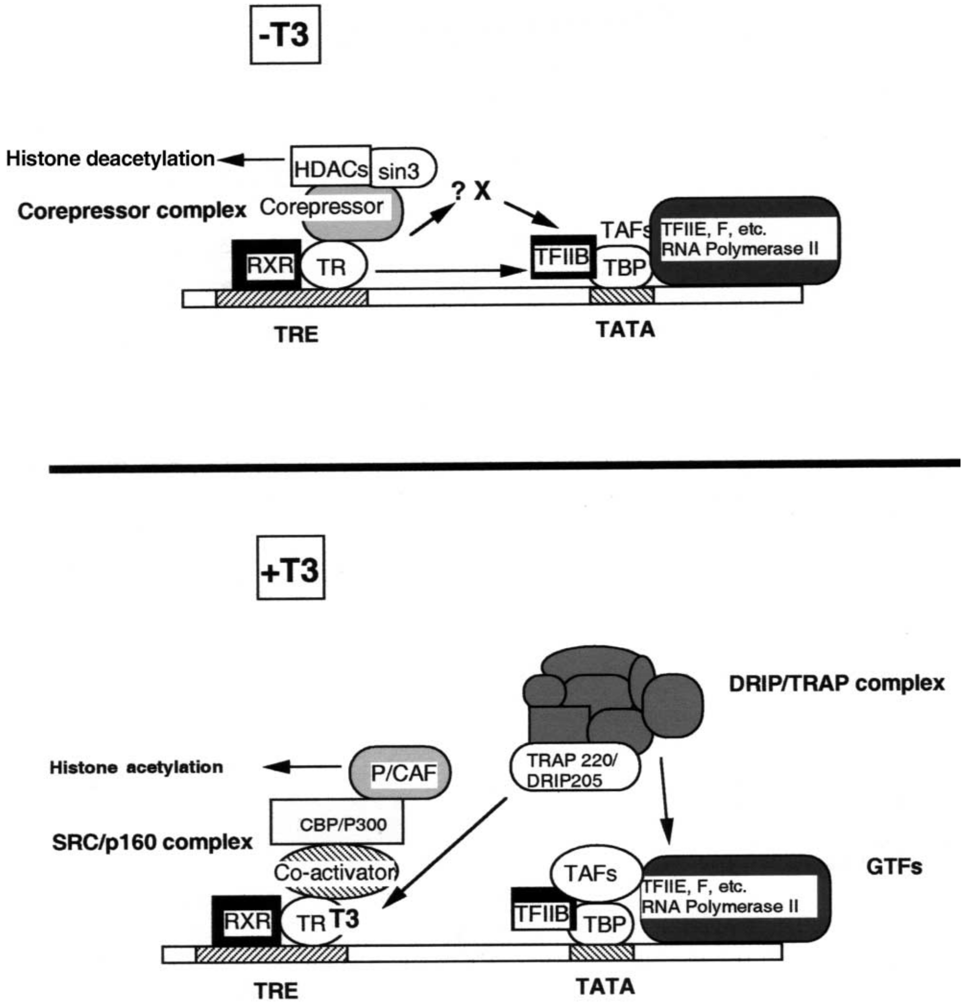
-T3
Histone deacetylation
HDACs
sin3
Corepressor complex
Corepressor
? X
RXR
TR
TFIIB
TAFs
TBP
TFIIE, F, etc.
RNA Polymerase II
TRE
TATA
+T3
DRIP/TRAP complex
TRAP 220/
DRIP205
Histone acetylation
P/CAF
CBP/P300
SRC/p160 complex
Co-activator
RXR
TR T3
TAFs
TFIIB
TBP
TFIIE, F, etc.
RNA Polymerase II
GTFs
TRE
TATA

FIGURE 3 Molecular model for basal repression in the absence of triiodothyronine (T3) and transcriptional activation in the presence of T3. Possible additional cofactors (X) remain to be identified. HDAC, Histone deacetylase; RXR, retinoid acid X receptor; TR, thyroid hormone receptor; TAFs, TBP-associated factors; SRC, steroid receptor co-activator; CBP, cAMP response element binding protein; P/CAF, p300/CBP-associated factor; DRIP, vitamin D-interacting protein; TRAP, thyroid hormone receptor-associated protein; TBP, TATA-binding protein. See text for additional details.

predominant isoform expressed in liver whereas TRα-1 is the major isoform expressed in heart) has fostered attempts to develop TRβ-specific thyroid hormone analogues that may have cholesterol-lowering effects but minimal cardiac toxicity.

## V. TR KNOCKOUT MODELS OF TR ISOFORMS

The selective ablation of TR isoforms in mice by homologous recombination has provided insight on the roles of TR isoforms in the development and function of various tissues. Two different groups have generated TRα knockout mice that have different phenotypes. The structure of the TRα gene is complex because it encodes TRα-1, c-erbAα-2 (which cannot bind T3), and rev-erbA (generated from the opposite strand encoding TRα); thus, the locus of homologous recombination will determine which isoforms will be knocked out. Transgenic mice in which both TRα-1 and c-erbAα-2 have been deleted (TRα −/−) have a more severe phenotype, involving hypothyroidism, intestinal malformation, growth retardation, and early death shortly after weaning. The early death can be partially rescued by T3 injection of pups. Transgenic mice that lack only TRα-1 (TRα-1 −/−) have a milder phenotype compared to TRα −/− mice because they have decreased body temperature and heart rate and a prolonged electrocardiogram ventricular depolarization/repolarization (QT) interval. These findings suggest that TRα-1 plays an important role in regulating cardiac function. The differences in the two knockout phenotypes could be due to specific functions of c-erbAα-2; however, specific knockout of c-erbAα-2 does not affect the survival of pups. Short TRα isoforms can be generated from an internal promoter within the seventh intron, which then have dominant negative activity on wild-type TR function. It thus is likely that these short TR isoforms may be responsible for the more severe phenotype of the TRα − /− knockout mice. In this regard, a TRα knockout mouse that lacks both TRα-1 and c-erbA α-2, and does not express the short TRα isoforms ($TR\alpha_{o/o}$), has a milder phenotype compared to $TR\alpha_{-/-}$.

TRβ knockout mice ($TR\beta_{-/-}$) display modest phenotypic changes, including elevated TSH and T4, thyroid hyperplasia, and hearing and retinal defects. These abnormalities in the hypothalamic–pituitary–thyroid (HPT) axis are similar to those seen in patients with the syndrome of resistance to thyroid hormone. Additionally, these findings suggest a critical role for TRβ in the development of the auditory and visual systems. Interestingly, ligand-independent elevation of TSH is normal in hypothyroid TRβ − /− mice, but the suppression of TSH by TH is impaired. Recently, TRβ-2 has been selectively knocked out. The mice had elevated levels of TH and TSH, implicating TRβ-2 as the major TR isoform regulating TSH production. Interestingly, the mice did not have any hearing defects, suggesting that TRβ-2 may not be required for auditory development or that its function can be compensated by TRβ-1.

Because the TRα-1 and TRβ knockout mice have relatively mild phenotypes, it is likely that the TR isoforms may have redundant transcriptional activity and can compensate for each other in most target genes. To examine the effects of abolishing all TR isoforms, TRα-1 − /− TRβ − /− double knockouts have been generated and, surprisingly, these mice are still viable. These mice have markedly elevated T4, T3, and TSH levels and large goiters. The mice also have growth retardation and decreased fertility, as well as impaired bone development and reduced bone mineral content. Furthermore, the mice have reduced heart rate and impaired control of body temperature, similar to the TRα-1 − /− mice. It is noteworthy that the effects on peripheral tissues generally are milder in the double-knockout mice compare to those seen in congenital hypothyroidism. It is possible that nongenomic effects of TH may be involved in double-knockout mice but not in mice with congenital hypothyroidism. It also is possible that lack of TRs is less deleterious than the presence of TRs during hypothyroidism. Because unliganded TRs bind to TREs and recruit corepressors to repress basal transcription of some positively regulated target genes, it is possible that critical target genes may be shut down in the hypothyroid mice whereas basal transcription is maintained in the double-knockout mice.

## VI. CONCLUSION

Tissue-restricted expression and knockout mice studies suggest that the multiple TR isoforms may have specialized roles, although cotransfection studies suggest that most of the known target genes can be coregulated. Future studies with microarrays, proteomics, and isoform-specific ligands, perhaps in conjunction with knockout mice, should help identify isoform-specific roles in the transcription of specific genes.

## Glossary

**basal repression** Target gene transcription repression by unliganded thyroid hormone receptor and its recruitment of corepressors.

**dominant negative activity** Blockade of thyroid hormone receptor transcriptional activity by a transcription factor or mutant receptor, typically by competitive binding to the thyroid hormone response element.

**nuclear hormone receptor superfamily** Nuclear receptor family that includes the steroid, vitamin D, retinoic acid, and thyroid hormone receptors.

**thyroid hormone response elements** Discrete enhancer sequences in the promoters of target genes to which thyroid hormones bind and regulate transcription.

**thyroid hormones** Triiodothyronine (T3) and thyroxine (T4); secreted by the thyroid gland and converted by peripheral deiodination.

**transactivation** Ligand-dependent transcription of target genes mediated by a thyroid hormone receptor and its recruitment of co-activators.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Estrogen Receptor Biology and Lessons from Knockout Mice • Thyroglobulin • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH)**

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# Thyroid Hormone Receptor, TSH and TSH Receptor Mutations

ROY E. WEISS AND PETER M. SADOW
*University of Chicago*

I. INTRODUCTION
II. TH RECEPTOR MUTATIONS AND RESISTANCE TO TH
III. TSH MUTATIONS
IV. TSH RECEPTOR MUTATIONS

**The thyroid hormone receptor interacts with the thyroid hormone triiodothyronine, which is secreted from the thyroid following interaction of thyroid-stimulating hormone and its receptor. Mutations in any of the genes regulating the production and function of these molecules lead to pathological conditions. In addition to the importance of these mutations as a cause of human disease, such errors of nature allow for an understanding of the structure and function of these molecules in normal physiology.**

## I. INTRODUCTION

At each juncture in the hypothalamic–pituitary–thyroid axis, there is an interaction between hormone and receptor that produces a specific biologic effect. Thyrotropin-releasing hormone (TRH), secreted from the hypothalamus, interacts with TRH receptors on the thyrotroph cell surface. This results in production and release of thyroid-stimulating hormone (TSH) from the pituitary. TSH, in turn, interacts with thyroid follicular cell surface receptors, resulting in synthesis and release of thyroid hormones (THs) from the thyroid gland. Finally, TH binds to nuclear TH receptors in target tissues for TH action. All of this is controlled by a series of negative feedback loops at the level of the hypothalamus and pituitary involving primarily TH. The focus of this article is on what has been learned from mutations in these hormones and their receptors in humans, how the mutations relate to human disease, and the structure and function of these molecules in normal physiology.

Abnormalities in either the hormone or the receptor can result in altered states of hormone sensitivity, such as resistance (loss of function) or hypersensitivity (gain of function). In the human thyroid axis, there are examples of both types of mutations. Resistance is defined as a condition of reduced or absent target tissue responsiveness to the hormone. Resistance manifests as higher than normal levels of the hormone without the expected biological effect. Resistance may be due to any of the following reasons: (1) a mutation in the hormone, resulting in altered biologic activity; (2) production of a substance, such as an antibody, that interferes with hormonal action; (3) an abnormal receptor due to a loss-of-function mutation, resulting in failure to bind the hormone or failure to activate the appropriate postreceptor response cascade; (4) an abnormality in any of the multiple cofactors involved in the process of hormone action; or (5) any postreceptor defect, such as abnormal G-protein, that could also result in hormone resistance. "Pseudoresistance" refers to lab assay results indicating excessive amounts of hormone when, in fact, the results are false, due to an interfering substance.

Hormone hypersensitivity is a condition of exaggerated effects of a hormone in the presence of normal to low levels of the hormone. Theoretically, this may be due to an increase in biological activity, as in a "superhormone," or to an abnormal constitutively activated receptor. Additionally, hypersensitivity can be due to a substance other than the hormone which stimulates the receptor.

## II. TH RECEPTOR MUTATIONS AND RESISTANCE TO TH

### A. Summary of TH Action

Thyroid hormone penetrates all body tissues and is believed to enter cells by a process of concentration-dependent passive diffusion after its dissociation from the serum hormone-binding proteins. Triiodothyronine (T3), the biologically active form of the hormone in blood, or generated from tetraiodothyronine (T4, thyroxine) within the cell, reaches its site of action, the cell nucleus. There it binds with DNA-bound specific molecules known as TH receptors (TRs). Hormone-bound TRs then interact with various cofactors that modify the receptor–ligand complex and allow it to regulate gene transcription.

### B. General Introduction to Nuclear Receptors

Thyroid hormone receptors belong to the nuclear hormone receptor superfamily, which includes receptors for the sex hormones, testosterone and estrogen, as well as for vitamin D, retinoic acid, and the peroxisome proliferation receptor. These receptors are characterized by a carboxy-terminal ligand-binding domain (LBD) and a more proximal DNA-binding domain (DBD). These receptors act as nuclear transcription factors by binding to specific sequences of DNA located in target genes. In the case of TH, these sequences are TH response elements (TREs). Some regions of the receptor are involved in interacting with a variety of cofactors that modify the receptor–hormone complex on the TRE, thus modulating transcriptional activity.

### C. The TR and Mode of Action

Mutations have been described that result in a failure to initiate TH-responsive transcription. Either the LBD is altered, preventing the recognition of the hormone, or the DBD or the hinge region is altered, inactivating the hormone–receptor complex. Mutations in TRs may result in the syndrome of resistance to TH. In a subject heterozygous for an abnormal TR allele, a dominant negative interaction occurs whereby the abnormal allele interferes with function of the normal allele, resulting in the phenotype of resistance.

### D. Clinical Aspects of Resistance to TH

Resistance to TH (RTH) is an inherited syndrome (usually autosomal dominant) of reduced target tissue responsiveness to TH. More than 700 cases in 250 families have been described that fit this definition. It is suggested that the incidence is 1:50,000. With the exception of a single family, inheritance is autosomal dominant. In practice, patients with autosomal dominant RTH are identified by a persistent elevation of serum free 3,5,3′,5′-tetraiodothyronine and free 3,5,3′-triiodothyronine levels without TSH suppression. In these RTH patients, this combination of abnormal laboratory test results occurs in the absence of intercurrent illness, drugs, or alterations of TH transport proteins in serum. More importantly, administration of supraphysiological doses of TH fails to produce the expected suppressive effect on the secretion of pituitary TSH and the anticipated metabolic responses in peripheral tissues.

The common features of RTH are (1) elevated serum levels of free T4 and free T3, (2) normal or slightly increased concentrations of serum TSH that responds to TRH stimulation, (3) absence of typical symptoms and metabolic consequences of TH excess, and (4) goiter.

### E. Molecular Basis of RTH: TRβ Mutations

Mutations in the gene encoding thyroid hormone receptor β (TRβ) have been described in the majority of families with RTH. All mutations affect the LBD or adjacent hinge region of the TRβ molecule and are distributed in three clusters (Fig. 1). In the *TRβ* gene, 43 mutations in 86 families have been found in cluster 1 (corresponding to amino acids 429–460), 49 mutations in 113 families have been found in cluster 2 (amino acids 309–383), and 16 mutations in 31 families have been found in cluster 3 (amino acids 234–282). A complete *TRβ* gene deletion has been reported in one family. Mutations have been identified in 64 codons of the *TRβ* gene, with 30 of these codons altered by more than one mutation. Of the 109 identified *TRβ* gene mutations, 71 are unique in

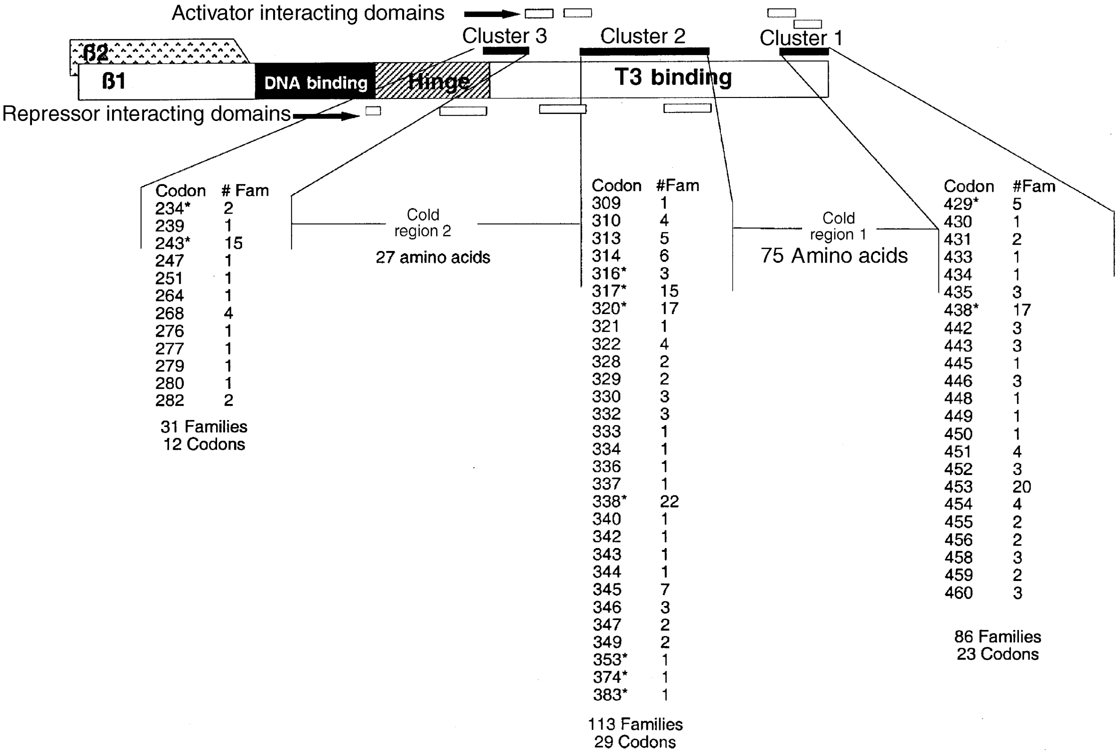
Activator interacting domains
Cluster 3
Cluster 2
Cluster 1
ß2
ß1
DNA binding
Hinge
T3 binding
Repressor interacting domains
Codon # Fam
234* 2
239 1
243* 15
247 1
251 1
264 1
268 4
276 1
277 1
279 1
280 1
282 2
31 Families
12 Codons
Cold region 2
27 amino acids
Codon #Fam
309 1
310 4
313 5
314 6
316* 3
317* 15
320* 17
321 1
322 4
328 2
329 2
330 3
332 3
333 1
334 1
336 1
337 1
338* 22
340 1
342 1
343 1
344 1
345 7
346 3
347 2
349 2
353* 1
374* 1
383* 1
113 Families
29 Codons
Cold region 1
75 Amino acids
Codon #Fam
429* 5
430 1
431 2
433 1
434 1
435 3
438* 17
442 3
443 3
445 1
446 3
448 1
449 1
450 1
451 4
452 3
453 20
454 4
455 2
456 2
458 3
459 2
460 3
86 Families
23 Codons

**FIGURE 1** Functional domains of the thyroid hormone receptor β-subunit and reported mutations in the *TRβ* gene. The mutated codon is listed along with the number of families known to have mutations in that particular codon. There may be more than one mutation in a particular codon. In several codons, identical mutations have been found in multiple families. The mutations are present in three clusters, separated by two regions in which natural mutations have not been reported. *TRβ1* and *TRβ2* have different ATG start sites and differ only in the amino terminus. Asterisks indicate a CpG dinucleotide.

that they occur in only one family, and 38 occur in multiple unrelated families. Using the single-letter amino acid code to describe the protein mutations, the five most frequent mutations in the *TRβ* gene correspond to R338W (19 families); A317T (14 families); R438H (11 families); R243Q (10 families), and P453T (9 families). Of the mutations described, 95 (87.2%) are due to a single-base-pair substitution resulting in a single-amino-acid substitution (92) or a premature stop codon (3). The remaining mutations are due to dinucleotide substitution (1), complete deletion (1), single-base-pair deletion (1), three-base-pair deletion (5), single-base-pair insertion (4), three-base-pair insertion (1), and seven-base-pair duplication (1). Of 109 mutations, 17 occur in CpG dinucleotides and these account for 43% of the families with *TRβ* gene mutations. In 37 of 236 families with RTH, the *TRβ* mutation has occurred *de novo* and the mutant allele can be traced to the normal progenitor of one of the two parents. In contrast to syndromes of vitamin D and androgen resistance, mutations in the DNA-binding region are not found in RTH.

No mutations have been reported in the *TRα* gene. Mice with deletion of TRα1 and TRα2 isotypes have hypersensitivity to TH. Therefore, the phenotype of a mutant TRα is unlikely to manifest as RTH and may explain the lack of observed mutations in this protein.

Normal TRβ is found in 10% of RTH patients. Patients with non-TRβ RTH and patients with TRβ mutations have identical phenotypes. Whereas the majority of patients with glucocorticoid resistance syndromes usually have mutations in the glucocorticoid receptor (GR), similar to RTH, some patients have been described with non-GR glucocorticoid resistance. Based on the observation of RTH in mice deficient in steroid receptor co-activator-1 (SRC-1), it is logical to postulate that abnormalities of co-activators or corepressors, known to be involved in hormone action, may also cause hormonal resistance.

## III. TSH MUTATIONS

Thyroid-stimulating hormone is a 30-kDa glycoprotein secreted by the thyrotroph; it is encoded on human chromosome 1. Similar to other glycoprotein hormones secreted by the anterior pituitary, TSH is composed of a common α-subunit noncovalently bound to a specific β-subunit. The CAGYC (C, cysteine; A, alanine; G, glycine; Y, tyrosine) region of the protein forms the "seat belt" part of the TSH β-subunit, which allows for interaction with the α-subunit. It is the unique β-subunit that confers the hormonal specificity. In some instances, as in a molar pregnancy, a condition in which there are very high levels of chorionic gonadotropin (CG), stimulation of the TSH receptor by CG α-subunit can cause hyperthyroidism. All reported mutations in the *TSHβ* gene result in significant hypothyroidism. The patients present with typical findings of central hypothyroidism, namely, low levels of T3 and T4 with inappropriately very low or absent concentrations of serum TSH. The low serum TSH concentrations can be understood based on the observation that TSHβ is degraded intracellularly. TSHβ degradation occurs when TSHβ is not bound to the α-subunit, and the mutation prevents this binding. Therefore, any abnormality of the protein in the CAGYC region would result in undetectable TSH. Whereas most cases of central hypothyroidism are associated with disruptions in other components in the pathways of the pituitary hormone axis, such as adrenocorticotropic hormone (ACTH)/cortisol or luteinizing hormone (LH)/follicle-stimulating hormone (FSH)/sex hormone, TSHβ mutations present as isolated central hypothyroidism. Attempts to stimulate pituitary secretion by thyrotropin-releasing hormone results in prolactin release but fails to result in TSH stimulation in patients with TSHβ mutations.

The first reported cases of TSHβ mutations were found in three (possibly related) Japanese families homozygous for a G to A substitution encoded in codon 29 of the *TSHβ* gene; the substitution results in a conversion of glycine to an arginine (G29R). The second abnormal TSHβ was found in two Greek families in which a G to T substitution encoded in codon 12 results in a premature stop codon and leads to the deletion of amino acid residues 12–118. A third variant described in four families is a 1-bp deletion in codon 105 that produces a frameshift. This causes Cys-105 to be converted to valine and the addition of eight nonsense amino acids. The fourth variant was described in a consanguineous Turkish family whose members were homozygous for a C to T transition at nucleotide 654 of the region encoding the TSH β-subunit, resulting in Gln-49 becoming a stop codon and premature termination of the protein. This removes the region necessary for α-subunit heterodimerization and no TSH is secreted by the thyrotrophs (Fig. 2).

## IV. TSH RECEPTOR MUTATIONS

The TSH receptor (TSHR) belongs to the superfamily of G-protein-coupled receptors, which are characterized by serpentine-like looping through the membrane, with seven-transmembrane segments. The molecule can be divided into three domains: extracellular, intramembranous, and intracellular. The extracellular domain consists of the 398-residue amino-terminal segment and three extracellular loops; the membranous domain consists of seven α-helices; and the intracellular domain consists of the carboxy-terminal amino acids of the molecule, with three intracellular loops. The specificity of the receptor for the ligand is encoded in the amino-terminal segment, which is responsible for ligand recognition. The carboxy-terminal segment contains the sites that interact with the cascade of G-proteins, leading to the generation of cyclic adenosine monophosphate (cAMP). The cAMP pathway, when stimulated, results in thyroid cell growth and TH secretion. The intramembranous domain is also coupled to the activation of the phospholipase C cascade. The latter requires TSH concentrations 5–10 times higher than are required for the cAMP pathway. The phospholipase C stimulation of diacylglycerol and inositol

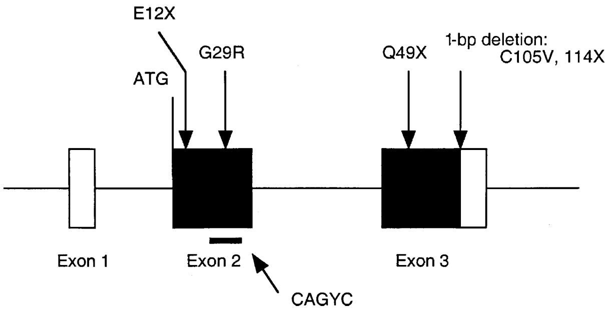

FIGURE 2 Thyroid-stimulating hormone gene mutations; organization of the *TSHβ* gene and reported mutations. The region encoding CAGYC (see text for discussion) is important for noncovalent interaction between the TSH β- and α-subunits.

phospholipase is mainly involved in the control of iodination and TH synthesis.

Abnormalities in the TSHR can result in either activation of the G-proteins in the absence of TSH, known as "gain-of-function" mutations, or reduced or absent activation of the G-proteins, known as "loss-of-function" mutations. Gain-of-function mutations in the TSHR usually result in hyperthyroidism and goiter through constitutive activity of the TSHR. This is due to a mutation activating the TSHR. Excessive amounts of TH released by the thyroid gland cause the symptoms associated with an overactive thyroid, including rapid heart beat (tachycardia), weight loss, jitteriness, fatigue, and goiter. Loss-of-function mutations in the TSHR usually cause hypothyroidism, manifesting as weight gain, tiredness, and dry skin. The hypothyroidism is caused by the resistance of the TSHR, resulting in failure of the thyroid gland to respond to TSH, despite high circulating levels of TSH. Gain-of-function and loss-of-function mutations have also been described for other glycoprotein hormone receptors, including LH, FSH, and parathyroid hormone.

To date, 39 gain-of-function mutations have been reported in the TSHR (Fig. 3). Only one of these mutations occurs in the 398-residue amino terminus; the rest occur in the third cytoplasmic loop or in the adjacent sixth transmembrane segment of the receptor. This observed clustering of mutations underlines the importance of this portion of the molecule. Of the mutations, 23 are found only as somatic mutations, 9 are found only as germ-line mutations, and 8 have been described as somatic in some families and germ-line in other families. The most common setting to observe these gain-of-function mutations is in patients with "toxic adenomas." These are nodules of the thyroid gland that autonomously secrete TH. In some series of studies, up to 80% of toxic adenomas have TSHR mutations, but other populations have a lower incidence, perhaps related to levels of environmental iodine. One TSHR mutation (L677V) was also associated with thyroid carcinoma.

A mutation of the TSHR has been determined to be the cause of familial gestational hyperthyroidism in one family whose members experienced hyperthyroidism only when pregnant. Investigation of this

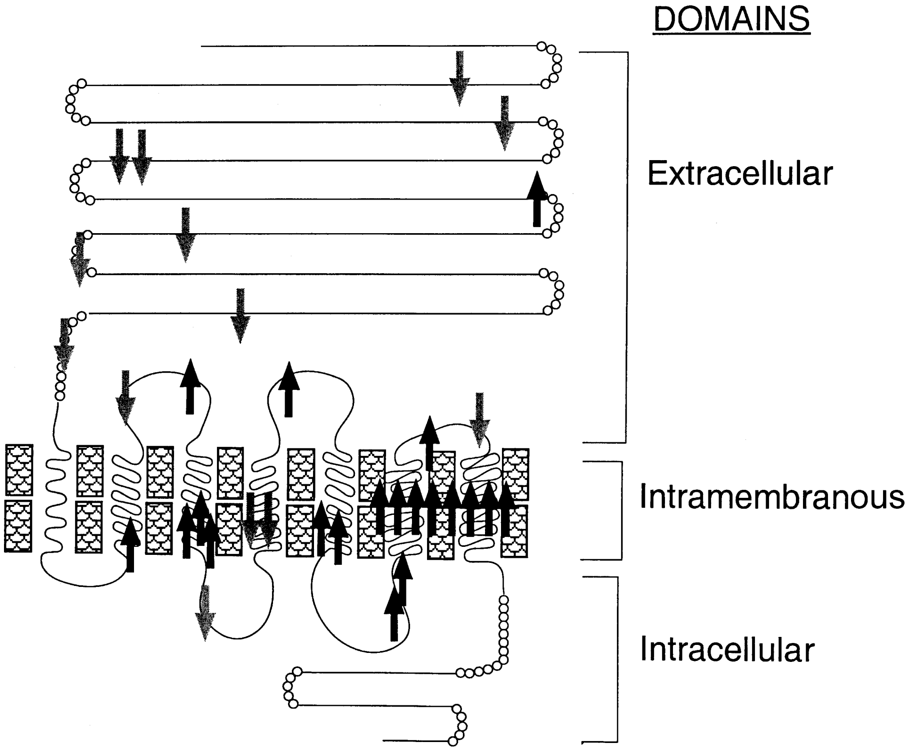

FIGURE 3 Organization of the thyroid-stimulating hormone receptor and reported mutations. Black arrows pointing up indicate gain-of-function mutations; gray arrows pointing down indicate loss-of-function mutations. Note that a majority of the gain-of-function mutations are in the intramembranous domains, whereas the loss-of-function mutations are in the extracellular domains.

family revealed a heterozygous mutation (K183R) located in the extracellular domain of the TSHR, causing chorionic gonadotropin to bind with higher affinity than TSH. However, the receptor also maintained normal responsiveness to TSH. Therefore, when the CG levels increased dramatically during pregnancy, the TSHR was activated, causing hyperthyroidism that resolved spontaneously when the CG levels decreased following delivery. This shows that some mutations can produce a promiscuous TSHR that responds to other hormones.

Cases involving 16 loss-of-function mutations in the TSHR have been described. In contrast to the gain-of-function mutations, a majority of the loss of function mutations are located in the amino-terminal extracellular domain, with the remainder in the extracellular loops of the and fourth intramembranous region. These mutations can result in either partial or complete resistance to TSH (RTSH). In all instances, TSH is elevated but does not cause goiter. In partial RTSH, there is a compensatory increase in TSH, stimulating the thyroid to produce just the needed amount of TH. In complete RTSH, the thyroid gland is completely insensitive to TSH and the patients are severely hypothyroid. Administration of TH to these patients appropriately suppresses their elevated TSH and they become euthyroid. This supports the notion that the defect in RTSH is in responding to TSH and that TH acts normally. Loss of function implies that both alleles are abnormal. Of the 14 families described, 8 were euthyroid and 6 were hypothyroid; RTSH occurred in 6 families homozygous for the TSHR mutations and 8 families were compound heterozygous.

## Acknowledgments

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## Glossary

**co-activators** Molecules that modulate gene transcription, usually through bound receptor; generally have histone acetyl transferase activity, which allows for unraveling of DNA and initiation of transcription.

**corepressors** Proteins that modulate gene transcription in the presence of receptor; generally stabilize the receptor complex on the response element of the target gene, recruiting histone deacetylase. When ligand is present, corepressor binding is unfavorable and the corepressor dissociates from the receptor–ligand–DNA complex, allowing for the recruitment of a co-activator.

**gain-of-function mutations** Gene alterations that render a receptor constitutively active, in the absence of hormone. In the thyroid-stimulating hormone receptor, such mutations usually result in hyperthyroidism.

**germ-line mutations** Gene alterations that are present in germ cells (ova or sperm) and are thus transmitted to the progeny.

**goiter** Enlargement of the thyroid gland; occurs in hypothyroid states, when thyroid cells are under TSH stimulation to produce more hormone, or in hyperthyroid states, when stimulating substances, usually antibodies, bind to the TSH receptor and stimulate thyroid function.

**hyperthyroidism** Condition of thyroid hormone excess; commonly associated with weight loss, tachycardia, excessive sweating, heat intolerance, moist skin, and fatigue; usually due to excessive thyroid gland production of thyroid hormone.

**hypothyroidism** Condition of thyroid hormone deficit; commonly associated with weight gain, slow mentation, decreased energy, cold intolerance, edema, and dry skin; usually due to failure of the thyroid gland to synthesize and release thyroid hormone.

**loss-of-function mutations** Gene alterations that reduce or abolish receptor activity; result in impaired ligand–receptor interaction. Such mutations in thyroid-stimulating hormone receptor produce resistance to TSH, which, when severe, causes hypothyroidism. Such mutations in thyroid hormone receptor produce resistance to thyroid hormone.

**resistance to thyroid hormone** Inherited syndrome of reduced target tissue responsiveness to thyroid hormone. Diagnostic criteria are elevated serum levels of free thyroid hormone, nonsuppressed TSH, and absence of classic signs and symptoms of thyrotoxicosis.

**resistance to thyrotropin** Inherited syndrome of reduced responsiveness of the thyroid gland to TSH. All patients have elevated serum levels of TSH with serum thyroid hormone concentrations that are normal (in compensated forms) or low (in noncompensated forms).

**somatic mutation** Gene alteration that occurs in any cell that is not destined to become a germ cell.

**thyroid hormone receptor** Nuclear receptor for triiodothyronine (T3, the active form of thyroid hormone). The two thyroid hormone receptor genes, α and β, are located on human chromosomes 17 (q21–q22) and 3 (p22–p24.1), respectively. Various splicing arrangements of these genes result in several isoforms of both α and β.

## See Also the Following Articles

**Co-activators and Corepressors for the Nuclear Receptor Superfamily • Parathyroid Hormone • Thyroid Hormone Receptor Isoforms • Thyroid Stimulating Hormone (TSH) • Thyrotropin Receptor Signaling • Thyrotropin-Releasing Hormone (TRH) • Thyrotropin-Releasing Hormone Receptor Signaling**

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# Thyroid Hormone Transport Proteins: Thyroxine-Binding Globulin, Transthyretin, and Albumin

Samuel Refetoff
*The University of Chicago*

- I. INTRODUCTION
- II. STRUCTURAL, PHYSICAL, AND BIOLOGICAL PROPERTIES
- III. GENE STRUCTURE AND TRANSCRIPTIONAL REGULATION
- IV. ACQUIRED DEFECTS
- V. INHERITED DEFECTS

**Thyroid hormones are transported in blood bound to serum proteins. They are in equilibrium with a minute fraction of free hormones immediately available to tissues. Acquired abnormalities and inherited defects of transport proteins can have a profound effect on the serum concentration of the hormones but little or no consequences on their metabolic effects. Recognition of such defects prevents unnecessary and often harmful therapeutic interventions.**

## I. INTRODUCTION

Thyroxine ($T_4$)-binding globulin (TBG) is the principal thyroid hormone transport protein; the other two are transthyretin (TTR) and human serum albumin (HSA). TBG transports 75% of serum $T_4$ and TTR and HAS carry 20 and 5%, respectively. TBG also transports 75% of serum triiodothyronine ($T_3$). The benefit of thyroid hormone associating with proteins is unknown since the absence or inherited loss of binding function of the proteins does not result in a demonstrable disadvantage. However, it has been speculated that thyroid hormone-binding proteins may (1) safeguard the body from the effects of abrupt fluctuations in hormone secretion; (2) protect against iodine wastage by imparting macromolecular properties to the small thyroid hormone molecules and thus reducing their loss in urine; (3) facilitate the uniform cellular distribution of $T_4$; and (4) target the hormone to sites of inflammation through its release through TBG cleavage by neutrophil-derived elastase.

Important changes in TBG, either quantitative or qualitative, result in profound alterations in the total thyroid hormone level in serum without disturbing the free hormone concentration. Thyroid function tests in these circumstances may be misinterpreted as indicative of hyper- or hypothyroidism and could result in unnecessary treatment. Many physiologic, pathologic, and genetic conditions, as well as certain drugs, can alter the concentration of TBG (see below). In addition, drugs can compete with the binding of iodothyronine ligands.

## II. STRUCTURAL, PHYSICAL, AND BIOLOGICAL PROPERTIES

### A. TBG

TBG is a 54 kDa acidic glycoprotein synthesized by the liver (see Table 1). It is composed of a single polypeptide chain of 395 amino acids and four heterosaccharide units with five to nine terminal sialic acids. The carbohydrate chains are not necess-

**TABLE 1 Properties of the Principal Thyroid Hormone-Binding Proteins in Serum**

| | TBG | TTR | HSA |
|---|---|---|---|
| Molecular weight (kDa) | 54[a] | 55 | 66.5 |
| Structure | Monomer | Tetramer | Monomer |
| Carbohydrate content (%) | 20 | | |
| Number of binding sites for $T_4$ and $T_3$ | 1 | 2 | Several |
| Association constant $K_a$ ($M^{-1}$) | | | |
| For $T_4$ | $1 \times 10^{10}$ | $2 \times 10^{8}$[b] | $1.5 \times 10^{6}$[b] |
| For $T_3$ | $1 \times 10^{9}$ | $1 \times 10^{6}$ | $2 \times 10^{5}$ |
| Concentration in serum (mean normal, mg/liter) | 16 | 250 | 40,000 |
| Relative distribution of $T_4$ and $T_3$ in serum (%) | | | |
| $T_4$ | 75 | 20 | 5 |
| $T_3$ | 75 | $<5$ | 20 |
| *In vivo* survival | | | |
| Half-life (days) | 5[c] | 2 | 15 |
| Degradation rate (mg/day) | 15 | 650 | 17,000 |

[a] Apparent molecular weight on acrylamide gel electrophoresis is 60 kDa.
[b] Value given is for the high-affinity binding site only.
[c] Longer half-life under the influence of estrogen.

ary for hormone binding but are required for the correct posttranslational folding and secretion of the molecule. However, secreted TBG that has been properly folded can be deglycosylated without loss of its hormone-binding and immunologic properties.

Serum TBG is stable at room temperature but undergoes rapid denaturation and loses its binding activity at temperatures above 55 °C and pH below 4. The half-life of TBG *in vivo* is approximately 5 days but that of asialo TBG is only 15 min.

TBG has a single iodothyronine-binding site with higher affinity for $T_4$ than $T_3$ (Table 1). In the euthyroid state, approximately one-third of TBG molecules carry thyroid hormone, mainly $T_4$. When fully saturated, TBG carries ~20 mg of $T_4$ per deciliter of serum. Normal serum concentration in adults ranges from 1.1 to 2.1 mg/dl. TBG is detected in the 12-week-old fetus, and in newborns and in children up to the age of 2–3 years, its concentration is 1.5-fold that in adults. Denatured TBG does not bind iodothyronines but can be detected with antibodies that recognize the primary structure of the molecule.

## B. TTR

TTR is a 55 kDa, highly acidic tetramer devoid of carbohydrate. It forms a complex with retinol-binding protein (RBP) and thus plays a role in the transport of vitamin A.

TTR is present in blood as a stable tetramer of identical subunits, each containing 127 amino acids. Together they form a symmetrical structure with a double-barreled hydrophobic channel forming the two iodothyronine-binding sites. Yet, TTR usually binds only one $T_4$ molecule because the binding affinity of the second site is greatly reduced through a negative cooperative effect. The presence of RBP does not interfere with $T_4$ binding and vice versa.

Despite the 20-fold higher concentration of TTR in serum relative to that of TBG, it plays a lesser role in iodothyronine transport. The first $T_4$ molecule binds to TTR with a $K_a$ that is approximately 100-fold higher than that for HSA and approximately 100-fold less than that for TBG. Relative to $T_4$, $T_3$ has lower affinity and tetraiodoacetic acid has higher affinity for TTR. Among the drugs that compete with $T_4$ binding to TTR are ethacrynic acid, salicylates, 2,4-dinitrophenol, and penicillin.

The average concentration of TTR in serum is 25 mg/dl and corresponds to a maximal binding capacity of 300 μg $T_4$. Changes in TTR concentration have relatively little effect on the concentration of serum iodothyronines. Only 0.5% of the circulating TTR is occupied by $T_4$. TTR has a relatively rapid turnover ($t_{1/2} = 2$ days). Hence, acute diminution in the rate of synthesis is accompanied by a rapid decrease of its concentration in serum.

## C. HSA

HSA is a 66.5 kDa protein synthesized by the liver. It comprises 585 amino acids, has a high cysteine content, and has a high proportion of charged amino acids but contains no carbohydrate. The three domains of the molecule can be depicted in a model as three tennis balls packaged in a cylindrical case.

HSA associates with a wide variety of substances including hormones and drugs possessing a hydrophobic region, and thus the association of thyroid hormone with HSA can be viewed as being nonspecific. Fatty acids and chloride ions decrease the binding of iodothyronine to HSA.

The biological $t_{1/2}$ of HSA is longer than that of TBG and TTR. HSA constitutes more than half of the total protein content in serum and it is thus the principal contributor to the maintenance of colloid osmotic pressure. Despite the high iodothyronine-binding capacity, the low affinity is responsible for the minor contribution of HSA to thyroid hormone transport. Thus, even the most marked fluctuations in HSA concentration, including analbuminemia, have no significant effect on thyroid hormone levels.

## III. GENE STRUCTURE AND TRANSCRIPTIONAL REGULATION

### A. TBG

TBG is encoded by a single-copy gene located on the long arm of the human X chromosome (Xq22.2) (see Fig. 1). The gene consists of five exons and has two polyadenylation sites giving rise to mRNAs of different sizes. The gene organization and amino acid sequences are similar to those of other members of the serine protease inhibitor family, which includes cortisol-binding globulin, $\alpha_1$-anti-trypsin, and $\alpha_1$-anti-chymotrypsin. An upstream sequence of 218 bp contains liver-specific enhancer elements and is required for the transcription of the TBG gene. The promoter has no estrogen receptor-binding sites and estradiol does not appear to have a direct effect on TBG gene transcription.

### B. TTR

TTR is encoded by a single-copy gene located on chromosome 18 (18q11.2–q12.1). The gene consists of four exons spanning 6.8 kb. Knowledge about the transcriptional regulation of the human TTR gene comes from studies of the mouse gene structural and sequence homology, which extends to the promoter region. In both species, a TATAA-box and binding sites for hepatocyte nuclear factor-1 (HNF-1), HNF-3, and HNF-4 are located within 150 bp of the transcription start site.

Although TTR in serum originates from the liver, TTR mRNA is also found in kidney cells, the choroid plexus, and pancreatic islet cells. TTR constitutes up

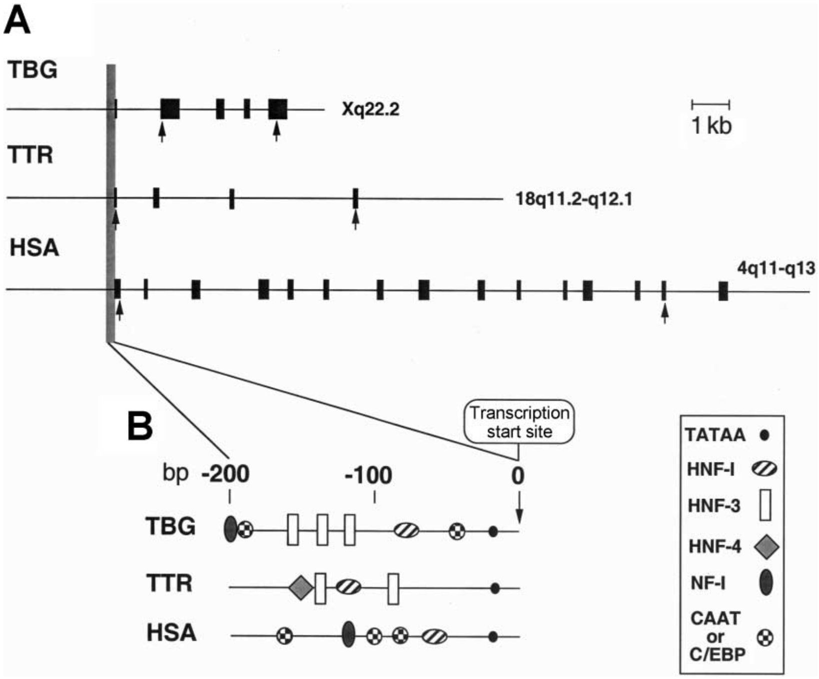

**FIGURE 1** Genomic organization and chromosomal localization of thyroid hormone serum-binding proteins. (A) Filled boxes represent exons. Locations of initiation codons and termination codons are indicated by arrows. (B) Structure of promoter regions with the location of *cis*-acting transcriptional regulatory elements.

to 25% of the total protein present in ventricular cerebrospinal fluid.

### C. HSA

HSA is encoded by a single-copy gene located on human chromosome 4 (4q11–q13). The gene contains 15 exons. The promoter region contains binding sites for hepatocyte-enriched nuclear proteins, such as HNF-1, CCAAT/enhancer-binding protein, and D-site binding protein, a member of the C/EBP family.

## IV. ACQUIRED DEFECTS

### A. TBG

Altered synthesis, degradation, or both are responsible for the majority of acquired TBG abnormalities. Severe terminal illness is undoubtedly the most common cause of an acquired decrease in TBG concentration. Interleukin-6 is a likely candidate for mediation of this effect. Altered rates of degradation are responsible for the decrease and increase of serum TBG concentration in thyrotoxicosis and hypothyroidism, respectively.

Partially desialylated TBG may be present in relatively higher proportion than intact TBG in the serum of some patients with severe liver disease and in a variety of nonthyroidal illnesses. Patients with carbohydrate-deficient glycoprotein syndrome show a characteristic cathodal shift in the relative proportion of TBG isoforms compatible with diminished sialic acid content.

Estrogen excess from an endogenous (hydatidiform mole, estrogen-producing tumors, etc.) or exogenous source is the most common cause of increased serum TBG concentrations. The levels of several other serum proteins such as corticosteroid-binding globulin and sex hormone-binding globulin are also increased. This effect of estrogen is mediated through an increase in the complexity of the oligosaccharide residues in TBG, prolonging its biological half-life.

### B. TTR

The reduction of serum TTR concentration surpasses that of TBG in major illness and protein-calorie malnutrition. This is caused by either a decrease in the rate of synthesis or an increase in the rate of degradation. Increased serum TTR concentration can occur in some patients with islet cell carcinoma.

## V. INHERITED DEFECTS

### A. TBG

With a single exception, the inheritance of TBG abnormalities, including TBG deficiency and TBG excess, follows an X-linked pattern. Clinically, patients present with euthyroid hyper- or hypothyroxinemia. TBG deficiency can present as partial deficiency (TBG-PD) or complete deficiency (TBG-CD), depending on the serum TBG level in hemizygotes. In addition, TBG variants can be characterized by their properties that include (1) immunologic identity; (2) isoelectric focusing (IEF) pattern; (3) rate of inactivation at various temperatures or pH; and (4) affinity for ligands. More precise characterization of TBG defects requires sequencing of the TBG gene.

#### 1. *Complete Deficiency*

TBG-CD is defined as an undetectable TBG concentration in hemizygotes (XY males or XO females) who express only the mutant allele. Obligatory carriers (mother or daughters of affected hemizygotes) have on the average a TBG concentration that is half that of normal individuals. On occasion, selective X-chromosome inactivation can cause a female heterozygote to manifest the hemizygous phenotype of TBG-CD. The prevalence of TBG-CD is approximately 1:15,000 newborn males.

Fifteen distinct mutations have been identified in subjects with TBG-CD (Fig. 2). Thirteen have truncated molecules due to an early stop codon. In 2 of these (TBG-K and TBG-Ja), nucleotide substitutions in introns caused abnormal splicing. In 2 other mutations, TBG-CD was caused by a missense mutations. The substitution of the normal Leu-227 with a Pro, in TBG-CD5, prevented secretion of the variant molecule due to aberrant posttranslational processing.

No mutations were found in the coding sequences or in the promoter regions of the TBG gene in two families with X-linked TBG-CD. The cause of TBG deficiency in these families remains unknown.

#### 2. *Partial Deficiency*

With a prevalence of 1:4000 newborns, partial deficiency is the most common inherited TBG defect. Yet, the precise genetic error has been identified in only a few families. The serum level of TBG in hemizygotes is detectable but reduced and levels in heterozygous females often overlap the normal range. This invalidates the assignment of genotype based solely on serum TBG concentration.

Seven variants manifesting as TBG-PD have been identified. Two of these, TBG-S and TBG-A, are

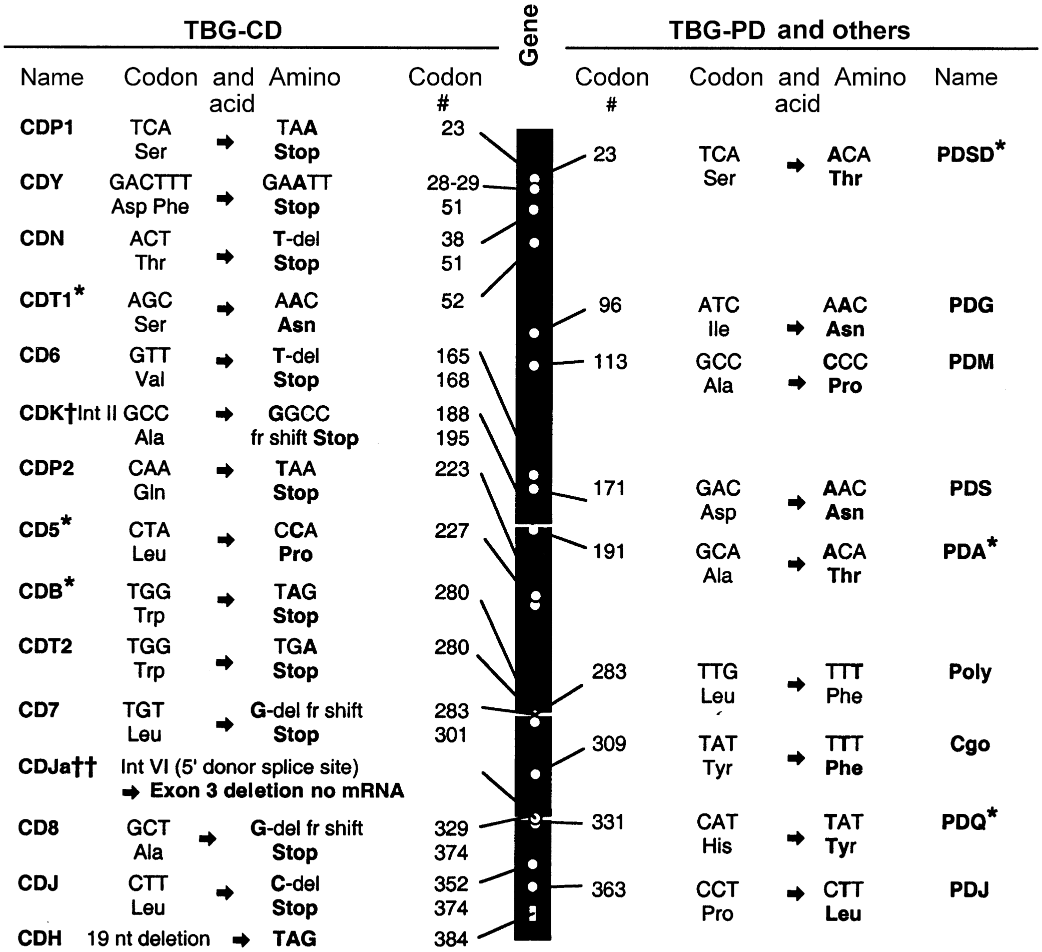
TBG-CD
Gene
TBG-PD and others
Name | Codon and Amino acid | Codon #
Codon # | Codon and Amino acid | Name
CDP1 TCA Ser → TAA Stop 23
CDY GACTTT Asp Phe → GAATT Stop 28-29 51
CDN ACT Thr → T-del Stop 38 51
CDT1* AGC Ser → AAC Asn 52
CD6 GTT Val → T-del Stop 165 168
CDK†Int II GCC Ala → GGCC fr shift Stop 188 195
CDP2 CAA Gln → TAA Stop 223
CD5* CTA Leu → CCA Pro 227
CDB* TGG Trp → TAG Stop 280
CDT2 TGG Trp → TGA Stop 280
CD7 TGT Leu → G-del fr shift Stop 283 301
CDJa†† Int VI (5' donor splice site) → Exon 3 deletion no mRNA
CD8 GCT Ala → G-del fr shift Stop 329 374
CDJ CTT Leu → C-del Stop 352 374
CDH 19 nt deletion → TAG 384
23 TCA Ser → ACA Thr PDSD*
96 ATC Ile → AAC Asn PDG
113 GCC Ala → CCC Pro PDM
171 GAC Asp → AAC Asn PDS
191 GCA Ala → ACA Thr PDA*
283 TTG Leu → TTT Phe Poly
309 TAT Tyr → TTT Phe Cgo
331 CAT His → TAT Tyr PDQ*
363 CCT Pro → CTT Leu PDJ

FIGURE 2 Mutations in the TBG gene and their locations. The TBG variants, in order of location, are as follows: SD, San Diego; CDP1, complete deficiency Portuguese 1; CDY, complete deficiency Yonago; CDN, complete deficiency Negev, formerly Bedouin (SDBe); CDT1, complete deficiency Taiwan 1; G, Gary; M, Montreal, CD6, complete deficiency 6; S, slow; CDK, complete deficiency Kankakee; A, Aborigine; CDP2, complete deficiency Portuguese 2; CD5, complete deficiency 5; CDB, complete deficiency Buffalo; CDT2, complete deficiency Taiwan 2; CD7, complete deficiency 7; Poly, polymorphic; Cgo, Chicago; CDJa, complete deficiency; CD8, complete deficiency 8, Quebec; CDJ, complete deficiency Japan; PDJ, partial deficiency Japan; CDH, Hatwichport. Asterisks indicate the coexistence of TBG-Poly. Dagger indicates a mutation in the acceptor splice site of intron II.

present in high frequency in some populations. TBG-S has a frequency of 5–16% in Black Africans. It has a characteristic cathodal shift on IEF due to loss of negative charge, caused by substitution of the normal Asp-171 with Asn. TBG-A was found in 40% of Australian Aborigines. It has a normal IEF pattern but is more heat-labile than the common type TBG (TBG-C). It was also found to have reduced affinity for $T_4$ (54%) and $T_3$ (30%), reducing the total $T_4$ and $T_3$ concentrations in serum out of proportion to that of TBG.

TBG-SD has a normal IEF pattern but is more - heat-labile and the affinities to $T_4$ and $T_3$ are reduced by ~50%. The mean TBG concentration in hemizygotes can be low or overlap the normal range. TBG variants that have normal serum concentrations but reduced affinity for $T_4$ produce erroneous values for the estimated free thyroxine index ($FT_4I$).

Three of the seven TBG-PD variants are characterized by decreased stability at 37 °C and hence increased levels of denatured TBG in serum that correlated inversely with the native TBG concentration. These are TBG-Q, TBG-M, and TBG-G. TBG-Q and TBG-M were found in French Canadians. Expression of TBG-G in COS-1 cells showed impaired secretion of the variant molecule, leading to excessive intracellular degradation. This is caused by improper folding of the molecule due to the presence of an additional carbohydrate chain at a new N-linked glycosylation site.

TBG-PDJ (Japan) has only a mild reduction in heat stability, suggesting that impaired secretion is responsible for the low TBG level.

Recently, a Japanese family with partial TBG deficiency, transmitted in an autosomal dominant pattern, was described. The sequences of the proband's TBG gene and promoter region were normal. An abnormality in a transcription factor that is important for TBG gene expression is a distinct possibility.

#### *3. TBG Variants Present in Normal Concentrations*

Four TBG variants with minimal or no alterations in serum concentrations have been identified. TBG-Poly with a silent polymorphism was found in 16% of French Canadians and 20% of Japanese. TBG-Cgo is heat- and acid-resistant. The substitution of the normal Tyr-309 with a Phe ties the internal α-helixes to the molecule, stabilizing its tertiary structure.

#### *4. TBG Excess*

Inherited TBG excess is a relatively rare cause of increased TBG. The prevalence of inherited TBG excess is estimated to be 1:25,000. TBG levels in hemizygous affected subjects range from 2.5 to 5 times the normal mean value. TBGs of several unrelated families were shown to have normal properties and no abnormalities were found in the coding sequence or promoter regions of the gene. Gene dosing studies revealed that gene duplication and triplication were the causes of TBG excess in two families. The latter was proven by fluorescence *in situ* hybridization. More recently, a double TBG gene dose was found in two individuals with TBG excess, whose mothers were normal, suggesting *de novo* gene duplication occurring in gametes during meiosis.

### B. TTR

Some of the TTR variants are responsible for the dominantly inherited familial amyloidotic polyneuropathy. TTR variants with reduced affinity for $T_4$ have little effect on the concentration of serum $T_4$. Only variant TTRs with a substantially increased affinity for iodothyronines produce significant elevations in serum $T_4$ and reverse $T_3$ concentrations. They account for 2% of subjects with euthyroid hyperthyroxinemia. The inheritance pattern is autosomal dominant.

A single family with elevated total $T_4$ concentration due to the replacement of the normal Ala-109 with a Thr has been described. The mutation increases its affinity for $T_4$, $rT_3$, tetraiodothyroacetic acid, and to a lesser extent $T_3$ and triiodothyroacetic acid. Crystallographic analysis of this variant TTR revealed an alteration in the size of the $T_4$-binding pocket. Another TTR gene mutation involving the same codon with a Val-109 has an increased affinity for $T_4$ that is of similar magnitude.

A more common defect found in subjects with TTR-associated hyperthyroxinemia is a point mutation in the TTR gene replacing the normal Thr-119 with Met. Despite an increase in the fraction of $T_4$ and $rT_3$ associated with this variant TTR, only a few subjects have serum $T_4$ levels above the upper limit of normal. Several TTR variants that do not alter the properties of the molecule and that are thus of no clinical significance have been found.

### C. HSA

#### *1. Familial Dysalbuminemic Hyperthyroxinemia*

Another form of dominantly inherited euthyroid hyperthyroxinemia, later to be linked to the albumin gene, is known as familial dysalbuminemic hyperthyroxinemia (FDH). It is the most common cause of an inherited $T_4$ increase in the Caucasian population, producing on the average a twofold increase in the serum total $T_4$ concentration. The prevalence varies from 0.01 to 1.8%, depending on ethnic origin, with the highest prevalence occurring among Hispanics. This form of FDH has not been reported in subjects of African or Asian origin. Falsely elevated free $T_4$ values, when estimated by standard clinical laboratory techniques, have often resulted in inappropriate treatments.

FDH is suspected when serum total $T_4$ concentration is increased without a proportional elevation in total $T_3$ level and nonsuppressed serum TSH. Half of affected subjects have also $rT_3$ values above the normal range (Table 2). Since the same combination of test results is found in subjects with the Thr-109 TTR variant, the diagnosis of FDH should be confirmed by the demonstration of an increased proportion of the total serum $T_4$ migrating with HSA. This form of FDH is caused by a missense mutation in codon 218 of the HSA gene replacing the normal Arg with a His. Its association with a $SacI^+$ polymorphism suggests a founder effect and is compatible with ethnic predilection of FDH.

TABLE 2 Serum Iodothyronines in FDH

| Variant | Serum concentration (fold of the normal mean) | | | Binding affinity ($K_a$) of the variant albumins as fold of the normal mean | |
|---|---|---|---|---|---|
| | $T_4$ (μg/dl) | $T_3$ (ng/dl) | $rT_3$ (ng/dl) | $T_4$ | $T_3$ |
| R218H | 16.0<br>(2) | 147<br>(1.2) | 29<br>(1.4) | 10–15 | 4 |
| R218P | 146<br>(18.2) | 253<br>(2.3) | 135<br>(6.1) | 11–13[a] | 1.1[a] |
| L66P | 8.7<br>(1.1) | 320<br>(3.3) | 22.3<br>(1) | 1.5 | 40 |

[a] Determined at saturation. Affinities are higher at the concentration of $T_4$ and $T_3$ found in serum.

Two other mutations of the HSA gene that increased the affinity of the molecule for iodothyronines have been recently identified. One is also located in codon 218 but the mutant amino acid is Pro. This variant HSA has a 90-fold increase in the affinity to $T_4$, resulting in serum total $T_4$ concentrations that are 22-fold the mean normal and the highest observed in any physiological or pathological circumstances. Corresponding increases of $rT_4$ and $T_3$ are on the average 5- and 2-fold the normal mean (Table 2). The other mutation, a replacement of the normal Leu-66 with a Pro, produces a 40-fold increase in the affinity for $T_3$ but only a 0.5-fold increase in the affinity for $T_4$. As a consequence, patients have hypertriiodothyroninemia but not hyperthyroxinemia. It should be noted that serum $T_3$ concentrations are falsely low, or even undetectable, when measured using an analogue of $T_3$ as a tracer rather than a radioisotope.

### 2. *Bisalbuminemia and Analbuminemia*

Variant HSAs, with altered electrophoretic mobility, produce "bisalbuminemia" in heterozygotes. $T_4$ binding has been studied in subjects from unrelated families with a slow HSA variant. In one study, only the slow-moving HSA bound $T_4$, while in another study, both did. The differential binding of $T_4$ to one of the components of bisalbumin may be due to enhanced binding to the variant component with a charged amino acid sequence.

Analbuminemia is extremely rare. $T_4$ transport has been studied in only two subjects with this homozygous condition. The virtual absence of HSA had no clear effect on the concentration of serum iodothyronines as judged by determination of protein-bound iodine, despite an increased binding capacity of TBG and TTR. TBG and TTR become normalized when serum HSA was restored to normal by multiple transfusions.

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## Glossary

**ligand** A functional group, atom, or molecule that is attached to the central atom of a coordination compound, particularly a nonmetallic substance that combines with another substance in solution to form a coordination compound.

**polyadenylation** A process by which a sequence of adenylic acid residues is added to the 3′-end of many eukaryotic mRNA molecules immediately after transcription.

**tetramer** A polymer that is assembled from four identical monomers.

**transcription** The formation of a nucleic acid molecule using another molecule as a template, particularly the synthesis of an RNA using a DNA template.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Iodine: Symporter and Oxidation, Thyroid Hormone Biosynthesis • Thyroglobulin • Thyroid Hormone Receptor Isoforms • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyroid Stimulating Hormone (TSH) • Thyrotropin-Releasing Hormone (TRH)**

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# Thyroid-Stimulating Hormone (TSH)

H. Mircescu, J. C. Goffart, and J. E. Dumont
*Université Libre de Bruxelles*

I. PHYSIOLOGICAL EFFECTS OF THYROID-STIMULATING HORMONE
II. BIOCHEMISTRY OF TSH ACTION: THE THYROTROPIN RECEPTOR
III. BIOCHEMISTRY OF TSH ACTION: TSH RECEPTOR-ACTIVATED CASCADES
IV. THYROTROPIN CONTROL OF THYROID GROWTH
V. THYROTROPIN ACTION IN DISEASE

**The subject of this article is the action of thyroid-stimulating hormone (TSH) on the thyroid gland. The main function of the thyroid gland is the synthesis and secretion of thyroid hormones. These hormones are necessary for body and brain development in the fetus and in the child. Throughout the lifetime of an individual, they set the level of metabolism of the body and its various organs. Thyroid gland function and growth are essentially controlled by two major physiological systems: TSH from the hypophysis and iodide from the diet. The first system functions as a thermostat maintaining the serum levels of thyroid hormones at a constant level by negative feedback regulation. Thyroid hormones negatively control the synthesis and secretion of TSH by specialized cells of the anterior pituitary lobe: the thyrotrophs. TSH stimulates the function and growth of the thyroid. Serum iodide, the main precursor of thyroid hormones, negatively controls the thyroid, ensuring that the gland becomes more efficient when the supply of its substrate in the diet declines. TSH stimulates by various mechanisms all steps of iodine metabolism and thyroid hormone synthesis and secretion by the gland. On a chronic basis, it exerts trophic control of the gland, maintaining or increasing its size, i.e., the number of cells it contains in relation to the long-term level of serum thyroid hormones.**

## I. PHYSIOLOGICAL EFFECTS OF THYROID-STIMULATING HORMONE

Thyroid-stimulating hormone (TSH) exerts two types of effects on the thyroid gland (see Fig. 1). When

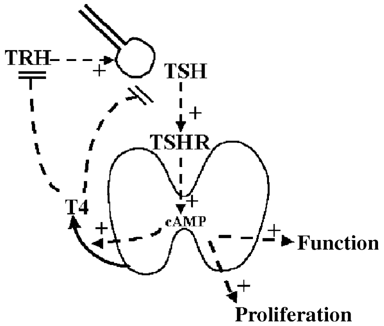

FIGURE 1 Pituitary control of the thyroid through TSH. TSHR, TSH receptor; TRH, TSH-releasing hormone; cAMP, 3′-5′ cyclic AMP.

administered acutely, it stimulates within minutes or hours thyroid hormone synthesis and secretion; in addition, its tonic level controls the growth and proliferation of the thyrocytes (see Fig. 2). The synthesis of thyroid hormones takes place at the apex of the thyrocyte, which faces a closed lumen: the follicular lumen. Synthesis requires (1) at the base of the cell, the trapping of iodide from the extracellular fluid by the $Na^+/I^-$ symporter (NIS); (2) at the apex of the cell, the export of iodide from the cell to the lumen through specialized channels; (3) the synthesis of a very large protein, thyroglobulin (MW 680,000), which will serve as a matrix for the synthesis of thyroid hormones; (4) the export by exocytosis of thyroglobulin in the lumen; (5) the generation of $H_2O_2$ by a specialized enzyme, THOX (thyroid $H_2O_2$-generating system); (6) the oxidation of iodide and its covalent linkage to the tyrosines of thyroglobulin by thyroperoxidase using $H_2O_2$ as oxidizing cofactor; (7) the coupling in iodothyronines [thyroxine (T4), triiodothyronine (T3)] of iodotyrosyls of thyroglobulin by thyroperoxidase. Iodide channels, THOX, and thyroperoxidase are all located in the apical membrane of the cell.

Secretion also takes place at the apex of the cell. It involves the endocytosis of lumenal thyroglobulin, its hydrolysis in secondary lysosomes, and the secretion by an unknown mechanism of the iodothyronines released from thyroglobulin. Endocytosis may involve the bulk endocytosis of thyroglobulin by macropinocytosis or micropinocytosis with selective binding of thyroglobulin by protein acceptors, such as megalin and asialoglycoprotein, and its subsequent uptake (Fig. 3).

TSH stimulates within minutes several steps of synthesis and secretion: iodide efflux in the follicular lumen, $H_2O_2$ generation and thyroglobulin iodination, iodotyrosine coupling, and the uptake of thyroglobulin by macropinocytosis and therefore secretion. Iodide transport at the base of the cell is regulated by the level of the transporter NIS: its synthesis in significant amounts in response to TSH requires an effect at the level of gene transcription and therefore requires several hours.

Other effects on transcription supplement the acute effects of TSH: thus, TSH enhances the synthesis of thyroperoxidase and, in some species, of THOX and thyroglobulin. The latter effects represent the functional counterpart of the differentiating effects of TSH in thyroid cell cultures. In such cultures, stimulation of the cells by growth factors such as EGF (epidermal growth factor), hepatocyte growth factor, or phorbol esters induces proliferation and the loss of thyroid-specific gene expression, i.e., of differentiation. TSH re-induces such expression; it has a differentiating action. Such effects, involving changes in the pattern of transcription, require several hours.

*In vivo* or *in vitro*, if administered for at least 24 h and at a high enough level, TSH also induces thyroid cell proliferation and consequently thyroid growth. Due to the negative feedback regulation of thyroid hormones on TSH secretion, any treatment decreasing thyroid hormone secretion by inhibiting its synthesis, such as anti-thyroid drugs or iodine deficiency, will thus lead to thyroid enlargement, i.e., goiter.

Although it promotes the growth and differentiation of thyrocytes, TSH is not necessary for these developments in the fetus. Decapitated fetuses or fetuses lacking TSH develop a normal thyroid, although the growth of this thyroid will be slowed in the second part of pregnancy. By far, the most important effect of TSH in the fetus is the induction of NIS and therefore of iodide transport.

## II. BIOCHEMISTRY OF TSH ACTION: THE THYROTROPIN RECEPTOR

Thyrotropin (7000 MW) is a heterodimer of an α-subunit, common to follicle-stimulating hormone,

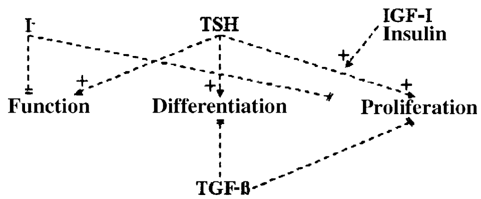

FIGURE 2 Effects of extracellular signals on human thyroid follicular cells.

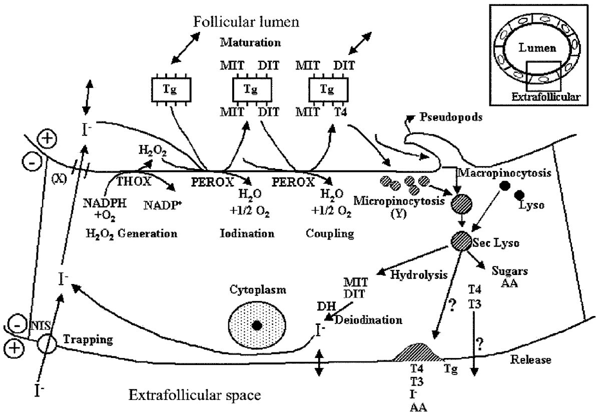

**FIGURE 3** Cellular physiology of the thyroid follicular cell. AA, amino acids; DH, iodotyrosine dehalogenase; DIT, diiodotyrosine; MIT, monoiodotyrosine; NIS, $Na^+/I^-$ symporter; PEROX, thyroid peroxidase; Tg, thyroglobulin; T4, thyroxine; THOX, thyroid $H_2O_2$-generating system; X, iodide apical channel; Y, acceptor involved in thyroglobulin uptake.

luteinizing hormone, and TSH, and a β-subunit specific to TSH. This glycoprotein is synthesized and secreted by the pituitary thyrotrophs. It is specifically recognized by a seven-transmembrane receptor expressed only in thyrocytes and, in some species, in some preadipocytes and adipocytes. The TSH receptor links the thyroid with its controlling system; i.e., it is its connection with the physiology of the cell. The TSH receptor is a classical seven-transmembrane receptor. It is a linear sequence of 764 amino acids from the exterior N-terminal to the interior C-terminal. Its external part contains 419 amino acids. Its doughnut-shaped structure recognizes and accommodates TSH. The membrane part of the receptor contains seven transmembrane α-helical segments, three extracellular loops, and three intracellular loops. The intracellular loops, especially the third loop, but also the second loop and the intercellular C-terminal, presumably interact with their GTP-binding protein effectors. The binding of TSH to the extracellular N-terminal induced a conformational change of the intramembrane part—a shift in the position of the α-helices—thus opening the intracellular loops for the effector GTP-binding proteins. The extracellular part of the receptor acts as an inhibitory domain, switching when bound to TSH from a negative to a positive regulator on GTP-binding proteins $G_s$, $G_q$, and $G_i$ (Fig. 4).

The TSH receptor activates at least three effectors: the GTP-binding proteins $G_s$, $G_q$, and $G_i$. $G_s$ itself activates adenylate cyclases, which generate cyclic AMP from ATP. $G_q$ activates phospholipase C β, which generates from phosphatidylinositol 3,5-phosphate the intracellular signals inositol 1,5-phosphate and diacylglycerol. $G_i$ inhibits adenylate cyclases and through its βγ-subunits may affect other signal transduction proteins. Although the TSH receptor is able to activate both $G_s$ and $G_q$, in some species such as dog it effectively activates only $G_s$. In human thyroid cells, it stimulates $G_s$ and $G_q$ and therefore stimulates two regulatory cascades: the cyclic AMP and the phospholipase C pathways.

## III. BIOCHEMISTRY OF TSH ACTION: TSH RECEPTOR-ACTIVATED CASCADES

Like other hormones, the TSH receptor exerts its effects through two biochemical cascades: the cyclic AMP pathway and the phospholipase C inositol–1,4,5-phosphate and diacylglycerol pathway.

The human thyroid cell generates cyclic AMP through adenylate cyclases III, VI, and IX. cAMP then binds to and activates at least two effector proteins: the two isoforms of protein kinase A (cyclic AMP-dependent protein kinase), which modulate the activity of target proteins through phosphorylation, and EPAC protein, a guanyl nucleotide exchanger protein that activates the small G-proteins Rap1 and Rap2, which modulate other proteins themselves. Until recently, only protein kinase A was considered, although only a few substrates have been identified in thyroid. One of these, CREB ($Ca^{2+}$/cAMP-response element-binding protein), is a transcription factor that is activated by phosphorylation, which might account for some transcriptional effects of cAMP. The role of the Rap activator is still unknown. Although most effects of TSH and its mediator of cAMP are currently ascribed to the action of PKA, it is surprising that, in fact, as the availability of more or less specific PKA inhibitors has followed the study of cAMP effects, such a role of PKA has only rarely been investigated.

In the human thyroid, TSH activates phospholipase C β and thus releases two intracellular signal molecules: diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$). This effect requires higher concentrations of TSH than the stimulation of adenylate cyclase. The role of diacylglycerol is to stimulate protein kinase C (PKC). This role can be inferred from the effects of phorbol esters, which behave as nonhydrolyzable analogues of diacylglycerol. Thus, the activation of PKC leads to the stimulation of $H_2O_2$ generation, protein iodination, and thyroid hormone synthesis. $IP_3$, on the other hand, presumably, as in other cells activates the release of $Ca^{2+}$ stored in the endoplasmic

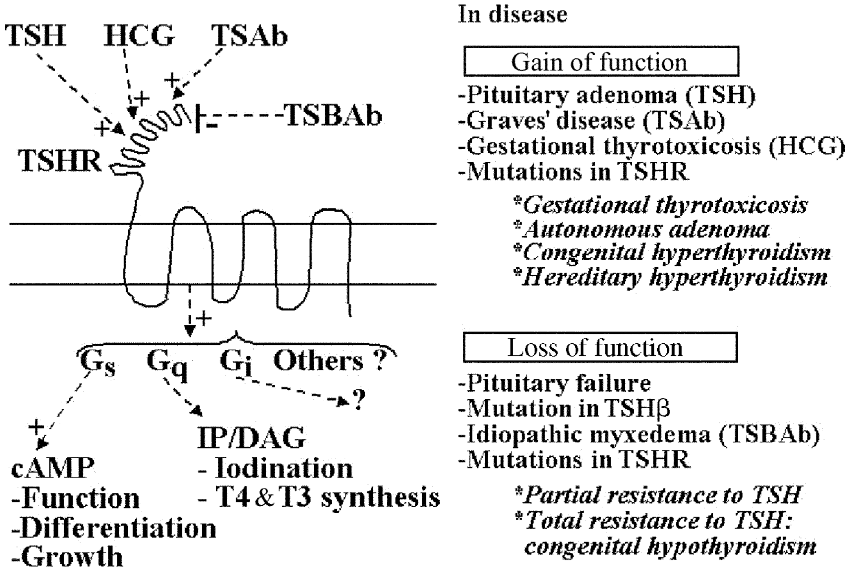
TSH
HCG
TSAb
+
+
+
TSBAb
TSHR
+
Gs
Gq
Gi
Others ?
?
+
IP/DAG
cAMP
- Iodination
-Function
- T4 & T3 synthesis
-Differentiation
-Growth
In disease
Gain of function
-Pituitary adenoma (TSH)
-Graves' disease (TSAb)
-Gestational thyrotoxicosis (HCG)
-Mutations in TSHR
*Gestational thyrotoxicosis
*Autonomous adenoma
*Congenital hyperthyroidism
*Hereditary hyperthyroidism
Loss of function
-Pituitary failure
-Mutation in TSHβ
-Idiopathic myxedema (TSBAb)
-Mutations in TSHR
*Partial resistance to TSH
*Total resistance to TSH: congenital hypothyroidism

FIGURE 4 The TSH receptor. HCG, human chorionic gonadotropin; TSAb, thyroid-stimulating antibodies; TSBAb, thyroid stimulation-blocking antibodies; $G_s$, $G_q$, $G_i$, GTP-binding proteins mediating the activation ($G_s$) or inhibition ($G_i$) of adenylate cyclase and the activation of phospholipase ($G_q$).

reticulum, leading to an increased influx of extracellular calcium in the cell. Such effects of $IP_3$ would account for the known effects of TSH on thyrocyte calcium metabolism. Free intracellular calcium in its turn, presumably mainly through calmodulin, activates a variety of enzymes in cascade. The main physiological effect of this cascade is the activation of $H_2O_2$ generation and consequently of protein iodination and iodothyronine synthesis. Thus, through both arms of the phospholipase C cascade, TSH stimulates these processes; cyclic AMP, in contrast, inhibits them. These effects take place within minutes.

TSH stimulates to various extents the expression of the genes corresponding to specific thyroid proteins. These effects are mediated mostly by cyclic AMP and presumably by protein kinase A phosphorylation of various transcription factors of the CREB, cAMP response element modulator (CREM), and activator protein-1 families. Thyroid-specific transcription factors (Pax8, paired box gene 8; TTF1 and TTF2, thyroid transcription factors 1 and 2) are necessary for these syntheses but their function is not regulated directly by TSH or cyclic AMP. The induction of thyroglobulin and NIS mRNA expression requires an intermediary protein synthesis, but the induction of thyroperoxidase does not. Although the expression of NIS and TPO is greatly enhanced by TSH, that of thyroglobulin is not, but it is decreased in the absence of normal TSH levels. Of course, in time, *in vivo*, the synthesis of most proteins is increased, reflecting the growth of the cell. General protein synthesis is increased in thyroid cells by insulin and insulin-like growth factor-I (IGF-I) acting mostly through the IGF-I receptor. The synthesis of most thyroid-specific proteins is decreased by growth factors such as EGF, reflecting the dedifferentiating action of these factors.

## IV. THYROTROPIN CONTROL OF THYROID GROWTH

Thyrotropin exerts a positive control on thyroid growth *in vivo*. In its absence, either because of a pituitary disease or because of a treatment with thyroid hormone, the gland becomes hypoplastic, with a decrease in the number and the size of the thyrocytes. Conversely, excess thyrotropin causes the hypertrophy of the thyroid, i.e., goiter. Inactivating or activating mutations of the TSH receptor similarly lead to thyroid atrophy or goiter. Antibodies blocking or activating the receptor have similar effects. The mechanism of the growth effect of thyrotropin has been studied *in vitro*. It corresponds to a DNA-synthesizing and mitogenic activity. It is mediated by cyclic AMP and can be mimicked by agents increasing intracellular cyclic AMP levels such as forskolin. It requires the activation of protein kinase A but this activation is not sufficient to trigger it. The role of EPAC and Rap in this action is under study. The effect of thyrotropin requires the action of IGF-I on its receptor. The respective roles of TSH and IGF-I can be summarized as follows: IGF-I provides the necessary cyclins for the cyclin–cyclin-dependent kinase complexes, which relieve the inhibition by retinoblastoma (RB) proteins of DNA synthesis; TSH, through cyclic AMP, activates these complexes.

Although thyrotropin growth-promoting action is the main controlling factor of thyroid size after birth, it is not required for thyroid differentiation and development in early fetal life. The thyroid differentiates, migrates to its normal location, and develops, albeit to a lesser extent, in the absence of the hypophysis or when the TSH receptor is inactivated.

## V. THYROTROPIN ACTION IN DISEASE

Different diseases cause abnormalities in thyrotropin action. Lack of thyrotropin action through decreased thyrotropin synthesis or secretion occurs in various pituitary diseases through congenital hypopituitarism, a defect in TSH synthesis, or pituitary destruction by an adenoma. Inactivating defects of the TSH receptor have similar effects. In all of these cases, the thyroid becomes inactive, thus causing a decrease in thyroid hormone secretion and hypothyroidism and causing atrophy of the thyroid.

Conversely, hyperstimulation of the TSH by excessive pituitary secretion, by stimulating antibodies, or by congenital activating mutations of the TSH receptor causes hyperthyroidism and goiter.

Somatic activating mutations of the TSH receptor generate a hyperactive adenoma that, because of its excessive thyroid hormone secretion, will depress TSH blood levels and induce the quiescence of the unaffected thyroid cells.

Antibodies to the TSH receptor, depending on whether their action is stimulatory or inhibitory, will cause similar diseases: Graves' disease and hyperthyroidism with the thyroid-stimulating antibodies and hypothyroidism with the thyroid stimulation-blocking antibodies.

## Glossary

**NIS** $Na^+/I^-$ symporter that actively transports three $Na^+$ ions for two iodide ions.

## See Also the Following Articles

**Environmental Disruptors of Thyroid Hormone Action • Iodine: Symporter and Oxidation, Thyroid Hormone Biosynthesis • Parathyroid Hormone • Thyroid and Reproduction • Thyroid Hormone Action on the Heart and Cardiovascular System • Thyroid Hormone Action on the Skeleton and Growth • Thyroid Hormone Receptor Isoforms • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyrotropin-Releasing Hormone (TRH) • Thyroid Hormone Transport Proteins**

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# Thyrotropin Receptor Signaling

Leonard D. Kohn[*] and Minho Shong[†]

[*]*Edison Biotechnology Institute and Ohio University* •
[†]*Chungnam National University, Korea*

I. INTRODUCTION
II. THE TSH RECEPTOR—STARTING POINT OF SIGNAL GENERATION
III. THE SIGNALS THEMSELVES
IV. TSHR SIGNALING AND TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION
V. FEEDBACK CONTROL OF TSHR SIGNALING

**The functional role of the thyroid is to synthesize and secrete the thyroid hormones necessary for the normal metabolic homeostasis of every cell**

**in the human body. This process is under the primary control of the pituitary glycoprotein hormone, thyrotropin (TSH), but requires the coordination of multiple genes and the activities of multiple gene products. This article explains the interaction of TSH with its receptor to govern thyroid gland growth and function.**

## I. INTRODUCTION

In addition to stimulating the synthesis and secretion of thyroid hormones, thyroid-stimulating hormone (TSH) regulates thyroid cell function and growth. TSH acts via a single molecular entity on the surface of the cell, the TSH receptor (TSHR). TSH interacting with this receptor accomplishes its complex array of duties by using a multiplicity of signals that are coordinated with the signals of other receptors, in particular, the insulin/insulin-like growth factor-I (IGF-I) signal system.

The thyroid is composed of a multiplicity of follicles, wherein thyrocytes surround a central lumen. Each follicle is surrounded by a vascular network that imports and exports raw materials or product from the thyrocyte and follicle. The primary function of the thyroid is to synthesize thyroglobulin (TG), the thyroid hormone precursor; the TSHR signal transduction system is devoted to its synthesis, storage, iodination, and degradation.

Humans ingest iodide, the key component of thyroid hormones, in episodic bursts. TSH/TSHR, by its control of the sodium iodide symporter (NIS), regulates the ability of ingested iodide to be scavenged from the bloodstream and concentrated within the thyrocytes surrounding the follicular lumen. TSH/ TSHR is involved in iodide transport and secretion into the follicular lumen; Pendrin is one porter now known to be important for this process. TG, to which the iodide is coupled and on which iodotyrosine residues are converted to thyroid hormones, is synthesized, glycosylated, phosphorylated, and vectorially transported to the follicular lumen. Thyroid peroxidase (TPO), which is necessary to iodinate the TG and couple iodotyrosine residues to form thyroid hormones, is synthesized, inserted into the apical membrane facing the follicular lumen, and coupled to a system to generate the hydrogen peroxide necessary for the TPO-dependent iodination process. The iodinated TG is stored and then transported to the lysosome where it is degraded to form thyroid hormones. The thyroid hormones are finally secreted into the bloodstream both to maintain a steady-state level necessary to achieve normal homeostasis and to respond in stress situations. These processes are not synchronized, since the thyroid follicles are functionally heterogeneous. The TSHR and its signaling process control and coordinate all of these steps by regulating gene expression, by posttranslational activation of genes, and by inducing morphologic changes in the cell important for TG degradation and thyroid hormone release.

The TSHR also controls the growth of the thyrocytes. In this case, it is now clear that TSH/ TSHR signaling is a preconditioning prelude for insulin, IGF-I, and possibly other growth factors to cause cell cycle progression. Moreover, TSH/TSHR signaling exerts a negative control on major histocompatibility (MHC) and intracellular adhesion molecule 1 gene expression to preserve self-tolerance. This is necessary to avoid an autoimmune response when TSHR-induced positive regulatory signals increase the multiplicity of genes and gene products needed for growth and function.

The concept that all of this is accomplished by a single signal transduction system, the adenylate cyclase system, and a single signaling molecule, cyclic AMP (cAMP), has been eliminated. Although some clinicians may still consider this signal the sole cause of Graves' hyperthyroidism and goiter. Graves' disease is caused by autoantibodies to the TSHR that cause its signal generation system to function excessively.

## II. THE TSH RECEPTOR—STARTING POINT OF SIGNAL GENERATION

The TSH receptor is a member of the G-protein-coupled family of receptors with seven transmembrane domains. The receptor has two parts (Fig. 1), a long hydrophilic region that binds TSH followed by a region with seven hydrophobic, membrane-spanning domains, similar in sequence to other G-protein-coupled receptors and its sister receptors, the lutropin/chorionic gonadotropin receptor (LH/CGR) and follicle-stimulating hormone receptor (FSHR). Unlike many receptors in this family that couple to a single G-proteins, i.e., the α- or β-adrenergic receptors, TSHR, LH/CGR, and FSHR couple to more than one G-protein, thereby activating both the phosphatidylinositol 4,5-biphosphate ($PIP_2$) and cAMP cascades (Fig. 1).

The TSHR hydrophilic extracellular domain is longer than that of the LH/CGR or FSHR by approximately 60 residues (Fig. 1). These are located in a long ≥50-residue insert in the region of residues 300–400 of the TSHR and a short insert involving

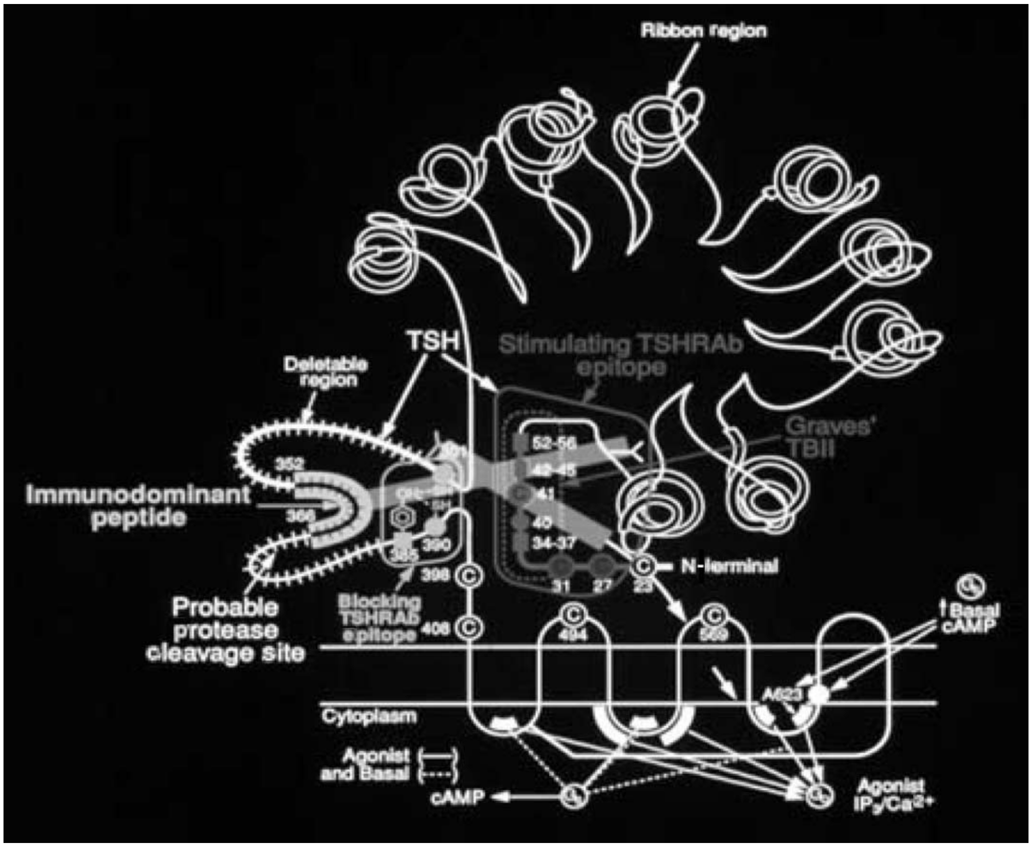
Ribbon region
TSH
Stimulating TSHRAb epitope
Deletable region
Graves' TBII
52-56
42-45
41
40
34-37
31
27
23
352
366
Immunodominant peptide
401
390
385
398
408
N-terminal
494
569
Probable protease cleavage site
Blocking TSHRAb epitope
Basal cAMP
A623
Cytoplasm
Agonist and Basal
cAMP
Agonist IP3/Ca2+

**FIGURE 1** Putative model of TSHR. Determinants for blocking TSHR Abs and stimulating TSHR Abs are approximated to make up the TSH site. The former are implicated in high-affinity TSH binding and in the expression of disease by patients with idiopathic myxedema and hypothyroidism. The latter are implicated in patients with Graves' disease and hyperthyroidism. The loop between residues 303 and 382 is separated from the remainder of the external domain, since residues within it can be deleted with no loss in receptor function. This loop includes residues 352–366, which constitute the immunogenic peptide used to produce a specific antibody to the receptor. The hatched lines denote the regions of the receptor in close approximation based on antibody-peptide inhibition studies and by studies of TSHR relationships with the LH/CGR. The first, second, and third intracytoplasmic loops, particularly Ala-623 and the N- and C-terminal 5 residues of the third loop, are identified as the critical link for hormone and TSHR Ab coupling to $G_q$ and the $PIP_2$ cascade. All loops interact with $G_s$ to regulate constitutive or basal cAMP levels. The middle of the second cytoplasmic loop is coupled to agonist-increased cAMP signaling via $G_s$.

residues 38–47 (Fig. 1). The former region can be deleted with little loss of TSHR signaling; modification of the latter causes a loss in signal generation. TSH binds to multiple parts of this extracellular domain; however, the high-affinity binding site has important residues between residues 280 and 400. Binding to the extracellular domain perturbs the transmembrane domain where the signal is generated and induces a conformational change that converts the receptor to its agonist state.

TSHR residues 8–165 can be replaced by the equivalent N-terminal portion of the LH/CGR with no significant loss of TSHR signal generation; in contrast, stimulating TSHR autoantibodies from patients with Graves' disease largely lose their ability to increase cAMP levels or activate the $PIP_2/Ca^{2+}$/arachidonate signal system. The residues in this region that are important for coupling to the cAMP and $PIP_2/Ca^{2+}$/arachidonate signals appear to be different; critical sites linked to cAMP signaling lie predominantly between residues 90 and 165, whereas several residues important for the $PIP_2/Ca^{2+}$/arachidonate signal lie in the region between residues 30 and 90. The probability is that the two regions on the N-terminus, residues 30–60 and 90–165, act together in a conformational epitope, approximating helices and creating the agonist face of the TSH-binding site (Fig. 1).

Like adrenergic receptors (ARs), mutation of each of the extracellular loops of the transmembrane domain results in a loss of TSH-stimulated and TSHR autoantibody activities as a result of a conformational change rather than abnormal receptor synthesis, processing, or incorporation into the bilayer. The short cytoplasmic peptides of the transmembrane domain of the TSHR differ in length, are relatively nonhomologous among receptor types, contain one or more potential phosphorylation sites, and couple to $G_q$, which signals the $PIP_2$ cascade, as well $G_s$, which initiates the cAMP cascade.

Residues in the first, second, and third cytoplasmic loops are important for TSH and Graves' immunoglobulin G (IgG) induction of the $PIP_2$ signal, but also control constitutive cAMP levels (Fig. 1). Thus, all the first, second, and third cytoplasmic loop mutations affecting agonist-induced signaling also have decreased basal or constitutive cAMP levels and in some cases are hot spots that result in constitutively high basal cAMP signaling, i.e., in a receptor conformation that will bind and activate a G-protein in the absence of ligand. The latter mutations have been associated with functioning adenomas in patients; similar mutations in the LH/CGR are associated with precocious puberty. The middle region of the second cytoplasmic loop, particularly residues 525–527, is involved in agonist-induced $G_s$ coupling, whereas the entire loop is important for agonist-induced $G_q$ coupling.

The amino acid sequence in the second cytoplasmic loop, as an example, is identical in rat, human, and dog TSHR, suggesting that interactions and signals are likely to be applicable to all species of TSHR. The number of residues in the second cytoplasmic loop of TSH and LH/CGR is the same; further, the sequences of LH/CGR or FSHR are 60% identical and most differences are conservative substitutions. This suggests that the second cytoplasmic loop will share common signal transduction/G-protein interactions among the family of receptors. In the second cytoplasmic loop, the number of residues in the TSH, gonadotropin, and adrenergic receptors is the same, but the conformations of TSHR and ARs are very different with respect to helix, extended coil, or β-turn. This may contribute to the ability of the TSHR to couple to both $G_s$ and $G_q$, whereas AR or muscarinic receptors couple to only one type of G-protein.

Since it is unlikely that $G_q$ and $G_s$ couple simultaneously and since TSHR transfections result in an increased basal cAMP level, it is possible that TSHR transmembrane domains are in a partial agonist conformation in the absence of ligand. Alternatively, there are several TSHR forms, precoupled to $G_s$, to $G_q$, or to neither one, and an equilibrium exists among them. Mutations might influence this equilibrium by allowing interactions not usually evident in the absence of ligand; they might also alter the affinity of each for $G_s$ or $G_q$. This might explain curvilinear TSH-binding isotherms, i.e., high- and low-affinity binding sites, with low and high capacities, respectively. This also might explain why different concentrations of TSH are necessary to induce the cAMP and $PIP_2$ signals *in vitro*; i.e., the cAMP signal is linked to a high-affinity $G_s$-coupled TSHR form, whereas the $PIP_2$ signal is linked to a low-affinity $G_q$-coupled form.

The $P_1$ purinergic receptor regulates TSH and Graves' IgG induction of the cAMP and $PIP_2$ signals; $P_1$ purinergic agonists (phenylisopropyladenosine, PIA) inhibit TSHR-induced cAMP production but enhance inositol phosphate formation, despite the fact that they have little direct effect on either signal. The effect of PIA is mediated by a pertussis toxin-sensitive G-protein ($G_i$). The ability of TSH to modulate the cAMP signal system has also been related to pertussis toxin-sensitive ADP ribosylation of a $G_i$ family member.

## III. THE SIGNALS THEMSELVES

### A. Adenylate Cyclase–cAMP Signal

A close correlation exists between TSH binding and stimulation of the adenylate cyclase/cAMP signal. It induces TG, TPO, and NIS gene expression and is important in their posttranslational activation. This dual role is exemplified by NIS, where there is a clear-cut dichotomy between gene induction (RNA levels), protein, and iodide uptake (function). Little is still known about the mechanism by which TSH activates Pax-8, the critical transcription factor that regulates NIS gene expression, or about the mechanism by which TSH activates the NIS protein. Pax-8 is the homeoprotein encoded by the PAX-8 gene, a member of the HOX network of transcription factors. Recent work suggests that cAMP modulation of redox potential is important for the activation of Pax-8 and that cAMP-modulated protein phosphorylation is important for protein activation.

The increase in cAMP activates protein kinase A. More than one form of protein kinase A exists. These differ in their regulatory subunits and in thyrocytes from different species, perhaps contributing to signal differences. Despite overwhelming evidence that kinase A mediates cAMP activity and despite the identification of a multiplicity of phosphorylated proteins resulting from the TSH/TSHR/cAMP signal, a cohesive view remains an enigma.

TSH-increased cAMP has been linked to thyroid cell growth, and cAMP is a necessary but insufficient signal. Thus, in the absence of insulin/IGF-I, TSH increases cAMP perfectly well but does not induce growth. TSH was found to be a preconditioner for insulin/IGF-I regulation of cell cycle progression; the insulin/IGF-I effect is, at the least, related to the action of Akt or PKB, a downstream kinase of the insulin signaling system.

TSH/TSHR and cAMP, together with insulin/ IGF-I, increase 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, thereby increasing the formation of gerranylgerranyl phosphate. This eliminates p27Kip1 by accelerating its degradation; cyclin-dependent kinase 2 is activated, causing the cyclin cascade to proceed, and S6 kinase 1 (S6K1) is activated. Rho proteins necessary for S-phase development are also gerranylated and translocated to membranes during $G_{1/S}$ progression. The effect of gerranylgerranyl pyrophosphate is abolished with botulinum C3 exoenzyme, which specifically ADP ribosylates Rho proteins. The connection of TSHR/cAMP signaling to phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK1), and insulin/IGF-I-coordinated actions to increase S6 kinase activity is increasingly clear (Fig. 2).

TSH/TSHR/cAMP signaling causes morphologic changes in cells. The signals induce pseudopod and microvilli formation on the apical membrane adjacent to the follicular lumen and initiate the fluid pinocytosis process whereby colloid containing TG is engulfed and taken into the cell to lysosomes for degradation. They initiate the secretion process as lysosomes that had moved to the apical membrane to fuse with the pinocytotic vesicles return toward the basal membrane while digesting the TG and converting it to amino acids. Contractile and cytoskeleton proteins are altered during this process. Increased membrane fluidity needed for these processes may derive from TSHR/cAMP signaling inducing HMG CoA reductase to produce cholesterol, not simply gerranylgerranyl phosphate.

## B. $PIP_2/Ca^{2+}$/Arachidonate Signaling

TSH stimulates the hydrolysis of $PIP_2$ by phospholipase C. The best characterized functional effect of this signal is on iodide efflux from the apical membrane and $H_2O_2$ generation important for TPO activity, iodination of TG, and iodotyrosine coupling. The importance of this pathway in thyrocyte growth is recognized clinically. Thus, two populations of TSHR antibody (Ab)-stimulating autoantibodies exist, one increasing cAMP levels and the second increasing $PIP_2/Ca^{2+}$/arachidonate signaling. The two together maximally increase growth and are associated with the largest goiters.

Only approximately 50% of TSH-increased cytosolic $Ca^{2+}$ levels is phospholipase C-dependent; the remaining 50% is linked to phospholipase A2 activation. Phospholipase A2 produces arachidonic acid; cyclooxygenase-, lipoxegenase-, and expoxygenase-derived metabolites of arachidonic acid are important end products regulating cell activity, i.e., prostaglandins and leukotrienes.

Currently, the importance of this pathway focuses on TSH-regulated hydrolysis of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-biophosphate and phosphatidylinositol 3,4,5-triphosphate. The complexity of the effects of TSH action and its connection to insulin/IGF-I regulated Akt (PKB) is illustrated in a report by Rameh and Cantley (1999).

## C. PI3K Signaling

Recently, pituitary glycoprotein hormone receptors, including the TSHR, have been shown to activate PI3K-dependent signaling pathways to regulate the growth of target endocrine glands. Signaling in thyrocytes is TSHR-specific, is mediated by the cAMP signal, and involves the p85 subunit of PI3K that is bound to the TSHR as well as $G_s$ (Fig. 2). PDK1 is a major regulator for transmitting PI3K-dependent signaling pathways to downstream kinases, such as Akt/PKB and S6K1, in response to TSH and insulin/IGF-I in thyroid cells (Fig. 2). TSH preferentially activates S6K1 signaling pathways compared to Akt/PKB in thyroid cells (Fig. 2). However, insulin independently activates two PDK1 downstream signaling pathways, Akt/PKB and S6K1, thus exhibiting synergistic actions with TSH on S6K1 activation (Fig. 2). The inhibition of S6K1 by rapamycin results in inhibition of $G_{1/S}$ cell cycle progression by TSH/insulin *in vitro*. S6K1 is also involved in the regulation of thyroid follicle activity, for example, uptake of colloid, which is regulated by endogenous TSH *in vivo*.

## D. Other Signals

TSH/TSHR-increased ADP ribosylation is not mediated by cAMP and has been related to modulation of $G_s$. Thus, the cholera toxin subunit has an intrinsic ADP-ribosyltransferase activity that

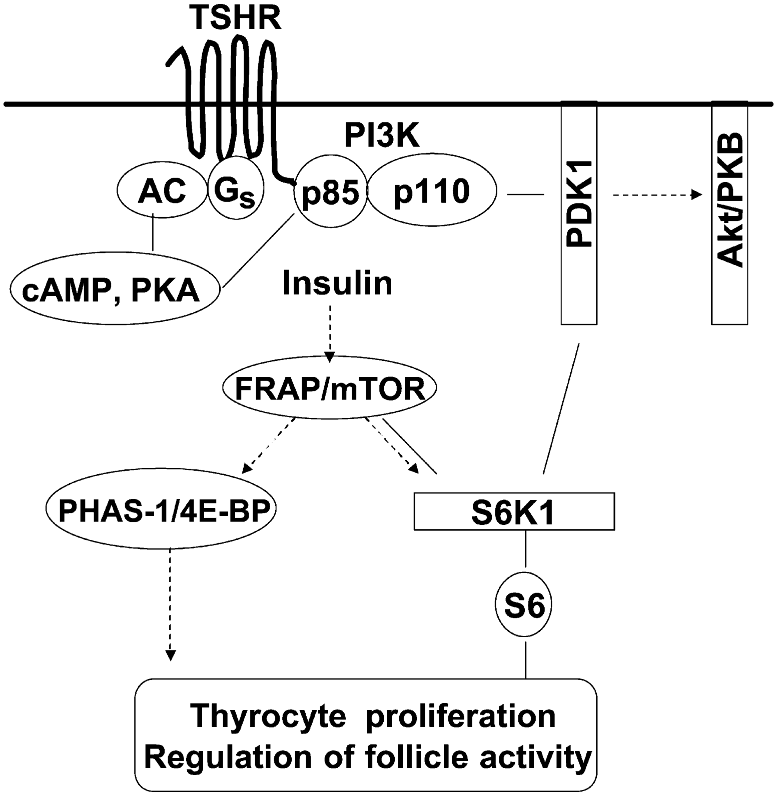

FIGURE 2 TSHR and PI3K signaling: involvement of PDK1 and link to insulin/Akt. TSHR is associated with the p85 regulatory subunit of PI3K and its PI3K activity is induced by TSH and cAMP. TSH is able to translocate PDK1 into the plasma membrane and PDK1 preferentially phosphorylates S6K1, but not Akt/PKB. However, insulin phosphorylates Akt/ PKB and PHAS-1/4E-BP1 in a PI3K- and FRAP/mTOR-dependent manner. Rapamycin inhibits the cooperative actions of PHAS-1/4E-BP1 and S6K1 and results in the inhibition of TSH-mediated follicle proliferation and activity. The solid lines represent the pathways preferentially activated by TSH and the dashed lines represent pathways activated by insulin but not by TSH in thyroid cells. PHAS-1, the small, heat- and acid-stable protein 4E-BPI; FRAP, flouride-resistant acid phosphates; mTOR, mammalian target of rapamycin.

ADP-ribosylates $G_s$, altering ATP/ATP hydrolysis and binding, and shifting $G_s$ to a "permanently activated" state. TSH activates a membrane ADP-ribosyltransferase imputed to act similarly and activates a deribosylating enzyme. Little is known about the significance of this potential signal. TSH/TSHR can regulate the membrane electrical potential and pH gradient via a non-cAMP mechanism. It does not seem to involve ion flux mechanisms exhibited by prototypical seven-transmembrane domain receptors such as rhodopsin.

## E. Signaling Crosstalk

Phorbol esters mimic or potentiate TSHR effects. There is clear feedback regulation between the cAMP and $PIP_2$ signal systems in FRTL-5 cells and transgenic animals. Catecholamines and $\beta_1$-adrenergic agents modulate TSHR signal transduction via cAMP. However, perhaps the least recognized yet most important receptor signal system able to regulate TSHR signaling is the transforming growth factor-β (TGF-β) signal.

TGF-β up-regulates upstream stimulatory factors (USF). As will be noted below, USF transcription factor sites are directly competitive with Pax-8 sites and reverse TSHR/ cAMP signal transduction by competing for Pax-8 binding, most notably on the NIS promoter. However, USF sites exist on TG, TPO, and the TSHR. TGF-β-induced Smad signaling can regulate TTF-1 action. Smad/TTF-1 complexes up-regulate Pendrin gene expression.

Basic fibroblast growth factor (bFGF) decreases TSHR mRNA levels, cAMP signal generation, and cAMP signal action. Despite this, bFGF increases thyrocyte thymidine incorporation and cell growth.

This appears to be explained by the ability of bFGF to activate the $PIP_2$ signal system, the $Ca^{2+}$ signal, and a novel tyrosine kinase activity that is able to activate the insulin/c-*ras* pathway. It mimics the crosstalk between the TSHR-induced $PIP_2$ and cAMP signal systems.

## IV. TSHR SIGNALING AND TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION

### A. TG, TPO, and NIS

A fundamental advance in TSHR signaling is dependent on the work of DiLauro and colleagues, who described the importance of tissue-specific thyroid transcription factors in the function of thyrocytes: thyroid transcription factor-1, (TTF-1), TTF-2, and Pax-8 (Fig. 3). TG synthesis is controlled by TTF-1, TTF-2, and Pax-8. Thus, there are three TTF-1 sites within the minimal promoter, A to C, from 5′ to 3′; the C site also binds Pax-8. TSH/TSHR/cAMP decreases TTF-1 gene expression and binding to the three TTF-1 sites but increases Pax-8 expression, phosphorylation, redox potential, and binding. This allows TG synthesis to persist or increase, despite decreases in TSHR, which is controlled by TTF-1 alone. TTF-2 is a forkhead protein controlled by insulin/IGF-I. This allows TG synthesis in the absence and in the presence of TSH, so TG can accumulate in the follicular lumen to act as an iodide trap.

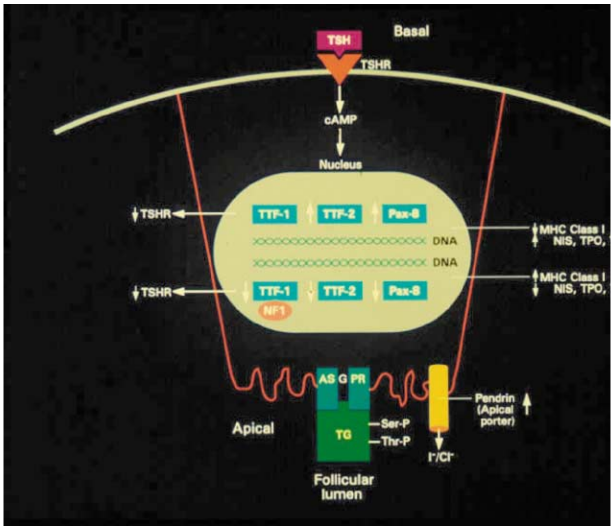

**FIGURE 3** TG counteracts TSH-increased Pax-8 and TTF-2 and is additive in suppressing TTF-1. Both TSH and TG therefore decrease TSHR. TSH increases NIS, TPO, and TG, whereas TG suppresses them. TSH decreases MHC class I, whereas TG increases class I as well as the Pendrin gene product. TG activity is mediated by binding to the ASGPR; TG activity is regulated by serine and threonine phosphorylation.

TPO gene expression is similarly controlled by TSHR/cAMP signaling, TTF-1, TTF-2, and Pax-8. NIS gene expression is predominantly up-regulated by Pax-8, but not TTF-1 or TTF-2. The Pax-8 site contains within it a consensus USF-binding site termed an E-box. USFs are up-regulated and phosphorylated by TGF-β; these compete for Pax-8 binding and down-regulate NIS gene expression, counteracting TSHR/cAMP signals. USF sites also exist in the TPO B site for TTF-1, the TG TTF-1/Pax-8 site, and the downstream but not upstream TSHR TTF-1 site. USF thus plays a role in controlling the TSH/TSHR/cAMP-signaled synthesis of genes important for triiodothyronine (T3) or tetraiodothyronine (T4) formation.

### B. TSHR

Signaling by TSHR/TSH/cAMP autoregulates TSHR gene expression. Thus, these signals decrease TSHR gene expression rather than up-regulate it, like TG, TPO, or NIS, because the TSHR has only a TTF-1 site and TSH/TSHR/cAMP decreases TTF-1 gene expression. Thus, gene expression of TSHR is decreased, whereas gene expression of NIS, TPO, and TG is increased because Pax-8 can replace the TTF-1. This may be an important feedback control for TSHR signaling. It is hypothesized that TSH/TSHR/ cAMP decreased TSHR gene expression is important for cell cycle progression; functional genes are expressed followed by growth signals.

### C. MHC Genes

MHC class I genes are negatively regulated by the TSHR-mediated cAMP signal. Insulin and IGF-I also are negative regulators of MHC class I gene expression in the thyroid. In the thyroid, hormonal suppression of class I genes appears to be a means of preserving self-tolerance in the face of hormone action to increase the expression of tissue-specific genes such as TG, TPO, and the TSHR. TG, TPO, and the TSHR are the autoantigens associated with autoimmune disease. Inappropriate class I expression in the thyroid, i.e., if induced by interferon, viruses, or some as yet unknown agent, is hypothesized to contribute to the generation of autoimmune disease by the loss of negative regulation.

This hypothesis has been supported by studies showing that TSHR, MHC class I, and MHC class II have common *cis* elements that are regulated by the same transcription factors. Moreover, MHC class I knockouts result in a loss in the ability to induce several autoimmune diseases. Finally, the thesis has been supported by the development of a mouse model of Graves' disease, the Shimojo model, wherein Graves' disease is induced by immunizing mice that have normal immune systems with fibroblasts overexpressing MHC class II and TSHR.

The model suggests that Graves' disease is initiated by an insult to the thyrocyte in an individual with a normal immune system. The insult, infectious or otherwise, causes double-stranded DNA or RNA to enter the cytoplasm of the cell. This causes abnormal expression of MHC class I as a dominant feature, but also aberrant expression of MHC class II, as well as changes in genes or gene products needed for the thyrocyte to become an antigen-presenting cell. A critical factor in these changes is the loss of normal TSHR/cAMP-induced negative regulation of MHC class I, MHC class II, and TSHR gene expression, which is necessary to maintain self-tolerance during TSHR-signaled increases in genes involved in growth and function. Self-tolerance of the TSHR is maintained in normals because there is a population of $CD8^+$ cells that normally suppresses a population of $CD4^+$ cells that can interact with the TSHR. This is a host self-defense mechanism that probably leads to autoimmune disease in persons with a specific viral infection or perhaps a genetic predisposition. The model is suggested to be important in explaining the development of other autoimmune diseases including systemic lupus and diabetes.

As noted above, TSHR/cAMP-induced inhibition of constitutive MHC class I gene expression requires the expression of several transcription factors. In addition, TSHR/cAMP inhibits upstream interferon-γ (IFN-γ) signaling pathways, in particular Janus kinase 1 (JAK1). TSH/TSHR-mediated inhibition of JAK1 is caused by TSH-induced suppressor of cytokine signaling-1 (SOCS-1) and SOCS-3. TSH/TSHR-induced SOCSs are negative regulators of IFN-γ-mediated signal transducer and activator of transcription 1 activation in thyroid cells. This TSH/TSHR-induced inhibitory cross talk of IFN-γ may also participate in thyrocyte survival in an autoimmune environment.

## V. FEEDBACK CONTROL OF TSHR SIGNALING

As noted above, the synthesis of TG is the primary function of the thyrocyte, since this is the scaffold upon which thyroid hormones are formed. TG is now recognized not to be simply an inert scaffold for T3/T4 formation; it is an important transcriptional regulator of thyroid function and T3/T4 formation (Fig. 3). TG stored in the follicular lumen is a feedback suppressor, counterregulating the actions of TSH, insulin, and IGF-I to up-regulate TG, TPO, NIS, and TSHR gene expression by specific down-regulation of the thyroid-specific transcription factors, TTF-1, TTF-2, and Pax-8. It simultaneously up-regulates the synthesis of the Pendrin gene product, thus maximizing TG iodination in the follicular lumen even in the suppressed state. TG suppression involves binding to the asialoglycoprotein receptor (ASGPR) on the apical membrane of the thyrocyte, which faces the follicular lumen. TG suppresses TTF-1 by decreasing nuclear factor 1 binding to two sites within 200 bp of the transcriptional start site. The feedback regulation process is an important determinant of follicular heterogeneity and up-regulates the MHC class I gene. This has been related to autoimmune phenomena associated with goiters where poorly iodinated TG accumulates.

*In vivo* evidence supporting these data includes findings that TG binding to the apical surface of thyrocytes facing the follicular lumen suppresses iodide uptake by NIS. TG also suppresses vascular endothelial growth factor (VEGF) gene expression, decreasing vascular permeability and further decreasing iodide uptake.

In a follicle rich in follicular TG, TG would suppress NIS and VEGF/VPF (vascular permeability factor) gene expression, as well as TG and TPO expression and synthesis. In this follicle, TSH would act predominantly to cause resorption and degradation of follicular TG and the secretion of thyroid hormones into the bloodstream. Because TSH-induced resorption/degradation of TG exceeds the rate of TSH-increased synthesis and replacement of TG in the colloid, the TG concentration in the lumen of this follicle will decrease. The decrease in follicular TG releases transcriptional suppression and reinitiates TSH-increased NIS, VEGF/VPF, TG, and TPO gene expression. Pendrin gene expression is maximally induced by a low concentration of TG. The increased vascular permeability, NIS, and Pendrin gene expression will increase iodide uptake; increased TG and TPO will contribute to the synthesis and storage of T3 and T4 most efficiently. When the accumulation of follicular TG reaches a certain level, TG suppression of gene expression would again dominate TSH-stimulated gene expression and the whole process would be repeated.

## Glossary

**Akt (PKB)** Insulin-regulated downstream serine/threonine kinase important in thyroid-stimulating hormone receptor-regulated signaling of growth and function.

**Graves' disease** Disorder caused by overexpression of thyroid-stimulating hormone receptor (TSHR) signaling, which is induced by TSHR autoantibodies.

**major histocompatibility genes** Expression in thyroid is regulated by thyroid-stimulating hormone receptor signaling and is important for preserving self-tolerance, i.e., preventing autoimmunity.

**Pendrin (PDS gene product)** An apical membrane iodide porter controlling iodide export from the thyrocyte into the follicular lumen.

**thyroglobulin** The primary protein synthesized by thyrocytes; it is both the precursor of thyroid hormone formation and a feedback regulator controlling thyroid-stimulating hormone receptor signaling.

**thyroid follicle** Structural component of the thyroid, composed of thyrocytes that surround a central lumen and whose function and growth are controlled by thyroid-stimulating hormone receptor signaling.

**thyroid peroxidase** The enzyme catalyzing the iodination of thyroglobulin and catalyzing the coupling of iodotyrosine residues to form thyroid hormones, whose activity is controlled by thyroid-stimulating hormone receptor signaling.

**thyroid transcription factor-1** Thyroid-restricted transcription factor controlling thyroid-specific gene expression, whose levels and activity are controlled by thyroid-stimulating hormone receptor signaling.

**thyroid transcription factor-2** Thyroid-restricted transcription factor controlling thyroid-specific gene expression, whose levels and activity are controlled by insulin signaling but which influences thyroid-stimulating hormone receptor signaling.

## See Also the Following Articles

**Thyroglobulin • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyrotropin-Releasing Hormone (TRH) • Thyrotropin-Releasing Hormone Receptor Signaling • Thyroid Stimulating Hormone (TSH)**

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# Thyrotropin-Releasing Hormone Receptor Signaling

Marvin C. Gershengorn

*National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health, Maryland*

I. INTRODUCTION
II. THREE-DIMENSIONAL STRUCTURE OF TRH-Rs
III. TRH-Rs AND G-PROTEINS
IV. TRH-R AND SIGNAL TRANSDUCTION PATHWAYS
V. CONSTITUTIVE SIGNALING BY TRH-Rs
VI. TRH DESENSITIZATION
VII. TRH-R INTERNALIZATION
VIII. SUMMARY

**Thyrotropin-releasing hormone signaling occurs primarily, if not exclusively, by binding of the hormone to one of two hormone-specific**

**receptor subtypes, TRH-R1 and TRH-R2, members of the large superfamily of G-protein-coupled receptors. The primary pathway of signal transduction is mediated by activation of $G_q$ or $G_{11}$ proteins; this, in turn, activates a series of further enzymatic reactions, leading to an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ activates $Ca^{2+}$/calmodulin protein kinase and is further augmented by activation of cell surface membrane channels that allow influx of extracellular $Ca^{2+}$. One end result of all of these activities is stimulation of hormone secretion and gene transcription in the anterior pituitary.**

## I. INTRODUCTION

Thyrotropin-releasing hormone (TRH; thyroliberin) is a tripeptide (*pyro*-glutamyl-histidyl-proline-amide; *pyro*-Glu-His-Pro-$NH_2$) that functions as a hormone, a paracrine regulatory factor, and a neurotransmitter/neuromodulator. TRH initiates some, if not all, of these effects by interacting with receptors on cell surfaces. A complementary DNA (cDNA) for a TRH receptor (TRH-R) was initially cloned from mouse pituitary tissue. Using nucleotide sequence information derived from the mouse pituitary receptor, cDNA clones of rat, human, bovine, and chicken TRH-Rs were isolated. There is very high sequence homology in the protein-coding regions of these TRH-Rs. A second subtype of TRH-R was subsequently cloned from rat and then mouse tissue. The two subtypes of TRH-Rs are now referred to as TRH-R1 and TRH-R2. The primary amino acid sequences and the putative two-dimensional topologies of the two mouse TRH-Rs are illustrated in Fig. 1. The two-dimensional topology is based on hydropathy analyses of the receptor proteins and, because TRH-Rs couple primarily to G-proteins (see Section III), on the consensus two-dimensional structure of all G-protein-coupled receptors (GPCRs). The two TRH-Rs are most similar in the transmembrane regions and differ significantly within the extracellular amino terminus and within the intracellular carboxyl terminus (see Section II).

The roles of TRH-Rs in normal physiology are only partly understood. Within the central nervous system, the two TRH-R subtypes exhibit distinct patterns of expression, signaling properties, and regulation, but similar ligand-binding characteristics, thus it is likely that they serve different physiological roles. TRH-R1 is the predominant receptor in the anterior pituitary gland and has a major regulatory role in stimulating thyrotropin (thyroid-stimulating hormone; TSH) synthesis and secretion and, thereby, plays a central role in thyroid hormone homeostasis. A role for TRH-R1 in regulation of prolactin secretion from the anterior pituitary is less clear. In the central nervous system, in which there is widespread but discrete expression of the two TRH-R subtypes, a number of pharmacological experiments have been performed in which effects of inhibiting or stimulating the TRH/TRH-R systems have been observed. Nevertheless, the physiologic roles of TRH-R1 and TRH-R2 in the central nervous system have not been clearly elucidated.

The emphasis here is on the molecular aspects of cellular signaling by TRH-Rs. The following issues are addressed: TRH-R structure, how TRH binds to TRH-Rs, which coupling proteins initiate TRH-R signaling, which transduction pathways mediate signaling, and what cellular mechanisms regulate TRH-R signaling.

## II. THREE-DIMENSIONAL STRUCTURE OF TRH-Rs

To understand the relationship between the three-dimensional structure of a receptor protein at an atomic level of detail and function is a major goal of receptor research. TRH-Rs, like all GPCRs, contain an extracellular amino terminus, three extracellular loops (ECLs), seven transmembrane-spanning helices (TMHs), three intracellular loops (ICLs), and an intracellular carboxyl terminus. A major advance in understanding the structure–function relationships of all GPCRs was made with the resolution of the structure of the inactive state of bovine rhodopsin by X-ray crystallography. Three-dimensional models of mouse TRH-R1 have been constructed based on data from molecular genetic experimental analyses, computer simulations, and the structure of rhodopsin. The model of TRH-R1 in the unliganded (unbound) state attempts to depict the overall structure of the receptor, including the intramolecular atomic interactions that constrain TRH-R in an inactive conformation. The model of TRH-bound TRH-R1 tries to illustrate the interactions between TRH and TRH-R1 that allow for high-affinity binding and, by comparison with the unliganded model, the changes in conformation that the receptor underwent on assuming the active state. These models are used, therefore, to generate hypotheses regarding TRH binding and the mechanism of TRH-R activation.

TRH-R1 is a cylindrical protein that traverses the cell surface membrane seven times, with the amino terminus and three loops outside of the cell membrane, facing the extracellular environment

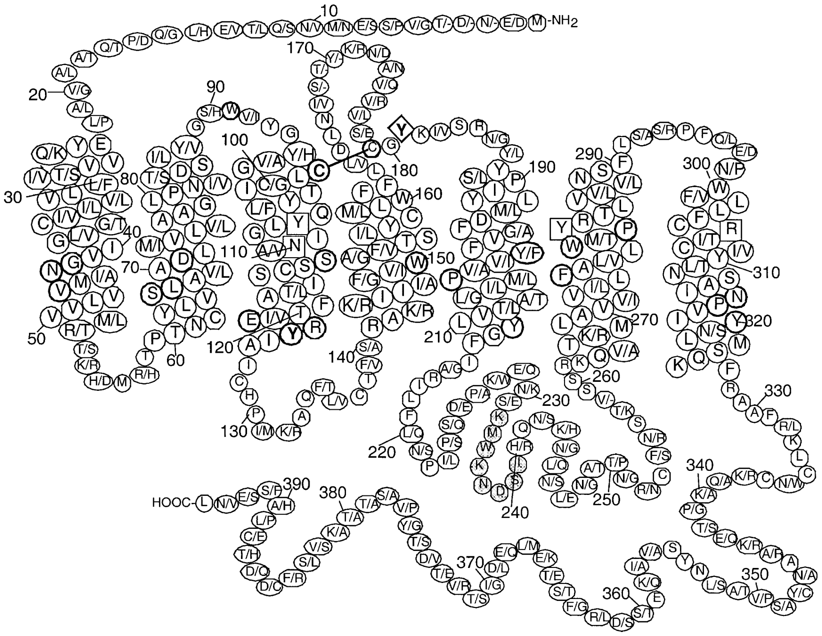

**FIGURE 1** Putative two-dimensional topologies of murine TRH-R1 and TRH-R2 sequences. The amino acids are numbered beginning with the first putative residue at the amino terminus. The amino terminus and three extracellular loops are at the top and the three intracellular loops and the carboxyl terminus are at the bottom. The sequences for both receptors (TRH-R1/TRH-R2) are represented by giving within each circle the specific single-letter amino acid symbols for each position; when the amino acid at any position is the same in both receptors, the symbol is given once, and when TRH-R2 does not have a corresponding residue, a hyphen is used (X/-). Residues that are important for binding TRH (see text) are presented within squares.

[i.e., ECL-1-ECL-3: connecting TMHs 2 and 3 (ECL-1), 4 and 5 (ECL-2), and 6 and 7 (ECL-3)], and three loops within the cell [i.e., ICL-1-ICL-3: connecting TMHs 1 and 2 (ICL-1), 3 and 4 (ICL-2), and 5 and 6 (ICL-3)] (Fig. 2). The "core" of the cylinder, which does not form a channel, has projecting into it hydrophilic and hydrophobic residues that form interhelical interactions, which hold the protein in its native conformation. The side chains of specific residues that are found in the core interact directly with TRH. In contrast, positioned on the surface of the cylinder there are principally hydrophobic residues that allow interaction with the lipid environment of the cell surface membrane.

Like binding pockets for small ligands in other GPCRs, the pocket for TRH is within the upper third of the TMH core of the receptor. A computer simulation of TRH-R predicts that there is water in the core of the unliganded receptor. It is noteworthy that evidence has been presented that TRH binds initially to residues in the ECLs of TRH-Rs and then moves through a "channel" formed by the ECLs to gain access to the binding pocket within the TMHs. (The waters that are present in the "channel" are shown in Fig. 2 as the lighter gray spheres.) A number of critical interactions between TRH and residues within the TMH core of the receptor are responsible for high-affinity binding (Fig. 3). These include the hydrogen bond (H-bond) between the C–O of the *pyro*-Glu moiety of TRH and the OH group of Tyr-106 in TMH-3 of TRH-R1; the H-bond between the NH of *pyro*-Glu and the C–O of Asn-110, which is one helical turn below Tyr-106 in TMH-3; the imidazole of the His residue of TRH, in close proximity to Tyr-282 in TMH-6, forming a stacking or hydrophobic interaction; the C–O group of the Pro-$NH_2$, which forms the H-bond with Arg-306 in TMH-7; and the guanidino group of Arg-306, which also forms an H-bond with the backbone C–O of *pyro*-Glu. All of these interactions are important for TRH binding because mutation of any of these residues within the receptor or change by chemical synthesis of any moiety of TRH leads to decreases in binding affinity. It is noteworthy in this regard that of literally hundreds of analogues of TRH that have been synthesized, only one, *pyro*-Glu-[*methyl*]His-Pro-$NH_2$, exhibits a higher affinity for TRH-Rs than does native TRH.

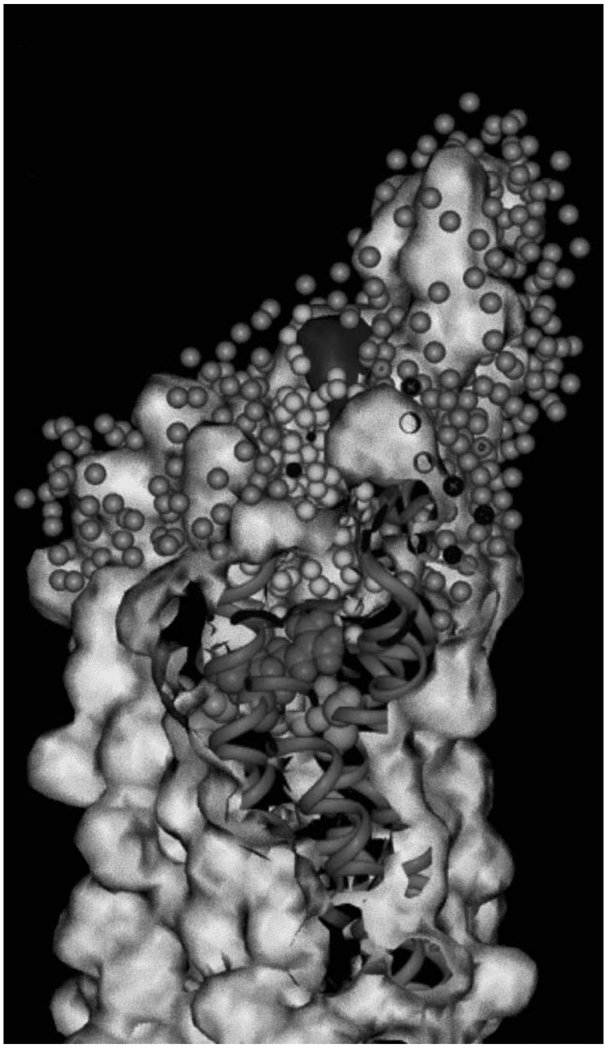

**FIGURE 2** Computer-simulated three-dimensional model of mouse TRH-R1. The model was constructed by computational techniques that include the extracellular loops obtained in primary hydration cell simulations (constructed by Avia Rosenhouse-Dantzker and Roman Osman, Mount Sinai School of Medicine). The model is surrounded by a surface to illustrate the path from the initial anchoring site at Tyr-181 into the transmembrane binding pocket defined by Arg-306, Tyr-282, and Tyr-106. The waters used in the primary hydration shell simulations are displayed as dark spheres. The waters that define the path from Tyr-181 into the transmembrane binding pocket are the lighter spheres.

Although TRH-R1 and TRH-R2 differ at approximately 30% of amino acid residues within the most conserved domains of the TMHs, all of the residues just described that appear to interact directly with TRH are present at the same positions within both receptor subtypes. It is, therefore, not surprising that a series of TRH analogues that differ from one another at all three amino acids of the tripeptide have been shown to bind to both receptors with similar hierarchies of affinities.

The conformational changes within the receptor that constitute activation of TRH-Rs are not known. Because the TRH binding pocket appears to be present in the extracellular third of the TMH bundle, it is assumed that the conformational changes in the TMHs that occur on agonist binding secondarily affect the G-protein-coupling domains on the cytoplasmic surface of the receptor protein. It is probable that the unoccupied receptor is restrained in an inactive state in the absence of TRH and that TRH binding releases these restraints. Interactions between residues in different TMHs have been suggested to fulfill this function because mutation of TMH residues that are predicted to dissociate these interactions leads to receptors that are active in the absence of agonist. For example, in the model of the unliganded state of TRH-R1, there is a hydrophobic stacking interaction between Phe-199 in TMH-5 and Trp-279 in TMH-6 that holds TMH-5 and TMH-6 close to one another. In the model of TRH-bound TRH-R1, this interaction is disrupted and helices 5 and 6 are further apart. Experiments in which either Phe-199 or Trp-279 is mutated to Ala lead to receptors that are constitutively active, and models for this demonstrate positioning of TMH-5 and TMH-6 further apart than in the unoccupied receptor model due to lack of interaction of the residues in positions 199 and 279. Thus, work providing initial insights into the changes that constitute TRH-R activation caused by binding of TRH is underway.

## III. TRH-Rs AND G-PROTEINS

The proposed structure of TRH-R1 is similar to that of all GPCRs and it is therefore likely that TRH-Rs couple to a heterotrimeric, signal-transducing G-protein(s) composed of α-, β-, and γ-subunits. Specific G-proteins that interact with TRH-R have been identified. TRH-Rs couple primarily to $G_q$ and $G_{11}$, which are pertussis-toxin-insensitive G-proteins (see later) that activate phosphoinositide-specific phospholipase C (PPI-PLC). One of the most important observations supporting this conclusion was the demonstration that antibodies directed toward the common carboxyl terminus of these two G-protein α-subunits inhibit TRH stimulation of this enzyme activity. Several lines of evidence have been presented that support the idea that TRH-Rs couple to other G-proteins in addition to $G_q$ and $G_{11}$. Bacterial toxins

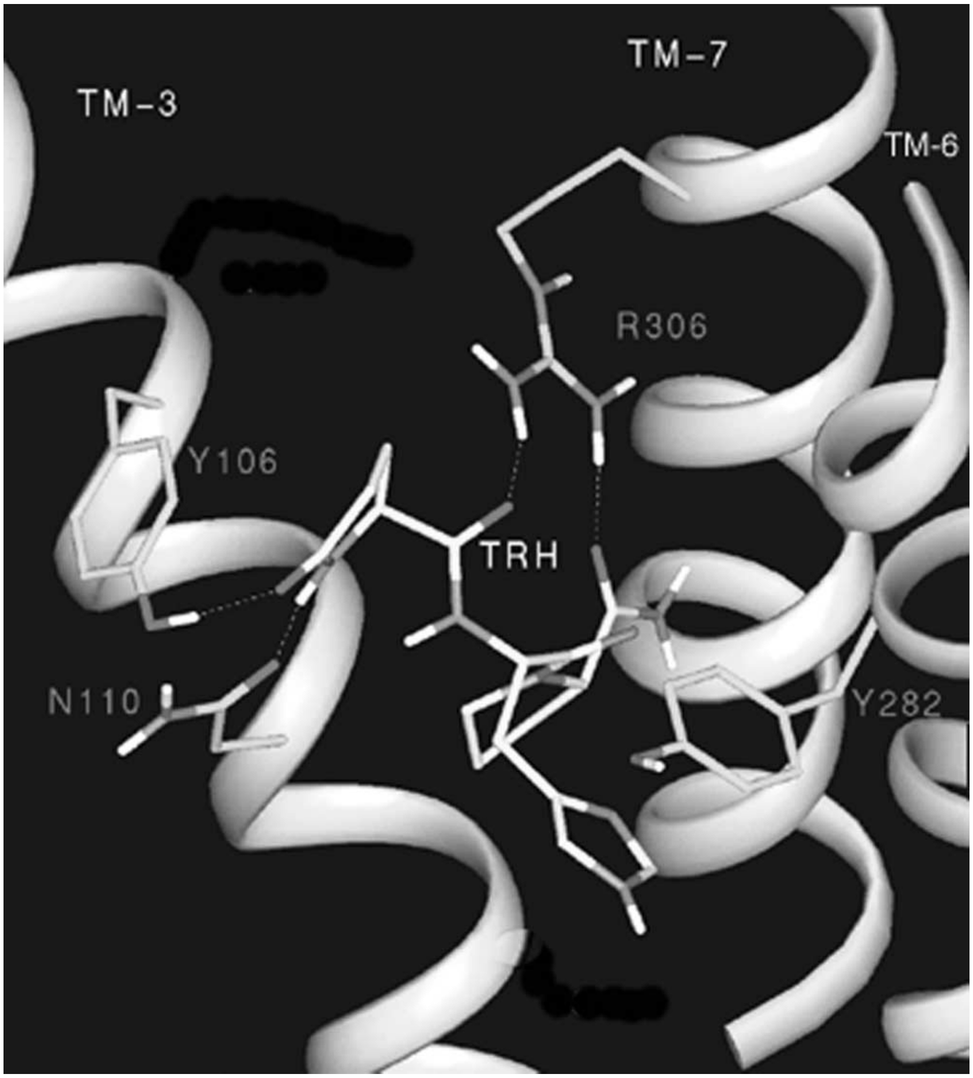

**FIGURE 3** Computer-simulated three-dimensional model of the binding pocket of a mouse thyrotropin-releasing hormone receptor subtype (TRH-R1). A close-up view of TRH (light gray bond representations) in the binding pocket, showing the proposed interactions with Tyr-106 and Asn-110 in transmembrane-spanning helix 3 (TM-3), Tyr-282 in TM-6, and Arg-306 in TM-7 (dark gray). The ribbons represent the backbones of the α-helices, which are the part of the receptor that spans the cell surface membrane.

that covalently modify certain G-proteins (pertussis toxin modifies proteins of the $G_i$ class and cholera toxin modifies proteins of the $G_s$ class), have provided evidence in studies of model systems that $G_{i2}$ and $G_{i3}$ can mediate TRH stimulation of voltage-sensitive calcium channels and that TRH-Rs can couple to $G_o$, which is a pertussis-toxin-sensitive G-protein that is found at high levels in the brain. The most controversial aspect of TRH-R/G-protein coupling involves $G_s$ or a $G_s$-like protein; $G_s$ is known to activate adenylyl cyclase and calcium channels and to inhibit sodium channels in different cell types. Evidence has been presented that both supports and refutes coupling of TRH-Rs to $G_s$ or $G_s$-like proteins. Thus, it appears that under specific conditions in model systems, TRH-Rs couple to $G_q$, $G_{11}$, $G_{i2}$, and $G_{i3}$, and, perhaps, to a $G_s$-like protein that does not activate adenylyl cyclase. Because the principal pathway for TRH-R signaling appears to be through the cascade initiated by phosphoinositide hydrolysis, it appears that coupling of TRH-R to $G_q$ and $G_{11}$ is of primary importance.

The regions of TRH-Rs that are involved in coupling to G-proteins include ICL-3 and the carboxyl-terminal tail. In both ICL-3 and the carboxyl tail, it is the regions just underneath the cell surface membrane that are important for coupling. For example, a large part of the middle portion of ICL-3 and a large part of the distal aspect of the carboxyl tail of TRH-R1 can be deleted without any effect on $G_{q/11}$ activation. Thus, TRH-Rs behave like most GPCRs and couple selectively to a limited number of G-proteins that are members of several G-protein families.

## IV. TRH-R AND SIGNAL TRANSDUCTION PATHWAYS

TRH-Rs predominantly utilize a ubiquitous second-messenger system that is initiated by the hydrolysis of phosphoinositides, which are phospholipids that contain the sugar *myo*-inositol as the polar head group, for signal transduction. When TRH binds to TRH-Rs, $G_q/G_{11}$ proteins are activated; in turn, a PPI-PLC that is activated primarily hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to generate two molecules that serve as second messengers, i.e., inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (1,2-DAG). $InsP_3$, which is water soluble, is released into the cytoplasm and diffuses away from the membrane whereas 1,2-DAG remains membrane bound. $InsP_3$ leads to release of intracellular stores of $Ca^{2+}$ into the cytoplasm and elevates the cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), and 1,2-DAG activates protein kinase C (PKC). These two second messengers then induce signal transduction via two parallel pathways.

A central aspect of $Ca^{2+}$ signaling stimulated by TRH is that increases in $[Ca^{2+}]_i$ are translated into changes in cellular function through a number of specific, $Ca^{2+}$-binding regulatory proteins or protein subunits. On binding $Ca^{2+}$, these proteins undergo conformational changes that regulate their activity. For example, calmodulin is a ubiquitous $Ca^{2+}$-binding protein that, on binding $Ca^{2+}$, binds to and activates several enzymes, including $Ca^{2+}$/ calmodulin-dependent multifunctional protein kinases, which phosphorylate a broad array of proteins and may mediate regulation of gene transcription, protein synthesis, and secretion.

TRH-R stimulation of $PIP_2$ hydrolysis leads to formation of $InsP_3$, which causes an elevation of $[Ca^{2+}]_i$ within seconds. $InsP_3$ rapidly diffuses in the cytoplasm away from the cell surface membrane to bind to receptors on the endoplasmic reticulum. $InsP_3$ receptors are $Ca^{2+}$ channels that "open" on binding $InsP_3$, allowing the flow of previously sequestered $Ca^{2+}$ from the lumen of the endoplasmic reticulum into the cytoplasm and thereby elevating $[Ca^{2+}]_i$. This component of elevation of $[Ca^{2+}]_i$ is rapid and transient. Within seconds of TRH activation of TRH-Rs in excitable cells, such as those in the anterior pituitary and nervous system, $Ca^{2+}$ channels within the cell surface membrane are activated by a mechanism that is incompletely understood. When "opened," these channels allow rapid influx of $Ca^{2+}$ down the electrochemical gradient from the extracellular space to the cytoplasmic compartment, thus also elevating $[Ca^{2+}]_I$. The increase in $[Ca^{2+}]_i$ secondary to stimulated $Ca^{2+}$ influx is more prolonged. TRH-stimulated increases in $[Ca^{2+}]_i$ can occur as a rapid but transient increase followed by a plateau phase, as increases in the frequency of oscillations ("spikes"), or as a combination of a transient increase followed by a plateau with superimposed oscillations (Fig. 4). $Ca^{2+}$ influx across the cell surface membrane is also increased when $Ca^{2+}$ stores in the endoplasmic reticulum are depleted during TRH stimulation. This has been termed "capacitative $Ca^{2+}$ entry" and may be more important in "nonexcitable" cells than in excitable cells. Thus, several different processes that lead to elevations in $[Ca^{2+}]_I$ or replenishment of intracellular $Ca^{2+}$ stores, or both, are initiated rapidly after TRH-R activation.

The other limb of the PPI pathway activated after TRH stimulation of $PIP_2$ hydrolysis is mediated by 1,2-DAG. 1,2-DAG, in combination with phosphatidylserine (PS) and, depending on the isoenzyme subtype, with or without a requirement for an elevation of $[Ca^{2+}]_i$, activates phospholipid-dependent PKC. PKC enzymes are a family of serine and threonine kinase isoenzymes that phosphorylate many proteins involved in regulating downstream cellular responses. In unstimulated cells, the level of 1,2-DAG in membranes is very low, but 1,2-DAG accumulates in response to TRH, causing within seconds a transient translocation of some isoforms of PKC to the plasma membrane. An important proximate effect of activation of PKC is one of negative feedback to inhibit PPI signaling, which may be mediated by phosphorylation of TRH-Rs (see Section VI). The more distal effects of activation of PKC by TRH signaling include stimulation of hormone secretion and gene transcription in the anterior pituitary.

Other protein kinases are also activated after TRH-R activation. The elevation of $[Ca^{2+}]_i$ caused by TRH stimulation leads to activation of multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase II. Evidence has been provided that this kinase has a role

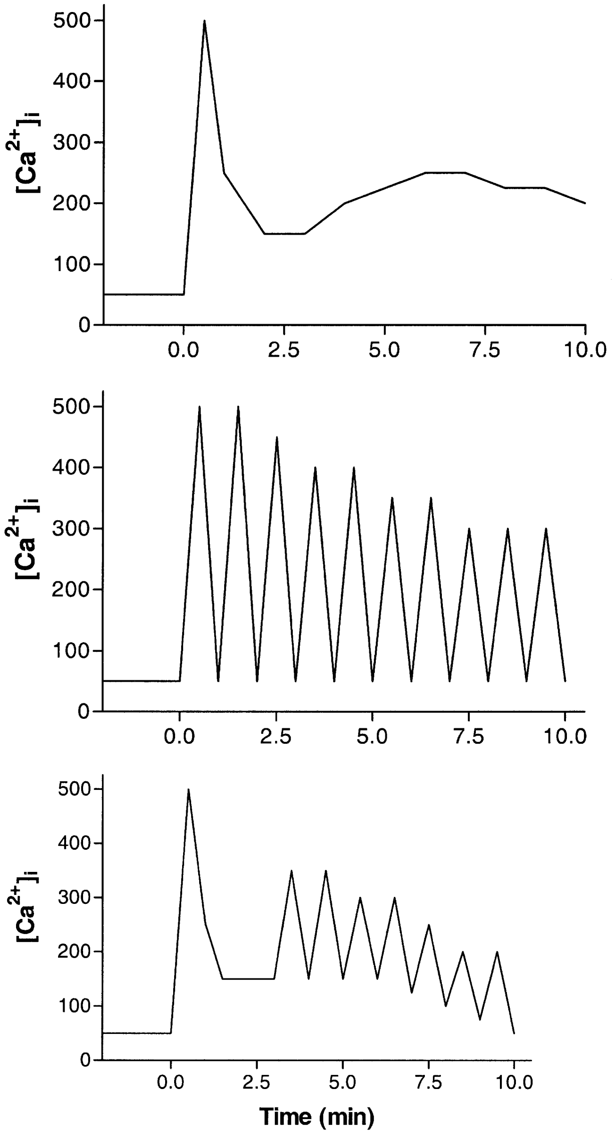

FIGURE 4 Three ways that TRH-stimulated elevations of $[Ca^{2+}]_i$ occur in pituitary cells. TRH was added to cells in tissue culture at time zero. See text for discussion.

in regulation of gene transcription in pituitary cells. TRH activates mitogen-activated protein kinases (MAPKs), a family of differentially regulated serine/threonine kinases, which also appears to lead to regulation of gene transcription. Activation of MAPK is in part secondary to PKC activation and is enhanced via the βγ subunit dimer of heterotrimeric G-proteins. The set of genes regulated by these protein kinases after TRH stimulation has only begun to be delineated. Although a number of signal transduction pathways initiated by TRH-R activation have been identified, cellular responses other than stimulation of hormone synthesis and secretion by the anterior pituitary are poorly understood.

## V. CONSTITUTIVE SIGNALING BY TRH-Rs

Constitutive (basal; i.e., agonist-independent) signaling is a well-documented characteristic of some native receptors and of receptors that are mutated and cause human disease. Both TRH-R subtypes, at least when they are expressed at high levels in cells in tissue culture, exhibit constitutive signaling. However, the level of basal signaling exhibited by TRH-R2 is much greater than that of TRH-R1. Indeed, in some assays in certain cell types, basal signaling by TRH-R1 cannot be measured. As with most native GPCRs, whether basal signaling occurs *in situ* under normal conditions and whether it has an important physiological role is not known for TRH-Rs. It is noteworthy that some drugs (for example, benzodiazepines) are able to bind to TRH-Rs and inhibit their basal signaling activities. Ligands that inhibit basal signaling are termed inverse agonists (or negative antagonists). Inverse agonists may be used in future studies to determine whether basal signaling by TRH-Rs is physiologically relevant.

## VI. TRH DESENSITIZATION

Persistent stimulation by a constant level of TRH, as with many agonists that act at GPCRs, leads to a diminished response over time. This phenomenon of TRH-induced desensitization is an important regulatory mechanism that may be mediated at several steps in the signal transduction pathway. Perhaps the most important mechanisms of desensitization involve effects on the receptor. These include receptor phosphorylation, which decreases G-protein coupling, and receptor down-regulation, which is a decrease in the number of receptors on the cell surface. These two types of desensitization can often be dissociated because uncoupling of receptors from G-proteins occurs rapidly, within seconds to minutes, whereas receptor down-regulation is usually slower, requiring several hours.

TRH stimulation of $InsP_3$ formation rapidly causes desensitization. (TRH-induced elevation of $[Ca^{2+}]_i$ is also rapidly desensitized, but this may be due in part to depletion of $Ca^{2+}$ stores.) Although not proved, rapid TRH desensitization likely involves receptor phosphorylation, which may be caused by the action of PKC or by a member of the family of GPCR-specific kinases. The Ser or Thr residues that are likely to be phosphorylated are within ICL-2 and ICL-3 and in the carboxyl tail of TRH-R. These proposals are based on observations that pharmacologic activation of PKC (for example, by phorbol myristate acetate) and overexpression of GPCR kinases inhibit TRH stimulation of $InsP_3$ generation. TRH-induced TRH-R down-regulation can be observed after approximately 1 h of initiation of signaling. Down-regulation of TRH-Rs leads to diminished TRH responsiveness because the magnitude of the TRH response is usually directly related to the number of TRH-Rs expressed on the cell surfaces. TRH-R down-regulation may be caused by a number of extracellular regulatory factors, including TRH, thyroid hormones, and vasoactive intestinal polypeptide acting in pituitary cells. The number of TRH-Rs can be decreased by decreasing their rate of synthesis or by increasing their rate of degradation, or both. Changes in the rate of TRH-R protein degradation have not been studied.

Modulation of the rate of TRH-R synthesis secondary to decreases in the level of TRH-R mRNA has been shown by monitoring gene transcription and mRNA degradation. These studies were of TRH-R1 but may apply to TRH-R2 as well. Decreases in the levels of TRH-R mRNA can occur by decreasing the rate of TRH-R gene transcription or by increasing TRH-R mRNA degradation, or both. TRH causes a decrease in the level of endogenous TRH-R mRNA by increasing the rate of TRH-R mRNA degradation. This effect of TRH appears to involve activation of PKC and elevation of $[Ca^{2+}]_i$. Thus, it appears that TRH increases TRH-R mRNA degradation through its well-characterized signal transduction pathway, which is initiated by hydrolysis of $PIP_2$. TRH also decreases TRH-R mRNA levels by inhibiting TRH-R gene transcription; however, the signaling pathway that mediates this effect has not been demonstrated. In conclusion, down-regulation of the TRH-R number by a mechanisms that involves modulation of TRH-R protein turnover, although likely, has not been studied directly, whereas regulation of TRH-R synthesis is well documented and involves several distinct mechanisms that are initiated by different regulatory factors.

## VII. TRH-R INTERNALIZATION

Internalization (or endocytosis) of TRH-Rs occurs rapidly after TRH binds. This process serves to bring TRH and receptors into the cell, where either or both can be recycled to the cell surface or sorted to lysosomes for degradation. Although it has not been specifically shown with TRH-Rs, GPCR recycling allows for receptor resensitization because protein phosphatases associated with recycling vesicles can dephosphorylate receptors that have been acutely desensitized by phosphorylation. Unoccupied TRH-Rs are almost exclusively present on the cell surface. TRH/TRH-R complexes are internalized via clathrin-coated vesicles by a mechanism that involves arrestin and perhaps dynamin. The majority of TRH and TRH-Rs recycle to the cell surface membrane under most conditions studied. On binding TRH, TRH-R2 internalizes at a much faster rate than does TRH-R1. The reason for this difference is not clear. It is thought that proteins that reside in the cell surface membrane and undergo internalization contain a "signal" that directs them to plasma membrane clathrin-coated pits, from which coated vesicles pinch off during the initial steps in internalization. Evidence has shown that two distinct domains within the carboxyl terminus of TRH-R1 are necessary for TRH-stimulated TRH-R internalization. Although it has been suggested that coupling to G-proteins is necessary for internalization, it is not clear whether there is coupling to proteins of the internalization machinery in the absence of coupling to G-proteins.

## VIII. SUMMARY

The primary pathway of TRH signal transduction is mediated by activation of $G_q$ or $G_{11}$ proteins; activation of PPI-PLC follows, leading to formation of $InsP_3$ and 1,2-DAG. $InsP_3$ elevates $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from the endoplasmic reticulum and 1,2-DAG activates PKC. The increase in $[Ca^{2+}]_i$ is further augmented by activation of cell surface membrane channels, allowing influx of extracellular $Ca^{2+}$. The elevation of $[Ca^{2+}]_i$ activates $Ca^{2+}$/ calmodulin protein kinase. Although increases in $[Ca^{2+}]_i$ and activation of these protein kinases are known to result in stimulation of anterior pituitary hormone secretion and gene transcription, the effects in the central nervous system are still poorly understood.

## Glossary

**agonist** Ligand that activates a receptor.

**$Ca^{2+}$/calmodulin-dependent protein kinase** Family of serine/threonine protein kinases; activated by $Ca^{2+}$ and calmodulin.

**constitutive signaling** Receptor signaling that occurs in the absence of agonist binding; also termed basal or agonist-independent signaling.

**1,2-diacylglycerol** Lipid product of phospholipase C-mediated hydrolysis of phospholipids; a neutral lipid with fatty acyl chains at positions 1 and 2; activates protein kinase C.

**G-proteins** Family of signal-transducing (or coupling) proteins made up of three subunits ($\alpha$, $\beta$, and $\gamma$); activated by members of the largest family of receptors and, in turn, activate ion channels and a number of effector molecules, including enzymes such as adenylyl cyclase and phospholipase C.

**G-protein-coupled receptors** Seven-transmembrane-spanning (heptahelical, or serpentine) cell surface receptors that bind extracellular regulatory molecules such as hormones, neurotransmitters, and growth factors, and bind and activate G proteins.

**inositol 1,4,5-trisphosphate** Sugar product of phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate; a hexahydroxy, cyclic sugar with phosphate groups at the 1, 4, and 5 positions; activates channels in the endoplasmic reticulum to release previously sequestered $Ca^{2+}$.

**ligand** Any molecule, such as a hormone, neurotransmitter, growth factor, or drug, that binds to a receptor.

**phospholipase C** Lipid-hydrolyzing enzyme that cleaves phospholipids at the 3 position to form two products—the head group, such as $InsP_3$, and 1,2-diacylglycerol.

**protein kinase C** Serine/threonine protein kinase that is activated by 1,2-diacylglycerol, phospholipids, and/or $Ca^{2+}$ downstream of activation of certain receptors.

## See Also the Following Articles

**Calmodulin • GPCR (G-Protein-Coupled Receptor) Structure • Heterotrimeric G-Proteins • Inositol Phosphate Signaling • Thyrotropin Receptor Signaling • Thyroid Hormone Receptor, TSH and TSH Receptor Mutations • Thyrotropin-Releasing Hormone (TRH)**

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# Thyrotropin-Releasing Hormone (TRH)

RONALD M. LECHAN[*] AND ANTHONY HOLLENBERG[†]

[*]*Tufts–New England Medical Center and Tufts University School of Medicine* • [†]*Harvard Medical School*

I. INTRODUCTION
II. BIOSYNTHESIS AND PROCESSING OF TRH
III. TRH GENE AND PROMOTER REGULATION
IV. ANATOMY OF THE HYPOTHALAMIC TRH TUBEROINFUNDIBULAR SYSTEM
V. FEEDBACK REGULATION OF HYPOPHYSIOTROPIC TRH BY THYROID HORMONE
VI. REGULATION OF HYPOPHYSIOTROPIC TRH BY COLD EXPOSURE AND SUCKLING
VII. REGULATION OF HYPOPHYSIOTROPIC TRH BY FASTING AND INFECTION (NONTHYROIDAL ILLNESS SYNDROME)
VIII. EXTRAHYPOTHALAMIC FUNCTIONS OF TRH

**Thyrotropin-releasing hormone (TRH) arises from posttranslational processing of a large precursor protein but it is only one of several other potentially biologically active peptides derived from the same precursor. The biosynthesis of TRH is dependent on feedback regulation of thyroid hormone, mediated by its interaction with the β2 thyroid hormone receptor and negative thyroid hormone-responsive elements in the TRH gene. Whereas TRH originating in neurons of the hypothalamic paraventricular nucleus contributes to the hypothalamic tuberoinfundibular system and regulates anterior pituitary secretion, the TRH gene is expressed in many other regions of the brain and**

**in peripheral tissues where TRH and other proTRH-derived peptides are expressed in a region- and tissue-specific manner to exert a variety of biologic actions.**

## I. INTRODUCTION

The maintenance of thyroid function is dependent on a complex interplay between the hypothalamus, anterior pituitary, and thyroid gland, as well as other factors that influence the function of these organ systems. This function is critical in achieving a constant level of free thyroid hormone in the bloodstream to support peripheral tissues by affecting protein synthesis and/or altering the metabolic activity of cells, as well as for brain development during fetal growth and early infancy. The hypothalamic peptide primarily responsible for this action by stimulating the biosynthesis and release of thyrotropin (TSH) from the anterior pituitary gland is thyrotropin-releasing hormone (TRH). TSH then stimulates the release of the thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) from the thyroid gland into the circulation; T4 and T3 in turn feed back on the hypothalamic TRH neurons and anterior pituitary thyrotropes, completing what is recognized as a classic example of a negative feedback loop system (Fig. 1). Elucidation of the normal anatomy and connectivity of the TRH neurons responsible for the secretion of TSH and characterization of the TRH gene has vastly improved the understanding of the normal physiology of hypophysiotropic TRH and the mechanisms by which the thyroid axis is modified under adverse conditions such as fasting, infection, and cold exposure.

TRH should not be considered to have an exclusive action on the secretion of TSH as its name suggests, however, as it also subserves a wide range of other biologic functions both within the central nervous system and in some peripheral tissues. The recognition that posttranslational products of the TRH prohormone include not only TRH but also other cryptic peptides that may have biologic activity further expands the diversity of the actions of the products of proTRH and raises the possibility that in some tissues, the primary product of posttranslational processing may not be TRH.

## II. BIOSYNTHESIS AND PROCESSING OF TRH

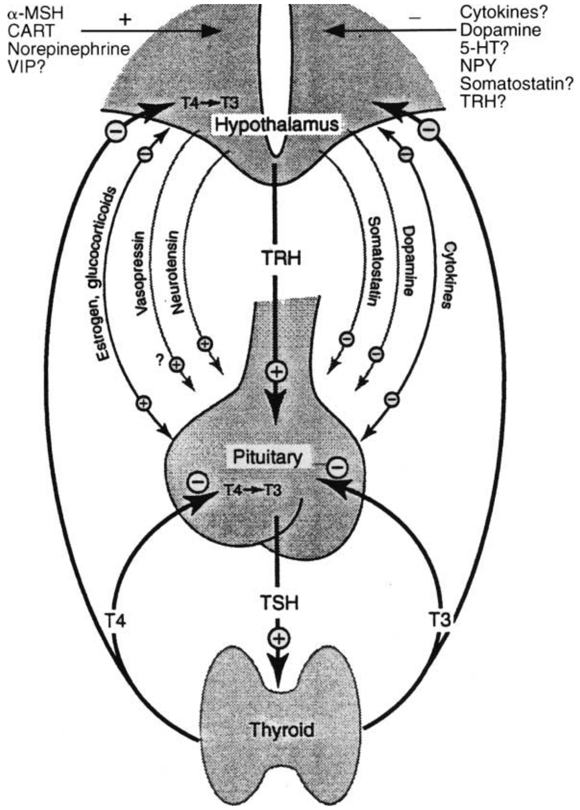

**FIGURE 1** Neuroregulatory control systems involved in the secretion of thyroid hormone. Boldface lines denote the negative feedback loop of thyroid hormone on TRH secretion from the hypothalamus and TSH secretion from the anterior pituitary. Both the hypothalamic TRH neurons and the anterior pituitary thyrotropes are impinged upon by numerous other potential regulatory influences that are activated under specific physiological or pathological conditions.

TRH (pyroGlu-His-ProNH$_2$, MW 362) arises by a mRNA-directed ribosomal mechanism and posttranslational processing of a larger precursor prohormone. The complete sequence of the TRH precursor, deduced from its cDNA, has been elucidated for frog, rat, mouse, and human and is schematically illustrated in Fig. 2. Common to each prohormone are multiple copies of a progenitor sequence for TRH, Gln-His-Pro-Gly, flanked on either side by paired basic amino acids, Lys-Arg or Arg-Arg, that are processing signals for carboxypeptidase B-like enzymes. The C-terminal glycine functions as a substrate for α-amidating enzymes that convert TRH-Gly to TRH and the N-terminal glutaminyl is modified by glutaminyl cyclase to result in the fully mature and biologically active TRH.

Rat preproTRH contains 255 amino acids and five copies of the TRH progenitor sequence and is approximately 88% homologous to the mouse preproTRH, which contains 256 amino acids and also gives rise to five copies of TRH by proteolytic

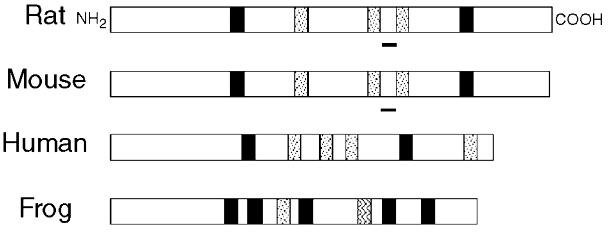

FIGURE 2 Schematic representation of rat, mouse, frog, and human preproTRH. Each sequence contains multiple copies of a TRH progenitor sequence (Gln-His-Pro) preceded by Lys-Arg and followed by Lys-Arg or Arg-Arg. Unique to the frog is one TRH progenitor sequence that is preceded by Arg-Arg and followed by Lys-Arg. The cryptic peptides preproTRH$_{160-169}$ and preproTRH$_{178-199}$ are located between the third and the fourth progenitor TRH sequences and between the fourth and the fifth progenitor TRH sequences, respectively.

processing. The human preproTRH contains 242 amino acids and six copies of the progenitor sequence for TRH. Frog brain has at least three different TRH preprohormones ranging between 224 and 227 amino acids and contains seven copies of the TRH progenitor sequence. Common to all species are the repeating progenitor sequences of TRH in the prohormone, presumably important in magnifying the response to signals that trigger TRH secretion.

In addition to the sequences that give rise to TRH, the TRH precursor contains several cryptic peptides between the TRH progenitor sequences and the N- and C-terminal flanking peptides that may be biologically active. At least one peptide, preproTRH$_{160-169}$, a decapeptide containing the amino acid sequence Ser-Phe-Pro-Trp-Met-Glu-Ser-Asp-Val-Thr that follows the third TRH sequence in the rat prohormone, is also present in mouse preproTRH and has been isolated from bovine hypothalamus. This peptide is released from the medial hypothalamus into the system of veins that conveys releasing hormones to the anterior pituitary, the portal capillary system, and specific binding sites for the decapeptide have been identified in the anterior pituitary as well as in other regions in the brain. PreproTRH$_{160-169}$ has little effect on anterior pituitary secretion by itself, but in the presence of TRH, it potentiates the secretion of TSH by increasing TSHβ mRNA in thyrotropes. The action of preproTRH$_{160-169}$ is probably not directly on thyrotropes, however, as receptors have been identified only in folliculostellate cells, a specialized glial cell of anterior pituitary origin that is not known to secrete TSH. Presumably, therefore, the effect of preproTRH$_{160-169}$ to potentiate the action of TRH on TSH secretion is indirect via paracrine interactions between the processes of folliculostellate cells and anterior pituitary thyrotropes.

In addition to TSH secretion, preproTRH$_{160-169}$ enhances both the synthesis and the secretion of prolactin and possibly growth hormone release. PreproTRH$_{160-169}$ also potentiates the effect of TRH to induce gastric acid secretion when the two peptides are simultaneously microinjected into the dorsal motor nucleus of the vagus.

PreproTRH$_{178-199}$, which separates the fourth and fifth progenitor TRH sequences, is also released from the rat median eminence but as yet, no receptors for this peptide have been identified. Claims for biologic activity of preproTRH$_{178-199}$ were based on its ability to act as a corticotropin-inhibiting factor, blocking both ACTH release and proopiomelanocortin (POMC) mRNA transcription, but these claims are still controversial. PreproTRH$_{178-199}$ may also have a number of other potential actions including inhibition of growth hormone secretion and neuroendocrine responses to stress, antidepressant activity, and neuroprotection against cerebral ischemia. PreproTRH$_{178-184}$ and preproTRH$_{186-199}$, posttranslational products of preproTRH$_{178-199}$, may have prolactin-releasing activity.

Despite the potential for the above rodent proTRH-derived peptides to have biologic activity, most of these sequences are not present in the human or frog preproTRH, raising questions as to their importance across animal species. Hydropathy profiles of the rat, human, and frog preproTRH sequences, however, do show a peak hydrophobicity over the region corresponding to the C-terminal flanking peptide. In particular, this region has remarkable resemblance over a 47- to 50-amino-acid span in the rat, mouse, and human prohormone. In the human, this sequence contains within it the sixth progenitor TRH that may have arisen from evolutionary changes between the fifth TRH coding sequence and the stop codon. No data that establish a definite biologic action of the C-terminal flanking peptide have been reported, however. Several preproTRH sequences have been identified in extracts of human hypothalamus including preproTRH$_{141-149}$, preproTRH$_{158-183}$, and preproTRH$_{192-224}$. PreproTRH$_{141-149}$ (referred to as human thyrotropin-releasing hormone-associated peptide 3 or hTAP-3) is also present in human serum and corresponds in position to the intervening sequence preproTRH$_{160-169}$ in the rat prohormone.

Processing of proTRH to its final products is region specific and dependent on the type of processing enzymes in the cell. In the hypothalamus, preproTRH is fully processed to yield all five copies of TRH and each of the spacer peptides. In contrast, in the olfactory lobes, C-terminal extended forms of TRH, $\text{preproTRH}_{154-169}$, and $\text{preproTRH}_{172-199}$ are the predominant end products, suggesting incomplete processing at Arg-Arg residues. Furthermore, in the midbrain periaqueductal gray, the N-terminal cryptic peptide $\text{preproTRH}_{83-106}$ is selectively increased during opiate withdrawal while TRH and the C-terminal peptide are reduced.

Two enzymes that are critical for the processing of proTRH are the proconvertase enzymes, PC1 and PC2. These enzymes cleave at the C-terminal end of single or paired basic amino acid residues and the remaining basic amino acids are removed by carboxypeptidases. PC1 appears to be capable of processing the entire proTRH precursor to mature TRH, whereas PC2 processes only specific regions of the prohormone. Along these lines, it is of interest that both PC1 and PC2 are present in the majority of TRH neurons in the paraventricular nucleus (PVN), whereas primarily PC2 is expressed in the olfactory lobes and periaqueductal gray. Accordingly, differences in the expression of PC1 and PC2 in different regions of the brain may be responsible for the differential processing of proTRH observed in these regions.

A schema for the processing of proTRH is shown in Fig. 3. In this model, the 26 kDa prohormone is cleaved at one of two sites to generate either 15 and 10 kDa fragments ($\text{preproTRH}_{25-151}$ and $\text{preproTRH}_{160-255}$) or 9.5 and 16.5 kDa fragments ($\text{preproTRH}_{25-112}$ and $\text{preproTRH}_{115-255}$), respectively. This initial cleavage occurs in the Golgi apparatus. Subsequently, the 15 kDa N-terminal fragment is processed in vesicles to a 6 kDa intermediate ($\text{preproTRH}_{25-74}$) and a 3.8 kDa peptide ($\text{preproTRH}_{77-106}$), and the 10 kDa fragment produces a 5.6 kDa intermediate ($\text{preproTRH}_{160-199}$) and a 5.4 kDa intermediate corresponding to the C-terminal peptide, $\text{preproTRH}_{208-255}$. The 5.6 kDa fragment is then further processed to $\text{preproTRH}_{160-169}$ and $\text{preproTRH}_{178-199}$. $\text{PreproTRH}_{178-199}$ can then be further processed to the smaller peptides, $\text{preproTRH}_{178-184}$ and $\text{preproTRH}_{186-199}$, mediated by the action of PC2. Alternatively, processing of the 9.5 kDa N-terminal fragment is proposed to yield $\text{preproTRH}_{25-50}$, $\text{preproTRH}_{53-74}$, and $\text{preproTRH}_{83-106}$, and the 16.5 kDa C-terminal fragment is proposed to yield $\text{preproTRH}_{160-199}$ and $\text{preproTRH}_{208-255}$. Following cleavage at all paired basic residues, the mature TRH (pGlu-His-Pro-$NH_2$) is generated by amidating the C-terminal end of the tripeptide using the glycine residue in the immediate precursor to TRH, TRH-Gly, as a substrate for the α-amidating enzyme, PAM, and cyclization of the N-terminal glutaminyl to pGlu by glutaminyl cyclase.

## III. TRH GENE AND PROMOTER REGULATION

The murine, rat, and human TRH genomic structures are identical, with each structure containing three exons and two introns (Fig. 4); exon 1 encodes the 5′-untranslated region, whereas exon 2 and 3 contain the sequence that encodes the preproTRH message. The DNA sequences of the exons are well conserved across species, but the sequences of the introns are poorly conserved. The promoter region of the TRH gene is located immediately 5′ to exon 1 and presumably is the major locus where regulation of the gene takes place. *In vivo* studies in transgenic mice using TRH promoter fragments linked to a luciferase reporter gene suggest that sequences within the proximal promoter as well as the first exon are critical for TRH expression in the hypothalamus. It remains unclear, however, which regions of the TRH gene allow for its expression in other tissues, such as the pituitary, pancreas, or heart.

The TRH promoter region is heralded by the presence of a conserved TATAA-box, allowing for transcriptional initiation by RNA polymerase II. Just proximal to the TATAA-box is an important regulatory element, termed Site 4 (TGACCTCA), that is conserved in the mouse, rat, and human promoters (Fig. 4). This sequence is important for basal expression of the TRH promoter in many different mammalian cell lines when assessed using transfection studies. In addition, Site 4 serves as a multifunctional binding site for the transcription factor cyclic AMP-response element-binding protein (CREB) and thyroid hormone receptors (TRs), including TR homodimers and heterodimers of TR and the retinoic acid X receptor (RXR). It appears that Site 4 cooperates with a second weak TR-binding site 11 bp 3′ to Site 4 that binds both TR homodimers and TR/RXR heterodimers. Taken together, Site 4 appears to be a critical sequence within the TRH promoter that is preserved across species and controls both basal and regulated expression of the TRH gene.

The importance of Site 4 in the regulation of TRH expression was first demonstrated in functional studies in mammalian cell lines that showed that it was required for down-regulation of TRH promoter constructs by T3 in the presence of the TR. T3

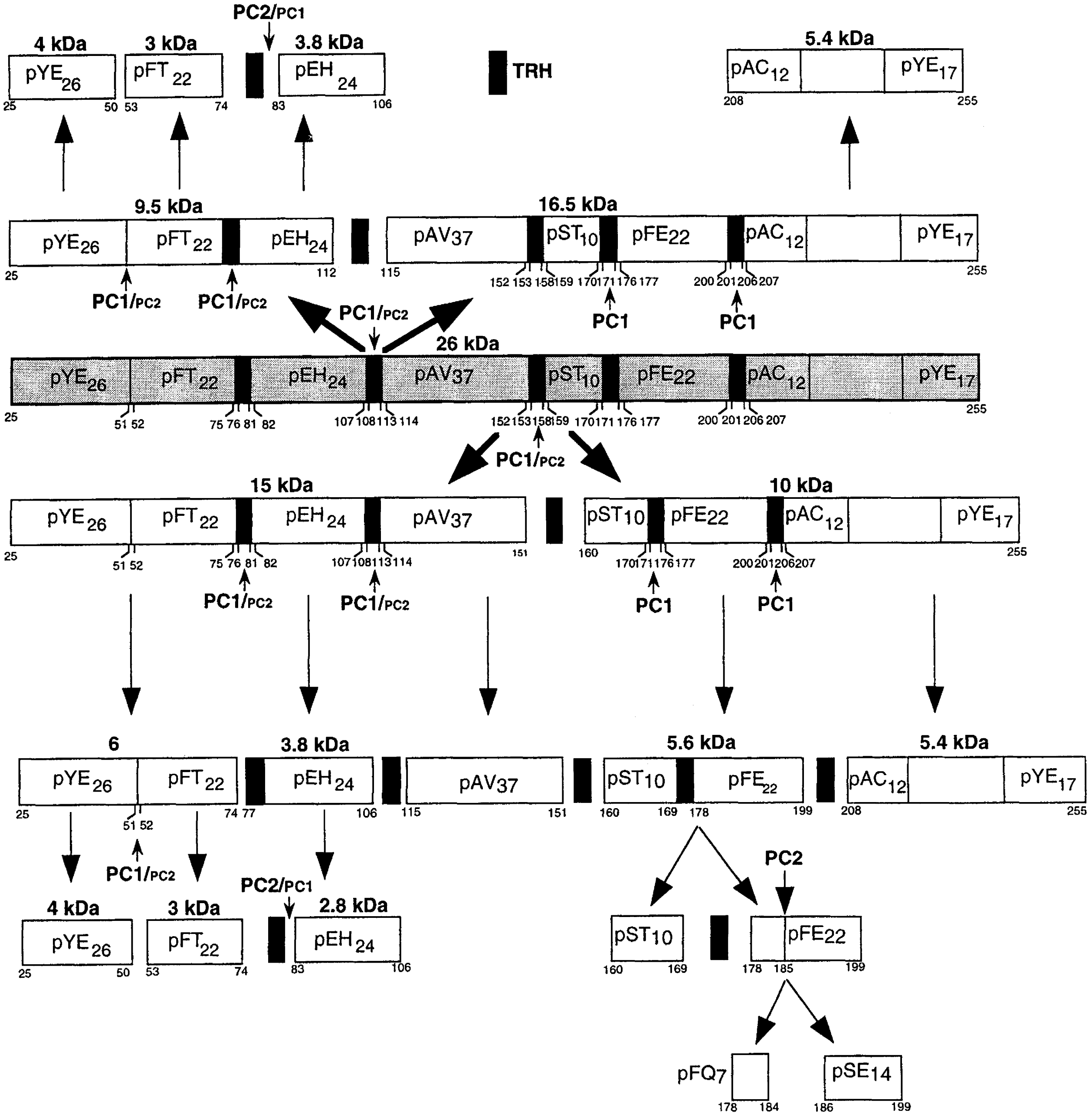
PC2/PC1
4 kDa
3 kDa
3.8 kDa
5.4 kDa
pYE26
pFT22
pEH24
TRH
pAC12
pYE17
9.5 kDa
16.5 kDa
pAV37
pST10
pFE22
PC1/PC2
PC1
26 kDa
15 kDa
10 kDa
6
5.6 kDa
2.8 kDa
PC2
pFQ7
pSE14

FIGURE 3 Proposed schema for processing of preproTRH. Reprinted from Nillni and Sevarino (1999), with permission from The Endocrine Society.

mediates its effects on gene expression via the three TR isoforms (TR$\alpha_1$, TR$\beta_1$, and TR$\beta_2$). TR$\alpha_1$ is expressed in many tissues but plays only a small role in negative regulation by T3. Its actions are critical for the effects of T3 in the heart, bone, and small intestine. The TR$\beta$ isoforms are products of a single gene and differ only in their amino-terminal regions. TR$\beta_1$ mediates the effect of T3 in the inner ear, liver, and kidney, and recent genetic studies have confirmed that TR$\beta_2$ is critical for negative regulation of the TRH, TSH$\alpha$ subunit, and TSH$\beta$ subunit genes by T3. The molecular mechanism governing negative regulation of the TRH promoter by T3 has not been firmly established. Site 4 and its surrounding region can bind TR$\beta_2$, which appears to be necessary for negative regulation. However, it is not clear which co-factors

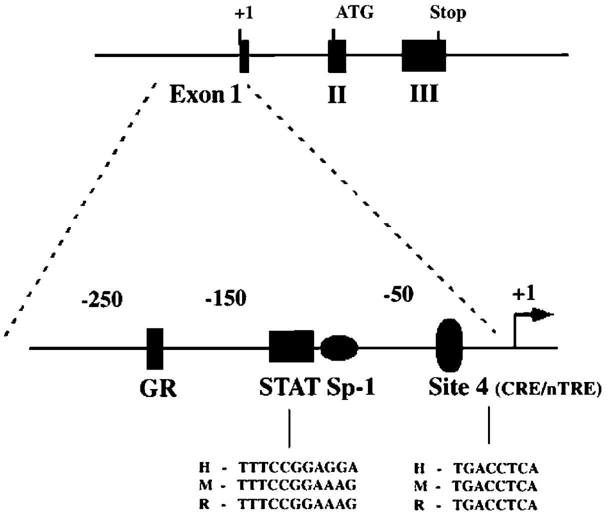

FIGURE 4 Genomic and promoter structure of TRH. The murine, rat, and human TRH genes are composed of three exons and two introns. The coding sequence for the precursor protein is present on exons 2 and 3. As depicted, the TRH promoter region precedes the transcription start site in exon 1. The proximal 250 bp sequences of the human, mouse, and rat promoters are similar and share the indicated transcription factor-binding sites. The sequences of the STAT element and Site 4 across species are indicated.

are involved. On genes positively regulated by T3, the TR isoforms are bound constitutively to thyroid hormone-response elements (TREs). In the absence of T3, they recruit the nuclear co-repressors (nuclear receptor co-repressor and silencing mediator for retinoid and thyroid hormone receptor) that repress transcription of target genes. The presence of T3 causes the release of co-repressors and the recruitment of co-activators such as SRC-1, which lead to transcriptional activation. The role of co-repressors and co-activators in the negative regulation of the TRH gene remains less clear, given that the recruitment of co-activators by TR$\beta_2$ in the presence of T3 would be expected to activate rather than repress TRH expression. However, they also appear to be important because mutations in TRs that prevent binding of either co-repressors or co-activators without altering DNA binding are unable to mediate negative regulation. In addition to Site 4, a second region within the TRH gene present within the first 55 bp of exon 1 may be important in mediating the effects of T3. This region interacts with TR$\beta$ monomers and its removal in functional studies causes a loss of negative regulation by T3.

Site 4 can also be bound by CREB. CREB binding and TR binding appear to be mutually exclusive and there is no evidence that they interact on Site 4. Thus, it is possible that they in fact compete for Site 4. CREB is bound constitutively to target promoters, usually as a homodimer or as a heterodimer with other members of the ATF-1 family. When phosphorylated by protein kinase A after activation of the cAMP signaling cascade by activators such as forskolin, PCREB recruits the co-activator CREB-binding protein (CBP), which allows for transcriptional activation of the TRH gene. CBP functions as a histone acetyl transferase, allowing modification of surrounding histones to enhance transcription. Mutation or deletion of Site 4 prevents the activation of TRH promoter constructs by forskolin. The phosphorylation of CREB, therefore, is likely a key mechanism for positive regulation of TRH gene expression, and by competing with PCREB for Site 4, TR$\beta_2$ could prevent activation of the TRH promoter. Binding of PCREB to Site 4 also has an important role in mediating the effects of melanocortin signaling on the TRH gene (see below).

Activation of the TRH gene also occurs via a canonical signal transducer and activator of transcription (STAT)-binding site in the murine and rat promoter 5′ to Site 4. A similar site in the human promoter located between $-150$ and $-125$ has also been identified, suggesting the importance of this sequence across animal species (Fig. 4). A number of STAT isoforms (STAT1, STAT3, and STAT5) have been identified and appear to be ubiquitously expressed. In the inactivated state, STAT proteins are present in the cytoplasm. Activation occurs after a cell surface receptor (usually a member of the cytokine receptor family) is bound by its ligand. This leads to a cascade that results in recruitment and activation of Janus tyrosine kinase (JAK), which in turn leads to recruitment and phosphorylation of the STAT isoforms. Tyrosine phosphorylation allows phospho-STAT to dimerize and enter the nucleus to bind to its target regulatory elements such as that found in the TRH promoter. STAT activates transcription by recruiting co-activators such as CBP. The leptin receptor also activates transcription via the JAK/STAT signaling pathway and is able to significantly enhance TRH promoter activity in transfection experiments in mammalian cells. This direct action of leptin on the TRH promoter is the best example to date of a downstream transcriptional target of the leptin signaling pathway. The presence of such a STAT site also suggests that the TRH promoter is poised to respond to other cytokine signaling pathways that could engage TRH neurons in the paraventricular nucleus (PVN) or elsewhere.

The TRH promoter also contains binding sites for the glucocorticoid receptor (GR) and Sp-1. The glucocorticoid receptor is also a member of the nuclear receptor family, and Sp-1 is a widely expressed transcription factor that binds to GC-rich motifs in target promoters. Their role in its regulation are less well understood. The GR-response element is located just upstream of the STAT element and appears to be functional in the pituitary, where glucocorticoids enhance TRH gene expression (Fig. 4). However, glucocorticoids appear to be inhibitory to TRH gene expression in the PVN. There are multiple potential Sp-1 sites within the promoter. One is located adjacent to the STAT site and is required for full induction by leptin.

## IV. ANATOMY OF THE HYPOTHALAMIC TRH TUBEROINFUNDIBULAR SYSTEM

The origin of TRH-containing neurons that regulate anterior pituitary TSH secretion is the hypothalamic PVN, a triangular, midline nuclear group symmetrically located on either side of the dorsal portion of the third ventricle (Fig. 5A). The PVN is composed of two major parts: magnocellular neurons located in more lateral portions of the nucleus and parvocellular neurons located in more medial portions of the nucleus. The magnocellular component gives rise to the hypothalamic neurohypophyseal tract that carries vasopressin and oxytocin to the posterior pituitary. The parvocellular component has a number of subcompartments including anterior, medial, periventricular, ventral, dorsal, and lateral parvocellular subdivisions. TRH-synthesizing neurons are found primarily in the anterior, medial, and periventricular parvocellular subdivisions (Fig. 5B) and to a lesser extent in the dorsal and lateral subnuclei. However, only TRH neurons in medial and periventricular parvocellular subdivisions project to the median eminence (often referred to as hypophysiotropic neurons because they are involved in regulation of the anterior pituitary) and thereby are functionally distinct from the TRH neurons in the anterior parvocellular subdivision. Further support for the anatomical diversity of medial and periventricular parvocellular subdivision TRH neurons is the presence of the peptide cocaine- and amphetamine-regulated transcript (CART) in hypophysiotropic TRH neurons but not in other TRH neuronal populations.

Axons from hypophysiotropic TRH neurons in the PVN project to the median eminence, one of five so-called “circumventricular organs” in the mammalian brain that lie outside of the blood–brain barrier. Here, they terminate in the external zone of the median eminence where they have access to the fenestrated capillaries of the portal system (Fig. 5C). Some fibers also descend in the pituitary stalk to terminate in the posterior pituitary and may provide an alternative pathway by which TRH can regulate anterior pituitary secretion through the vascular channels (short portal veins) that connect the posterior pituitary to the anterior pituitary.

By residing in the PVN, TRH-producing neurons are situated in a region highly enriched by afferent inputs from other regions in the brain. These inputs are important to establish the setpoint for feedback regulation of hypophysiotropic TRH by thyroid hormone under basal conditions and during times when it is necessary to increase or decrease circulating levels of thyroid hormone (described below). Synaptic contacts between axon terminals and both the perikarya and the dendrites of TRH-producing neurons have been observed by ultrastructural analysis, including both the symmetric type, suggesting inhibitory control, and the asymmetric type, suggesting excitatory control. Symmetric synapses containing small and large electron-lucent vesicles tend to predominate on TRH perikarya, whereas both symmetric and asymmetric contacts containing small, round and oval, clear vesicles predominate on TRH dendrites. Central modulation of hypophysiotropic TRH neurons through afferent projections may also occur in the external zone of the median eminence, which could influence tuberoinfundibular TRH release by axo-axonal associations, but true axo-axonic synapses have only rarely been recognized in the mammalian median eminence.

Although several regions of the brain project to the PVN, at least two regions provide the major input to hypophysiotropic TRH neurons, brainstem catecholamine neurons and the hypothalamic arcuate nucleus (Fig. 6A). Axon terminals containing catecholamines give one of the most conspicuous innervations to TRH neurons in the PVN arising primarily from C1/A1 groups in the medulla, contributing approximately 20% of all synapses on these cells. These axons establish mostly asymmetric synapses with both perikarya and dendrites of TRH neurons, suggesting an excitatory function. Noradrenergic axon terminals have also been identified in close apposition to TRH axons in the external layer of the rat median eminence, indicating that catecholamine inputs may influence TRH hypophysiotropic neurons not only through direct effects on their perikarya and dendrites but also on their axon terminals. The trajectory of catecholamine-contain-

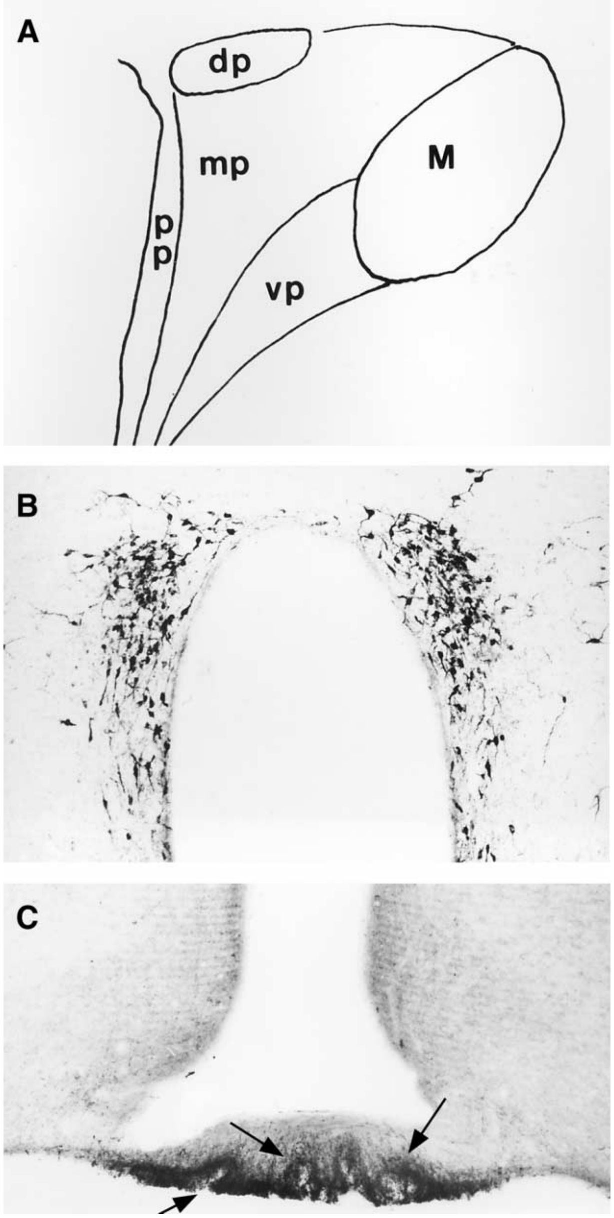

**FIGURE 5** (A) Location of the major subdivisions in the PVN showing its major subdivisions. pp, periventricular parvocellular subdivision; mp, medial parvocellular subdivision; dp, dorsal parvocellular subdivision; vp, ventral parvocellular subdivision; M, magnocellular division. (B) TRH neurons originate in the medial and periventricular parvocellular subdivisions of the PVN and (C) terminate in the external zone of the median eminence in contact with capillaries of the portal vascular system (arrows).

ing axons from the brainstem is to course rostrally in the dorsal and ventral noradrenergic bundles to ultimately enter the median forebrain bundle in the hypothalamus before terminating in the PVN (Fig. 6B). Transection of the noradrenergic bundle at the level of the midbrain results in marked depletion of the catecholamine input to the PVN, in keeping with their origin from the brainstem.

At least four different peptides of arcuate nucleus origin, α-melanocortin-stimulating hormone (α-MSH), CART, neuropeptide Y (NPY), and agouti-related peptide (AGRP), are contained in axon terminals in synaptic contact with TRH hypophysiotropic neurons (Fig. 6A,C). Neurons of the arcuato-PVN pathway project their axons primarily ipsilateral to the PVN and, like the catecholamine axon trajectory from the brainstem, course through the medial forebrain bundle prior to entering the PVN. In the rat, α-MSH and CART arise from the same neurons located in lateral portions of the arcuate nucleus, whereas NPY and AGRP are both contained in a distinct population located in more medial portions of the nucleus. A similar organization is also present in the human brain, suggesting the evolutionary importance of this neuroregulatory control system. Indeed, in the rat and mouse, pharmacologic ablation of the arcuate nucleus not only results in profound reduction in the number of axons contacting TRH neurons in the PVN that contain these neuropeptides, but also reduces circulating levels of TSH and thyroid hormone.

Similar to the catecholamine innervation, asymmetric specializations between axons containing α-MSH/CART and TRH neurons in the PVN are observed, suggesting an excitatory function, whereas axon terminals containing NPY and AGRP establish primarily symmetric synaptic specializations, suggesting an inhibitory function. The latter associations are sometimes so dense as to completely envelop the perikarya and first-order dendrites of TRH neurons. In addition to the arcuate nucleus, however, which constitutes more than 80% of the NPY innervation to TRH neurons in the PVN and 100% of the AGRP innervation, brainstem catecholaminergic neurons may also contribute to the NPY innervation of hypophysiotropic TRH neurons in a minor way, particularly to their distal dendrites. Nevertheless, a major NPY projection pathway from the brainstem to the PVN does exist, but is primarily targeted to CRH neurons in the PVN. This target-specific innervation of the PVN by axons containing NPY originating from at least two discrete sources in the brain may be a mechanism by which a single peptide can exert independent actions on discrete populations of neurons under different physiologic conditions.

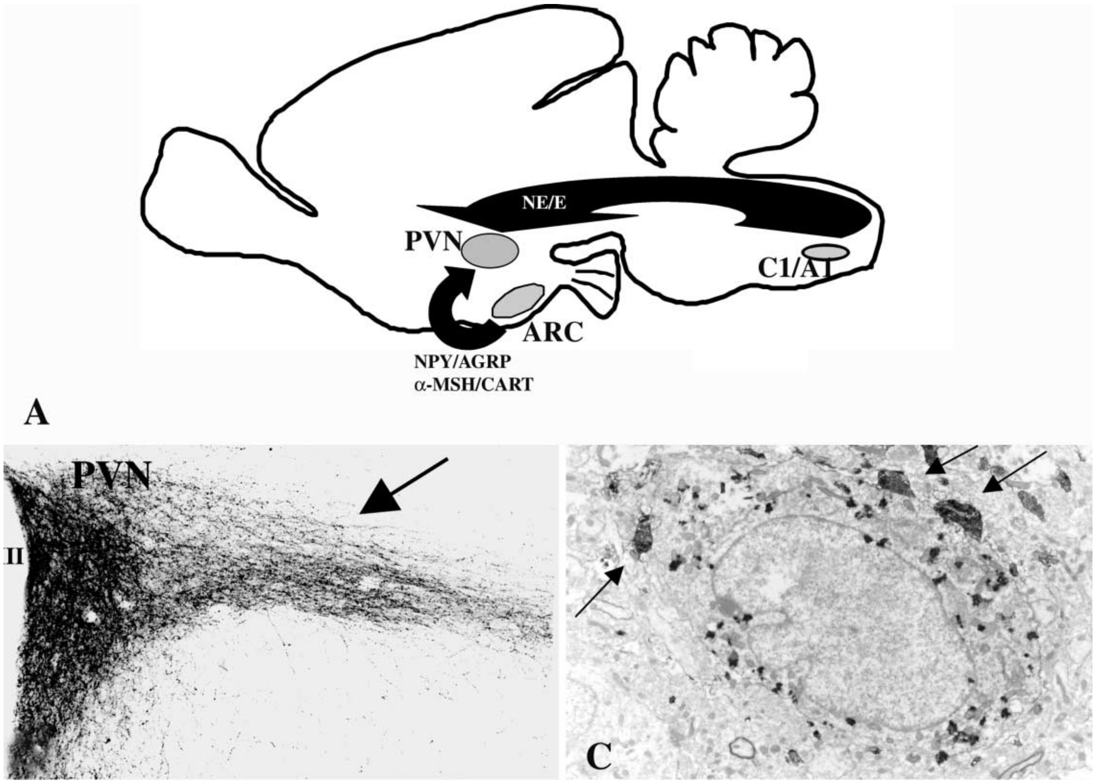

**FIGURE 6** (A) Sagittal drawing of the rat brain showing two major sources of innervation of TRH neurons in the hypothalamic PVN: the hypothalamic arcuate nucleus (ARC) that carries the neuropeptides NPY, AGRP, α-MSH, and CART to the PVN and brainstem C1/A1 cell groups that carry the catecholamines norepinephrine (NE) and epinephrine (E) to the PVN. (B) Neurons arising in the arcuate nucleus and brainstem innervate the PVN by traversing the medial forebrain bundle (arrow). (C) Electron micrograph showing that these axons (arrows) establish synaptic contacts with the cell body and dendrites of TRH neurons.

## V. FEEDBACK REGULATION OF HYPOPHYSIOTROPIC TRH BY THYROID HORMONE

The anatomical specificity of the TRH neuronal population in the medial and periventricular parvocellular subdivisions of the PVN is associated with a functional specificity of these cells with respect to feedback regulation by thyroid hormone. When circulating levels of thyroid hormone fall below normal values, perikaryal size and the content of proTRH and proTRH mRNA increases in these neurons, but not other TRH neurons in the forebrain (Figs. 7A and 7B). This increase is accompanied by a decline in the content of TRH in the median eminence due to an increase in the secretion of TRH into the portal blood for conveyance to the anterior pituitary. Conversely, increased circulating levels of T4 cause marked suppression of proTRH mRNA in the PVN and a reduction in the secretion of TRH into the portal plexus, establishing an inverse relationship between thyroid hormone and the biosynthesis and secretion of hypophysiotropic TRH.

The increase or decrease in the amount of hypophysiotropic TRH secreted into the portal system for conveyance to the anterior pituitary as dictated by variations in circulating levels of thyroid hormones is important in establishing the setpoint for feedback regulation by thyroid hormone on anterior pituitary TSH secretion. When portal blood TRH concentrations are low, TSH can be suppressed by less T4 circulating in the bloodstream (reduced setpoint), whereas high portal blood TRH concentrations raise the setpoint for feedback regulation by thyroid hormone. Thus, thyroidectomized animals with lesions of the hypothalamus that include the PVN can suppress TSH with smaller doses of T4 than animals with an intact hypothalamus, whereas chronic intrathecal infusion of TRH can elevate TSH and circulating free and total thyroid hormones.

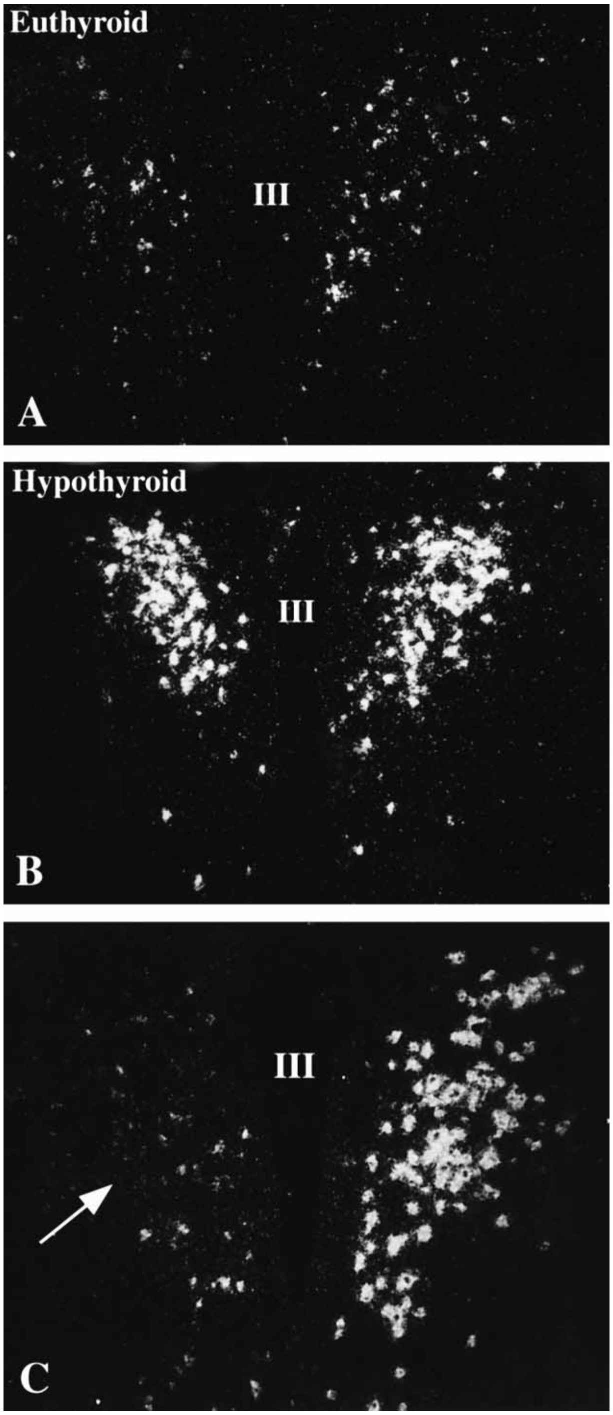

FIGURE 7 *In situ* hybridization autoradiographs of proTRH mRNA in the PVN of (A) euthyroid and (B) hypothyroid animals. Note the marked increase in hybridization signal in (B). (C) Effect of stereotaxic placement of thyroid hormone adjacent to one side of the PVN in a hypothyroid animal. Marked asymmetry of hybridization signal is apparent with diminished signal on the side of the implant (arrow).

The selectivity of TRH neurons in the medial and periventricular parvocellular subdivisions of the PVN to negative feedback regulation by thyroid hormone is an intrinsic property of these cells, mediated by the presence of thyroid hormone receptors in these neurons and the high vascular flow to the PVN. Stereotaxic implantation of microcrystals of T3 adjacent to one side of the PVN in hypothyroid animals, therefore, leads to marked inhibition of proTRH mRNA on that side, but not on the opposite side (Fig. 7C). Although TRH neurons in the PVN contain all of the known thyroid hormone receptors in their nucleus, TRα1 and TRβ2 are the most highly expressed in these cells. However, TRβ2 is the most important thyroid hormone receptor isoform responsible for thyroid hormone-mediated negative feedback regulation of TRH gene expression in hypophysiotropic neurons. Thus, mice with targeted deletion of TRβ2 alone show no significant increase in proTRH mRNA concentration in response to PTU-induced hypothyroidism or a decrease in proTRH mRNA concentration in response to the exogenous administration of T3.

The source of nuclear T3 responsible for feedback regulation of TRH neurons in the PVN differs from the source of T3 in other regions of the central nervous system (CNS) such as the cerebral cortex and anterior pituitary, where the majority of T3 arises from the intracellular monodeiodination of T4 to T3 by type II iodothyronine 5′-monodeiodinase (D2). This is because the PVN contains little, if any, D2 activity or D2 mRNA, indicating that cells in this region are not capable of intracellular conversion of T4 to biologically active T3. Thus, hypophysiotropic TRH neurons in the PVN must receive T3 from an exogenous source, directly from the bloodstream, from the cerebrospinal fluid, from adjacent glia, or by transneuronal transport. The possibility that feedback regulation by thyroid hormone on TRH neurons in the PVN is mediated exclusively by circulating levels of T3 alone is unlikely since the systemic infusion of graded doses of T3 to hypothyroid animals that restore plasma levels of T3 to normal do not suppress proTRH mRNA levels in the PVN to euthyroid levels. Only after constant infusion with higher concentrations of T3 that raise plasma T3 into the supranormal range is there an apparent reduction in hybridization signal for proTRH mRNA to euthyroid levels. Thus, the calculated plasma level of T3 required to suppress proTRH mRNA to normal in the absence of T4 is approximately 1.6 times euthyroid levels, suggesting that like other regions of the brain, feedback regulation of thyroid hormone on

TRH neurons in the PVN is partially dependent on circulating T4.

One potential source of T4 to T3 conversion is the base of the third ventricle where contained among the ependymal cells are specialized glial cells or tanycytes, which contain one of the highest concentrations of D2 mRNA in any region of the brain. Tanycytes extend apical blebs into the CSF and cytoplasmic extensions into the neuropil that envelop the portal capillaries in the median eminence and blood vessels in the hypothalamic arcuate nucleus. Thus, tanycytes may provide a cytoplasmic conduit between the bloodstream and the CSF, allowing bidirectional movement of substances between the vascular and CSF compartments. “D2 tanycytes,” therefore, may have an important role in the conversion of T4 to T3 either by the uptake of T4 from the CSF or from vascular compartments in the median eminence/arcuate nucleus and then the delivery of T3 to the CSF. By volume transmission, T3 would have access to extracellular spaces in the brain, such as the hypothalamic PVN, where it could mediate actions directly on hypophysiotropic TRH neurons. Alternatively, tanycytes may release T3 directly into the arcuate nucleus through tanycyte–neuronal interactions, where it could influence the activity of arcuate nucleus neurons that have known projections to TRH neurons in the PVN.

## VI. REGULATION OF HYPOPHYSIOTROPIC TRH BY COLD EXPOSURE AND SUCKLING

Thyroid hormone levels and TSH are acutely elevated by cold exposure in several animal species due to the increased secretion of TRH in the median eminence. This response is accompanied by a rapid increase in proTRH mRNA in the PVN, peaking within 30 to 60 min of the stimulus, suggesting that the response is mediated at least in part at the level of TRH gene transcription. A particularly intriguing finding regarding the acute cold-induced stimulation of the thyroid axis is that proTRH mRNA is increased at a time when circulating levels of thyroid hormone are high. This is in contrast to the mechanism of inverse feedback regulation by thyroid hormone as discussed above where proTRH mRNA would be expected to be inhibited. Thus, cold exposure may override the normal mechanism for feedback regulation by altering the setpoint for inhibition of TRH gene expression by T3.

The mechanism whereby the setpoint for feedback inhibition by thyroid hormone changes with cold exposure and suckling is still uncertain but there is strong evidence to suggest a role of catecholamines. Norepinephrine stimulates the release of TRH from hypothalamic preparations in culture, and depletion of catecholamines with inhibitors of catecholamine biosynthesis, such as α-methyl-para-tyrosine, or selective catecholamine neurotoxins, such as 6-hydroxydopamine, abolishes the response of the hypothalamic–pituitary–thyroid axis to cold exposure. In addition, the TSH response to acute cold exposure does not occur within the first 10 days after birth in the rat when the hypothalamic norepinephrine innervation is still immature. As noted previously, the PVN receives dense, afferent input from catecholamine-containing neurons in the brainstem that form asymmetric-type synaptic interactions on the cell body and dendrites of TRH neurons, suggesting an excitatory role. Norepinephrine may also stimulate TRH secretion by interacting with α-1 receptors directly in the median eminence.

Suckling increases proTRH mRNA in the PVN within 30 to 60 min of the response but for the primary purpose of increasing prolactin secretion from the anterior pituitary. TSH and thyroid hormone levels do not increase, perhaps due to the simultaneous release of oxytocin, which can attenuate TRH-induced TSH secretion. Although serotonergic afferents from the midbrain raphe may mediate this response, the mechanism for the increase in TRH gene expression remains elusive.

## VII. REGULATION OF HYPOPHYSIOTROPIC TRH BY FASTING AND INFECTION (NONTHYROIDAL ILLNESS SYNDROME)

Infection and fasting also alter the normal mechanisms of feedback regulation by circulating levels of thyroid hormone on hypophysiotropic TRH. In these circumstances, however, there is a fall in thyroid hormone levels but a seemingly paradoxical reduction of proTRH mRNA in the PVN, reduced secretion of TRH into the portal blood, and low or inappropriately normal plasma TSH levels rather than the anticipated increase in all of these parameters as seen in primary hypothyroidism. Thus, these conditions override the normal feedback mechanism described above and induce a state of central hypothyroidism, commonly referred to as “nonthyroidal illness” or the “sick euthyroid syndrome” in human. By reducing thyroid thermogenesis and preserving nitrogen stores, this mechanism is an important adaptive response to reduce energy expenditure until the adverse stimulus has been removed.

The state of central hypothyroidism induced by fasting is orchestrated by a circulating cytokine of white adipose tissue origin, leptin, which declines with fasting and is restored to normal levels by refeeding. Thus, if leptin is administered exogenously to fasting animals, the reduction in circulating levels of thyroid hormone, TSH, and hypophysiotropic proTRH mRNA in the PVN is prevented (Fig. 8). The primary action of leptin on the hypothalamic–pituitary–thyroid (HPT) axis is mediated primarily by the hypothalamic arcuate nucleus via the arcuato-PVN pathway. If the arcuate nucleus is destroyed, not only is the response of the thyroid axis to fasting abolished, its response to the exogenous administration of leptin is lost as well. The arcuate nucleus, therefore, serves as a critical locus to mediate the effects of leptin on the HPT axis and in so doing presumably establishes the setpoint for feedback sensitivity of proTRH-producing neurons in the PVN to thyroid hormone, lowering the setpoint when leptin levels are suppressed during fasting.

At least two anatomically distinct populations of neurons in the arcuate nucleus with opposing functions, the α-MSH-producing neurons that co-express CART and the NPY-producing neurons that co-express AGRP, are responsible for the actions of leptin on hypophysiotropic TRH. When leptin levels are suppressed during fasting, expression of the genes encoding POMC, the precursor protein of α-MSH, and CART are reduced simultaneously with a marked increase in the genes encoding NPY and AGRP. As suggested by the appearance of the synaptic specializations between axons containing α-MSH and CART and TRH neurons, both peptides, when administered intracerebroventricularly to fasting animals, activate proTRH gene expression and each can increase the suppressed levels of proTRH mRNA in hypophysiotropic neurons induced by fasting. Suppression of α-MSH and CART by fasting, therefore, is an important component of the effect of fasting on the thyroid axis, and their increase with refeeding may partly explain the recovery of the thyroid axis to normal. Since the TRH gene contains a multifunctional cAMP-response element that is activated by the phosphorylated form of CREB (PCREB) and inhibited by thyroid hormone bound to its receptor, and all melanocortin receptors couple to stimulatory G-proteins, an increase in cAMP generation may have the effect of decreasing the sensitivity of the TRH gene to feedback inhibition by thyroid hormone by increasing PCREB. Nevertheless, in contrast to the systemic administration of leptin, which restores the thyroid axis to normal in fasting animals, α-MSH is capable of only partly restoring circulating thyroid hormone levels and TSH, and CART has no effect on these hormones, suggesting that other central regulatory mechanisms also participate in this response.

It is likely that NPY has an important role in resetting the hypothalamic–pituitary–thyroid axis during fasting since fasting up-regulates NPY gene expression simultaneously with a decrease in POMC/-CART gene expression and NPY receptors couple to inhibitory G-proteins that reduce cAMP. An inhibitory effect of NPY is suggested by the morphologic specializations between NPY-containing axon term-

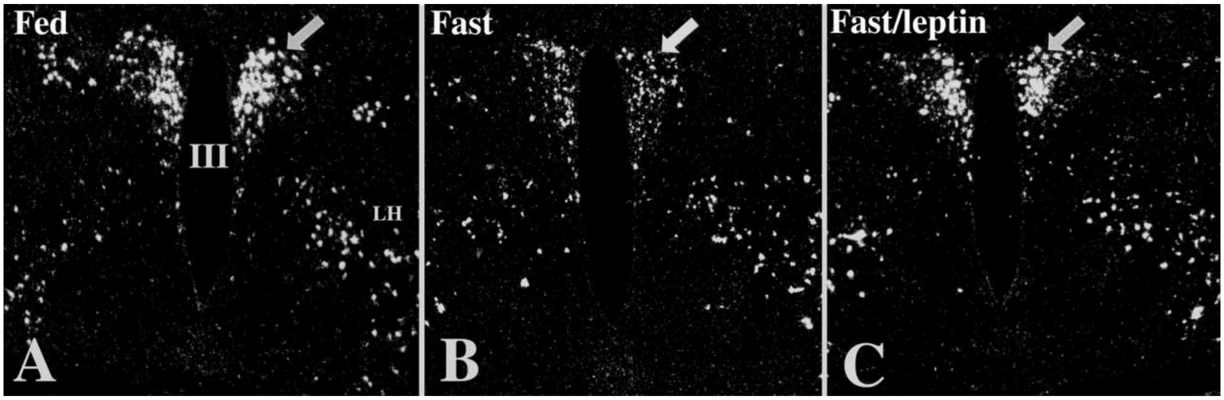

FIGURE 8 *In situ* hybridization autoradiographs of proTRH mRNA in the PVN (arrow) of (A) normal fed and (B) fasting animals. Note the marked reduction in hybridization signal by fasting. (C) ProTRH mRNA levels are restored to normal in fasting animals administered leptin.

inals and TRH neurons in the PVN. Indeed, the exogenous administration of NPY to normal, fed rats reduces circulating thyroid hormone and TSH levels and induces a greater than 50% reduction in proTRH mRNA in PVN neurons. The inhibitory action of NPY is mediated primarily by NPY Y1 and NPY Y5 receptors, with little or no effect of NPY Y2 receptors. NPY may also antagonize the activating effect of α-MSH on hypophysiotropic TRH neurons at a postreceptor level by preventing the phosphorylation of CREB.

Since the NPY-deficient transgenic mouse retains typical homeostatic responses to fasting, including a fall in thyroid hormone levels, it is likely that inhibitory factors other than NPY also contribute to the regulatory responses of fasting on the HPT axis. A likely candidate is AGRP, which is co-expressed in the same arcuate nucleus neurons and axon terminals that innervate TRH neurons in the PVN and which is also a potent inhibitor of proTRH mRNA when administered exogenously to fed animals. As AGRP is an endogenous antagonist at melanocortin receptors, its primary role may be to prevent the stimulatory effects of α-MSH on the TRH gene. Not all TRH neurons in the PVN receive contacts by α-MSH-containing axon terminals, however, indicating that AGRP may also function as an inverse agonist to constitutively active melanocortin receptors that may be uniformly expressed in hypophysiotropic TRH neurons or at a separate, as yet unknown receptor.

Although a decrease in POMC and CART gene expression contributes to fasting-induced suppression of the HPT axis, this mechanism does not appear to participate in other causes of nonthyroidal illness syndromes such as infection. In fact, following the administration of endotoxin, both POMC mRNA and CART mRNA increase in the arcuate nucleus despite suppression of proTRH mRNA in the PVN, and circulating levels of leptin are increased. Presumably, therefore, a set of regulatory controls over the hypothalamic–pituitary–thyroid axis that is different than that observed during fasting must be utilized under these conditions, superceding the stimulatory action of these substances on hypophysiotropic TRH.

The inhibitory effect of infection on hypophysiotropic TRH neurons is likely multifactorial and may involve direct and indirect effects of cytokines other than leptin such as interleukin-1, interleukin-6, tumor necrosis factor, and interferon. Supporting a direct action of cytokines on hypophysiotropic TRH neurons is the presence of a STAT-binding site in the promoter of the TRH gene. The potential contribution of cytokine-inducible inhibitors of signaling such as the SOCS (suppressor of cytokine signaling) proteins that prevent activation of the JAK/STAT pathway should also be considered. Glucocorticoids, which rise dramatically following endotoxin administration, may also contribute to the abnormal feedback responses to thyroid hormone. Both the normal circadian variation and the pulsatile secretion of TSH can be abolished by glucocorticoids, and glucocorticoids can reduce proTRH mRNA in the PVN, although the mechanism is unclear.

## VIII. EXTRAHYPOTHALAMIC FUNCTIONS OF TRH

Not all populations of TRH neurons subserve a hypophysiotropic function. As noted above, even within the PVN itself, TRH neurons in the anterior and dorsal parvocellular subdivisions do not project to the median eminence and instead are probably involved in the regulation of autonomic function and behavior. There are many TRH-rich regions in the brain including such diverse groups as the lateral hypothalamus, preoptic region, olfactory lobes, periaqueductal gray, dorsal motor nucleus of the vagus, and spinal cord, to name only a few. Rather than influence anterior pituitary TSH secretion, these neurons have a number of independent functions (depending upon their location), as described in Table 1. The spinal cord, for example, contains one of the highest concentrations of TRH in the CNS, exceeding even that in the hypothalamus. Here, TRH is located in axon terminals in the intermediolateral column and ventral horn (Fig. 9), originating from a large population of neurons in the brainstem medullary raphe. The dense concentration of TRH

**TABLE 1 Nonhypophysiotropic Effects of TRH/TRH-Derived Peptides**

| | |
|---|---|
| Analgesia | Increased gastric acid secretion |
| Anticonvulsant activity | Increased gastrointestinal motility |
| Arousal | Increased glucagon secretion |
| Cerebral vasodilation | Increased locomotor activity |
| Diaphoresis | Increased respiration |
| Facilitation of memory | Inhibition of food intake |
| Facilitation of motoneuron excitability | Neurotropic effects on spinal cord motoneurons |
| Hypertension | Peripheral vasoconstriction |
| Improved memory | Reduced pancreatic exocrine secretion |
| Increased blood pressure | Tachycardia |
| Increased body temperature | Tremor |

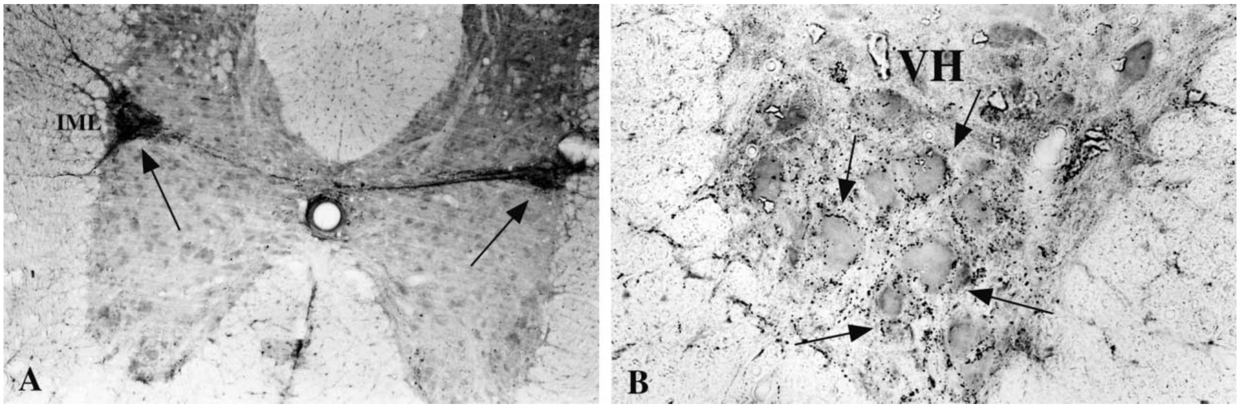

**FIGURE 9** TRH terminal fields in the spinal cord (A) intermediolateral column (IML) [arrows in (A)], and (B) ventral horn (VH). Arrows in (B) show dense terminal fields in association with α-motoneurons.

in the intermediolateral column mediates some of the autonomic effects of TRH including cardiovascular function. Ventral horn TRH may act as a facilitatory (excitatory) modulator of lower motoneuron function but could also have a tropic role, stimulating the outgrowth of motoneurons and promoting recovery following traumatic or ischemic spinal injuries. The effect of TRH on gut motility and gastric acid secretion is mediated by neurons in the dorsal motor nucleus of the vagus. TRH in the hippocampus exerts an anticonvulsant action, whereas TRH in the lateral hypothalamus and septum is involved in arousal and locomotor activity. Of particular interest are regions in the brain such as the midbrain periaqueductal gray, reticular nucleus of thalamus, and olfactory bulbs, where products of the TRH prohormone other than TRH itself may be the primary end products of proTRH processing. In the periaqueductal gray, for example, opiate withdrawal results in preferential accumulation of preproTRH$_{83-106}$ and may be involved in nociception or recovery from the physical manifestations of opiate withdrawal.

TRH (and/or TRH mRNA) has also been identified in anterior pituitary somatotropes, where it may contribute to basal secretion of TSH by a paracrine mechanism, and in many peripheral tissues including the retina, thyroid (parafollicular cells), heart, adrenal medulla, epididymis, testis (Leydig cells), placenta (syncytiotrophoblast cells), ovary, spleen, gastrointestinal tract, and pancreas (beta cells). The demonstration that mice with targeted deletion of the TRH gene develop diabetes has implicated TRH or products of proTRH in islet cell development and/or glucose homeostasis.

## Glossary

**cyclic AMP-response element-binding protein** A nuclear transcription factor mediating intracellular signaling systems that activate cyclic AMP.

**deiodinase** One of three different enzymes (D1, D2, and D3) responsible for removal of iodine from tyrosine residues to activate or inactivate thyroid hormone.

**hypophysiotropic** That pertaining to regulation of anterior pituitary secretion.

**paraventricular nucleus** A collection of small (parvocellular) and large (magnocellular) neurons in the hypothalamus that contain the neurons of origin of the thyrotropin-releasing hormone tuberoinfundibular system.

**proconvertase enzymes** Proteolytic enzymes of the subtilisin/kexin-like family that participate in the conversion of peptide precursors to their final biologically active forms by cleaving at the C-terminal basic amino acids.

**promoter** The region of a gene that directs regulation of transcription of its specific mRNA.

**retinoid acid X receptor** A member of the nuclear receptor family capable of forming heterodimers with thyroid hormone receptors.

**signal transducers and activators of transcription** A family of transcription factors mediating the effects of cytokines.

**tuberoinfundibular system** The collection of neurons in the brain and their axon trajectory that terminate in the neural–hemal contact zone of the hypothalamic median eminence.

## See Also the Following Articles

**Parathyroid Hormone • Thyroglobulin • Thyroid Hormone Receptor Isoforms • Thyroid Stimulating Hormone (TSH) • Thyrotropin Receptor Signaling • Thyrotropin-Releasing Hormone Receptor Signaling**

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# TNF

See *Tumor Necrosis Factor*

# Transcortin and Blood-Binding Proteins of Glucocorticoids and Mineralocorticoids

Agnès Emptoz-Bonneton, Catherine Grenot,
and Michel Pugeat
*INSERM ERM 03 22*

I. INTRODUCTION
II. STRUCTURE, BIOCHEMISTRY, AND BIOSYNTHESIS OF CBG
III. CBG GENE
IV. PHYSIOLOGICAL VARIATIONS
V. PATHOLOGY AND FAMILIAL CBG DEFICIENCY
VI. ROLE OF CBG

**Transcortin or corticosteroid-binding globulin (CBG) is the specific plasma transport protein that binds glucocorticoid hormones and regulates their biological disposal to target cells. Analysis of its primary structure, gene organization, and chromosomal location shows a close relationship with α1-anti-trypsin as well as other serine protease inhibitors. During acute inflammatory stress, the fall in circulating CBG levels enhances the bioavailability of cortisol and further amplifies the acute stimulation of adrenal function. In addition to its role as a steroid transport protein, there are indications of a possible role played by CBG in the release of glucocorticoids at inflammation sites and in**

**steroid hormone targeting through the binding of CBG–steroid complexes to cell membranes.**

## I. INTRODUCTION

In blood, hydrophobic steroid hormones circulate while bound to proteins. Albumin binding is the nonspecific, high-capacity transport system that is generally nonsaturated in normal physiological circumstances, whereas corticosteroid-binding globulin (CBG), also referred to as transcortin, represents a specific high-affinity but low-capacity transport system for cortisol, the main glucocorticoid hormone secreted by adrenal glands in humans. No specific binding protein for aldosterone, which is the main mineralocorticoid hormone secreted by the adrenals, has been identified thus far.

Most vertebrate species examined have circulating CBG with varying serum concentrations and steroid-binding specificities. In each species, the binding specificity of CBG is correlated with the biological activity of glucocorticoids. In humans, CBG-binding affinity is much higher for glucocorticoids (cortisol, corticosterone, and deoxycortisol) and progesterone than for mineralocorticoids (aldosterone and deoxycorticosterone) (Table 1). The binding capacity of CBG is close to the highest physiological cortisol concentration, and more than 90% of circulating cortisol is bound to CBG. Thus, according to the free hormone hypothesis, CBG, by limiting cortisol access to target cells, regulates cortisol biodisposal.

## II. STRUCTURE, BIOCHEMISTRY, AND BIOSYNTHESIS OF CBG

Human CBG is a monomeric glycoprotein of 50–55 kDa, as estimated by polyacrylamide gel electrophoresis under denaturing conditions, with one steroid-binding site per molecule. The mature protein is a 383-amino-acid polypeptide, derived from a 405-residue precursor polypeptide.

**TABLE 1** **Affinity ($K_a$) of Adrenal Steroids for CBG at 37°C**

| | $K_a$ (liter·mol$^{-1}$) |
|---|---|
| Cortisol | $76 \times 10^6$ |
| Corticosterone | $76 \times 10^6$ |
| Deoxycortisol | $76 \times 10^6$ |
| Cortisone | $7.8 \times 10^6$ |
| Aldosterone | $1.9 \times 10^6$ |
| Deoxycorticosterone | $45 \times 10^6$ |

CBG comprises five N-linked oligosaccharide chains per molecule, of which three are biantennary and two triantennary. A pregnancy-specific variant containing only triantennary oligosaccharide chains has been described, but does not affect steroid-binding activity. These carbohydrate chains potentially influence the biological half-life of the protein and may play a role in the interaction of CBG with the membrane receptors that are reported to be present at the surface of some target cells.

CBG is a member of the serine protease inhibitor (SERPIN) superfamily and shares a high degree of sequence homology with α1-anti-trypsin (α1AT), α1-anti-chymotrypsin (AACT), and another plasma transport protein, thyroxin-binding globulin (TBG). The role of α1AT (structurally and genetically the closest to CBG) is to protect tissue from hydrolysis by elastase, released by activated neutrophils during inflammation. CBG is specifically cleaved *in vitro* by neutrophil elastase at a single site in the neighborhood of the steroid-binding site. This cleavage induces a conformational change in the molecule, resulting in a 10-fold decrease in cortisol-binding affinity and the subsequent release of cortisol from its steroid-binding site.

Circulating CBG is synthesized by the liver. However, small amounts of CBG are also present in tissues such as pituitary corticotrophs, lung, kidney, uterus, and testis. It is not yet clear whether the presence of CBG at these sites is due to local synthesis or to sequestration from blood circulation. The physiological significance of this extrahepatic production of CBG, which probably has no influence on the plasma CBG concentration but could alter the local bioavailability of steroids that bind to CBG, has also not yet been established.

## III. CBG GENE

Human CBG is encoded by a single gene located on the long arm of chromosome 14, at position 32.1 (14q32.1). This region, the SERPIN gene cluster, includes five other SERPIN genes: α1AT, AACT, kallistatin, protein C inhibitor, and ATR, an anti-trypsin-related sequence.

The human CBG gene (*Cbg*) comprises five exons distributed over approximately 19 kb, with the complete coding sequence for CBG spanning exons 2 to 5 (Fig. 1). *Cis*-acting sequence elements identified in rat *Cbg* promoter (*pCbg*) and highly conserved in the human *pCbg* indicate that hepatocyte nuclear factor-1β and hepatocyte nuclear factor-3α (HNF-1β and HNF-3α), CCAAT-binding protein, D-site-bind-

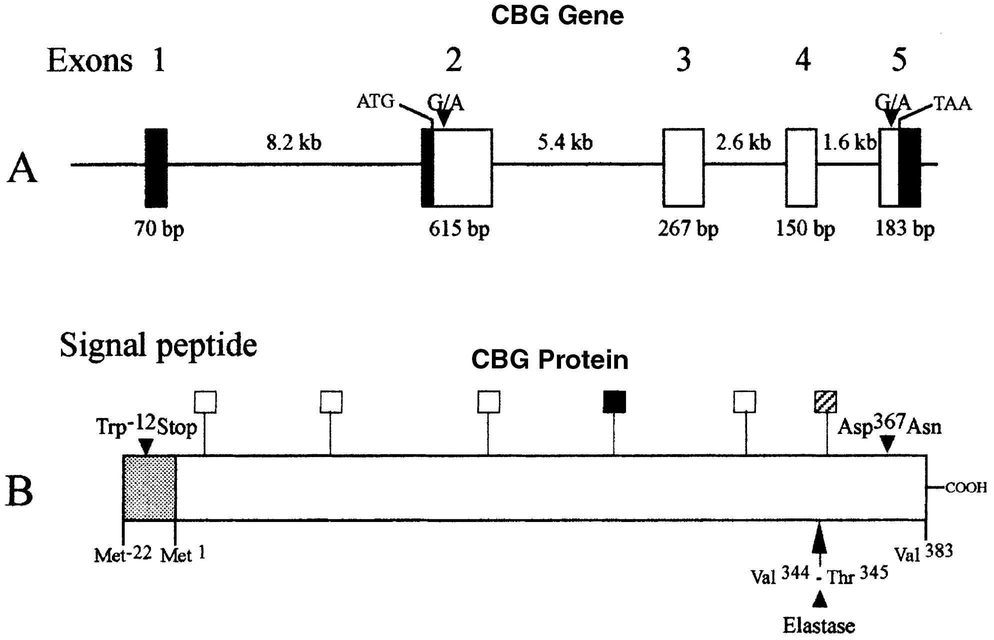

FIGURE 1 Schematic representation of the human CBG gene and CBG protein. (A) The human CBG gene comprises five exons (boxes), numbered 1–5; the black areas correspond to 5′- and 3′-untranslated regions; the size of the exons [in base pairs (bp)] is indicated at the bottom; the size of the introns [in kilobases (kb)] is indicated at the top. The start codon (ATG) and stop codon (TAA) are indicated at the top. The two known mutations inducing CBG deficiency are indicated by arrows (CBG null, c.121G → A in exon 2; and CBG Lyon, c.1254G → A in exon 5). (B) Human CBG and its signal peptide (shaded area). The consensus sites for N-linked carbohydrate chains are indicated by squares; the evolutionarily highly conserved site, essential for the production of CBG with steroid-binding activity, is shown as a black square, and the site that appears to be partially utilized is shown as a diagonally striped square. The position of the elastase cleavage site is indicated by an arrow. The amino acid substitutions corresponding to the mutations in the CBG gene are indicated by arrowheads (CBG null, Trp$^{-12}$Stop; and CBG Lyon, Asp$^{367}$Asn).

ing protein, and interleukin-6 (IL-6) can contribute to the regulation of *Cbg*. It has also been demonstrated recently that HNF-1α and HNF-4 control both the chromatin structure and the gene activity of the entire α1AT/CBG gene locus within the SERPIN gene cluster at 14q32.1.

## IV. PHYSIOLOGICAL VARIATIONS

In mammals, CBG biosynthesis varies considerably during development, independently of maternal hepatic CBG biosynthesis. Fetal hepatic CBG mRNA and plasma CBG levels increase during mid to late gestation and decrease shortly before birth (rabbit, rat, and mouse) or immediately postnatally (sheep). Thereafter, the production of CBG by the liver increases during the first weeks of postnatal life. This decrease in hepatic CBG biosynthesis around the time of birth may control the amount of glucocorticoids available to tissues, especially to the lungs, the glucocorticoid-dependent maturation of which is critical to neonate survival at birth.

In humans, CBG levels are low in cord blood, rise rapidly during the first week of life, and then rise steadily during infancy (Fig. 2). Maximum plasma CBG levels are observed at 4 to 6 years of life. A decrease of 30% is observed before puberty (between 6 and 12 years), and then the level of plasma CBG decreases gradually from 12 to 18 years and remains remarkably stable throughout adult life, except in women during pregnancy. CBG levels rise from the 9th week to the 24th week of pregnancy, reach values two or three times as high as basal levels, and return to nonpregnant levels 1 week after delivery. By analogy to animal models, in which CBG variations during pregnancy reflect changes in CBG mRNA levels, this increment has been said to be associated with the inducing effect of estrogens on CBG biosynthesis rather than to a decreased clearance rate.

Gender differences in CBG levels have been reported: CBG values have a bimodal distribution in

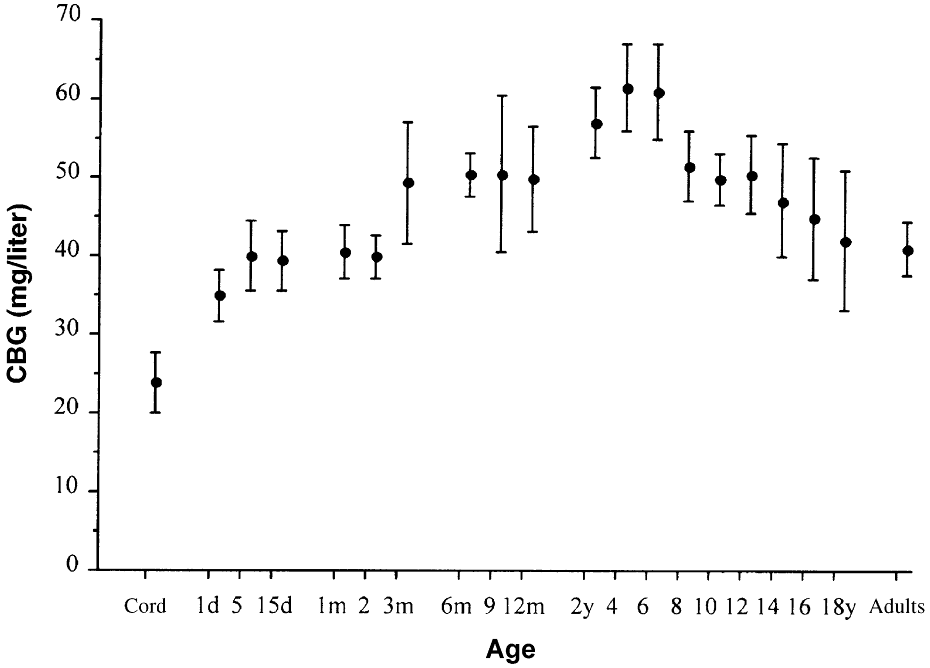

FIGURE 2 Plasma CBG levels (±SEM) according to age from birth to adulthood (cord, cord blood; d, days; m, months; y, years).

men but not in women. No diurnal fluctuation in plasma CBG levels is observed in humans, in contrast to diurnal variations in plasma glucocorticoid levels, and the level of CBG remains unchanged with aging.

## V. PATHOLOGY AND FAMILIAL CBG DEFICIENCY

### A. Acute Inflammation

Acute inflammation (documented in sepsis, burn injury, and cardiac surgery) induces a rapid and prolonged decrease in plasma CBG concentration. This two-step decrease can be explained by (1) an increased CBG clearance rate after proteolytic cleavage by neutrophil elastase and (2) IL-6 repression of hepatic CBG biosynthesis, as suggested by *in vitro* and *in vivo* experiments (Fig. 3). During inflammation, CBG depletion enhances the bioavailability of cortisol and further amplifies the acute stimulation of adrenal function escaping the normal cortisol negative feedback regulation.

### B. Glucocorticoid Excess

Glucocorticoid excess (Cushing's syndrome or glucocorticoid treatment) is associated with low CBG levels, most likely by reduced CBG gene transcription rate, as demonstrated in adult rats. In contrast, the increased cortisol levels observed during depressive syndrome or anorexia nervosa are not associated with any significant decrease in CBG levels.

### C. Insulin-Dependent Diabetes Mellitus

In insulin-dependent diabetes mellitus, CBG levels tend to be elevated and return to normal after insulin treatment. Obese subjects with glucose intolerance have higher CBG levels than do lean or normally glucose-tolerant obese subjects, and CBG levels are negatively correlated with the insulin response to intravenous glucose. These results are in line with evidence that insulin is a potent inhibitor of hepatic CBG production, as demonstrated on a human hepatoma (HepG2) cell line (Fig. 3).

### D. Thyroid Disease

In thyroid diseases, CBG levels have a tendency to be negatively correlated with free thyroid-hormone levels; patients with thyroxin excess have low CBG plasma concentrations and, conversely, hypothyroid patients have high CBG levels. Treatment of hyper- or hypothyroidism normalizes CBG concentrations. The relationship between CBG and thyroid function is unclear.

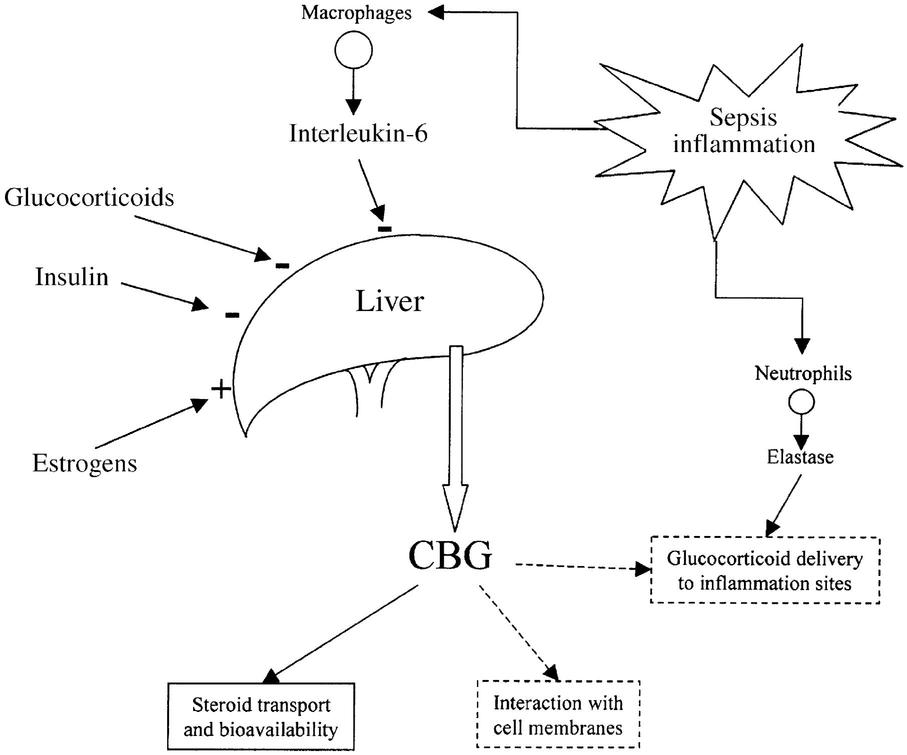
Macrophages
Interleukin-6
Sepsis inflammation
Glucocorticoids
Insulin
Liver
Estrogens
Neutrophils
Elastase
CBG
Glucocorticoid delivery to inflammation sites
Steroid transport and bioavailability
Interaction with cell membranes

FIGURE 3 Schematic representation of CBG biosynthesis regulation and of the roles played by CBG (dashed lines indicate hypothetical roles).

### E. Diseases Involving Protein Metabolism

In diseases involving protein metabolism alterations, such as liver cirrhosis and nephrotic syndrome, the CBG plasma concentration is decreased proportionally to the decrease in albumin concentration.

### F. Familial CBG Deficiency

Familial CBG deficiency is a rare occurrence. Recently, two independent mutations (one in exon 2 and one in exon 5) have been described in patients with unexplained fatigue, obesity, low blood pressure, and abnormally low basal and adrenocorticotropic hormone (ACTH)-stimulated cortisol concentrations despite normal free urinary cortisol and ACTH levels. The low cortisol concentrations in CBG-deficient patients illustrate the crucial effect of CBG on the metabolic clearance rate of cortisol, whereas the normal functioning of the hypothalamo–pituitary–adrenal axis indicates that negative cortisol feedback on ACTH secretion is exerted by the free cortisol fraction, which is in the normal range in these patients. Interestingly, obesity is a common clinical feature in CBG-deficient subjects and may be explained by enhanced cortisol activity on adipose tissue, since it has recently been shown that these patients have low CBG mRNA expression in preadipocytes.

## VI. ROLE OF CBG

### A. Steroid Transport and Bioavailability

The main circulating cortisol fraction is bound to CBG (90 to 95%), suggesting that CBG may provide a buffer reservoir of cortisol in the vascular compartment that can become rapidly available to the free and active hormone pool by simple dissociation from CBG-binding sites. During pregnancy, increased CBG levels may optimize this cortisol reservoir function of CBG.

CBG protects cortisol from peripheral metabolism and thus reduces the rate of synthesis required by the adrenal to maintain a given level of unbound or biologically active hormone.

### B. Glucocorticoid Delivery to Inflammation Sites

As a member of the SERPIN family, CBG is specifically cleaved by elastase released at the surface of activated neutrophils. It is unlikely that elastase cleavage of CBG occurs in the general circulation because of the large surplus of α1AT (the main inhibitor of elastase). Nevertheless, at inflammation sites, CBG cleavage can occur at the surface of activated neutrophils that produce superoxide anions inactivating α1AT. Thus, large amounts of glucocorticoid could be released directly to inflammatory cells.

## C. CBG Interaction with Cell Membranes

Although the intracellular presence of CBG can be attributed to *de novo* synthesis in some cases, it can only be explained by sequestration from blood circulation in many other instances, and this may involve a process of cellular internalization. In support of this notion, a specific interaction has been demonstrated between CBG and binding sites on the plasma membrane of various cell types, and the binding is influenced by the conformation of CBG and by the structure of its carbohydrate chains. Furthermore, a CBG–receptor complex has been characterized on placental plasma membranes. It has also been shown that CBG binding to trophoblast cells increases intracellular cyclic AMP levels. Taken together, these findings suggest that CBG could be involved in the guided transport of steroid hormones to target cells and transmembrane transfer of hormones and/or hormonal signals.

## Glossary

**free hormone hypothesis** Plasma-specific binding proteins sequester circulating hormones and therefore only the non-protein-bound fraction ( = free) of hormones is available for movement out of capillaries and for cell biodisposal, where it may either initiate a biological response or be metabolized and cleared from the circulation.

**interleukin-6 (IL-6)** One of the major cytokines secreted by macrophages. IL-6 induces the synthesis of hepatic acute-phase proteins and activates the hypothalamic–pituitary–adrenal axis during inflammation.

**serine protease inhibitors (SERPIN)** Superfamily of proteins that undergo a relaxed/stressed transition state on binding with serine protease to form an inactive complex. The subgroup including corticosteroid-binding globulin, α1-anti-trypsin, and α1-anti-chymotrypsin derives from a common ancestral gene by gene duplication.

## See Also the Following Articles

**Glucocorticoid Biosynthesis • Glucocorticoid Effects on Physiology and Gene Expression • Glucocorticoid Receptor, Natural Mutations of • Glucocorticoid Resistance • Heterodimerization of Glucocorticoid and Mineralocorticoid Receptors • Interleukin-6 • Mineralocorticoid Biosynthesis • Mineralocorticoid Effects on Physiology and Gene Expression • Mineralocorticoid Receptor, Natural Mutations of**

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# TRH

See *Thyrotropin-Releasing Hormone*

# TSH

See *Thyroid Stimulating Hormone*

# Thymic Stromal Lymphopoietin (TSLP)

Steven D. Levin[*‡], David J. Rawlings[*], Steven F. Ziegler[†], and Andrew G. Farr[*]

[*]*University of Washington* ● [†]*Virginia Mason Research Center, Seattle, Washington* ● [‡]*ZymoGenetics, Inc., Seattle*

I. CLONING AND CHARACTERIZATION OF TSLP
II. CHARACTERIZATION OF THE TSLP RECEPTOR COMPLEX
III. SIGNAL TRANSDUCTION THROUGH THE TSLP RECEPTOR
IV. FUNCTION OF TSLP *IN VITRO*
V. BIOLOGY OF TSLP *IN VIVO*
VI. CONCLUSIONS

**Thymic stromal lymphopoietin was first identified as an activity in supernatants from the thymic stromal cell line, Z210R.1. This cell line was derived from medullary thymic epithelium from an adult Balb/C mouse. Specifically, conditioned media from Z210R.1 cells supported the outgrowth of B lymphocytes from fetal liver precursors, and this activity was determined to be distinct from IL-7 and other known cytokines.**

## I. CLONING AND CHARACTERIZATION OF TSLP

Using a cDNA library from Z210R.1 cells and a transient expression strategy, Sims *et al.* isolated a cDNA clone whose product supported the growth of the fetal liver-derived pre-B-cell line NAG8/7 in a bioassay. The cDNA encoded a protein of 140 amino acids, including the 19-amino-acid leader sequence. The mature polypeptide contained seven cysteine residues and three potential N-linked glycosylation sites. Further examination showed that the protein was predicted to fold into the canonical four-helix bundle structure common to members of the cytokine family. Expression studies have shown that thymic stromal lymphopoietin (TSLP) mRNA is most highly expressed in thymus and lung, with lower levels found in a wide variety of tissues. The *Tslp* gene was localized to mouse chromosome 18.

Recently, a cDNA clone encoding human TSLP was isolated using database search methods. The predicted amino acid sequence of the cDNA showed 43% identity with the mouse TSLP sequence and was also predicted to form a four-helix bundle structure. For both mouse and human TSLP, the most closely related member of the cytokine family was interluekin-7 (IL-7), consistent with the similarities in the biological properties of these cytokines (see below).

## II. CHARACTERIZATION OF THE TSLP RECEPTOR COMPLEX

Using radiolabeled TSLP, Park and colleagues identified two classes of TSLP-binding sites, high affinity (ranging from $7 \times 10^9\ M^{-1}$ to $1.0 \times 10^{10}\ M^{-1}$) and low affinity ($< 4 \times 10^8\ M^{-1}$). All of the cell lines that were capable of binding TSLP were of hematopoietic origin, including those derived from the B-cell, T-cell, and monocyte lineages. No binding of the mouse protein was found on any human cell line tested, suggesting that TSLP binding was species-specific. Interestingly, all cell lines found to bind TSLP were also capable of binding IL-7. A direct expression cloning strategy was used to isolate cDNA clones that encode a protein capable of binding TSLP. Using either database searching or a signal-sequence trap, three other groups also isolated cDNA clones shown to encode the mouse TSLP receptor (TSLPR). In all three cases, the cDNA clones were predicted to encode a 359-amino-acid polypeptide with a resemblance to members of the cytokine receptor family. Homology studies showed that the common cytokine receptor γ-chain (γc) was most closely related to the deletion of the TTP gene or deletion of the UA-rich element causes TNF-dependent systemic inflammatory disease.

Probably all of the TLRs, when activated, stimulate TNF production in some measure. Other stimuli, including physical stimuli such as ultraviolet light, can also activate TNF production, albeit in far lower quantities. Other cytokines may also activate TNF synthesis under some conditions. However, TNF occupies a position near the apex of the cytokine cascade that occurs following macrophage activation, and it has a greater propensity to induce the synthesis of other cytokines than to be induced. The proximal cause of TNF production in diseases such as rheumatoid arthritis and Crohn's disease is not known.

## III. STRUCTURE

TNF, a homotrimer, is synthesized as a transmembrane protein but is efficiently released from membrane anchorage by the action of a matrix metalloproteinase, TNFα-converting enzyme (TACE), concurrent with synthesis, or, in some instances, at the cell surface. A trimeric quaternary structure is essential for biological activity, insofar as the protein must stimulate a conformational response in a dimeric receptor, which engages two of three identical active sites on the ligand surface. Bivalent inhibitors of TNF activity are highly effective because they occupy two of the three binding sites, leaving only one site available for receptor binding.

Paralogous proteins in the TNF superfamily are also trimeric, although some are heteromers [e.g., lymphotoxinβ (LTβ)], and most remain stably attached to the cell membrane. In this regard, the sole exception is lymphotoxinα (LTα), which is entirely secreted. The three-dimensional structure of TNFα and LTα have been solved crystallographically. Likewise, the liganded and unliganded p55 TNF receptor ectodomain structure has been solved. These studies contribute to a model of receptor activation events.

## IV. RECEPTORS AND SIGNAL TRANSDUCTION

The TNF protein binds to two receptors, both of which have elongated extracellular domains with four cysteine-rich repeat motifs. Different numbers of these repeats are observed in paralogous members of the TNF receptor family, and, indeed, define the family. The type I (p55) TNF receptor is distinguished from the type II (p75) TNF receptor by the presence of a "death domain" motif in the cytoplasmic domain of the former. The death domain permits heterotypic interaction with signaling molecules such as Fas-associated death domain (FADD), TNF receptor-associated death domain (TRADD), and receptor-interacting protein (RIP), which have similar domains of their own.

Death domains ultimately elicit cell death by means of caspase recruitment and activation. FADD, in particular, is known to activate caspase-8, with ensuing activation of more distal cell apoptotic machinery. Among the critical targets for caspase cleavage is polyadenosine diphosphate (polyADP)-ribose polymerase (PARP), which is believed to kill cells when activated by consuming cellular nicotinamide adenine dinucleotide (NAD). Other targets, yet unknown, may also participate in cell killing.

The TNF signal transduction pathway is complex and, in part, is mimicked by the *imd* (immunodeficient) pathway in *Drosophila melanogaster*, which is responsible for sensing gram-negative bacterial infection. The *Drosophila* genome does not encode orthologues of TNF or either TNF receptor, but the *imd* gene encodes a facsimile of RIP, one of the transducers of TNF signals in mammals, and mutations of *imd* render flies susceptible to infection. RIP and *Drosophila* FADD signal a *Drosophila* death effector domain-like domain (DREDD), a homologue of caspase-8, *Drosophila* TAK-1, and kenny, a homologue of IκB kinase γ (IKKγ), to activate Relish, a homologue of NF-κB. *Galere*, a gene encoding a homologue of Tab2, also affects signaling through the pathway, and at the apex, a sensing protein similar to mammalian peptidoglycan recognition proteins (PGRPs) is required to transduce bacterial signals across the membrane.

In mammals, TRADD and RIP engage the strongly proapoptotic p55 TNF receptor and signal to activate proteins of the caspase cascade, as well as NF-κB. FADD, which serves the Fas receptor, is activated by interaction with TRADD. The p75 TNF receptor can, under some circumstances, mediate cell death as well, although the mechanism by which it does so is unclear. It is best known to engage members of the (TNF receptor-associated factor (TRAF) family, which leads to the activation of NF-κB. In mice, p75 stimulates lymphoid proliferation, although it also contributes to the net toxic effect of TNF.

Some of the proinflammatory effects of TNF are undoubtedly mediated by both receptors. Gene knockout work, however, has established that the preponderance of the toxicity of TNF is mediated through the p55 receptor, as is the tumoricidal effect. Receptor-selective TNF mutants have been fashioned and used to dissociate cytotoxic from growth-stimulatory effects of the two receptor subtypes.

## V. BIOLOGICAL EFFECTS

Shock and inflammation are caused by TNF, which to a large extent mediates the effects of microbial toxins such as LPS. At the same time, TNF offers protection against a wide array of pathogens, including viruses (some of which have fashioned systems for TNF inhibition), gram-positive bacteria, mycobacteria, and protozoa. It is quite clear that this immunoprotective, innate immune function is the central raison d'être of TNF.

Some chronic inflammatory and autoimmune disorders are clearly orchestrated by TNF, insofar as TNF blockade has ameliorative effects, and biologically significant amounts of TNF can be found within involved tissues or in biological fluids that surround them. Both the toxic and beneficial effects of TNF are mediated by its proinflammatory actions, which include neutrophil activation, a procoagulant effect on the vascular endothelium, stimulation of proteolytic enzyme synthesis, and enhancement of microbicidal activity (including enhancement of oxidative radical production) within various cells. Hence, both the benefits and liabilities of inflammation are embodied within this single molecule and the signals that it initiates.

A developmental role for the TNF type I receptor is seen in its requirement for normal development of germinal centers and follicular dendritic cell clusters in the spleen and other peripheral lymphoid organs. LTα signaling as a part of the LTα/LTβ heteromer via the LTβ receptor has an even more pronounced role in this regard, acting to organize lymphoid tissue throughout the body. The tumorolytic effect that gave TNF its name is now seen as the combined result of the procoagulant activity that TNF exerts with disproportionate efficacy in tumor vasculature, and the apoptotic effect that it exerts on sensitive tumor cells. It is apparently something of a curiosity in a biological sense, insofar as mice lacking TNF do not seem to be more susceptible to the *in vivo* development of tumors.

## VI. THERAPEUTIC MEASURES THAT INVOLVE TNF

Once regarded primarily as an antineoplastic drug—biotechnology's premiere reply to cancer—TNF proved to be far too toxic in humans to permit routine use for induction of remissions in patients with metastatic neoplasia. Isolated limb perfusion with TNF has been used with some success to treat localized tumors, such as melanomas that have not yet produced visible metastases. The greatest therapeutic success has been witnessed in the arena of TNF inhibition. Although TNF blockade has not shown significant efficacy as a means of treating septic shock, it has been highly effective in the management of selected chronic inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and ankylosing spondylitis. Psoriatic arthritis has also been approached with TNF blockade, with promising results. Inhibition of TNF activity is generally not considered to be effective therapy for multiple sclerosis, ulcerative colitis, osteoarthritis, heart failure, or systemic lupus erythematosus. Indeed, TNF blockade appears to induce signs and symptoms of SLE in a small proportion of patients. This observation, taken together with the fact that several autoimmune diseases involve defects in cell death pathways, suggests that TNF-mediated apoptosis is normally required for the removal of cells that cause certain types of autoimmunity.

Although macromolecules with high specificity have been used as the most effective means of blocking TNF activity, other approaches aimed at preventing TNF signaling or preventing TNF synthesis have also been entertained. A number of small molecular antagonists of LPS signaling have been described, and some of them may also block TNF production under other circumstances. Among these, thalidomide, pentoxifylline, p38 MAPK inhibitors, and glucocorticosteroids have been studied extensively. Each class of agents displays numerous side effects, however, and in some cases, poor efficacy. Specific inhibitors of TNF or TNF receptor processing have not yet been used for clinical effect.

## VII. GENETIC VARIABILITY AT THE TNF LOCUS

Although no major coding variants of the TNF gene have been reported, mutations affecting promoter sequences have been identified and exist at fairly high (polymorphic) frequency in the normal population. Numerous attempts have been made to link these polymorphisms to human disease phenotypes and, in particular, to infectious disease susceptibility or outcome. The results have been conflicting; to date, no solid proof of any relationship between a given TNF isoform and any specific phenotype has ever been made. In all events, it is possible to explain associations by linkage disequilibrium; in the most rigorous analyses, no difference in gene expression has been shown to result from mutational differences within the promoter region.

## VIII. PRESENT CHALLENGES IN TNF RESEARCH

The TNF field has reached a high degree of maturity. Nonetheless, there remains much uncertainty about fine details of the TNF signaling pathways, and there are inviting possibilities that bear on the selective exploitation of these pathways. Small molecular TNF mimetic drugs might yet see a role in cancer chemotherapy, provided that their effects are channeled so as to split the proapoptotic effect of the cytokine from the proinflammatory effects. In addition, approximately 30 paralogous genes encode all of the members of the TNF receptor and ligand families in mammals. To some extent, cooperativity and receptor "sharing" occur, so that a higher degree of complexity is observed in specific target cell populations. Considerable work must still be devoted to deciphering the pathways that are involved.

## Glossary

**apoptosis** Programmed cell death.

**Crohn's disease** Form of inflammatory bowel disease in which TNF is inappropriately produced and contributes to disease.

**death domain** Signature motif of the type I TNF receptor, responsible for inducing programmed cell death. Also found in other members of the TNF receptor superfamily.

**endotoxic shock** Severe, acute disorder caused by gram-negative infection; excess TNF is produced in response to bacterial lipopolysaccharide as a result of signaling via Toll-like receptor 4. Marked by fever, hypotension, and inadequate tissue perfusion.

**lipopolysaccharide** Principal glycolipid (also called endotoxin) of the outer membrane of gram-negative bacteria. The most potent known inducer of TNF synthesis.

**lymphotoxin** The closest paralogue of TNFα, but produced by different cells; also referred to as LTα and as TNFβ; also important for normal immune development and function.

**rheumatoid arthritis** Autoimmune inflammatory disorder, affecting joints and other tissues, in which TNF is inappropriately produced and contributes to disease.

**Toll-like receptors** Primary sensors of the innate immune system; named after the *Toll* gene found in *Drosophila*, provide essential signals for TNF production under conditions of infection.

**tumor necrosis factor** Proinflammatory cytokine mediator essential for normal immune development and function; the factor first discovered to be produced primarily by macrophages, also referred to as TNFα and as TNF.

## See Also the Following Articles

**Apoptosis • Anti-Inflammatory Actions of Glucocorticoids • CC, C, and CX$_3$C Chemokines • CXC Chemokines • Defensins • Flt3 Ligand • Glucocorticoids and Autoimmune Diseases • Interferons: α, β, ω, and τ • Interleukin-1 (IL-1) • Osteoporosis: Pathophysiology • Pro-Inflammatory Cytokines and Steroids**

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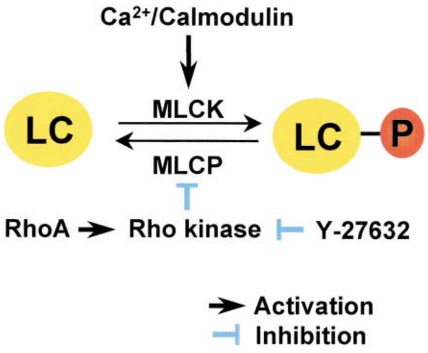

**FIGURE 2** The ratio of activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) affects contraction and relaxation in smooth muscle. Increased intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) activates calmodulin/MLCK, resulting in increased light chain (LC) phosphorylation and contraction. Inhibition of MLCP by Rho kinase linked to receptor activation sensitizes myosin light chain phosphorylation to $[Ca^{2+}]_i$.

The intracellular $Ca^{2+}$ concentration remains the mainstay for control of myometrial contraction. In the uterus, in the absence of extracellular $Ca^{2+}$ the force of contraction is $< 5\%$ of that obtained in the presence of extracellular $Ca^{2+}$. Thus, in myometrium, it appears that influx of $Ca^{2+}$ through voltage-dependent $Ca^{2+}$ channels in the sarcolemma is the main source of activating $Ca^{2+}$ for initiation of contraction. However, the intimate relationship between the release of $Ca^{2+}$ from the sarcoplasmic reticulum and the influx of $Ca^{2+}$ through $Ca^{2+}$-induced $Ca^{2+}$-release mechanisms confounds the precise localization of activating $Ca^{2+}$ sources. In addition, the release of $Ca^{2+}$ from inositol trisphosphate ($IP_3$)-dependent $Ca^{2+}$ stores provides additional layers of $Ca^{2+}$ regulation. Particular pathways of excitation–contraction coupling differentially affect $Ca^{2+}$ homeostasis. Elevation of $[Ca^{2+}]_i$ sets in motion the activation of the contractile elements by $Ca^{2+}$/calmodulin-dependent phosphorylation of myosin. Alterations in the $Ca^{2+}$ sensitivity of force may arise from effects on regulatory light chain phosphorylation or other contractile proteins.

In myometrial tissues, electrical depolarization results in rapid and synchronous depolarization of smooth muscle cells, influx of extracellular $Ca^{2+}$, and force development. Increases in $[Ca^{2+}]_i$ precede light chain phosphorylation and force development with immediate increases in $[Ca^{2+}]_i$ after electrical stimulation. After a period of latency (200 ms), significant increases in regulatory light chain phosphorylation and force occur, and these values decrease as a function of time despite maintenance of force. In uterine smooth muscle, maintenance of force in the presence of declining levels of light chain phosphorylation is not sustained for long periods of time. Nevertheless, the recruitment of nonphosphorylated cross-bridges into force-bearing structures may be an important mechanism for force generation in uterine smooth muscle during pregnancy.

## B. Activation of Contraction in Uterine Smooth Muscle During Pregnancy

In myometrium from nonpregnant women, spontaneous contractions ($\sim 10$ per hour) are associated with cyclic increases in $[Ca^{2+}]_i$ and phosphorylation/dephosphorylation of myosin. Myosin light chain phosphorylation increases from 0.10 to 0.49 mol phosphate per mole of light chain within 10% of the time required to develop the maximal force of spontaneous contraction. During the declining phase of the contraction, force and light chain phosphorylation dissociate. Experiments from a number of laboratories indicate that, although myosin light chain phosphorylation plays an important role in the initiation of uterine contraction, other cellular mechanisms are involved in the regulation of contraction and relaxation, particularly during pregnancy. In most mammalian species, maximal force generation capacity is increased in myometrial tissues during pregnancy. In myometrium from pregnant women, similar levels of force per cross-sectional area develop in nonpregnant and pregnant women. However, the rate and extent of light chain phosphorylation during contraction are significantly diminished in tissues from pregnant women. The extent of myosin light chain phosphorylation in contracting myometrial tissues from pregnant women is very low. Modifications in $[Ca^{2+}]_i$ may be responsible for these observations because they are not due to changes in the amounts of contractile proteins or the activities of myosin light chain kinase or phosphatase. Other signal transduction pathways may be involved.

## C. Mechanisms of Uterine Smooth Muscle Relaxation

Decreases in $[Ca^{2+}]_i$, brought about by $Ca^{2+}$ extrusion or uptake into the SR, result in inactivation of MLCK, regulatory light chain dephosphorylation by myosin phosphatase, and muscle relaxation. Decreases in $[Ca^{2+}]_i$ are mediated predominantly by decreases in membrane potential and extrusion of cytoplasmic $Ca^{2+}$ by plasma membrane $Ca^{2+}$ pumps or uptake of

$Ca^{2+}$ into the sarcoplasmic reticulum by SR $Ca^{2+}$ ATPases.

Recently, the search for mediators of uterine relaxation that maintain uterine quiescence during pregnancy has been intense. For many years, cyclic AMP (cAMP) was identified as a mediator of uterine smooth muscle relaxation, and the mechanism of relaxation has been shown to occur primarily through a lowering of $[Ca^{2+}]_i$. In addition, activation of soluble guanylate cyclase by nitric oxide leading to increased tissue levels of cyclic GMP (cGMP) has been identified as a major mechanism of cGMP-induced vasorelaxation through activation of cGMP-dependent protein kinase. Activation of cGMP-dependent protein kinase also results in decreased free intracellular $Ca^{2+}$ and increased myosin phosphatase activity in vascular smooth muscle. Although evidence supporting a role for this signaling pathway in maintenance of uterine quiescence during pregnancy has been published, experimental evidence from other studies conducted both *in vivo* and *in vitro* indicates that mechanisms other than cGMP-induced activation of cGMP-dependent protein kinase regulate myometrial relaxation during pregnancy.

### D. Role of Other Thin Filament-Associated Proteins

Two thin filament-associated proteins, h-caldesmon and calponin, are believed to modify contractility by inhibiting actin-activated myosin ATPase activity and, in the case of caldesmon, by tethering actin to myosin and inhibiting the velocity of actin/tropomyosin filaments in the presence of nonphosphorylated myosin. Caldesmon is a basic protein associated with actin filaments. In uterine smooth muscle cells, caldesmon is the most abundant calmodulin-binding protein. Calponin is a smooth muscle-specific, thin-filament protein with biochemical properties very similar to those of caldesmon. However, the subcellular distribution of these two proteins differs in smooth muscle cells. The total amounts of caldesmon are increased four- to fivefold in pregnant myometrium. Although the total amount of calponin does not increase in pregnant myometrium, calponin is redistributed in pregnant myometrium with increased amounts in the cytoplasmic fraction and decreased amounts in the myofilaments, suggesting a lower affinity for cytoskeletal and myofilament proteins during pregnancy. At this time, it is not clear whether these changes in thin filament proteins are involved in the regulation of uterine contractility during pregnancy.

### E. Anatomic and Cellular Considerations of Uterine Contractility

Myometrium belongs to the broad classification of smooth muscles termed phasic smooth muscle. Phasic smooth muscle is characterized by transient increases in force generation in response to $K^+$, initiation of force by action potentials inherent in the muscle cell, and differential expression patterns of contractile proteins. In general, phasic contractile activity of the myometrium (either spontaneous contractions or in response to $K^+$) is a response to the underlying electrical activity of the muscle cells. As such, contraction and relaxation of myometrium result from the cyclic depolarization and repolarization of muscle cell membranes, and the driving force for contractility is extracellular $Ca^{2+}$. The action potentials (voltage- and time-dependent changes in membrane ionic permeabilities) are characterized by depolarization and repolarization phases. The depolarization phase is due to an inward current carried predominantly by $Ca^{2+}$ ions and $Na^+$ ions. The outward current (repolarization) is carried by $K^+$ ions consisting of fast voltage-dependent and slow $Ca^{2+}$-activated components. Thus, the frequency and intensity of myometrial contractions are proportionate to the consistency and duration of action potentials in each muscle cell and the total number of cells that are active. The number of cells activated in response to the action potential is thereby determined by the propagation of the electrical impulses in myometrial cells, i.e., low-resistance pathways for current spread. Intercellular channels specified as gap junctions link cells by allowing the passage of inorganic ions and small molecules from one cell to another. Uterine contractions are thereby controlled by intracellular signal transduction mechanisms and by intercellular communications involving the synthesis, organization, size, and gating of gap junctions.

## II. REGULATION OF CA$^{2+}$ SENSITIVITY OF THE CONTRACTILE ELEMENTS

### A. $Ca^{2+}$Sensitivity of Myosin Light Chain Phosphorylation

#### *1. Regulation of Myosin Light Chain Phosphorylation by Phosphorylation of Myosin Light Chain Kinase*

The sensitivity of MLCK to activation by $Ca^{2+}$/calmodulin is diminished upon phosphorylation at a regulatory site A. As predicted, phosphorylation of MLCK has been shown to desensitize light chain phosphorylation to $[Ca^{2+}]_i$. Initial investigations on the phosphorylation-dependent desensitization of MLCK focused on the possibility that phosphorylation of MLCK by cAMP may be a potential mechanism whereby increases in cAMP and activation of protein kinase A lead to the relaxation of smooth muscle. In human myometrium and other smooth muscles, however, cAMP does not cause relaxation via phosphorylation of MLCK. Nevertheless, MLCK is phosphorylated at site A by calmodulin kinase II, resulting in desensitization of the contractile elements to elevated levels of intracellular $Ca^{2+}$. During the initiation of contraction and rapid phase of light chain phosphorylation (between 0.5 and 2 s in electrically stimulated tissues), there are no changes in MLCK activation properties. Thereafter, MLCK is phosphorylated and the $Ca^{2+}$ sensitivity of light chain phosphorylation is diminished.

#### *2. Regulation of Myosin Light Chain Phosphorylation by Myosin Phosphatase Activity*

The $Ca^{2+}$ sensitivity of myosin regulatory light chain phosphorylation is affected by myosin phosphatase activity. Protein serine/threonine phosphatases are regulated by protein–protein interactions with formation of oligomeric complexes that direct phosphatase catalytic subunits toward specific substrates by association with regulatory proteins. In addition, phosphatase subunits and their inhibitor proteins are regulated by phosphorylation. Smooth muscle myosin light chain phosphatase is a holoenzyme consisting of three subunits: a 38 kDa catalytic subunit (PP1C), a myosin-targeting subunit (MYPT, 110–133 kDa), and a small 20 kDa subunit. The small subunit has no established function and is not required for either catalytic activity or activation of the catalytic subunit.

Two well-described myosin phosphatase inhibitory pathways may result in increased levels of myosin phosphorylation and force in response to contractile agonists. Although most of the work in this area has been conducted in other muscles, recent studies suggest that these pathways may be involved in the regulation of myometrial contractions as well. The first is through the small GTPase RhoA, in which GTP-bound RhoA translocates to the membrane and activates Rho kinase. Activation of Rho kinase results in inhibition of myosin phosphatase activity either through direct phosphorylation of the myosin-binding subunit or through other indirect mechanisms. Dissociation of $PP1_C$ from the M complex occurs by phosphorylation of MYPT. Dissociation of the catalytic subunit from the myosin-targeting subunit results in the inhibition of phosphatase activity. The second signaling pathway, present in only some smooth muscles, involves phosphorylation of a phosphatase inhibitor, CPI-17 (PKC-potentiated phosphatase inhibitor protein-17 kDa), CPI-17 phosphorylation inhibits the catalytic subunit of myosin phosphatase. Currently, there is no information regarding the role of CPI-17 in uterine smooth muscle.

In myometrium, the expression of RhoA protein does not change during pregnancy. Expression of Rho kinase, however, increases in the uterus during pregnancy. The potential role of the RhoA/Rho kinase pathway in oxytocin-induced uterine contraction has been reported. A Rho-kinase inhibitor, Y-27632, inhibits oxytocin-induced rat uterine contraction on day 21 of pregnancy in a concentration-dependent manner. In rat myometrium, Y-27632 has no effect on oxytocin-induced increases in intracellular $Ca^{2+}$; yet, it is reported that oxytocin-induced increases in light chain phosphorylation are attenuated. Oxytocin increases the phosphorylation of MYPT, and the Rho-kinase inhibitor Y-27632 reduces phosphorylation of the phosphatase subunit by oxytocin. These results indicate that agonist-induced contractions involving G-protein-coupled receptors in the uterus may involve $Ca^{2+}$ sensitization through Rho-kinase-associated inhibition of myosin phosphatase. In addition to the well-described up-regulation of oxytocin receptors during pregnancy, up-regulation of Rho kinase in the myometrium during pregnancy may augment responsiveness to oxytocin.

### B. Myosin Light Chain Phosphorylation/Force Relationship

Conditions in which small increases in light chain phosphorylation result in large increases in force have been most frequently reported when smooth muscles are stimulated with activators of protein kinase C (PKC). In human myometrium, it appears that PKC augments agonist-induced contractions. In some smooth muscles, activators of PKC act to cause a slow, forceful contraction, but light chain phosphorylation is not increased. In rat myometrium, phorbol esters inhibit contraction by inhibiting increases in $[Ca^{2+}]_i$. It should be noted that PKC activators may also stimulate contractions with concomitant increases in $[Ca^{2+}]_i$ and phosphorylation of myosin light chain in some smooth muscles from some species.

Several laboratories working with a variety of smooth muscle types have reported that force may be desensitized (rather than sensitized) to increases in light chain phosphorylation. For example, both arterial and uterine muscles are relaxed upon addition of high external $Mg^{2+}$ without reduction in myosin light chain phosphorylation, and high concentrations of sodium nitroprusside have been shown to relax arterial smooth muscle without decreases in light chain phosphorylation. It has been proposed that complex interactions of the contractile proteins with thin filament regulatory proteins may alter the myosin light chain phosphorylation/force relationship, particularly in myometrium during pregnancy.

## III. MODULATION OF UTERINE CONTRACTILITY BY STEROID HORMONES AND PREGNANCY

### A. Estrogen and Progesterone

It has been suggested that the contractile phenotype of uterine smooth muscle is increased by estradiol treatment and decreased by progesterone. The concept that estrogen promotes a “contractile” state and progesterone gives rise to “quiescence” suggests that the tissue functions as one or the other of these physiological states depending on the hormonal milieu. In general, procontractile mechanisms are expressed and function under estrogen domination, and prorelaxant mechanisms act under the influence of progesterone. Many examples in the literature suggest that this paradigm may be operative in myometrial cells. Estrogen results in increased expression of oxytocin receptors, connexin-43, and smooth muscle myosin. Estrogen also acts to increase smooth muscle-specific expression of cGMP-dependent protein kinase, and this protein is involved in smooth muscle relaxation. Progesterone opposes the effect of estrogen on many, but not all, estrogen-regulated myometrial proteins. Thus, in most species at term, progesterone withdrawal, together with increasing levels of estrogen, leads to increased expression of oxytocin receptors, cervical collagenase, and formation of gap junctions, thereby providing effective contractions of labor and cervical ripening.

In ovariectomized animals, estrogen increases the expression of oxytocin receptor mRNA, even if combined with progesterone. Importantly, however, progesterone inhibits estrogen-induced increases in oxytocin-binding sites. It has been shown that progesterone inhibits oxytocin receptor binding and oxytocin-induced increases in the generation of intracellular second messengers though nongenomic mechanisms. The precise mechanisms of steroid-induced modulation of G-protein-linked receptor signaling remain to be determined.

### B. Pregnancy

This article has alluded to the effects of pregnancy on the contractile machinery of uterine smooth muscle. Although beyond the scope of this article, it should be noted that the uterus, like other smooth muscles, is regulated by the expression of a number of G-protein-coupled receptors. The best characterized of uterine G-protein-coupled receptors is the high-affinity oxytocin receptor.

Unlike the closely related vasopressin receptors, oxytocin receptor expression is highly regulated in a tissue-specific manner. Specifically, prior to parturition, uterine oxytocin receptors are increased up to 100-fold. Although, in the human, increases in oxytocin receptor are less dramatic than in many other species (approximately 2-fold), virtually every mammalian species exhibits changes in the expression of uterine oxytocin receptors before or during parturition. Moreover, expression of the receptor decreases dramatically within 24 h of parturition. Temporal expression patterns in the uterus are unique compared with patterns in the brain, mammary gland, and kidney. In addition, uterine stretch seems to be required for dramatic parturition-associated changes in uterine oxytocin receptor gene expression. These findings suggest that myometrial cell-specific transcriptional factors regulate oxytocin receptor gene expression.

## IV. SUMMARY

There have been major advances in the understanding of the regulation and physiological functions of contractile proteins in myometrium in recent years. Phosphorylation of the regulatory light chain of myosin by $Ca^{2+}$/CaM-dependent MLCK plays a pivotal role in activation. The simple view that contractile force in smooth muscle is proportionate to $[Ca^{2+}]_i$ and myosin light chain phosphorylation has been modified as additional experiments have provided insights into the mechanisms of regulation of the contractile elements. It is apparent that, although $[Ca^{2+}]_i$ is undoubtedly the master mediator of regulation, additional pathways act to modulate the regulatory components, particularly during pregnancy. The sensitivity of myosin light chain phosphorylation to $[Ca^{2+}]_i$ can be shifted by second messengers modulating the activities of MLCK and myosin phosphatase. MLCK is phosphorylated, which desensitizes its activation by $Ca^{2+}$/CaM; protein phosphatase activity toward myosin is also regulated by phosphorylation and dephosphorylation of regulatory subunits or cytosolic inhibitors. The dependence of force on myosin phosphorylation is established in the steady state of tonic contractions; however, in uterine smooth muscle, transient increases and decreases in light chain phosphorylation parallel cyclic increases in contractile force. Pregnancy can shift the relationship between regulatory light chain phosphorylation and force development, indicating that the number of cross-bridges can be altered independent of myosin light chain phosphorylation. The physiologic importance and relative contributions of other regulatory components for uterine smooth muscle function *in vivo* are not entirely clear. Moreover, the role of these mechanisms in various hormonal states and pregnancy remains to be defined. The uterus exhibits physiologic properties unique from other smooth muscle types characterized by unique ligand receptors, electrical properties, and $Ca^{2+}$ homeostatic mechanisms. In addition, there is growing appreciation for differences in contractile protein contents and differing patterns of expressed contractile protein isoforms in the uterus during pregnancy. The future holds exciting new information that will increase understanding of the regulatory processes in uterine contractility.

## Acknowledgments

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## Glossary

**G-protein** Any of a class of guanine nucleotide-binding proteins associated with the cytoplasmic face of the plasma membrane of mammalian cells; proteins involved in transmitting signals from certain types of hormone and neurotransmitter receptors to intracellular pathways.

**protein kinase** An enzyme that phosphorylates proteins, specifically phosphorylating the amino acids serine, threonine, and tyrosine.

**sarcoplasmic reticulum** A membranous organelle system of muscle cells, composed of vesicular and tubular components, that stores calcium ions involved in muscle contraction.

## See Also the Following Articles

**Oxytocin/Vasopressin Receptor Signaling • Placental Development • Progesterone Action in the Female Reproductive Tract**

## Further Reading

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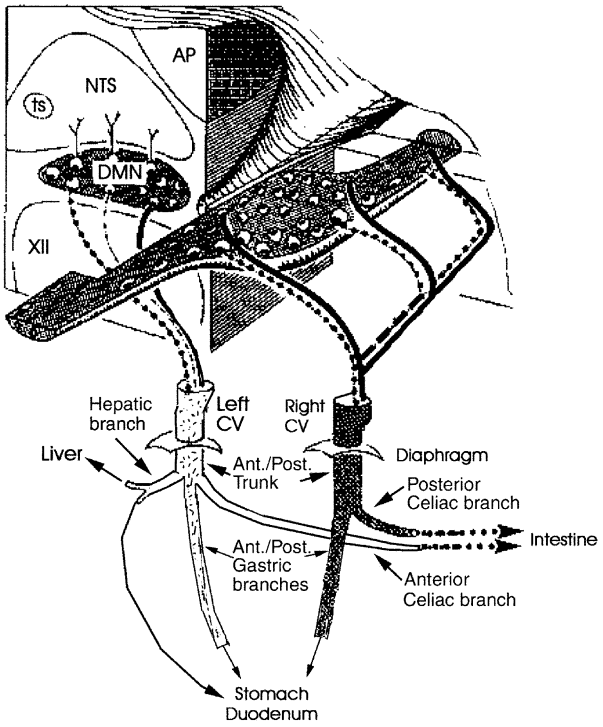

FIGURE 1 Rostrocaudal schematic representation of DMN neurons providing vagal innervation of the stomach through the gastric and hepatic branches. Dendritic projections of DMN motoneurons into the nucleus tractus solitarius are also shown. AP, area postrema; CV, cervical vagus; NTS, nucleus tractus solitarius. Reprinted with permission from Powley, T. L., Berthoud, H.-R., Prechtl, J. C. and Fox, A. E. (1991). Fibers of the vagus regulating gastrointestinal function. *In* "Brain–Gut Interactions" (Y. Taché and D. Wingate, eds.), pp. 73–82. Copyright CRC Press, Boca Raton, Florida.

the lowest threshold for synaptic activation, DMN neurons projecting to the fundus have smaller and shorter after-hyperpolarization and a higher frequency of action potential firing than neurons projecting to the cecum through the vagal celiac branch. Within the medial column, there is also a site-specific organization of neurons. Those projecting to the fundus are more laterally located and those innervating the antrum/pylorus are more medially positioned.

Neurons in the DMN also display dendritic fields that are spread in the horizontal plane with each column harboring spatial separation. Of particular significance are the dendrites of DMN neurons that extend to the overlying nucleus tractus solitarius (NTS) with the highest density in the subnucleus gelatinosus of the dorsal medial NTS, just rostral to the obex where gastric vagal afferent fibers project. This wiring provides the anatomic basis for gastric vago-vagal reflexes whereby signaling from gastric vagal afferent endings in the dorsal medial NTS feed back onto DMN neurons to modulate vagal outflow to the stomach and thereby regulate gastric functions.

Immunohistochemistry combined with retrograde tracing studies revealed that the neurochemical phenotypes of DMN neurons projecting to the stomach are mainly cholinergic. In addition, nitric oxide (NO), a recognized neuronal messenger molecule detected by NO synthase (NOS), is present in 12% of medial neurons of the caudal DMN projecting to the fundus. Nitric oxide innervation may play an important role in vagally mediated gastric reflex relaxation.

## C. Projections to the DMN from Brain Nuclei Involved in Regulating Gastric Functions

DMN neurons receive direct neural input from many brain nuclei. Tracing studies revealed that the parvocellular part of the paraventricular nucleus of the hypothalamus (PVN), the central amygdala, the locus coeruleus, and the bed nucleus stria terminalis are an interconnected continuum of "prevagal neurons" that send direct projections to influence DMN neurons. In the medulla, the DMN receives direct projections from a number of NTS neurons as well as from specific nuclei located in the ventral regions, namely, the raphe pallidus (Rpa), the raphe obscurus (Rob), and the parapyramidal regions (PPR). The biochemical coding of identified neurons projecting from the PVN to the dorsal vagal complex (DVC) includes a large number of peptides such as bombesin-like peptide, somatostatin, enkephalins, corticotropin-releasing factor (CRF), neuropeptide Y, vasopressin, and oxytocin.

Consistent with these anatomical circuits for regulatory process, the activation of these specific hypothalamic, limbic, pontine, and medullary neurons influences the neuronal activity of DVC neurons and results in the vagal-dependent alterations of gastric motor and secretory function. In addition, peptides shown to project from the PVN to the DVC, such as bombesin, CRF, and somatostatin, when applied directly into the DVC alter gastric function through vagal pathways.

## D. Vagal Efferent Innervation of the Stomach

The vagal preganglionic projections originating from DMN neurons end in the enteric nervous system, predominantly in the myenteric plexus located between the circular and the longitudinal muscular layers. The prevailing concept is that, due to their small number, vagal efferent fibers provide parasympathetic input to the gut through projections to selected "mother cells/command enteric neurons." However, this concept has been recently challenged. There is now convincing evidence that vagal efferent terminals encircle or make putative contacts with all the ganglia in the myenteric plexus and, to a lesser extent, in the submucosal plexus of the stomach. Electrophysiological probing is also consistent with the notion that a high percentage of gastric myenteric neurons receive direct synaptic fast excitatory postsynaptic potential input from the vagus nerve. Therefore, the current understanding of an interface between vagal efferent fibers and myenteric neurons supports the existence of a dense network of direct interactions between the extrinsic and the enteric nervous systems.

The neurochemical phenotypes of gastric myenteric neurons receiving vagal efferent input encompass serotonin (5-HT)-, vasoactive intestinal peptide (VIP)-, NOS-, gastrin-releasing peptide (GRP)-, and GRP/VIP-containing neurons. Moreover, 60–70% of myenteric neurons in the stomach are cholinergic and surrounded by cholinergic positive fibers, showing the importance of vagal efferent cholinergic/nicotonic input to postganglionic cholinergic myenteric neurons innervating the muscular and mucosal layers of the stomach.

## III. BRAIN MEDULLARY THYROTROPIN-RELEASING HORMONE AND VAGAL REGULATION OF GASTRIC FUNCTIONS

The combined use of neuroanatomical, electrophysiological, and pharmacological techniques provided increasingly detailed understanding on the role of medullary thyrotropin-releasing hormone (TRH) in the vagal regulation of gastric function. These studies greatly expanded initial observations from the 1980s that injection of TRH into the cerebrospinal fluid (CSF) at the level of the cisterna magna induced a vagal-dependent stimulation of gastric acid secretion in rats. Currently, TRH-containing projections to the DVC are the only brain peptidergic circuit known to have physiological relevance in the central vagal stimulation of gastric function.

### A. Neuroanatomical and Electrophysiological Evidence

Tracing studies revealed that neurons in the Rpa, Rob, and PPR are the exclusive source of the TRH fibers innervating the DVC. Immunoelectron microscopy showed that TRH immunoreactive fibers make direct synaptic contacts with dendrites of DMN neurons projecting to the stomach. Likewise, in humans, TRH immunoreactive fibers represent the most prominent neuronal network compared with that of 12 other neuropeptides investigated. In addition, there is a strong anatomical relationship between TRH nerve terminals in the DVC and the localization of TRH receptors. Earlier studies pointed out that the highest concentration of TRH-binding sites occurs within the medial DMN where gastric preganglionic motor neurons are located and in the subnucleus gelatinosus of the NTS where gastric vagal afferents project. This was further confirmed by the mapping of TRH receptor gene distribution after the cloning of TRH receptor subtype 1 and subtype 2 ($TRH_1$ and $TRH_2$). $TRH_1$ mRNA, but not $TRH_2$ mRNA, is highly expressed in DMN and NTS neurons. Electrophysiological reports demonstrated that TRH directly stimulates the firing rates of DMN neurons by increasing the inward cationic current and reducing the calcium-dependent after-hyperpolarizing current. TRH also indirectly activates DMN neurons by inhibiting the activity of the NTS neurons that are responsive to gastric distention. Indeed, electrophysiological evidence shows that distention of the stomach and/or duodenum increased the firing rate of the majority of NTS neurons and decreased the firing rate of most DMN neurons, suggesting suppressive input onto DMN neurons from gastric distention-responsive NTS neurons. The activation of DMN neurons by central injection of TRH stimulates vagal efferent outflow to the stomach as monitored electrophysiologically in the cervical vagus as well as in the gastric vagal branch. It also induces a widespread activation of myenteric neurons in the gastric corpus and antrum as shown by Fos expression, a marker of neuronal activation.

### B. Gastric Responses to Central TRH-Induced Vagal Stimulation

TRH injected into the CSF of the cisterna magna or brain ventricle or directly into the DMN results in a vagal-dependent and atropine-sensitive stimulation of gastric secretions (acid, pepsin, mucus, histamine, prostaglandin, NO, serotonin, and gastrin), gastric motor function (motility, transit), and gastric mucosal blood flow. The gastric acid secretion in response to central TRH results from the activation of cholinergic postganglionic neurons, which directly stimulate parietal cell secretion through interaction with muscarinic receptors localized on these cells. In addition, the vagally mediated increase of gastric histamine release also stimulates the parietal cells. The gastric hyperemia in response to a maximally effective dose of TRH injected intracisternally is mediated by the cholinergic activation of peripheral NO pathways. This increase in blood flow is independent of the release of established vasoactive substances, such as histamine, calcitonin gene-related peptide (CGRP), VIP, and prostaglandins (Fig. 2).

By contrast, under conditions of submaximal increase of vagal efferent activity, induced by low doses of TRH injected into the cisterna magna, the gastric acid response is largely blunted or abolished. This is related to the action of vagally released anti-acid secretory transmitters, namely, prostaglandins, CGRP, and 5-HT. However, despite this minimal acid response, there is a robust gastric hyperemia resulting from the vagal cholinergic-dependent activation of capsaicin-sensitive CGRP/NO vasodilatory pathways without the involvement of prostaglandins. These observations support the notion that various transmitters are released in the stomach by central vagal cholinergic stimulation. Their interplay varies with the degree of vagal activation and contributes to the differential patterns of gastric responses elicited by low or high levels of vagal efferent drive (Fig. 2).

## C. Roles of Endogenous Medullary TRH in the Vagal Regulation of Gastric Functions

The use of specific TRH antibody and $TRH_1$ receptor antisense oligodeoxynucleotides as well as the monitoring of changes in medullary TRH gene expression provided valuable approaches to examine the physiological role of endogenous medullary TRH in the absence of selective TRH receptor antagonists. There is now convincing evidence that this peptide participates in the central vagal stimulation of gastric function during the digestive process, specific stress conditions, and thyroid-related endocrine alterations.

The cephalic phase of gastric acid secretion is mediated by centrally driven vagal-dependent pathways as initially demonstrated by Pavlov's pioneering studies in dogs. Medullary TRH plays an important role in the cephalic phase of gastric acid secretion as shown by the dampening of the gastric acid response to sham feeding by pretreatment into the cisterna

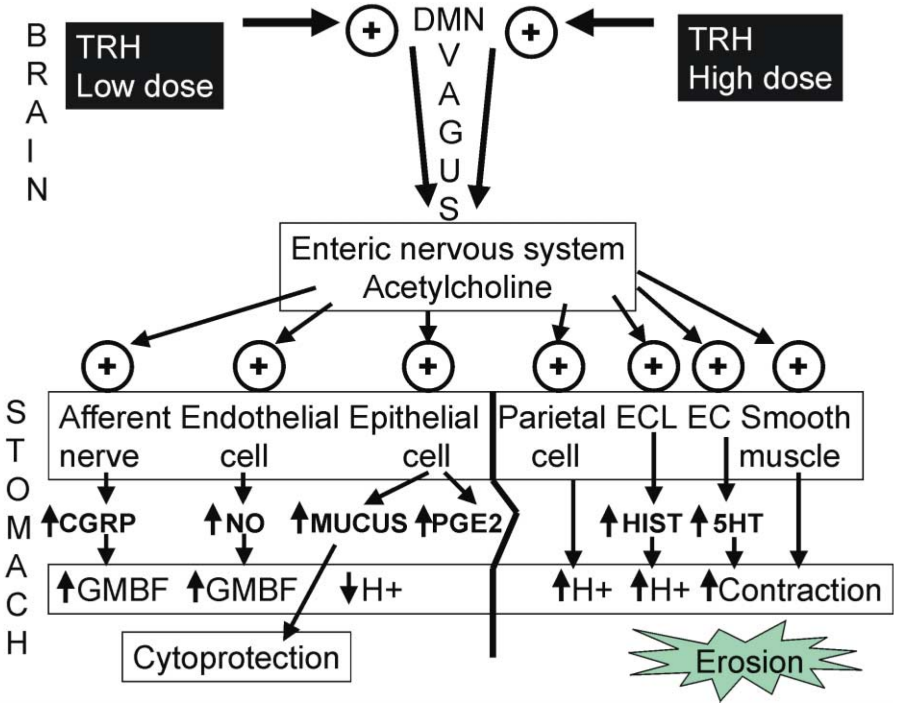

**FIGURE 2** Summary of experimental studies showing the gastroprotective and ulcerogenic actions of central vagal stimulation induced by low and high doses of TRH, respectively, and related transmitters involved in the dual gastric responses. CGRP, calcitonin gene-related peptide; EC, enterochromaffin cells; ECL, enterochromaffin-like cells; GMBF, gastric mucosal blood flow; HIST, histamine; NO, nitric oxide.

magna with antisense oligodeoxynucleotides targeted to the $TRH_1$ receptor. Another established role of the vagus was in the modulation of the gastric mucosa to withstand damaging agents such as strong acid or ethanol. Low doses of TRH injected into the cisterna magna or DVC confer gastric protection against ethanol-induced mucosal lesions. The gastric protection is brought about by medullary TRH inducing a vagal cholinergic-dependent release of gastric prostaglandins and NO, as well as the activation of a local effector function of capsaicin-sensitive splanchnic afferents containing CGRP and related gastric hyperemia (Fig. 2). Likewise, the endogenous release of TRH in the DVC induced by the activation of TRH cell bodies in the Rpa is also gastroprotective. Other phenomena, such as the vagal-dependent adaptive gastric protection whereby a mild gastric irritant reduced the damaging effect of a strong irritant, are also mediated by TRH in the brain medulla. In view of these findings, the cephalic phase of gastric secretion, which results in a mild vagal stimulation, may have beneficial effects on the gastric mucosa by triggering these mucosal protective mechanisms. Conversely, a deficient cephalic phase (nongustatory appreciation) may facilitate the damaging effect of ulcerogenic stimuli due to the reduction or absence of vagally recruited protective mechanisms.

By contrast, a high and sustained level of vagal activation is known to cause gastric hemorrhagic erosions. There is now evidence that intracisternal injection of a maximal acid secretory dose of TRH and maximal chemical stimulation of TRH-synthesizing neurons in the Rpa also result in the development of gastric erosions through the activation of vagal cholinergic pathways. Moreover, this gastric erosive response is potentiated when gastric prostaglandins, also released under these conditions of vagal stimulation, are blocked by an inhibitor of prostaglandin synthesis. Acute exposure of fasted rats to cold (an experimental model known since the 1960s to reliably induce vagal-dependent stimulation of gastric acid secretion, motility, and gastric lesions) is now clearly linked with the activation of medullary TRH pathways. Acute cold exposure increased TRH gene expression in the medullar nuclei selectively in the Rpa, Rob, and the PPR along with Fos expression, which is indicative of cell activation and gene transcription. TRH antibody injected into the cisterna magna reduced cold exposure-induced vagal-dependent gastric acid secretion and mucosal lesion formation. Antisense oligodeoxynucleotides targeted to the $TRH_1$ receptor, given centrally, prevented acute cold exposure-induced vagal cholinergic acceleration of gastric emptying. Collectively, these findings highlighted the dual role of medullary TRH, which, depending upon the differential level of activity, confers to the gastric mucosa a vagal cholinergic-mediated prevalence of protective or erosive mechanisms.

Another important role of medullary TRH is related to the alterations of autonomic activity linked with diseased thyroid states. Indeed, the synthesis of TRH gene expression in the Rpa, Rob, and PPR neurons is regulated by the feedback inhibition of thyroid hormones as established for TRH neurons located in the PVN. There is recent evidence that hypothyroidism significantly increased Fos expression in TRH-synthesizing neurons as well as TRH mRNA expression in the Rpa, Rob, and PPR neurons. Conversely, hyperthyroidism reduced TRH gene expression in these neurons. The localization of thyroid hormone receptors in these medullary nuclei indicates that the feedback regulation of TRH gene expression is mediated by a direct action of thyroid hormone on TRH neurons. The alterations of TRH neuroanatomical circuitry by hypo/hyperthyroidism along with the established effects of TRH to stimulate vagal outflow provide new insight into the understanding of autonomic disorders associated with altered thyroid states. Functional studies support the notion that hypothyroidism associated with increased TRH gene expression in raphe neurons is also linked with increased vagal drive to the viscera.

## D. Modulation of Medullary TRH Action by Other Brain Peptides

There is growing evidence that TRH excitatory action on DMN neurons occurs in concert with other modulatory influences exerted by other neuropeptides or neurotransmitters innervating the DVC. Neuroanatomical support for this interaction came with the co-localization of TRH with substance P (SP) and 5-HT immunoreactivity in neurons of the Rpa, Rob, and PPR projecting to the DVC. These substances modulate the activities of the vagal preganglionic motor neurons as they are co-released with TRH in the DVC. In particular, SP immunoreactive fibers innervate the entire lengths of the DMN and the NTS in rats and humans. Retrograde labeling studies identified DMN neurons projecting to the stomach located at the rostral level of the obex that are in contact with terminals containing SP fibers. In addition, gastric-projecting preganglionic vagal motor neurons expressed neurokinin-1 ($NK_1$) receptor. The biological consequence of activation of $NK_1$ receptors in the DMN is the reduction of the gastric secretory and motor responses to exogenous or endogenous TRH. Therefore, co-released SP with TRH in the DVC dampens the excitatory action of TRH in the DMN. By contrast, 5-HT, which is also co-localized with TRH in medullary raphe nuclei and PPR projecting into the DVC, potentiates TRH stimulatory action through 5-$HT_2$ receptors within the DVC. A number of brain peptides co-injected with TRH into the DMN have been reported to inhibit the vagal-dependent stimulation of gastric function by TRH. These include interleukin-1, opioid peptides, adrenomedulin, CGRP, bombesin, and calcitonin, providing pharmacological evidence of their central action to influence vagal outflow to the gut.

## IV. BRAIN BOMBESIN-LIKE PEPTIDES AND VAGAL INHIBITION OF GASTRIC FUNCTIONS

Thirty years ago, Erspamer *et al.* isolated the 14-amino-acid peptide bombesin from extracts prepared from the skin of the European frog, *Bombina bombina*. Earlier studies showed that bombesin-like immunoreactivity (-LI) was widely distributed in the brain and that central injection of bombesin exerted potent centrally mediated actions on thermoregulation, glucoregulation, and gastric function in rats. In addition, other reports showed the presence of bombesin-LI in the gut of rodents that fostered research to identify a mammalian bombesin counterpart. This resulted in the late 1970s in the characterization of a 27-amino-acid peptide displaying strong homology with the carboxyl-terminus of bombesin and potent gastrin-releasing activity and was accordingly named gastrin-releasing peptide. With these observations, bombesin/GRP was added to the list of peptides co-existing and acting both in the brain and in the gut, giving support to the emerging concept of the peptidergic brain–gut axis.

### A. Neuroanatomical Evidence

The neuroanatomical substrate role for the bombesin-LI terminal fields in the modulation of vagal outflow to the stomach came from the identification by retrograde tracing of bombesin-LI in cell bodies within the medial parvocellular part of the PVN projecting to the DVC. These projections constitute an important source of bombesin-LI terminals in the DVC with the strongest labeling in the medial NTS. A similar distribution of bombesin-LI in the rat DVC was confirmed with an N-terminal antibody selective for GRP and bombesin gene expression by *in situ* hybridization. Ultrastructural analysis identified a large number of bombesin-LI-labeled nerve terminals making mostly axo-dendritic synaptic contact on medium and small dendrites in the DMN and medial NTS. These data indicated that bombesin action was directly exerted on postsynaptyic neurons. Autoradiographic studies also revealed a high to moderate density of binding sites for $^{125}$I-[Tyr$^4$]bombesin in the caudal NTS.

### B. Biological Role

Bombesin was the first peptide shown to act in the brain to inhibit gastric function. The peptide administered into the cisterna magna induced a potent, dose-related, and long-lasting inhibition of acid secretion in several mammalian species including the rabbit, cat, dog, and rodents. The specificity and potency of bombesin action were established as 30 unrelated natural neuropeptides tested under the same conditions in rats and dogs were found to be inactive or less potent.

Consistent with bombesin action being mediated by altering vagal outflow to the stomach, bombesin-responsive hindbrain nuclei include the DVC. When microinjected at low doses (0.6–6.2 pmol), bombesin inhibited the gastric acid and contractile responses to the TRH analogue RX 77368 co-injected into the DVC. In addition, the delayed gastric emptying induced by central injection of bombesin was completely prevented by ganglionic blockade and vagotomy but not adrenalectomy in rats and dogs. The dose ranges at which central injection of bombesin delayed gastric emptying also correlated well with those inhibiting vagal outflow as monitored by electrophysiological recording of gastric vagal efferent discharges.

The biological actions of bombesin-like peptides are mediated by their binding to high-affinity receptors. Four members of the bombesin receptor family have been cloned: the mammalian neuromedin B-preferring receptor (neuromedin B receptor), the GRP-preferring receptor (GRP receptor), the bombesin subtype 3 (bombesin-3), and the amphibian bombesin subtype 4 (bombesin-4) receptors. The receptor subtype involved in bombesin anti-secretory action is unlikely to involve the neuromedin B receptor since neuromedin B injected into the CSF did not influence gastric acid secretion. The pharmacological characterization of bombesin-3 receptor showed that litorin, ranatensin, and bombesin have a low affinity and that [Phe$^{13}$]bombesin has no affinity for this subtype. Since these peptides also act centrally to inhibit acid secretion, this rules out a possible mediation through bombesin-3 receptor. Therefore, it is likely that the GRP receptor is involved in bombesin action. The bombesin/GRP receptor antagonist *N*-acetyl-$GRP_{20-26}-O-CH_3$ injected at a 52:1 antagonist:agonist ratio blocked intracisternal bombesin-induced anti-secretory action.

Taken together, these neuroanatomical and functional studies support an inhibitory action of bombesin-LI peptides in the DVC to regulate gastric function through vagal pathways. Additional actions of bombesin are also exerted at sites influencing sympathetic activity and the activation of this pathway contributes to the potent centrally mediated antisecretory effect of bombesin injected intracisternally or into the rostroventral medulla. The elucidation of the physiological importance of bombesin-like peptide-induced regulation of gastric function through vagal pathways will be forthcoming with the development of more selective and specific GRP receptor antagonists and/or the use of GRP receptor subtype gene knockout mice.

## V. BRAIN CRF RECEPTORS AND VAGAL INHIBITION OF GASTRIC MOTOR RESPONSE TO STRESS

The characterization of the 41-amino-acid peptide CRF in the 1980s and, more recently, of the CRF-related family members urocortin, urocortin II, and urocortin III, as well as the cloning of CRF receptor subtypes 1 ($CRF_1$) and 2 ($CRF_2$) and the development of specific $CRF_1/CRF_2$ receptor antagonists provided key tools to unravel the neurochemical basis of the stress response. Evidence has emerged that the activation of brain CRF receptors triggers almost the entire repertoire of behavioral, neuroendocrine, autonomic, immunological, and visceral responses characteristic of stress in rodents and primates. In particular, the activation of brain CRF receptors modulates autonomic outflow and plays a role in stress-related autonomic alterations of gut function.

### A. Activation of Brain CRF Receptors and Vagal Inhibition of Gastric Motor Function

Several reports consistently established that CRF and related peptides injected into the CSF act in the brain to inhibit gastric emptying of a solid or liquid meal and gastric contractility in rats and dogs. CRF and related peptides also act in the brain to inhibit gastric acid secretion and somatostatin release. These actions are mediated through modulation of vagal pathways. Brain sites responsive to CRF to inhibit gastric motor function are located in nuclei established to influence parasympathetic outflow to the viscera, namely, the PVN and DVC. In particular, CRF microinjected into the DVC blocked exogenous or endogenous TRH-induced vagal stimulation of gastric function. Functional mapping of brain neuronal activity using Fos expression also showed that central injection of CRF inhibits cold exposure-induced activation of DMN neurons and increases NTS neuronal activity. Moreover, direct electrophysiological recording of gastric vagal efferent discharges showed that intracisternal injection of CRF and related peptides inhibits gastric vagal efferent discharges. Finally, vagotomy prevented intracisternal CRF-induced inhibition of gastric motor function.

CRF actions in the medulla to decrease vagal outflow to the stomach may be primarily mediated by the $CRF_2$ receptor. This is supported by functional studies showing that selective $CRF_2$ receptor antagonists blocked central CRF- or urocortin-induced inhibition of gastric motor function. In addition, mapping studies of the distribution of CRF receptor gene expression in the medulla showed the presence of $CRF_2$ receptors particularly in the NTS. The network of CRF immunoreactive fibers in the NTS suggests that CRF may act through activation of NTS inhibitory input to the DMN preganglionic neurons.

### B. Role of Brain CRF Receptors in Stress-Related Alterations of Gastric Motor Function

Activation of brain CRF receptors plays a role in the vagally mediated alterations of gastric function evoked by stress. Delayed gastric emptying is a common pattern of response to exposure to various acute stressors such as operant avoidance, water avoidance, radiation, handling, acoustic stimulation, hemorrhage, abdominal or cranial surgery, tail shock, trunk clamping, wrap restraint at room temperature, swimming, and anesthetic exposure in experimental animals (mice, rats, guinea pigs, dogs, and/or monkeys). Likewise, in healthy subjects, anger, fear, labyrinthine stimulation, painful stimuli, preoperative anxiety, or intense exercise results in a slowing of gastric transit.

Various CRF receptor antagonists injected into the CSF or the PVN at doses preventing the biological actions of centrally injected CRF blocked the delayed gastric emptying resulting from exposure to various forms of acute stress. These include those elicited by immunological agents (intravenous or intracisternal injection of interleukin-1β), physico-psychological factors (partial restraint, forced swimming), exogenous chemical stimulation (short anesthetic), and body injury (abdominal or cranial surgery or peritoneal irritation). These findings provide new venues for understanding brain pathways contributing to gastric stasis in response to an acute stress including the underlying mechanisms of postoperative ileus. It is also noteworthy that the intercommunications between the immune and hypothalamic CRF systems impact the central regulation of gastric motor function.

## VI. VAGAL INHIBITION OF GASTRIC FUNCTION BY POSTPRANDIAL RELEASE OF THE GUT PEPTIDE, PEPTIDE YY

Peripherally originating gut peptides can enter the brain and act in specific brain nuclei outside the blood–brain barrier to regulate gastric functions. Recent studies have established that peptide YY (PYY) is a representative peptide with this mechanism of action. PYY is a 36-amino-acid hormone that was originally isolated from the pig intestine and localized in the open-ended L-type endocrine cells of the terminal ileum and colon in the rat, dog, and human. PYY is released postprandially via extramural neural or endocrine mechanisms that originate in the foregut and by intralumenal nutrients. However, the basal release of PYY seems to be partly regulated by tonic vagal activity. $PYY_{1-36}$ and $PYY_{3-36}$ are the two molecular forms of PYY that are abundant in the blood. Circulating PYY displays a profound inhibitory action on gastric emptying and acid secretion. Recent studies have revealed two important mechanisms involved in the inhibitory action of PYY on gastric acid secretion, one of which is its central action on vagally mediated regulation of gastric functions.

### A. Peripheral PYY Is a Potent Inhibitor of Vagally Mediated Gastric Acid Secretion

Intravenous infusion of PYY at doses reproducing circulating levels induced by food ingestion inhibits gastric acid secretion in several experimental animals and in humans irrespective of acid secretion being stimulated by peripheral (pentagastrin and liver extract) or central vagal (insulin, baclofen, and TRH) mechanisms. However, earlier observations indicated that PYY was more potent at inhibiting gastric acid secretion induced by central–vagal secretagogues than peripheral secretagogues. It was first speculated that PYY acts by inhibiting acetylcholine release from vagal nerve fibers rather than by inhibiting the action of acetylcholine on the parietal cell. This viewpoint was further supported by studies in rats showing that administration of PYY had no effect on bethanechol-induced acid output, but inhibited baclofen-induced acid output. Baclofen is a γ-aminobutyric acid receptor agonist that stimulates gastric acid output through atropine-sensitive and vagally mediated pathways. The demonstration that vagotomy markedly impaired the anti-secretory potency of peripherally infused PYY indicated that PYY inhibitory action on gastric acid secretion required the integrity of vagal innervation.

### B. Neuroanatomical Basis for PYY Action in the DVC

Recent studies revealed that PYY acts in the medullary DVC particularly at the level of the area postrema (AP) located close to the surface of the fourth ventricle (Fig. 1) and portions of the NTS are defined as circumventricular organs where the blood–brain barrier is incomplete. These regions can therefore act as portals of entry for circulating peptide hormones. PYY-binding sites are present in the AP and DVC. Specific binding sites for both $^{125}$I-[Leu$^{31}$Pro$^{34}$]PYY ($Y_1$ agonist) and $^{125}$I-$PYY_{3-36}$ ($Y_2$ agonist) have been detected in the NTS and AP. A recent study revealed that $Y_1$, $Y_2$, and $Y_4$ receptor subtype mRNAs are located in the AP, NTS, and the DMN with a high level of expression in the AP and DMN. Labeled PYY, injected intravenously at doses that produce a blood concentration of PYY within the range observed after a meal is consumed, binds specifically to the region of the rat brainstem containing the DVC. Likewise, peripheral injection of PYY activates NTS and AP cells as shown by Fos expression. Collectively these data provide neuroanatomical evidence supporting a receptor-mediated action of PYY in the brain medulla.

### C. Central Action of Peripheral PYY in Inhibiting Gastric Function

The centrally mediated action of peripheral PYY in inhibiting vagally stimulated gastric acid secretion was observed in studies using immunoneutralization with a PYY polyclonal antibody. PYY infused intravenously inhibited in a dose-dependent manner the acid response to TRH analogue injected intracisternally. PYY or the $Y_2$ agonist $PYY_{3-36}$ injected intracisternally reproduced the anti-secretory effect observed after peripheral administration of the peptides. In addition, intravenous injection of PYY antibody that shows a 35% cross reaction with $PYY_{3-36}$ by radioimmunoassay completely prevented the inhibitory effect of intravenous infusion of PYY. When injected intracisternally, the PYY antibody reversed not only the inhibition of gastric acid secretion induced by centrally administered PYY but also the inhibition elicited by PYY infused peripherally. These results strongly indicated that peripheral PYY acts within the area of the AP/NTS outside of the blood–brain barrier to inhibit central-vagally stimulated gastric acid secretion. The role of $Y_2$ receptors in mediating the anti-secretory action of PYY is further supported by the strong expression of $Y_2$ receptor mRNA in the AP and NTS. The central action of circulating PYY released postprandially to suppress vagally stimulated gastric response induced by TRH may have relevance in the dampening of the cephalic phase of gastric secretion in response to a meal.

## VII. SUMMARY

Brain medullary TRH is a physiologically important stimulant of vagal efferent discharges to the stomach and thereby stimulates gastric secretory and motor functions and blood flow. Medullary TRH also regulates the gastric mucosal resistance against injury with a dual action, protective or ulcerogenic in relation to the intensity of the vagal activity. The central action of this peptide is to function in the course of normal physiological digestive activities, in particular during the cephalic phase of the gastric response to a meal. Impaired thyroid levels feed back on the regulation of medullary TRH gene expression and impact on autonomic regulation of the viscera through TRH–vagal pathway. Within the brain, several selective nuclei send inputs to the DVC neurons and modulate the action of TRH through the release of peptides and transmitters. There is evidence that TRH action is modulated by inhibitory actions of medullary SP and PVN–DVC projections containing bombesin-LI, whereas medullary 5-HT potentiates TRH excitatory action in the DVC. In addition, postprandially released gut peptides, such as PYY, may impact on the cephalic phase of gastric secretion through a direct action on the medullary area outside of the blood–brain barrier.

New information is emerging about the stress-related inhibition of gastric motor function through the activation of brain CRF receptors, which suppressed vagal outflow to the stomach. The brain–gut peptidergic interaction is part of the mechanisms through which digestive function is coordinated and adjusted in response to internal and external environmental changes.

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## Glossary

**dorsal vagal complex** Association of two medullary nuclei: the dorsal motor nucleus of the vagus and the nucleus tractus solitarius. The dorsal motor nucleus contains cell bodies with axons that form the vagal innervation of the gut. The nucleus tractus solitarius contains cell bodies of interneurons and of neurons projecting to other brain areas as well as terminal fibers of vagal afferents originating from cell bodies in the nodose ganglia; fibers from cell bodies located in the other parts of the brain (central amygdala, hypothalamus) are also present in the nucleus tractus solitarius.

**enteric nervous system** Network of neuronal cells and fibers embedded within the gut wall that serve as a relay for signals to and from the brain or spinal cord. This network can respond to stimuli from various sensory receptors and generate changes in neuronal activity independent of the central nervous system.

**hypothalamus** A region in the forebrain that contains control centers for homeostatic regulation of pituitary hormone secretions, behavior, and visceral functions.

**peptides** Molecules composed of small numbers of amino acids (fewer than 100).

**thyrotropin-releasing hormone (TRH)** A tripeptide amide originally isolated from the hypothalamus and expressed in many extrahypothalamic brain nuclei, especially the medulla. Hypothalamic TRH regulates thyroid function by releasing pituitary thyrotropin hormone, whereas medullary TRH regulates autonomic function by acting as an excitatory neurotransmitter on autonomic regulatory neurons.

**vagus nerve** The 10th cranial nerve that emerges from the brainstem and passes through the neck and thorax to the abdomen, innervating visceral organs including the heart, lungs, and digestive organs; it plays a major role in the parasympathetic regulation of gut functions.

## See Also the Following Articles

**Appetite Regulation, Neuronal Control • Bombesin-like Peptides • Cholecystokinin (CCK) • Gastrointestinal Hormone-Releasing Peptides • Motilin • Peptide YY**

• **Thyrotropin-Releasing Hormone (TRH)** • **Vasoactive Intestinal Peptide (VIP)**

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# Vascular Endothelial Growth Factor B (VEGF-B)

ULF ERIKSSON AND XURI LI
*Ludwig Institute for Cancer Research, Stockholm*

I. DISCOVERY
II. PROTEIN STRUCTURE
III. GENE STRUCTURE AND REGULATION
IV. TISSUE DISTRIBUTION
V. RECEPTORS
VI. BIOLOGICAL ACTIVITIES AND PATHOLOGY
VII. FUTURE DIRECTIONS

**Vascular endothelial growth factor (VEGF)-B was found serendipitously as a partial mouse cDNA clone encoding a VEGF-related peptide. The partial cDNA was then used to isolate full-length mouse and human cDNA clones from mouse and human cDNA libraries.**

## I. DISCOVERY

A full-length cDNA that encoded a homologue of vascular endothelial growth factor (VEGF) was discovered, and in analogy with the nomenclature of the related platelet-derived growth factors (PDGFs), the new protein was denoted VEGF-B. Independently, other researchers found the same gene when attempting to identify the locus involved in multiple endocrine neoplasia type 1 (MEN1). The protein encoded by this gene was designated VEGF-related factor and was later excluded from being involved in MEN1.

## II. PROTEIN STRUCTURE

Two isoforms of mouse and human VEGF-B have been identified. Both isoforms are secreted proteins and have 167 ($\text{VEGF-B}_{167}$) and 186 ($\text{VEGF-B}_{186}$) amino acid residues, respectively. The isoforms have an identical amino-terminal domain of 115 amino acid residues, excluding the signal sequence, whereas the two different carboxyl-terminal domains are not related to each other. The highly conserved pattern of eight cysteine residues found in VEGFs and PDGFs, involved in intra- and intermolecular disulfide bonding, was present in the common amino-terminal domain. Both human and mouse VEGF-B isoforms lack the consensus sequence for N-linked glycosylation (-Asn-Xxx-Thr/Ser-), unlike the structurally related factors of the PDGF/VEGF family. However, VEGF-$B_{186}$ is O-glycosylated in the unique carboxyl-terminal domain, which is rich in serine and threonine residues. Pairwise comparisons of the amino acid sequences showed that mouse VEGF-$B_{167}$ is $\approx 43\%$ identical to mouse $VEGF_{164}$, $\approx 30\%$ identical to human placenta growth factor (PlGF), and $\approx 20\%$ identical to mouse PDGF-A and PDGF-B.

The two VEGF-B isoforms are produced as disulfide-linked homodimers and under reducing conditions the molecular mass of secreted VEGF-$B_{167}$ is 21 kDa. The secreted O-glycosylated VEGF-$B_{186}$ isoform has an apparent molecular mass of 32 kDa, and the unmodified intracellular form of VEGF-$B_{186}$ has a molecular mass of 26 kDa.

The different carboxyl-terminal domains of the two isoforms of VEGF-B affect their biochemical and cell biological properties. The highly hydrophilic carboxyl-terminal domain of VEGF-$B_{167}$ is related to the corresponding region in several isoforms of VEGF, with several conserved cysteine residues and stretches of basic amino acid residues. VEGF-$B_{167}$ will remain cell-associated upon secretion by binding to pericellular heparan sulfate proteoglycans. The cell association is likely to occur via its unique basic carboxyl-terminal region, as noted for the highly basic splice variants of VEGF.

The carboxyl-terminal domain of the VEGF-$B_{186}$ isoform is rather hydrophobic with several conserved alanine, proline, serine, and threonine amino acid residues, and its characteristics contrast with those of the hydrophilic and basic carboxyl-terminal domain in VEGF-$B_{167}$. The amino acid sequence of the carboxyl-terminus of this isoform has no significant similarity with other known amino acid sequences. On secretion, VEGF-$B_{186}$ does not remain cell-associated. Instead, it is proteolytically processed, and the processing regulates the biological properties of the growth factor.

Both isoforms of VEGF-B form disulfide-linked heterodimers with VEGF when co-expressed in transfected cells, but it has not been established whether naturally occurring VEGF·VEGF-B heterodimers exist. It is known that VEGF forms naturally occurring heterodimers with PlGF, and such heterodimers display functional properties distinct from those of both VEGF and PlGF homodimers. Similarly, VEGF·VEGF-B heterodimers may have unique functional properties.

Heterodimers of VEGF-$B_{167}$·VEGF remain cell-associated, whereas homodimers of $VEGF_{165}$ are secreted from cells in a soluble form. In contrast, heterodimers of VEGF-$B_{186}$ and VEGF are freely secreted. Thus, VEGF-$B_{167}$ determines the release of heterodimers from cells, and heterodimerization of VEGF with either of the two isoforms of VEGF-B might therefore control the release and bioavailability of VEGF·VEGF-B heterodimers. Whether the VEGF-B polypeptides act as homodimers, as heterodimers with VEGF, or as both is not known.

The ability of VEGF-B isoforms to affect the release of VEGF·VEGF-B heterodimers from the producing cells is intriguing since the two factors are co-expressed in many tissues, most prominently in heart and muscle.

## III. GENE STRUCTURE AND REGULATION

The human VEGF-B gene is localized to chromosome 11q13, close to the MEN1 locus. The mouse and human genes for VEGF-B are almost identical and both span approximately 4 kb of DNA. The genes are composed of seven exons and their exon–intron organization is similar to that of the VEGF and PlGF genes. The common amino-terminal domain in the two isoforms of VEGF-B is encoded by exons 1–5, and differential use of the remaining three exons gives rise to the two isoforms. The transcript for VEGF-$B_{167}$ is generated by exons 1–5, exon 6b, and exon 7. In contrast, the transcript encoding VEGF-$B_{186}$ is generated by the use of an alternative splice acceptor site in exon 6, leading to an insertion of 101 bp (exon 6a) that introduces a frameshift mutation and termination of the coding region in exon 6b (Fig. 1). In VEGF and PlGF, several isoforms are encoded by the use of alternative splice acceptor sites and different combinations of exons in the 3′-regions of the genes, but the corresponding transcripts are translated using the same reading frame. The use of partially overlapping but different reading frames is rare among higher eukaryotes, but is frequently used by different viruses.

The expression of the two isoforms of VEGF-B appears to be strictly regulated. In most adult and embryonic tissues, the transcript encoding VEGF-$B_{167}$ accounts for more than 80% of the total level of expression of VEGF-B. However, in several primary tumors and tumor cell lines, the expression of transcripts encoding VEGF-$B_{186}$ is up-regulated. The differential expression of VEGF-B isoforms would contribute to a genetically controlled mechanism involved in the release of VEGF·VEGF-B heterodimers in co-expressing tissues.

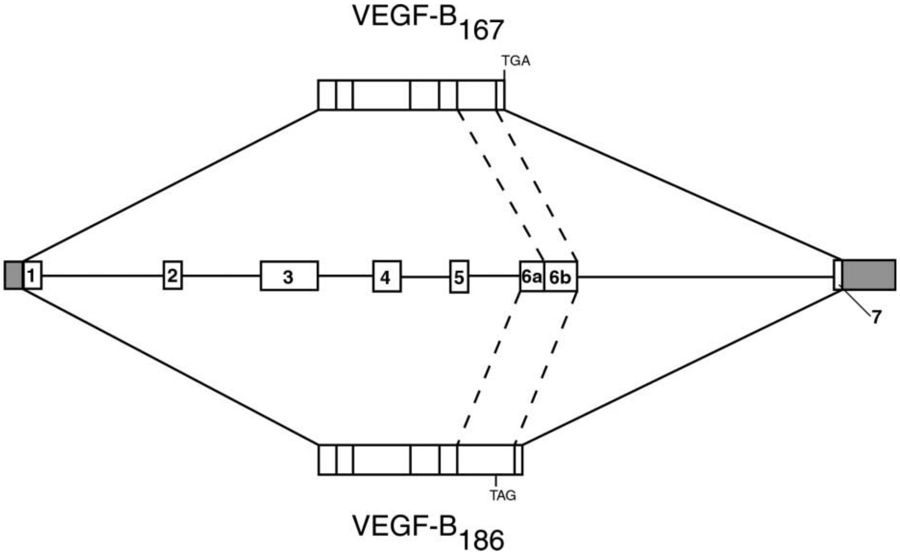

**FIGURE 1** Schematic structure of the gene encoding VEGF-B. The genomic region harboring the seven coding exons spans approximately 4 kb. Alternative use of the splice acceptor site in exon 6 generates the transcripts for the two VEGF-B isoforms. Translation terminates in exon 7 in VEF-$B_{167}$ and in exon 6b in VEGF-$B_{186}$.

The gene for VEGF-B does not contain binding sites for hypoxia-inducible factors, and accordingly, VEGF-B mRNA expression is not regulated by hypoxia, unlike VEGF expression. Instead, the expression levels of VEGF-B are remarkably stable and not influenced by a variety of other stimuli including several growth factors and cytokines.

## IV. TISSUE DISTRIBUTION

In adult mouse and human tissues, VEGF-B is abundantly expressed as a 1.4 kb mRNA in heart, brain, skeletal muscle, and kidney, and lower levels are present in most other tissues. VEGF-B and VEGF mRNAs are co-expressed in many tissues, such as heart, skeletal muscle, pancreas, and prostate, generating the possibility that VEGF·VEGF-B heterodimers may occur *in vivo*.

VEGF-B is expressed during embryonic development and is widely distributed. It is prominently expressed in the developing cardiac myocytes and less abundantly expressed in several other tissues, including developing muscle, bone, pancreas, adrenal, and the smooth muscle cell layer of several larger vessels. On embryonic day (E) 11.5–12.5, VEGF-B was strongly expressed in the developing heart. Later, on E14, VEGF-B was expressed in most tissues of the embryo, although most prominently in heart, spinal cord, and cerebral cortex. On E17, VEGF-B expression was concentrated in the heart, brown fat, and spinal cord. Throughout embryonic development, no expression of VEGF-B was detected in endothelial cells. Based on the expression pattern, VEGF-B was suggested to have a role in vascularization of the heart, skeletal muscles, and other tissues and act via paracrine interactions between endothelial cells and surrounding tissue cells.

## V. RECEPTORS

Two receptors have been identified for the two isoforms of VEGF-B, the vascular endothelial growth factor receptor (VEGFR)-1 and the co-receptor neuropilin-1 (NP-1). VEGFR-1 is a cell surface receptor tyrosine kinase containing seven immunoglobulin-like domains in the extracellular portion and an intracellular tyrosine kinase domain. In addition to VEGF-B, VEGFR-1 is a receptor for VEGF and PlGF. VEGF-B and PlGF form a subgroup of VEGFR-1-specific ligands since VEGF also binds to VEGFR-2, the main mitogenic VEGF receptor. The first three immunoglobulin-like domains are sufficient for VEGF-B binding, and the binding site is identical to, or at least overlaps, the binding site for VEGF and PlGF. VEGFR-1 binds to the common growth factor domains present in both VEGF-B isoforms. The receptor is expressed on the endothelium of blood vessels during embryonic development and in adult tissues, and it is also expressed by monocytes, macrophages, and certain stem cell populations. Targeted deletion of VEGFR-1 has shown that it is crucial for vascular development during embryogenesis.

Embryos homozygous for VEGFR-1 deletion die at E9–11 due to extensive accumulation of endothelial cells in the developing vessels. In contrast, embryos with a deletion of the intracellular tyrosine kinase domain of VEGFR-1 are viable. These data suggest that the extracellular ligand-binding portion of the receptor is sufficient to support normal development of the vasculature.

NP-1, the second receptor for VEGF-B, is also an isoform-specific co-receptor for some isoforms of VEGF and PlGF and is a receptor for semaphorins/collapsins involved in axonal guidance. In addition to its neuronal expression, NP-1 is present in the endothelial cells of blood vessels and in mesenchymal cells surrounding the blood vessels during embryonic development, as well as in certain other nonneuronal tissues. Embryos with targeted deletion of NP-1 die of cardiovascular failure at E10.5–12.5, and overexpression of NP-1 under the β-actin promoter is lethal due to severe anomalies of both the nervous system and the cardiovascular system.

NP-1 binds to both VEGF-B isoforms via the carboxy-terminal domains. In VEGF-$B_{167}$, the interaction is mediated by the heparin-binding exon 6B-encoded domain, which is homologous to the NP-1-binding domain of $VEGF_{165}$. VEGF-$B_{186}$, the non-heparin-binding isoform, binds to NP-1 following proteolytic cleavage of the unique carboxy-terminal domain that unmasks the binding epitope. The binding epitope has been mapped to the first 12 amino acid residues of the carboxy-terminal domain.

## VI. BIOLOGICAL ACTIVITIES AND PATHOLOGY

VEGF-B is poorly, if at all, mitogenic for endothelial cells. This property accompanies the weak ligand-induced activation of VEGFR-1. Initially, it was reported that conditioned medium from cells transfected with an expression vector generating VEGF-$B_{167}$ stimulated DNA synthesis in primary cultures of endothelial cells. However, at least part of this mitogenic activity is probably contributed by the formation of VEGF·VEGF-B heterodimers as most *in vitro* grown cell lines express VEGF endogenously.

The poor mitogenic effect of VEGF-B on endothelial cells seen *in vitro* is also reflected in *in vivo* studies. Transgenic mice overexpressing VEGF-B under strong promoters show no obvious vascular phenotypes, and mice carrying a targeted deletion in the VEGF-B gene develop normally. No gross abnormalities can be seen in such mice, even in organs in which normal expression of VEGF-B is high, such as heart, muscle, and kidney. These results have shown that VEGF-B is not required for embryonic angiogenesis. Similar results have been shown in mice deficient in PlGF, the functional homologue of VEGF-B, suggesting that neither VEGFR-1-specific ligand is a critical regulator of embryonic vessel development. This is in contrast to the essential role of VEGF in vasculogenesis and angiogenesis.

VEGF-B is expressed in most tumors and tumor-derived cell lines analyzed. Given its poor mitogenic capacity on endothelial cells, VEGF-B is unlikely to directly control the growth of the tumor vasculature like VEGF. Instead, the effects of VEGF-B may be indirect and related to the recruitment of stem cells, progenitor cells, and inflammatory cells to the tumors (see below).

Recent results have suggested that the receptor for VEGF-B, VEGFR-1, is important in the recruitment and mobilization of hematopoietic stem cells and endothelial progenitors from the bone marrow. Such cells are likely to have important functions in therapeutic angiogenesis whereby new vessels are generated or existing vessels are remodeled in ischemic tissues to allow increased blood flow. Furthermore, these cells also contribute to pathological vessel growth in several diseases, including cancer and retinopathies, and contribute to inflammatory conditions, such as atherosclerosis and arthritis. VEGF-B is also able to induce an endothelial cell phenotype of mesenchymal stem cells from the bone marrow. Given that expression of VEGF-B is widespread, and rather abundant in some tissues, a role of VEGF-B in stem cell biology and inflammation is likely.

## VII. FUTURE DIRECTIONS

The exciting finding that VEGFR-1 and its ligands control the recruitment and mobilization of hematopoietic stem cells and endothelial progenitor cells and the differentiation of mesenchymal stem cells suggests that they provide important tools for the therapeutic modulation of vessel growth in cardiovascular disease, cancer, retinopathies, inflammation, atherosclerosis, and hematopoiesis. Clearly, more extensive studies of these molecules, and particularly VEGF-B, in various experimental models are warranted.

## Glossary

**angiogenesis** Sprouting and growth of blood vessels from preexisting blood vessels.

**stem cells** Primitive cells that have an unlimited capacity to divide and that can differentiate into different functional cell types.

**vasculogenesis** *De novo* formation of blood vessels from mesodermal precursors.

## See Also the Following Articles

**Angiogenesis • Cancer Cells and Progrowth/Prosurvival Signaling • Corpus Luteum: Regression and Rescue • Epidermal Growth Factor (EGF) Family • Estrogen Receptor (ER) Actions through Other Transcription Factor Sites • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor D (VEGF-D)**

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# Vascular Endothelial Growth Factor D (VEGF-D)

Marc G. Achen and Steven A. Stacker
*Ludwig Institute for Cancer Research, Melbourne*

I. DISCOVERY AND ALTERNATIVE NAMES
II. PROTEIN STRUCTURE
III. BIOSYNTHESIS
IV. RECEPTORS
V. BIOLOGICAL ACTIVITIES
VI. GENE STRUCTURE AND REGULATION
VII. TISSUE DISTRIBUTION
VIII. PATHOLOGY
IX. FUTURE DIRECTIONS

**Vascular endothelial growth factor D (VEGF-D) is a member of the VEGF family of growth factors. These growth factors are secreted glycoproteins that contain a cysteine knot motif. Human VEGF-D is mitogenic for vascular endothelial cells *in vitro* and can induce the growth of both blood vessels and lymphatic vessels *in vivo*. VEGF-D is expressed at many sites in the developing embryo including lung and kidney mesenchyme, skin, liver, heart, and limb buds, likely playing a role in inducing the growth of blood and lymphatic vessels in these regions. In adult tissues, VEGF-D is expressed in the vascular smooth muscle of blood vessels, where it may play a role in the repair of blood vessels after vascular damage.**

## I. DISCOVERY AND ALTERNATIVE NAMES

Vascular endothelial growth factor D (VEGF-D) is the most recently discovered mammalian member of the VEGF family of growth factors, which consists of the secreted glycoproteins VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, placenta growth factor, and VEGF-like molecules encoded by viruses and present in snake venoms. VEGF family members form part of a structural superfamily of growth factors containing a cysteine knot motif in which six conserved cysteine residues contribute to a three-dimensional fold involving an unusual clustering of three cysteine bridges intertwined to resemble a knot. VEGF-D was originally reported as c-*fos*-induced growth factor because it was identified as a protein that was downregulated in fibroblasts deficient in c-*fos*. Subsequently, the protein was renamed VEGF-D when its capacity to bind and activate VEGF receptors was demonstrated. VEGF-D is most closely related to VEGF-C (these proteins share 48% amino acid identity throughout the entire molecule and 61% identity within the central VEGF homology domain); similarities in the structure, proteolytic processing, and receptor binding of these two growth factors indicate that they form a subfamily within the VEGF family.

## II. PROTEIN STRUCTURE

Human VEGF-D and mouse VEGF-D are 354 and 358 amino acids in length, respectively (including the signal sequence for protein secretion), are 87% identical in amino acid sequence, and contain three potential N-linked glycosylation sites. VEGF-D is initially synthesized as a precursor protein containing N- and C-terminal propeptides in addition to a central VEGF homology domain (VHD) (Fig. 1). The VHD contains the known receptor-binding regions and shares homology with all VEGF family members. The free N-terminal propeptide has an apparent molecular weight of approximately 10 kDa as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, whereas the VHD is approximately 21 kDa and the C-terminal propeptide is approximately 29 kDa. The C-terminal propeptide contains numerous cysteine residues, many of which are arranged in motifs resembling those of the Balbiani ring 3 protein (BR3P) ($CysX_{10}CysXCysXCys$). Approximately 50 of these motifs are found in BR3P, a cysteine-rich protein synthesized in the larval salivary glands of the midge *Chironomus tentans*. It has been speculated that the cysteine residues in the C-terminal propeptide of VEGF-D may have a role in intermolecular interactions (as is the case for the BR3P motifs), possibly modulating the bioavailability, localization, or biological half-life of the growth factor.

Two distinct isoforms of mouse VEGF-D, VEGF-$D_{358}$ and VEGF-$D_{326}$, which differ in the structure of the C-terminal propeptide, have been reported. Alternative use of an RNA splice donor site in exon 6 of the mouse *VEGF-D* gene produces the two different protein isoforms. The two isoforms are both expressed in a wide range of adult mouse tissues and embryonic stages of development. Both isoforms are proteolytically processed in a fashion similar to human VEGF-D to generate a range of secreted derivatives (Section III). The isoforms are differently glycosylated when expressed *in vitro*. Hence, RNA splicing, protein glycosylation, and proteolysis are mechanisms for generating structural diversity of mouse VEGF-D. Multiple isoforms of human VEGF-D, generated by alternative RNA splicing, have not been reported.

## III. BIOSYNTHESIS

Studies carried out with VEGF-D *in vitro* demonstrated that the N- and C-terminal propeptides are proteolytically cleaved from the VHD in a step-wise

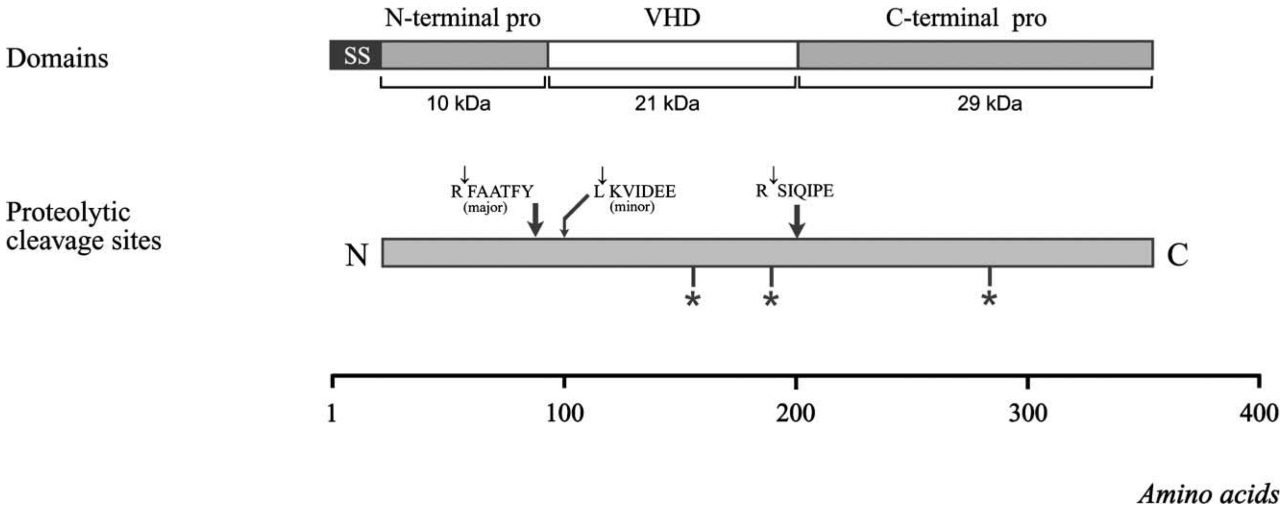

**FIGURE 1** Schematic representation of the domain structure of human VEGF-D. SS denotes signal sequence for protein secretion and pro denotes propeptide. Proteolytic cleavage sites are indicated by arrows and potential N-linked glycosylation sites by asterisks. Apparent molecular weights, indicated under the domains, were determined by SDS–PAGE under reducing conditions.

fashion after secretion from the cell to ultimately generate a mature form consisting of dimers of the VHD (Fig. 2A). The VHD dimers are assumed to be anti-parallel in nature based on the three-dimensional structure of VEGF, and the association between them is predominantly noncovalent. Proteolytic processing at the N-terminus of the VHD occurs at multiple sites in a region that appears to be prone to proteolysis, whereas cleavage at the C-terminus occurs at a unique site (Fig. 1) that is located at the same position in VEGF-C. Expression of VEGF-D in mammalian cells *in vitro* leads to the production of a mixture of unprocessed, partially processed, and fully processed forms that accumulate in the cell culture medium. Predominant forms observed by SDS–PAGE are the free N-terminal propeptide (~10 kDa), the free C-terminal propeptide (~29 kDa), the free VHD (~21 kDa), the VHD linked to the N-terminal propeptide (~31 kDa), and full-length material (~58 kDa). Analysis of VEGF-D purified from mouse lung demonstrated that this growth factor is proteolytically processed *in vivo*.

## IV. RECEPTORS

The receptors for human VEGF-D identified thus far are VEGFR-2 (also known as KDR in human and Flk1 in mouse) and VEGFR-3 (also known as Flt4) (Fig. 2B). These are cell surface receptor tyrosine kinases that are closely related in structure, contain seven Ig-like domains in their extracellular regions, and are bound, cross-linked, and activated by VEGF-D. VEGFR-2 is localized on the endothelium of blood vessels during embryonic development but is generally down-regulated in adult tissues. In contrast, VEGFR-3 is localized on lymphatic endothelium in adult tissues. During embryogenesis, VEGFR-3 is initially expressed on a wide range of vessels but subsequently becomes restricted to developing veins and then to the lymphatics. An extensive range of

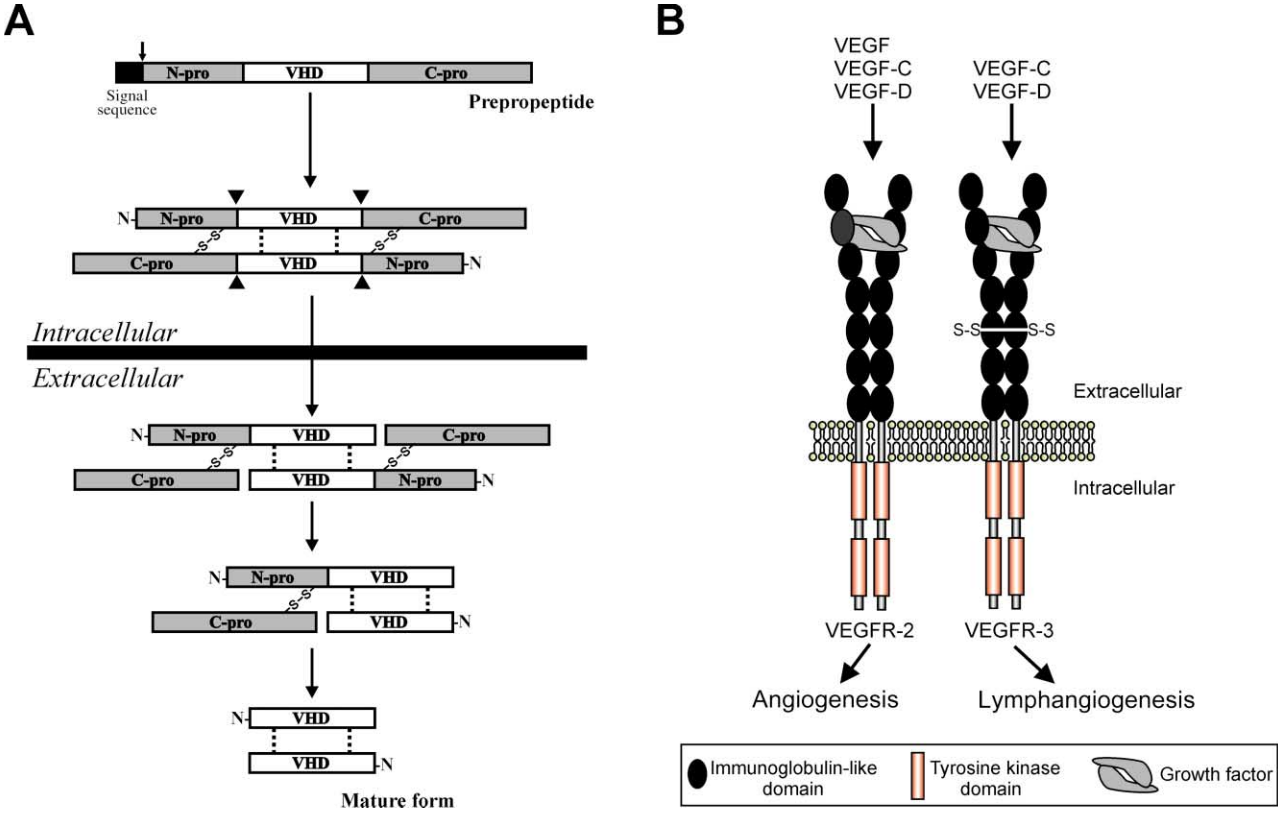
A
Signal sequence
N-pro
VHD
C-pro
Prepropeptide
N
Intracellular
Extracellular
Mature form
B
VEGF
VEGF-C
VEGF-D
VEGF-C
VEGF-D
S-S
S-S
Extracellular
Intracellular
VEGFR-2
VEGFR-3
Angiogenesis
Lymphangiogenesis
Immunoglobulin-like domain
Tyrosine kinase domain
Growth factor

**FIGURE 2** Model for biosynthesis of VEGF-D (A) and schematic representation of receptor interactions (B). (A) Stepwise proteolytic processing gives rise to a mature form consisting of dimers of the VHD. Arrowheads indicate sites of proteolytic cleavage; -S-S-, disulfide bridges; N-Pro, the N-terminal propeptide; C-pro, the C-terminal propeptide; dotted lines, noncovalent interactions. (B) Mammalian VEGF family members dimerize VEGFR-2 and VEGFR-3 to induce angiogenesis and lymphangiogenesis, respectively.

*in vivo* studies have indicated that VEGFR-2 signals for angiogenesis, whereas VEGFR-3 signals for lymphangiogenesis.

Both VEGFR-2 and VEGFR-3 are crucial for vascular development during embryogenesis. Embryos homozygous for *VEGFR-2* gene inactivation, which die at approximately 9 days post coitum (dpc), lack both hematopoietic precursor cells and endothelial cells, indicating that this receptor plays a crucial role in early vasculogenesis. In contrast, VEGFR-3 is not required for vasculogenesis as mouse embryos lacking this receptor have both hematopoietic and endothelial cells; however, large vessels become abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at 9.5 dpc. On the basis of these findings, it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. As the *VEGFR-3* mutant mice die before the lymphatic vessels emerge, it may be necessary to generate conditional knockout *VEGFR-3* mice in order to test the role of VEGFR-3 in the development of the lymphatic vasculature.

Unexpectedly, mouse VEGF-D, in contrast to human VEGF-D, fails to bind mouse VEGFR-2 but activates VEGFR-3. Mutation of amino acids in mouse VEGF-D to those in the human homologue indicated that residues important for the VEGFR-2 interaction are clustered at, or are near, the receptor-binding surface predicted from the structure of VEGF. The different receptor-binding specificities of mouse and human VEGF-D indicate that this growth factor may have different biological functions in mouse and human.

Analyses of the interaction of human VEGF-D with immobilized receptor extracellular domains revealed that proteolytic processing modulates VEGF-D function as the mature form of human VEGF-D binds VEGF-2 and VEGFR-3 with 290- and 40-fold greater affinity, respectively, than does unprocessed VEGF-D. Therefore, proteolytic processing is essential for generation of VEGF-D, which binds these receptors with high affinity. VEGF-D activates both VEGFR-2 and VEGFR-3 as the treatment of cells expressing these receptors with VEGF-D induces the phosphorylation of tyrosine residues on these receptors.

## V. BIOLOGICAL ACTIVITIES

Human VEGF-D is mitogenic for vascular endothelial cells *in vitro* and can induce both angiogenesis and lymphangiogenesis *in vivo*, although the predominant response to this growth factor depends on the timing and location of its application. For example, VEGF-D was demonstrated to be angiogenic in the adult rabbit cornea and in a mouse tumor model. When expressed during embryonic development in the epidermis of mouse skin, under the control of the *keratin-14* gene promoter, VEGF-D induced lymphatic hyperplasia/lymphangiogenesis, but not angiogenesis, in the underlying dermis. The biological response induced by VEGF-D, be it angiogenesis or lymphangiogenesis, most likely depends on the proximity of blood vessels and lymphatic vessels expressing VEGFR-2 and/or VEGFR-3 to the site of application of the growth factor. VEGF-D also induced lymphangiogenesis in a mouse tumor model. Importantly, the tumor lymphangiogenesis induced by VEGF-D promoted metastatic spread via the lymphatics, suggesting that the route of metastatic spread of a tumor is dependent on the capacity of tumor-derived growth factors to induce angiogenesis and/or lymphangiogenesis.

Some members of the VEGF family, such as VEGF and VEGF-C, potently induce a rapid and transient increase in the permeability of microvessels to macromolecules. However, the mature form of human VEGF-D did not exhibit any activity in the Miles vascular permeability assay. This suggests that VEGF-D is unlikely to induce edema in a therapeutic setting and that activation of VEGFR-2 alone may not be sufficient to induce vascular permeability.

## VI. GENE STRUCTURE AND REGULATION

The gene encoding human VEGF-D consists of seven exons, is approximately 50 kb in size, and is located on the X chromosome at position Xp22.1. The mouse *VEGF-D* gene is similar in structure and is also located on the X chromosome. The *VEGF-D* gene is highly homologous to that for VEGF-C, further illustrating the relatedness of these two growth factors. It is clear that the genes for VEGF-D and VEGF-C arose from duplication of a common ancestor gene. Although it is known that c-*fos* induces *VEGF-D* gene expression, few studies have addressed the regulation of this gene. VEGF-D gene expression is induced by cell–cell contact mediated by cadherin-11, but unlike VEGF-C, VEGF-D is not up-regulated by interleukin-1β, tumor necrosis factor α, or serum. Distinct mechanisms of gene regulation occur among VEGF family members to enable independent expression during blood and lymphatic vessel growth and development.

The mechanism by which the transcripts for mouse VEGF-$D_{358}$ and VEGF-$D_{326}$ are generated can be explained by the structure of exon 6 of the mouse *VEGF-D* gene. The VEGF-$D_{358}$ transcript is generated by a splice event from within exon 6 to the beginning of exon 7. In contrast, the transcript for VEGF-$D_{326}$ arises when this splice event does not occur. Therefore, the 3′ region of exon 6 is represented in the VEGF-$D_{326}$ transcript but not in the VEGF-$D_{358}$ transcript. Such alternative splicing events have not been reported for the human *VEGF-D* gene.

## VII. TISSUE DISTRIBUTION

VEGF-D is expressed at many sites in the developing embryo including lung and kidney mesenchyme, skin, liver, heart, and limb buds. As this growth factor can induce both angiogenesis and lymphangiogenesis, it is likely that it plays a role in attracting the growth of blood and lymphatic vessels into these regions of the embryo during development. In adult tissues, VEGF-D is localized in the vascular smooth muscle of blood vessels. Although VEGFR-2 is not strongly expressed on the endothelium of blood vessels in adult tissues, it can be up-regulated in response to various forms of vascular stress or damage. As VEGF-D is an activating ligand for VEGFR-2, the VEGF-D produced by vascular smooth muscle may play a role in repair of blood vessels after vascular damage.

## VIII. PATHOLOGY

VEGF-D is expressed in a range of human tumors including malignant melanoma, glioma, and breast and lung carcinomas. In non-small-cell lung carcinoma, VEGF-D was detected in tumor cells and the endothelium of nearby vessels. Furthermore, mRNA for VEGF-D was detected in the tumor cells but not in the endothelium, indicating that VEGF-D is produced by tumor cells and accumulates in nearby endothelium due to receptor-mediated uptake. These findings suggest a paracrine model by which VEGF-D derived from tumor cells promotes tumor angiogenesis and lymphangiogenesis. Direct demonstration of such a role was established by studies of VEGF-D action in a mouse tumor model that indicated that this growth factor can induce tumor angiogenesis, lymphangiogenesis, and metastatic spread via lymphatic vessels. Therefore, VEGF-D may be a useful target for anti-cancer therapy designed to block metastatic spread via the lymphatics.

## IX. FUTURE DIRECTIONS

Recent studies of VEGF-D action in tumor models suggest that inhibition of VEGF-D may be of use to block metastatic spread. Neutralizing monoclonal antibodies, peptidomimetic inhibitors of receptor binding, and small-molecule inhibitors of the catalytic domains of VEGFR-2 and VEGFR-3 must be carefully tested in animal models of tumor development to establish the utility of targeting the signaling pathways in which VEGF-D is involved. Other possible clinical utilities for VEGF-D include induction of endothelial cell mitogenesis for prevention of restenosis, induction of collateral vessel formation for treatment of diseases involving ischemia, and induction of lymphangiogenesis/lymphatic hyperplasia for treatment of primary and secondary lymphedema. Testing the utility of VEGF-D in these clinical contexts is a matter of high priority.

## Glossary

**angiogenesis** Growth of blood vessels.

**lymphangiogenesis** Growth of lymphatic vessels.

**lymphatic system** An open-ended network of vessels that collect fluid from tissue spaces and ultimately drain it into the venous system.

**lymphedema** Swelling of tissue due to accumulation of lymphatic fluid.

**vasculogenesis** *De novo* formation of blood vessels from mesodermal precursors.

## See Also the Following Articles

**Angiogenesis • Cancer Cells and Progrowth/Prosurvival Signaling • Corpus Luteum: Regression and Rescue • Epidermal Growth Factor (EGF) Family • Estrogen Receptor (ER) Actions through Other Transcription Factor Sites • Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) • HGF (Hepatocyte Growth Factor)/MET System • Platelet-Derived Growth Factor (PDGF) • Vascular Endothelial Growth Factor B (VEGF-B)**

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# Vasoactive Intestinal Peptide (VIP)

Bahri Karaçay and M. Sue O'Dorisio
*University of Iowa College of Medicine*

I. VASOACTIVE INTESTINAL PEPTIDE
II. VIP EXPRESSION DURING DEVELOPMENT
III. VIP RECEPTORS
IV. MECHANISM OF VIP ACTION (SIGNAL TRANSDUCTION PATHWAY)
V. BIOLOGICAL EFFECTS OF VIP
VI. CONCLUSION AND PERSPECTIVES

**Vasoactive intestinal peptide is a 28-amino-acid hormone belonging to the secretin/glucagon superfamily of peptides. Through receptor-specific interactions, vasoactive intestinal peptide activates signal transduction pathways that confer vasodilatory effects throughout the body. In its roles as neurotransmitter, hormone, and cytokine, this peptide also plays a role in oncologic disease; further studies may lead to important therapeutic applications.**

## I. VASOACTIVE INTESTINAL PEPTIDE

### A. Discovery of VIP

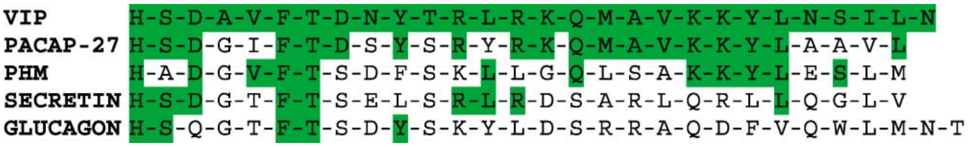
Vasoactive intestinal peptide (VIP), first discovered in porcine duodenum, was initially considered to be a gastrointestinal hormone. The peptide was named for its profound and long-lasting vasodilatory effects in laboratory animals. This initial identification, however, belies a much broader function of VIP in the central and peripheral nervous systems as well as in the immune system. Today we know that VIP functions as a neurotransmitter, a hormone, and a cytokine through neuroendocrine and neuroimmune axes. VIP regulates intestinal water and electrolyte secretion; vaginal fluid secretion; pituitary hormone, neuronal growth factor, and lymphocyte cytokine release; and glycogenolysis in the liver and cerebral cortex. Through its vasodilatory effect, VIP modulates the vascular reactivity for penile erection, influences cervical and vaginal blood flow, and induces bronchodilation. One or more of these functions is essential to the role of VIP in oocyte maturation, neurogenesis, neuroprotection, and immune homeostasis.

### B. Structure of the Peptide

VIP is a 28-amino-acid peptide with structural similarity to other gastrointestinal hormones, including secretin, glucagon, pituitary adenylate cyclase-activating polypeptides (PACAP-37 and PACAP-38), gastric inhibitory peptide (GIP), growth hormone-releasing hormone (GHRH), peptide histidine isoleucine (PHI; in pigs and rodents), and peptide histidine methionine (PHM; in humans) (Fig. 1). Thus, VIP belongs to the secretin/glucagon superfamily of peptides. The sequence is identical in human, porcine, bovine, sheep, goat, rabbit, rat, and mouse VIP, suggesting strict amino acid conservation during evolution.

### C. Structure of the VIP Gene

The approximately 9-kb gene encoding human VIP is located on chromosome 6q24. The VIP gene consists of six introns and seven exons. Exon sizes vary from 89 to 165 bp. Isolation and sequence determination of the cDNA for human VIP reveal that the messenger RNA encodes a second peptide, PHM (PHI in rodents). PHM is encoded by exon four and VIP is encoded by exon five (Fig. 2). The prepro-VIP consists of 170 amino acids; during proteolytic



**VIP** H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N
**PACAP-27** H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L
**PHM** H-A-D-G-V-F-T-S-D-F-S-K-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-M
**SECRETIN** H-S-D-G-T-F-T-S-E-L-S-R-L-R-D-S-A-R-L-Q-R-L-L-Q-G-L-V
**GLUCAGON** H-S-Q-G-T-F-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T

**FIGURE 1** Structures of the secretin/glucagon family of peptides. VIP, Vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine. Areas of amino acid sequence homologies with VIP are shaded. PACAP-27, PHM, secretin, and glucagon share 68, 44, 37, and 20% amino acid homology with VIP, respectively.

cleavage, five peptides are formed: prepro-VIP(22–79) (N-terminal flanking peptide), peptide histidine isoleucine/methionine (PHI/PHM)(81–107), prepro-VIP (111–122) (bridging peptide), VIP(125–152), and prepro-VIP(156–170) (C-terminal flanking peptide). Alternative splicing generates a C-terminally extended PHM, a peptide designated as PHV-42 that has been shown to be a potent smooth muscle relaxer (Fig. 2). The amino acid homology between VIP and PHM (37%) is less than the degree of similarity between VIP and PACAP (68%). The evolutionary process leading to the generation of different precursors for secretin/glucagon superfamily members probably includes serial gene duplication accompanied by exon loss events. The fact that two different peptides (VIP and PHM) are encoded by the same messenger RNA suggests cosynthesis of the two peptides in the same tissue. However, colocalization is not always found in the same cells in the brain. The differential regulation of these two peptides may be due to differences at translational or posttranslational levels.

## D. Regulation of Gene Expression

Cell-type-specific expression of the human VIP gene requires several cis-acting sequences located at the 5′ flanking region of VIP. Among these, a 425-bp tissue-specifier element (TSE) located between −4.6 and −4.0 kb and a region with multiple positive- and negative-acting elements, along with a cyclic adenosine monophosphate (cAMP) response element located between −1.55 kb and −904 bp, play important roles in regulation of VIP gene expression (Fig. 2). The ubiquitously expressed POU-homeodomain proteins Oct-1 and Oct-2 as well as AP-2/Ets transcription factors physically interact with the TSE and play a central role in regulation of VIP gene expression. Accurate spatial expression of the

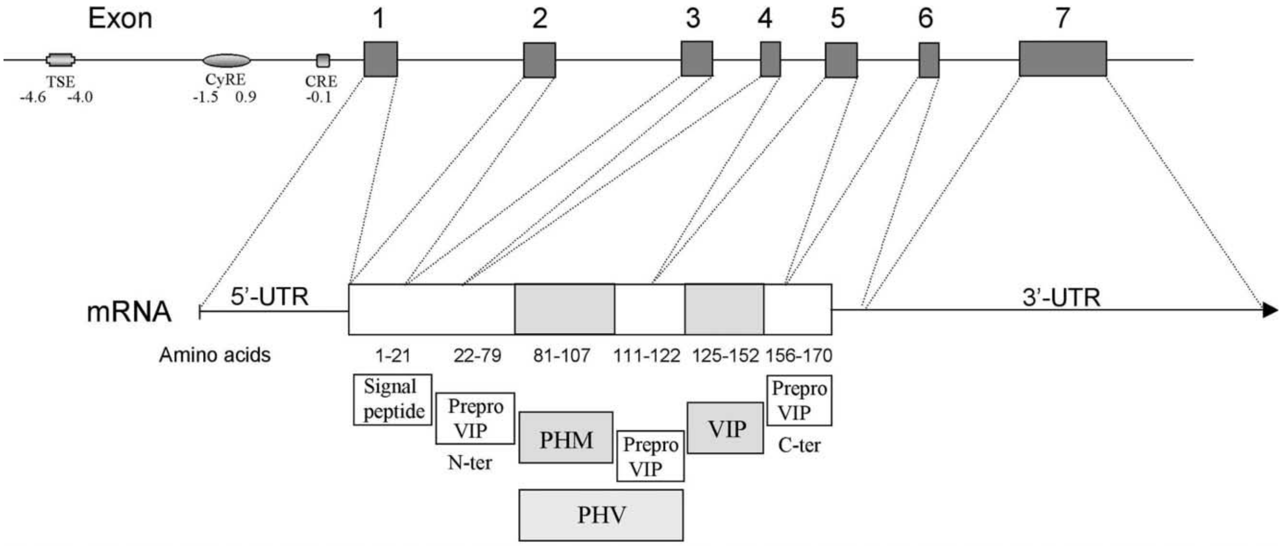


**FIGURE 2** Schematic representation of the human VIP gene structure, mRNA transcript, and peptide products. The VIP gene is approximately 9 kb in size and is located on human chromosome 6q24. Exon sizes vary from 89 to 165 bp. PHM, Peptide histidine methionine; PHV, peptide histidine valine; VIP, vasoactive intestinal peptide; TSE, tissue-specifier element; CyRE, cytokine response element; CRE, cyclic AMP response element; UTR, untranslated region.

VIP gene requires combinatorial effects of the cis-elements within the TSE and the proximal 1.55-kb 5′ flanking sequence. The proximal promoter region contains cis-acting elements such as E-boxes, MEF-2-like motifs, and a cytokine-responsive element. Interactions between the TSE and proximal promoter elements lead to either transcriptional repression or activation of VIP gene expression in different cell lineages, resulting in varying levels of VIP message in different cell types.

The spatial expression pattern of VIP is remarkably well conserved between rodents and human genes. Consistent with this, analysis of 5′ flanking regions of mouse and human VIP genes demonstrates a high level of conservation (80%) immediately upstream of the transcription initiation site that also includes the cAMP response element (Fig. 2). A higher level of conservation (91%) has been found between human and mouse genes within a 210-bp fragment located more than 1.1 kb upstream from the transcription start site; this sequence contains cis-acting elements such as PEA-3 and NGF–IL-6. Studies aimed at determining whether 5′ flanking sequences of the VIP gene could recapitulate the endogenous gene expression have demonstrated that a 5.2-kb promoter region of the human VIP gene is able to direct reporter gene expression to the small intestine of transgenic mice. In a later study, a 2-kb promoter fragment was shown to direct the expression of a reporter gene to tissues in a pattern similar to the endogenous VIP gene expression profile. However, the transgene expression was also detected in smooth muscle and Schwann cells, in which endogenous VIP mRNA is rare. When a 16.5-kb upstream sequence of the mouse VIP gene was fused to β-galactosidase, reporter gene expression was targeted to neurons in the esophagus, stomach, small intestine, and colon, where endogenous VIP is present. However, this reporter gene was not expressed in brain, including the regions that contain high levels of VIP such as the cerebral cortex, thalamus, hippocampus, amygdala, and suprachiasmatic nucleus. These results demonstrate that gene regulation at the level of transcription is of fundamental importance for VIP gene expression, but the results also suggest that additional studies are necessary to provide a better understanding of *in vivo* regulation of VIP gene expression. Recent studies demonstrate that VIP gene expression is also regulated at the posttranscriptional level; elements that regulate RNA stability are localized at the 3′ untranslated region of the VIP message. The presence of different sizes of VIP mRNA with varied half-lives, along with competition studies employing heterologous RNA stability elements, suggest an important role for tissue-specific posttranscriptional regulation of VIP levels (Fig. 2). The multiple levels of control of VIP gene expression provide a highly specific spatial expression pattern.

## II. VIP EXPRESSION DURING DEVELOPMENT

Developmental regulation of the VIP gene has not been studied in great detail. Although samples from different species have been used, most of the data regarding VIP expression have been obtained from rats. Early studies demonstrated no VIP expression in the central nervous system (CNS) of rats until birth, when a rapid increase in expression is observed. However, later studies employing *in situ* hybridization and histochemistry detected VIP message as early as embryonic day 14 (E14) in the hindbrain of a mouse. This may be due to the increased sensitivity of the method used, or to the interspecies differences between mice and rats. After birth, rat VIP mRNA increases in most brain regions until the level and distribution reach the expression profile of the adult. In the cortex, however, VIP message level increases until postnatal day 14 but decreases after postnatal day 21 until it reaches adult levels, suggesting that VIP may be involved in the development of the cortex. VIP is expressed earlier in the peripheral nervous system than in the CNS. *In situ* hybridization detects VIP message in the embryonic body in E14 rat embryos. By E16, VIP message is detectable in the sphenopalatine ganglion, aorta, and intestine. Contrary to late appearance of VIP message in the CNS during development, binding sites for VIP appear earlier (E13 in rats and E9 in mice). VIP binding sites are particularly abundant in brain regions where rapid cell division takes place, such as neuroepithelial regions of the brain or the intermediate medial thalamus. The observation that maternal VIP levels peak during midgestation raises the possibility that maternal VIP may be involved in early embryonic development, when embryonic VIP binding sites are apparent but embryonic VIP is still undetectable.

In the adult central nervous system, VIP is expressed in the cerebral cortex, hypothalamus, amygdala, hippocampus, and corpus striatum at high levels. Potentiation of cAMP levels in the cortex results in promotion of glycogenolysis. Localization of binding sites for VIP in cortex by *in vitro* autoradiography suggests that VIP may function in the energy metabolism of the cortex. Processes of some VIP-containing neurons penetrate the cortical surface, enter the pial membranes, and make contacts with cerebral blood vessels. VIP binding sites are abundant throughout the cortex, with the highest binding in layers of the cortex exhibiting greatest dendritic arborization of VIP neurons (layers I, II, IV, and VI). *In situ* hybridization reveals vasoactive intestinal peptide receptor 1 (VPAC1) message in the cortex of rat, suggesting that VPAC1 may mediate the effects of VIP in cerebral cortex. VIP neurons are also present in the hippocampal formation and send their axons to nearby pyramidal and granule cells, where VIP receptors are expressed. These neurons receive input from the γ-aminobutyric acid (GABA)-ergic septohippocampal pathway and diagonal band. *In vitro* binding studies localize VIP binding throughout the hippocampal formation, with higher levels in the molecular layer of the dentate gyrus. Thus, VIP may play a role in the regulation of electrical activity in the hippocampal formation.

Within the hypothalamus, the suprachiasmatic nucleus (SCN) has the highest density of VIP neurons and VIP binding sites. Both VIP peptide and message levels vary over the day/night cycle, with peak levels occurring at night. Microinjection of VIP or PACAP into the rodent SCN shifts the circadian pace maker whereas VIP antagonists and oligonucleotides disrupt the circadian cycle. Overexpression of VPAC2 in the SCN alters the circadian phenotype of mice, leading to a quicker resynchronization in transgenic mice compared to wild-type animals. VPAC2-overexpressing mice also exhibit a shorter circadian period in constant darkness. Some early studies focused on VIP regulation of prolactin release from the anterior pituitary. Subsequent studies have reported a role for VIP as an important modulator of other pituitary hormones, such as adrenocorticotropic hormone (ACTH), growth hormone, and luteinizing hormone (LH). VIP may also regulate anterior pituitary GABA concentrations.

## III. VIP RECEPTORS

### A. Receptor Subtypes

VIP binds with high affinity to specific G-protein-coupled receptors with seven transmembrane domains. Two receptor subtypes with different affinities for VIP have been isolated and designated as vasoactive intestinal peptide receptors 1 and 2 (VPAC1 and VPAC2) (Table 1). VPAC1 was first isolated from rat lung. The human homologue has also been cloned and expressed in different cell lines. VPAC1 consists of 457 amino acids with an estimated molecular mass of 52 kDa. Murine VPAC1 has 93 and 83% identity with previously cloned rat and human counterparts, respectively. The powerful fluorometric quantitative polymerase chain reaction (PCR) assay has demonstrated that expression of VPAC1 is highest in the small intestine and colon of the mouse, followed by the liver and the brain. VPAC1 message was also detected in thymus, spleen, lung, kidney, gonads, adrenal gland, spinal cord, and heart. Human, mouse, and rat VPAC1 genes map to syntenic regions of human chromosome 3p21.33–p21.31, the distal region of mouse chromosome 9, and to rat chromosome 8q32.

**TABLE 1 Receptor-Specific Agonists and Antagonists for VPAC1 and VPAC2**

| | $IC_{50}$ | | |
|---|---|---|---|
| Ligand | VPAC1 | VPAC2 | Activity |
| VIP | 1 n$M$[a] | 3–4 n$M$[a] | VPAC1 agonist, VPAC2 agonist |
| [Lys$^{15}$, Arg$^{16}$, Leu$^{27}$]VIP$_{(1-7)}$GRF$_{(8-27)}$-NH$_2$ | 1 n$M$ | — | VPAC1 agonist |
| [Arg$^{16}$]chicken secretin | 2 n$M$ | — | VPAC1 agonist |
| [Acetyl-His$^{1}$, D-Phe$^{2}$, lys$^{15}$, Arg$^{16}$]VIP$_{(3-7)}$GRF$_{(8-27)}$-NH$_2$ | 1–10 n$M$ | 3 μ$M$ | VPAC1 antagonist |
| RO25-1392 | — | 10 n$M$ | VPAC2 agonist |
| RO25-1553 | — | 1 n$M$ | VPAC2 agonist |
| PACAP-27 | 1 n$M$ | 10 n$M$ | VPAC1 agonist, VPAC2 agonist |
| PACAP-38 | 30 n$M$ | 2 n$M$ | VPAC1 agonist, VPAC2 agonist |
| Secretin | 1.5 μ$M$ | — | VPAC1 agonist |
| GRF | — | 5–30 μ$M$ | VPAC2 agonist |
| VIPhyb | 0.5 μ$M$ | | VPAC1 agonist |
| VIP$_{(10-28)}$ | 1 μ$M$ | — | VPAC1 antagonist |

[a] Value is $K_d$ instead of $IC_{50}$.

A second high-affinity VIP receptor has been cloned from rat olfactory bulb and designated as VPAC2. VPAC2 has 438 amino acids with an estimated molecular mass of 47 kDa. The VPAC2 gene was mapped to human chromosome 7q36.3. Northern blot analysis shows that the rat VPAC2 gene is expressed in lung, stomach, and intestine, with lower levels in telencephalon, diencephalon, brain stem, cerebellum, and olfactory bulb. VPAC2 message is also detected in rat thymus, spleen, pancreas, adrenal gland, heart, placenta, and pituitary. The two receptors have similar affinities for VIP (Table 1) when expressed in cell lines, with an $IC_{50}$ of 1 and 3 n*M* for VPAC1 and VPAC2, respectively. Both receptors also recognize the related peptide PACAP. VPAC1 has similar affinity for PACAP-27 and PACAP-38 ($IC_{50}$, 1 n*M*), but VPAC2 has different affinities for PACAP-27 and PACAP-38 ($IC_{50}$, 10 and 2 n*M*, respectively). A PACAP-selective receptor (PAC1) has been isolated, cloned, and shown to have a strong affinity for both PACAP-27 and PACAP-38 ($IC_{50}$, 1 n*M*), but has 1000-fold less affinity for VIP ($IC_{50}$, 1 μ*M*).

## B. Receptor Pharmacology

Long-term goals to use VIP analogues for the treatment of cancer, immune disorders, nerve degeneration, and impotence have been hampered by a lack of receptor-specific agonists and antagonists. Similarly, basic research into understanding the biological functions of VIP and its receptors would be facilitated by the availability of specific agonists and antagonists. However, few synthetic VPAC1- and VPAC2-specific agonist or antagonists have yet been designed. Molecular cloning of VIP receptors has facilitated functional testing of several newly developed compounds. These potential peptide agonists and antagonists have been examined for their ability to mimic or inhibit the physiological actions of VIP (Table 1).

Stearyl-Nle-VIP (SNV) is a VIP analogue designed by the addition of a fatty acid moiety to VIP; this enables penetration of the analogue through membranes while maintaining binding and functional activity. SNV also contains a norleucine at position 17, which increases the stability of the molecule against oxidation and increases its lipophilicity. SNV is 100-fold more potent than VIP in providing neuroprotection to cerebellar granule neurons. SNV binds both VPAC1 and VPAC2 with similar affinities as compared to VIP (Table 1). This peptide has also been shown to be effective in potentiation of sexual performance in a variety of impotence models in rats. Toxicological studies in animals indicate that SNV is safe, making it a possible candidate for clinical studies.

Two VPAC1-specific agonists have been described. The VIP/GRF hybrid [$Lys^{15}$,$Arg^{16}$, $Leu^{27}$]-$VIP_{(1-7)}GRF_{(8-27)}$-$NH_2$ has an $IC_{50}$ value of 1 n*M* for VPAC1 but does not bind to the GRF receptor. [$Arg^{16}$]chicken secretin is an agonist at both VPAC1 and secretin receptors, but can be used as a highly selective VPAC1 agonist in tissues, such as brain, that do not express the secretin receptor. [Acetyl-$His^1$, D-$Phe^2$,$lys^{15}$,$Arg^{16}$]$VIP_{(3-7)}GRF_{(8-27)}$-$NH_2$ acts as a selective VPAC1 antagonist ($IC_{50}$, 1–10 n*M*). $IC_{50}$ values of $^{125}$I-labeled VIP binding inhibition by this antagonist, also known as PG 97-269, were 10 and 2 n*M* for rat and human VPAC1, compared to 2 and 3 μ*M* for rat and human VPAC2.

RO25-1553, a VPAC2-specific agonist, was designed with a lactam ring that is introduced within the VIP peptide between positions 21 and 25. It exhibits a high potency and long duration of action. It is a selective agonist for VPAC2, having an affinity for this receptor ($IC_{50}$, 1 n*M*) that is 1000-fold higher than for the VPAC1 receptor ($IC_{50}$, 1 μ*M*). It exhibits anti-inflammatory effects using the VPAC2 signal transduction pathway in lipopolysaccharide (LPS)-stimulated human monocytes, leading to inhibition of tumor necrosis factor α (TNFα) and interleukin-12 (IL-12) synthesis.

A VIP cyclic analogue RO25-1392 is also highly potent and specific for VPAC2 (300-fold greater affinity than for VPAC1). It increases the intracellular concentration of calcium and cAMP. It also down-regulates VPAC2 expression when tested on Chinese hamster ovary cells stably expressing recombinant human VPAC1 or VPAC2. Several VIP antagonists have also been developed using VIP or a peptide from the same family as part of their structure, including [4-Cl-D-$Phe^6$,$Leu^7$]VIP, $VIP_{(10-28)}$, neurotensin$_{(6-11)}$-$VIP_{(7-28)}$ (also designated VHA), stearyl-$Nle^{17}$-hybrid antagonist (SNH), and [Ac-$Tyr^1$, D-$Phe^2$]-growth hormone-releasing factor(1–29) amide. VHA inhibits VIP-mediated cAMP formation or VIP-associated maintenance of neuronal survival in spinal cord cultures. A new derivative of this antagonist yields the stearyl-VIP-hybrid antagonist, stearyl-$Nle^{17}$-neurotensin$_{(6-11)}$-$VIP_{(7-28)}$; it has been shown to be 100-fold more potent in producing neuronal death than the parent VHA molecule and also a growth inhibitor in lung cancer. Although several candidate compounds have been developed within the past few years, the need for specific agonists and antagonists for each VIP receptor remains unfulfilled.

Despite the lack of extensive studies, endogenous peptide fragments of natural VIP, such as $VIP_{(4-28)}$ or

$VIP_{(10-28)}$, may play roles as agonist or antagonist, providing another level of regulation for the VIP–VPAC signal transduction pathway.

## IV. MECHANISM OF VIP ACTION (SIGNAL TRANSDUCTION PATHWAY)

An increase in the intracellular concentrations of cyclic 3′, 5′-adenosine monophosphate is generated by activation of a membrane-associated enzyme, adenylate cyclase, that is stimulated on binding of VIP to either VPAC1 or VPAC2 (Fig. 3). VIP binding to its receptors has been shown to elevate cellular cAMP levels through a guanosine triphosphate (GTP)-regulated coupling of the receptor to a stimulatory guanine nucleotide-binding ($G_s$) protein. In turn, cAMP binds cooperatively to two sites on the regulatory subunit of protein kinase A (PKA). This binding results in release of the active catalytic subunit that phosphorylates its substrate (the serine in the context X-Arg-Arg-X-Ser-X), present in a number of cytoplasmic and nuclear proteins such as cAMP response element binding (CREB) protein or CREMτ. Activated PKA modulates the function of transcription factors that bind to cis-acting elements present in the promoter regions of cAMP-induced

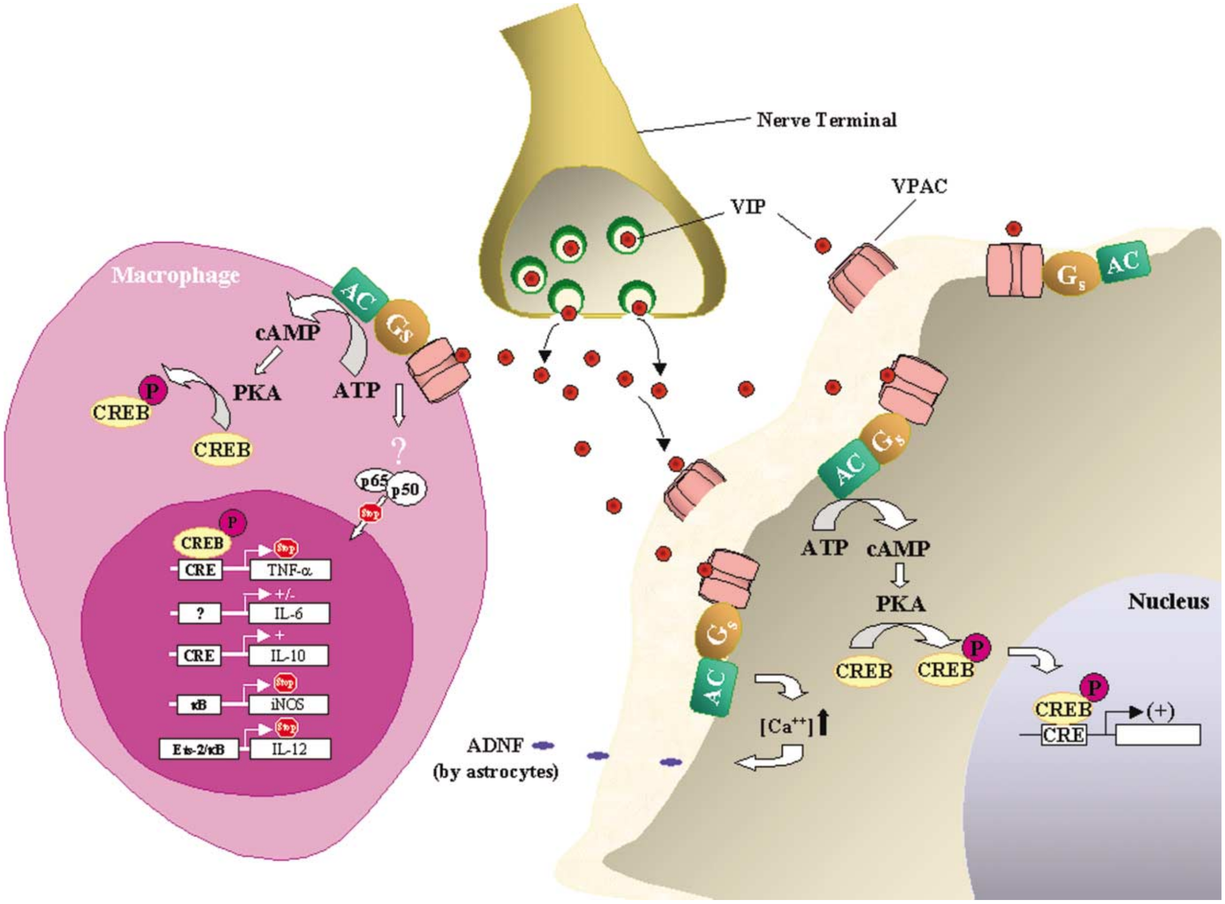


**FIGURE 3** Signal transduction pathway activated by vasoactive intestinal peptide (VIP) binding to receptor VPAC1 or VPAC2. Binding of VIP initiates VPAC1 or VPAC2 association with membrane-bound G-protein ($G_s$), activating adenylate cyclase (AC) and generating cAMP, which, in turn, binds to a regulatory subunit in protein kinase A (PKA). The active catalytic subunit of PKA is then released to phosphorylate its substrate, cAMP response element (CRE) binding protein (CREB). Phospho-CREB binds to cis-acting elements present in the promoter regions of cAMP-induced genes. Through this signal transduction pathway, VIP stimulates synthesis and secretion of neuroprotective molecules such as the cytokine interleukin-1α (IL-1α), activity-dependent neurotropic factor (ADNF), and activity-dependent neuroprotective protein (ADNP) from astrocytes. In macrophages, VIP inhibits the production of proinflammatory factors tumor necrosis factor α (TNFα), IL-6, IL-12, and nitric oxide (iNO) and stimulates the production of the anti-inflammatory cytokine IL-10. A cAMP-independent pathway of VIP is involved in the nuclear factor κB (NF-κB; p65/p50), the inactivation of which affects the transcription of the TNFα, IL-12, and iNOS genes.

genes. Most of the cAMP-responsive genes have one or more copies of cAMP response elements (CREs) within their promoters. The consensus CRE is constituted by the palindromic sequence TGACGTCA (Fig. 3). CRE binding protein, a member of the basic region/leucine zipper (bZip) transcription factor family and the first to be cloned, also binds to CRE. CREB cloning has been followed by cloning of many other proteins that have the characteristic bZip domains. Although heterodimerization is possible among the members, some specific combinations are more common than others. Certain CRE binding factors, such as ATF-2, ATF-3, and ATF-4, can heterodimerize with the bZip oncogenes Fos and Jun, allowing the cAMP/PKA pathway to interact with the diacylglycerol/protein kinase C pathways. Depending on the CRE binding factors, this pathway may result in the activation or repression of the expression of downstream genes.

In some neuronal preparations, inositol phosphate production and protein kinase C activation has been observed, suggesting that VIP signal transduction may follow alternative pathways in addition to adenylate cyclase. After VIP binds to its receptor, the peptide is rapidly internalized by receptor-mediated endocytosis. Most of the receptors are recycled and move back to the cell surface, although some are degraded in lysosomes. VIP peptide is cleaved and inactivated by tissue neutral endopeptidase 24.11, which is widely distributed in the peripheral circulation. VIP is also susceptible to trypsin and plasma-catalyzed cleavage.

## V. BIOLOGICAL EFFECTS OF VIP

### A. VIP in the Nervous System

VIP acts as a neurotransmitter in the nervous system; its presence and synthesis in neurons, its release from the nerve terminals in response to electrical stimulation, its target tissue response after neuronal stimulation, and its enzymatic degradation after binding to VPAC1 or VPAC2 are all characteristics of a true neurotransmitter. Furthermore, electrophysiological studies demonstrate that VIP can affect membrane potential and can act in concert with other neurotransmitters to modulate electrical responses. The effects of VIP on electrical activity can be either excitatory or inhibitory in the same brain region, suggesting that VIP modulates effects of other neuroactive substances. In early studies carried out on isolated spinal cords, VIP led to depolarization of the dorsal root terminals and motoneurons. However, VIP had both inhibitory and excitatory actions on neurons from preoptic, septal, and midbrain regions. VIP enhanced both the inhibitory effects of GABA and excitatory responses to acetylcholine. Although VIP binding to VPAC1 or VPAC2 has been shown to increase the cAMP levels in many cell types, the slow depolarization and decreased membrane resistance of retinal horizontal neurons on VIP treatment are not replicated by analogues of cAMP or forskolin (a lipophilic agent that causes an increase in intracellular cAMP levels). These results suggest that effects of VIP can be mediated through other signal transduction pathways or, alternatively, may be indirect. VIP shares some of the functional characteristics of neurotropins, which play fundamental roles in the nervous system, including neuroprotective neurotropins that are expressed after neural injury. VIP modulates cell proliferation, differentiation, neurite outgrowth, and neuronal survival. Early studies on the ability of VIP to prevent cell death associated with tetrodotoxin demonstrated that VIP stimulates secretion of neuroprotective molecules such as cytokine IL-1$\alpha$, a serine protease inhibitor (protease nexin I), and an extracellular stress protein, activity-dependent neurotropic factor (ADNF), from astrocytes (Fig. 3). Hence, many of the neurotropic and neuroprotective effects of the VIP have been attributed to the secretagogue action of the peptide. The survival-promoting action of VIP is mediated indirectly through nonneuronal astroglia via induction of glial cell neuroprotective proteins. Stimulation of high-affinity VIP receptors on astroglia results in the release of several neurotropic substances, including cytokines, protease nexin I, ADNF, and activity-dependent neuroprotective protein (ADNP). The active sites of ADNF and ADNP (ADNF-9 and NAP, respectively) have been shown to be effective in femtomolar concentrations. The neurotropic action of VIP occurs at very low peptide concentrations (0.1 n*M*) and appears to be correlated with mobilization of calcium and the translocation of specific isoenzymes of protein kinase C. On the other hand, cAMP increases are associated with neurotropic effects in developing sympathetic nervous system.

### B. VIP in Immunomodulation

The endocrine and immune systems are linked through a communication system that utilizes cytokines and neuropeptides. Studies employing immunohistochemistry and flow cytometry demonstrate that VIP is present in all lymphoid organs and in several lymphocyte subpopulations, including T and

B lymphocytes, and splenic lymphocytes. VIP is released by peptidergic nerve fibers that innervate primary and secondary lymphoid organs in mammalian species. VPAC1 and VPAC2 receptors have been detected in rodent and human immune systems by autoradiography. These receptors have been also detected in established cell lines of immune origin. Studies in rodents and humans demonstrate that VPAC1 and VPAC2 genes are differentially distributed and regulated in the immune system. Murine VPAC1 is expressed in stimulated and unstimulated thymocytes and in splenic CD4 and CD8 T cells. In contrast, VPAC2 is expressed on splenic T-cell subpopulations only following stimulation. Resting and LPS-stimulated murine B cells do not express either VIP receptor. VPAC1 is constitutively expressed in resting human T cells and monocytes. VPAC2 is expressed at very low levels in resting human T cells but is not detectable in resting monocytes. *In vitro* stimulation of T cells with soluble anti-CD3 plus phorbol myristic acid (PMA) induces a T-cell activation-dependent down-regulation of VPAC1.

*In vitro* studies have demonstrated that secretion of VIP is enhanced in mitogen-stimulated cell suspensions from different murine lymphoid organs. VIP secretion is also stimulated by corticosteroids and proinflammatory cytokines (IL-1, IL-6, and TNFα) produced by activated murine macrophages. VIP plays a role in modulation of the immune response through its effect on the expression of various key cytokines. VIP inhibits the production of proinflammatory factors TNFα, IL-6, IL-12, and nitric oxide and stimulates the production of anti-inflammatory cytokine IL-10 (Fig. 3). VIP may have inhibitory (inflammatory or endotoxemia models) or stimulatory (unstimulated macrophages or after low LPS doses) effects on IL-6 production, depending on the conditions. These results support the role of VIP as an immune homeostasis mediator. VIP has been shown to suppress collagen-induced arthritis in mice and to correct the associated cytokine imbalance. $T_H1$ and $T_H2$ cells are responsible for phagocyte-mediated host defense and phagocyte-independent host defense, respectively, and have relatively restricted cytokine production profile and effector functions. Enhancement of $T_H2$ function and suppression of $T_H1$ cells have been proposed as a therapeutic approach for rheumatoid arthritis. VIP suppresses $T_H1$ cell function and differentiation but enhances $T_H2$ function, as revealed by decreased interferon γ (IFNγ) and increased IL-4 production, respectively. VPAC1 was shown to be the major mediator of the anti-inflammatory effect of VIP.

VIP message is also detected within granulomas of murine schistosomiasis and expression is localized to granuloma T cells. In murine schistosomiasis, VIP invokes IL-5 release from granuloma T cells. Furthermore, VIP suppresses mitogen- and antigen-induced T-cell proliferation, possibly by inhibiting IL-2 production. The granuloma T cells express both VPAC1 and VPAC2 receptors. VIP also inhibits activation-induced cell death (AICD) *in vivo* and *in vitro* in peripheral T cells and T-cell hybridomas. The effect is dose dependent and is mediated through VPAC1 and VPAC2 receptors. A functional study has demonstrated that inhibition of AICD is achieved through inhibition of activation-induced FasL expression at protein and mRNA levels. VIP has also been shown to increase the survival of thymocytes against glucocorticoid-induced apoptosis, and this action of VIP is mediated by VPAC1. Phenotypic analysis shows that VIP, PACAP-27, and PACAP-38 protect predominantly CD4 + CD8 + thymocytes from glucocorticoid-induced apoptosis.

## C. VIP in Pulmonary and Cardiac Systems

Many systemic and pulmonary blood vessels are innervated by VIP immunoreactive nerve fibers that cause vascular smooth muscle dilation. VIP activation of adenylate cyclase has been observed in cerebral, heart, and coronary vessels and in the portal vein, aorta, mesenteric artery, and ovarian artery of mice. The vasodilatory effect of VIP in different species and tissues is not only due to increased cAMP but also to activation of lipoxygenase, nitric oxide synthase, or the guanylate cyclase and cyclooxygenase pathways. In some species, VIP effects may involve hyperpolarization of the vascular smooth muscle membrane, which reduces calcium influx and intracellular $Ca^{2+}$ concentration. The relative contribution of these various mediators to the vasodilatory effects of VIP is not known; however, interaction between different mediators may modulate VIP action. A possible example of this interaction is seen in gastrointestinal smooth muscle, in which relaxation is stimulated by both cAMP- and cGMP-dependent pathways. The effects of VIP on coronary arteries have been studied in isolated vascular tissue, in intact hearts, and *in vivo* in rodents as well as humans; VIP is shown to have a significant dilatory effect in each species. VIP has a stronger vasodilatory effect on arteries than on veins, possibly due to higher receptor density on the arteries. In ventricular myocytes, VIP binding to its receptor results in increased levels of cAMP, which can stimulate protein kinase A activity. This, in turn, enhances calcium channel phosphorylation and L-type calcium currents, leading to increased intracellular calcium concentration. As a result, cardiac myocyte tension rate and extent of contraction are enhanced. An increase in cAMP can also lead to sequestration of intracellular calcium via decreased troponin affinity for calcium, which subsequently enhances the rate and extent of myocyte relaxation. In this manner, VIP can increase cardiac myocyte contraction and relaxation. *In vivo* studies have also demonstrated that both administered and endogenous VIP can increase coronary blood flow. The vasodilatory effect of VIP is not limited to coronary arteries; similar effects have been observed in cerebral arteries as well as vessels of the eyes, thyroid, and pancreas. A vasodilatory effect of VIP was also confirmed by using a monoclonal anti-VIP antibody that significantly attenuated VIP-induced vasodilation in the *in situ* hamster cheek pouch.

VIP is present in nerves innervating the airway smooth muscles and pulmonary and nasal vessels. VIP produces prolonged relaxation of airway smooth muscles and mimics the electrophysiological changes produced by noncholinergic, nonadrenergic nerve stimulation. VIP has been shown to prevent lung injury and to improve survival in different experimental models of acute respiratory distress syndrome. Protective effects of VIP include inhibition of transcription factor nuclear factor κB (NF-κB) activation (Fig. 3) and inhibition of caspase activity coupled with an increase in antiapoptotic Bcl-2 protein. Both receptor subtypes, VPAC1 and VPAC2, are expressed by several lung cell lines. VPAC2 is expressed in airway epithelial, glandular, and immune cells of the lung but not in airway and vascular smooth muscles. The absence of VPAC2 mRNA in vascular and airway smooth muscle myocytes along with presence of high-affinity VIP binding in the lung suggest that the effects of VIP on vasodilation and bronchodilation are mediated by VPAC1 in the lung.

## D. VIP in the Gastrointestinal System

VIP nerves have been demonstrated in the esophageal wall, mostly in the circular muscle layer. More VIP-containing neurons are located near the sphincter region. Both *in vitro* and *in vivo* studies show that VIP has a relaxing affect on the lower esophageal sphincter, the sphincter of Oddi, and the anal sphincter. In the stomach as well as the small and large intestines, VIP is involved in relaxation of smooth muscles. VIP is a major peptide with relaxant activity and is considered a transmitter of inhibitory motor neurons of the gut. The relaxation-inducing effect of VIP has also been demonstrated using VIP antiserum, which blocks this effect. VIP stimulates the secretion of ductal pancreatic and biliary bicarbonate and water and simultaneously inhibits gastric acid and pepsinogen secretion as well as absorption from the intestinal lumen. VIP also stimulates enzyme secretion from pancreatic acinar cells and regulates chloride secretion. All layers of the gut contain VIP fibers. VIP nerve fibers are most dense in the lamina propria, where they associate with blood vessels and come into contact with the surface epithelium. VIP acts on neurons and muscle cells to regenerate nitric oxide (NO). VIP inhibits the growth of certain colonic adenocarcinoma cell lines and both VIP and VPAC1 expression are elevated during the enterocytic differentiation of a colonic adenocarcinoma cell line *in vitro*.

## E. VIP in Oncology

In 1958, Verner and Morrison described a clinical syndrome that included the triad of pancreatic islet cell tumors, severe watery diarrhea, and hypokalemia. In 1973, Bloom and colleagues made the association between Verner–Morrison syndrome and vasoactive intestinal peptide. They reported that patients with this syndrome also had elevated plasma levels of VIP. These VIP-secreting tumors of the pancreas are also known as VIPomas, watery diarrhea/hypokalemia/hypochlorhydria syndrome, or pancreatic cholera syndrome. VIP is the major mediator of the diarrhea, as demonstrated by a rapid fall in VIP levels and cessation of diarrhea after tumor removal as well as the demonstration that intravenous injection of VIP in normal subjects can induce diarrhea. Some patients develop hypotension resulting from peripheral vasodilation, and severe hypertension may develop after tumor removal; hypotension is also induced by intravenous administration of VIP. These features are compatible with the known cardiovascular effect of VIP. A recent 15-year retrospective review of patients with VIPoma showed that VIP-secreting tumors are usually metastatic at the time of diagnosis. However, survival of the patients with VIPoma has improved due to supportive treatment and the use of chemotherapy.

In approximately 20% of the VIPoma cases, the tumor is too small to localize with computerized tomography (CT) scanning. However, all of these patients receive symptomatic relief of the watery diarrhea. However, in a recent Verner–Morrison syndrome case study, octreotide, an analog of somatostatin, was shown to be effective in reducing the serum VIP level of the patient and in decreasing water loss, but both CT scan and octreoscan failed to detect the tumor. The tumor was detectable in the pancreatic tail using $^{123}$I-labeled VIP scintigraphy. Surgical resection of the tumor resulted in complete remission of the syndrome. Recent data also indicate that $^{123}$I-labeled VIP receptor scintigraphy is clinically useful for the *in vivo* localization of adenocarcinomas, liver metastases, and certain endocrine tumors of the gastrointestinal tract. Although $^{123}$I-labeled VIP receptor scintigraphy is a highly promising method for imaging, the high uptake of the peptide by the lung does not allow therapeutic use of VIP labeled with isotopes such as $^{131}$I or $^{90}$Y. Thus, related compounds with different binding profiles, such as $^{99m}$Tc-labeled VIP conjugates, have been developed and currently are undergoing testing for clinical use. Further scintigraphic studies with patients need to be carried out using newly designed VIP-based radioligands with high specific activity.

In a study to evaluate whether VIP can be used as a tumor marker, serum levels of VIP were evaluated in a total of 135 patients; 45 patients had metastatic colorectal cancer, 45 suffered from metastatic pancreatic cancer, and 45 healthy volunteers served as controls. As opposed to pancreatic cancer and healthy controls, patients with metastatic colorectal cancer had elevated serum VIP levels that were statistically significant ($p < 0.0001$). Several pediatric tumors, including neuroblastoma, primitive neuroectodermal tumors (PNET), and tumors of the Ewing's sarcoma/peripheral PNET family, express either VIP or VIP receptors. High levels of expression of VIP and VIP receptors are a favorable prognostic factor in neuroblastoma, a tumor of neuroectodermal origin. Patients with high levels of VIP and associated diarrhea have a more favorable prognosis. VIP can also slow the rate of proliferation and induce *in vitro* differentiation of neuroblastoma cells. Both medulloblastoma (infratentorial PNET) and supratentorial PNET (tumors derived from neuroepithelial precursors) express VIP receptors as demonstrated by reverse transcriptase (RT) and PCR, high-affinity binding of $^{125}$I-labeled VIP on quantitative autoradiography, and in competitive binding assays. VIP also inhibits tumor cell proliferation in a dose-dependent manner in PNET cell lines.

VIP inhibits proliferation of small-cell lung carcinoma cells (SCLCs) in culture and dramatically suppresses the growth of SCLC tumor cell implants in athymic nude mice. The effect of VIP is mediated by a cAMP-dependent mechanism. High-affinity VIP binding sites are also detected in several breast cancer cell lines as well as breast cancer biopsy specimens as revealed by $^{125}$I-labeled VIP. Several breast cancer cell lines make and secrete immunoreactive VIP; cAMP levels are elevated in these cell lines following VIP treatment. Furthermore, VIPhyb, a VIP antagonist, inhibits breast cancer growth *in vitro* and *in vivo*. Retinoic acid treatment of breast carcinoma cell lines leads to the down-regulation of the VPAC1 receptor.

## VI. CONCLUSION AND PERSPECTIVES

The previously unknown role and contribution of VIP to certain biological processes have been discovered within the past few years. During the preparation of this article, for example, a possible role for VIP in autism was reported. In the first study, in which neuropeptide and neurotropin concentrations were determined, plasma concentrations of VIP were found to be higher at birth in children with autism or mental retardation without autism than in healthy control children. The roles of VIP in brain development and its functions as neurotransmitter and neuromodulator warrant more research on its role in autism.

Anti-inflammatory effects of VIP in the immune system have also recently been documented. These studies have led to the use of VIP in the treatment of inflammatory collagen-induced arthritis in mice, suggesting a new therapeutic approach for rheumatoid arthritis. VIP inhibits the production of proinflammatory factors TNF$\alpha$ IL-6, IL-12, and nitric oxide, and stimulates the production of anti-inflammatory cytokine IL-10.

Receptor targeting with radiolabeled peptides is rapidly developing in nuclear oncology. These naturally occurring peptides are clinically useful diagnostic imaging agents and have future implications for the treatment of tumors expressing target receptors. Radiolabeled VIP and VIP analogues are being developed and evaluated for clinical application because of observations that most human carcinomas express VIP receptors as measured by *in vitro* receptor autoradiography. The application of radiolabeled VIP ($^{123}$I-labeled VIP) is generally safe and scintigraphy results can be obtained within 2–4 h after injection of the peptide.

Availability of genomic and cDNA sequences for VIP peptide and its receptors will facilitate the disruption of corresponding genes in mice using homologous recombination techniques, thus uncovering the biological processes in which VIP is involved as well as the relative contributions of each receptor for VIP action. Together with the development of receptor-specific analogues, these studies should lead to development of alternative therapies that will take advantage of manipulating VIP-initiated signal transduction pathways. Although use of native VIP as a drug is limited due to its susceptibility to endopeptidases and its limited passage across biological membranes, several VIP analogues have been developed to overcome this problem and are under investigation for their possible use.

Vasoactive intestinal peptide clearly functions in multiple ways, including neurotransmission; neuromodulation, immunomodulation, bronchodilation, vasodilation, smooth muscle relaxation, and modulation of reproduction. One peptide that can fulfill multiple roles as a cytokine, neurotransmitter, and hormone must be a physiologic VIP, a "very important peptide."

## Glossary

**agonist** Peptide, hormone, or drug that binds to a specific receptor and activates its signal transduction cascade.

**antagonist** Peptide, hormone, or drug that binds to a specific receptor and blocks its signal transduction cascade.

**autoradiography** Visualization of radioactively labeled molecules on X-ray film.

**complementary DNA** Nucleic acid synthesized by reverse transcription of RNA; cDNA is complementary to the RNA template.

**G-proteins** Guanine nucleotide-binding trimeric proteins that reside in the plasma membrane and transduce a signal from a receptor–ligand complex to the signal transduction cascade.

**$IC_{50}$** Concentration of ligand that inhibits by 50% the binding of a homologous radiolabeled ligand to a specific receptor.

**$K_d$** Concentration of ligand that inhibits by 50% the binding of an identical radiolabeled ligand to a specific receptor.

**signal transduction** Process by which a ligand–receptor interaction activates a cascade of biochemical reactions, leading to a change in cellular activity.

**$T_H1$** A T helper lymphocyte that makes interleukin-2 and interferon $\gamma$, promoting a cellular immune response.

**$T_H2$** A T helper lymphocyte that makes interleukin-4, -5, -9, and -10, promoting humoral or allergic responses.

**transfection** Introduction of new genetic material into a cell.

**transgenic** Describes animals that are generated by insertion of new DNA into the germ line via DNA microinjection into the one-cell-stage embryo.

## See Also the Following Articles

**Gastrin • Gastrointestinal Hormone-Releasing Peptides • Glucagon Action • Growth Hormone-Releasing Hormone (GHRH) • Oocyte Development and Maturation • Peptide YY • Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Its Receptor • Secretin • Vagal Regulation of Gastric Functions by Brain Neuropeptides**

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# Vasopressin (AVP)

William E. Armstrong

*University of Tennessee College of Medicine*

I. INTRODUCTION
II. NEUROANATOMICAL LOCALIZATION AND CONNECTIONS OF AVP NEURONS
III. AVP ACTIONS AND RECEPTORS
IV. STIMULI FOR AVP RELEASE
V. ELECTROPHYSIOLOGY OF AVP NEURONS
VI. SUMMARY

**Vasopressin, also called antidiuretic hormone because of its actions on the kidney, is formed with an arginine at position 8 in all mammals but pigs. Arginine vasopressin is a 9-amino-acid polypeptide hormone that is synthesized in and released from mammalian hypothalamic neurosecretory cells. It functions in blood pressure regulation and water reabsorption by the kidneys.**

## I. INTRODUCTION

Arginine vasopressin (AVP) and the closely related peptide oxytocin (OT) were the first pituitary hormones for which the amino acid structures were characterized (Fig. 1). Each hormone consists of 9 amino acids with an $NH_2$ terminus; the amino acids form a ring via disulfide bonds at the two cysteine residues. These hormones are similar to peptides serving related functions in fishes, birds, and amphibians. In pigs, vasopressin is formed with a lysine substituted for arginine at position 8.

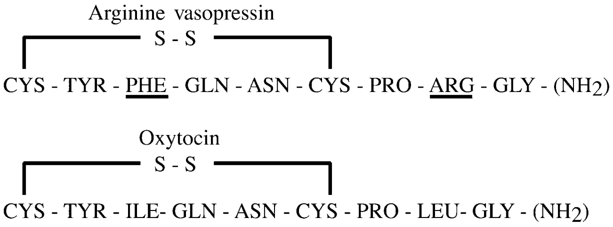
Arginine vasopressin
S - S
CYS - TYR - PHE - GLN - ASN - CYS - PRO - ARG - GLY - (NH2)
Oxytocin
S - S
CYS - TYR - ILE- GLN - ASN - CYS - PRO - LEU- GLY - (NH2)

**FIGURE 1** The amino acid sequences for arginine vasopressin and oxytocin. The nonapeptides share all but two amino acids, with substitutions at positions 3 and 8. Note the cysteine ring formed by disulfide bonds.

The production of neurohypophyseal hormones in brain tissue first was established by Ernst Bargmann and the Scharrers, who utilized Gomori-positive histochemistry (which reveals sulfide-rich elements) to demonstrate the axonal pathway from the hypothalamus to the neurohypophysis. These early studies have been confirmed and expanded with more advanced biochemical and immunohistochemical studies. AVP is synthesized in neurons of the supraoptic nuclei (SON) and paraventricular hypothalamic nuclei (PVN) of the central nervous system (CNS) (Fig. 2). Synthesis begins with a preprohormone complex containing a signal peptide; the latter is cleaved and the remaining protein is glycosylated to a prohormone in the Golgi apparatus. The prohormone is packaged into large (150–200 nm) neurosecretory vesicles, which are axonally transported to the neural lobe of the pituitary gland for release into the general circulation. The prohormone contains AVP, a large protein called neurophysin, and a 39-amino-acid glycopeptide (absent in the OT complex). These products are separated by several enzymes within the neurosecretory granules during axonal transport and are released as separate products. There is no generally accepted peripheral function for neurophysin or the glycopeptide, although each may have some biological activity. A natural "knockout" for the AVP gene, the Brattleboro rat, is characterized by a single base deletion. The Brattleboro rat has extreme polydipsia and polyuria and has been a model for diabetes insipidus.

Although in most situations AVP and OT are found in separate cells, many neurons can synthesize both peptides, as shown by extensive colocalization of mRNA when amplification procedures are employed. During enhanced hormone release, the amount of colocalization may be increased for OT and AVP as well as for other peptides, although in lesser quantities (e.g., neuropeptide Y), found in the same neurons. Synthesis is at least partly regulated by the stimuli that evoke AVP release. For example, osmotic stimulation increases transcription of AVP and mRNA abundance and increases the length of the RNA poly(A) tail. Transcription of AVP mRNA appears to be regulated in part by immediate-early genes such as c-*fos*, but other regulators, such as the cAMP response element binding protein, are

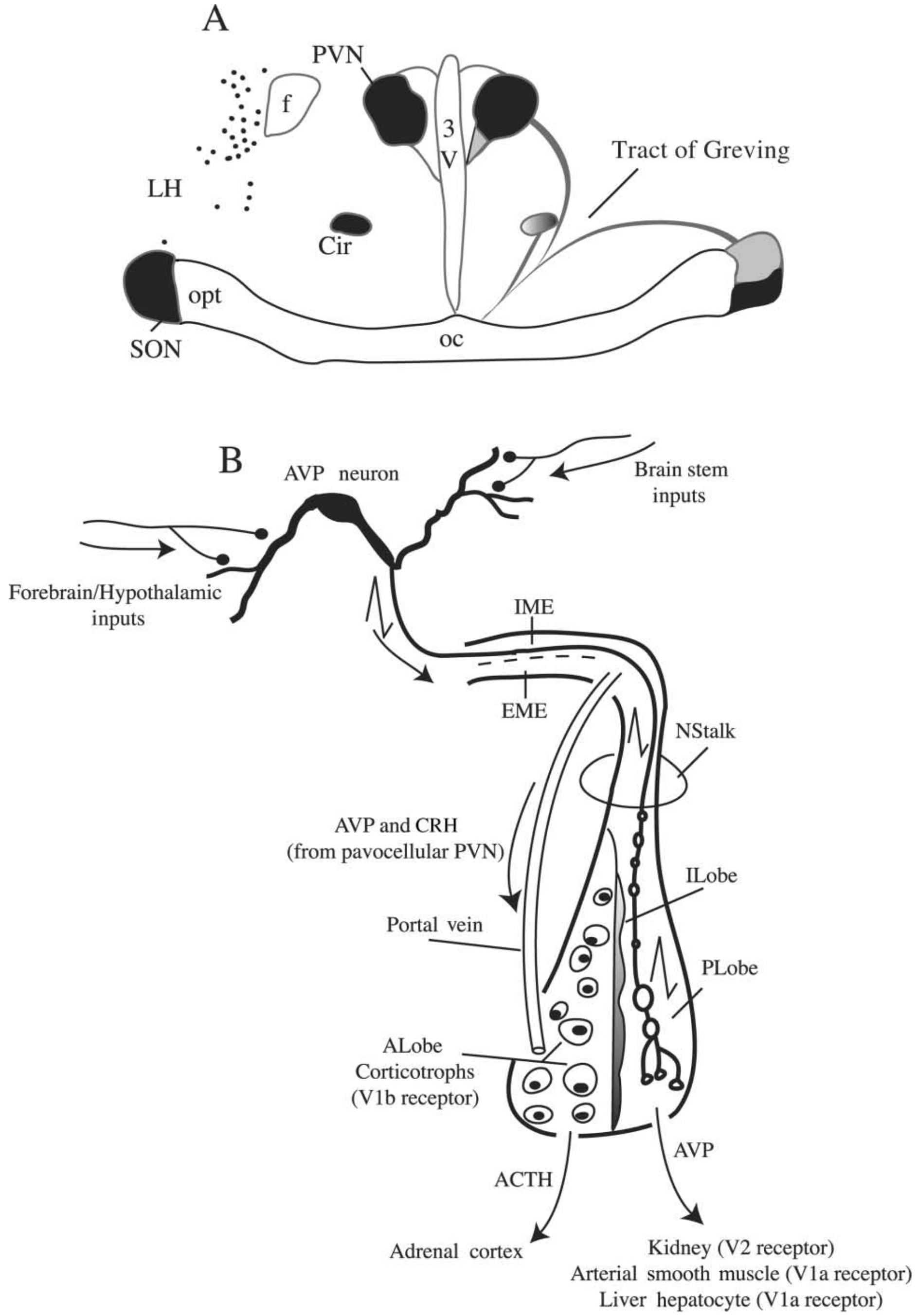
A
PVN
f
3
V
LH
Tract of Greving
Cir
opt
SON
oc
B
AVP neuron
Brain stem inputs
Forebrain/Hypothalamic inputs
IME
EME
NStalk
AVP and CRH (from pavocellular PVN)
ILobe
Portal vein
PLobe
ALobe Corticotrophs (V1b receptor)
AVP
ACTH
Adrenal cortex
Kidney (V2 receptor)
Arterial smooth muscle (V1a receptor)
Liver hepatocyte (V1a receptor)

**FIGURE 2** Anatomical location and connectivity of arginine vasopressin (AVP) neurons. (A) Drawing of a coronal section through the rat hypothalamus, illustrating the location of AVP and oxytocin neurons and schematizing their projection toward the pituitary. On the left, paraventricular hypothalamic nuclei (PVN) and supraoptic nuclei (SON) fiber tracts are named. On the right, AVP neuronal clusters are shown in black and oxytocin clusters are in gray. In addition to the PVN and SON, accessory neurosecretory nuclei are shown. At this plane, there are scattered neurons in the lateral hypothalamus (LH), and a cluster called the nucleus circularis (Cir). The axons from all nuclei collect in the tract of Greving, which eventually leads to the internal layer of the median eminence before projecting into the posterior (neural) lobe of pituitary. Abbreviations: f, fornix; oc, optic chiasm; opt, optic tract; 3V, third ventricle. (B) Schematic representation of an AVP neuron and its axonal projection to the posterior lobe of the pituitary. The neuron has a sparse dendritic tree but receives important brain stem synapses that relay vascular reflex signals; from the forebrain and rostral hypothalamus, synapses relay osmotic, vascular, and other stimuli. The single axon projects into the internal layer of

involved. These factors can be activated by neurotransmitter actions and/or increases in intracellular ($[Ca^{2+}]_i$).

## II. NEUROANATOMICAL LOCALIZATION AND CONNECTIONS OF AVP NEURONS

For neurohypophyseal release, the neurons synthesizing AVP are located in the SON, PVN, and a few accessory magnocellular nuclei (Fig. 2). Neurons in these regions send axons arching through the lateral hypothalamus (tract of Greving) to collect along the midline of the ventral hypothalamus in the internal lamina of the median eminence. These axons lead into the neural stalk and terminate in the posterior (or neural) lobe of the pituitary gland in varicosities, largely 1–2 μm in diameter, near fenestrated capillaries. Larger swellings (5–15 μm) called Herring bodies are more visible with histochemical stains but typically do not form endings per se. Herring bodies serve to store hormone but can also release it with prolonged stimulation. In addition to axon terminals and a dense capillary bed, the neural lobe contains a large number of modified astrocytes, called pituicytes, which have an intimate morphological relationship with the terminals. Pituicyte processes normally separate terminals from capillaries and can engulf terminals. The processes recede during periods of great hormone demand.

Magnocellular PVN and SON neurons have a relatively simple morphology (Fig. 2). A typical neuron has an egg-shaped somata 25–30 μm in diameter, two to three short dendrites with few branches, and a single axon arising from the soma or primary dendrite. A few axons may branch and form local connections (not shown in Fig. 2). This simple morphology computes to a relatively small surface area and, electrically speaking, a large input resistance. Thus, even distal synaptic inputs may reach the soma and exert significant control over the membrane potential and spike initiation. The dendrites are varicose, moderately spinous, and also contain AVP. Varicosities dot the axons along their course and form the classic "string of pearls" described in original Gomori-positive material.

Within the SON and PVN, AVP and OT neurons cluster into separate regions. In many species, a large cluster of AVP neurons characterizes the lateral wing of the PVN. Although all mammals, including humans, have a mix of OT and VP neurons in both the PVN and SON, the distribution and proportions vary widely across species. SON and PVN somata and dendrites receive a variety of synaptic inputs. Quantitatively, the most numerous synaptic terminals contain the excitatory amino acid glutamate or the inhibitory amino acid γ-aminobutyric acid (GABA). All of the sources of these two transmitters are not known, but two important contributors are the median preoptic nucleus and organum vasculosum of the lamina terminalis (OVLT), two areas known to participate in osmoregulation. Another strong forebrain input comes from the subfornical nucleus, also involved in water regulation. Although accounting for a minority of synapses, noradrenergic and other inputs arising from the ventrolateral medulla and nucleus of the solitary tract have great importance for cardiovascular functions. Additional inputs to the SON and/or the PVN include the locus coeruleus, the parabrachial nucleus, the bed nucleus of the stria terminalis, adjacent hypothalamic nuclei, and midbrain and/or hypothalamic dopaminergic neurons. Neurochemically, AVP neurons receive a rich variety of peptidergic and other neuromodulators, many of which colocalize with glutamate or GABA.

AVP also is found in other CNS neurons and synaptic terminals. Within the PVN, a parvocellular group of neurons synthesizes both AVP and corticotropin-releasing hormone (CRH). These neurons control the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary via projections to the portal vascular plexus of the external layer of the median eminence (Fig. 2). The suprachiasmatic nucleus contains AVP neurons involved in circadian rhythms. The bed nucleus of the stria terminalis and

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the median eminence and into the neural stalk before arborizing in the posterior lobe. AVP-containing vesicles are present in the many varicosities along the axon, the largest of which are called Herring bodies. The axons terminate near fenestrated capillaries, where released hormone enters the bloodstream. Action potentials from the neuron drive release at the terminals. The external layer of the median eminence also contains AVP terminals from axons of parvocellular PVN neurons that colocalize with corticotropin-releasing hormone (not shown). These neurons release these two hormones into the external layer of the median eminence, where portal veins carry the products into the anterior lobe of the pituitary. Abbreviations: ALobe, anterior lobe (adenohypophysis) of the pituitary; ACTH, adrenocorticotropic hormone; EME, external layer of the median eminence; ILobe, intermediate lobe of the pituitary; IME, internal layer of the median eminence; NStalk, neural stalk; PLobe, posterior (neural) lobe of the pituitary gland.

medial amygdaloid nucleus have widespread outputs; AVP neurons in these nuclei are highly responsive to gonadal steroids. The central effects of AVP on a variety of neuronal functions are well documented.

## III. AVP ACTIONS AND RECEPTORS

The pressor effects of AVP were discovered over 100 years ago. Shortly thereafter, the antidiuretic effects of AVP in kidney were appreciated, and, more recently, effects of AVP in liver, brain, anterior pituitary, and testis have been demonstrated. The receptors for AVP, now cloned, fall into three main classes: V1a, V1b, and V2 receptors. Along with the OT receptor, all are part of the G-protein-coupled superfamily. V1a receptors are coupled to G-proteins ($G_{q/11}$, $G_i$). Activation leads to increased phospholipases, production of inositol 1,4,5-trisphosphate and diacylglycerol, the activation protein kinase C, and the mobilization of intracellular calcium. V1a receptors also can increase $[Ca^{2+}]_i$ via extracellular influx by coupling to voltage-gated $Ca^{2+}$ channels (primarily L type). For vascular smooth muscle, V1a activation results in contraction and an increase in arterial blood pressure, and activation has a mitogenic effect as well. Because of the wide vascular distribution, V1a receptors can be found in most tissues, including the medullary vessels in the kidney. In the liver, AVP activation of V1a receptors on hepatocytes leads to gluconeogenesis and glycogenolysis. In the brain, V1a receptors are found on a variety of neurons, including autoreceptors on AVP cells, and on the pituicytes of the neural lobe. Although sensitivity to AVP can vary, even across vascular beds, AVP typically activates V1a receptors with $K_d$ values in the low nanomolar or high picomolar range. Occupation of a divalent binding site on the receptor, preferentially by $Mg^{2+}$, increases the affinity of AVP for the receptor.

The V1b receptor population is dense on corticotrophs of the anterior pituitary. Like V1a activation, yet with distinct intracellular pathways, V1b occupation leads to increased $[Ca^{2+}]_i$. Although the details of these messaging signaling differences are not fully known (e.g., cAMP may be increased with strong V1b activation), V1b receptors have a different pharmacological profile compared to V1a receptors, with a lower affinity for various V1a antagonists. V1b activation on corticotrophs leads to release of ACTH. AVP expression is low (even undetectable) in parvocellular PVN neurons but increases during stress or in the absence of negative glucocorticoid feedback (as with adrenalectomy). Because of the potency differences, AVP may normally serve as a cofactor augmenting the response of colocalized CRH. Like the V1a receptors, V1b receptors are found on neurons as well as in other tissues.

V2 receptors are the classic renal receptors and are concentrated on the collecting ducts and distal tubules of the kidney medulla. Activation of V2 receptors leads to a G-protein ($G_s$) stimulation of adenylyl cyclase, an increase in cyclic adenosine monophosphate (cAMP), activation of protein kinase A, and insertion of new water (aquaporin-2) channels. These new channels enhance reabsorption of water in the collecting ducts and tubules, producing concentrated urine. The affinity of AVP for the V2 receptor is in the low picomolar range.

## IV. STIMULI FOR AVP RELEASE

Increased plasma osmolality, decreased blood volume, and hypotension are the most effective and well-studied stimuli for the release of AVP. A low release threshold and the exquisite sensitivity of the kidney suggest that small osmolality perturbations near basal levels ($\sim$285 mOsm/kg $H_2O$) are finely controlled by AVP release (Fig. 3). The osmotic threshold for release is 1–3% in a well-hydrated subject, which can lead to an antidiuresis from the release of just a few picomoles of AVP. Suprathreshold stimulation results in a linear relationship between plasma osmolality of AVP release. Although a purely osmotic stimulus is apparently sufficient for AVP release, $Na^+$ is the most abundant and effective physiological osmotic particle, and brain $Na^+$ detection is a part of the normal response to hypertonicity. Release of AVP is triggered locally with the SON and PVN by these same stimuli. Locally released AVP can feed back on the neurons to modulate their electrical activity (see later).

The hypothesis first proffered by Verney some 60 years ago, that brain osmoreceptors were located near the SON, has been confirmed in a variety of studies and species. Indeed, the SON neurons are osmoreceptive. The mechanism is demonstrated to be a volume-dependent modulation of stretch-inactivated, nonselective cationic channels (SICs). Membrane stretching associated with hypotonic cell swelling decreases SIC channel openings. Cell shrinkage increases them, provoking membrane depolarization. Furthermore, extracellular $Na^+$ increases the permeability of SIC channels, and prolonged hypertonicity has been shown to increase $Na^+$ channel density in SON neurons. However, the intrinsic osmoreceptivity of the AVP neuron is insufficient to account for the full activation of SON neurons and

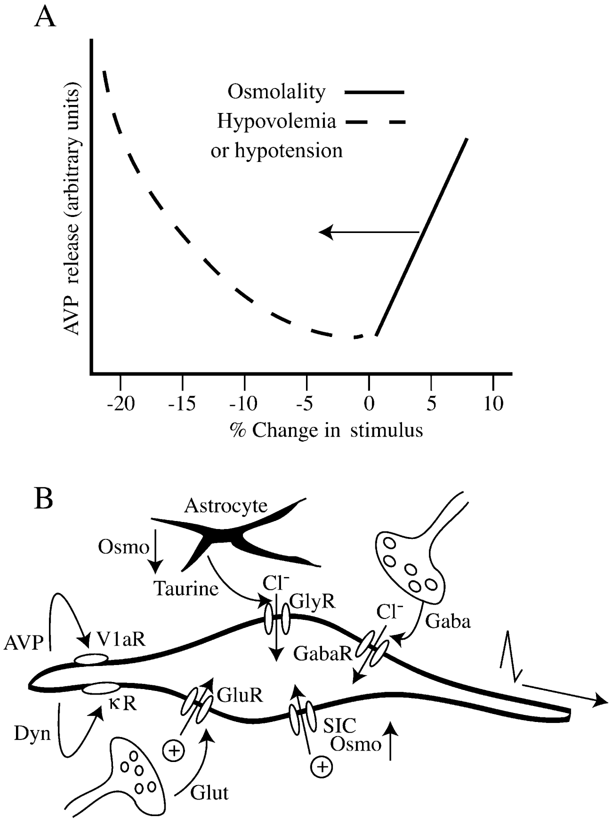

**FIGURE 3** Integration of vascular and osmotic signals by arginine vasopressin (AVP) neurons. (A) Schematic diagram illustrating the responsiveness of AVP neurons to both vascular (dashed line) and osmotic (solid line) stimuli. AVP release is linearly related to changes in osmotic pressure above threshold and is more sensitive to this than to vascular stimuli. Vascular stimuli are exponentially related to release, but ultimately can cause release of larger quantities of AVP compared to hypertonicity. The arrow shows that the osmotic curve is displaced to the left in the presence of hypovolemia/hypotension, such that release in response to osmotic stimuli is enhanced. (B) Neural, glial, and osmotic signals are integrated by AVP neurons and lead to changes in action potential activity. Osmotic stimuli have two effects: first, increased osmolality (Osmo)/$Na^+$ concentration opens stretch-inactivated cation (SIC) channels directly on the AVP cell, depolarizing it; second, decreases in osmolality increase the release of taurine from astrocytes in the supraoptic nuclei. Taurine increases $Cl^-$ influx through glycine receptors (GlyR). Thus, during hypertonicity, the neuron is released from this inhibitory stimulus in addition to being depolarized directly. Excitatory and inhibitory transmitters (and many modulators not shown) impinge on the AVP neuron. Some carry information from cardiovascular receptors, transducing blood volume and pressure signals, others carry information from additional osmoreceptive neurons. Increased activity is associated with increased somato-dendritic release of AVP and the colocalized peptide, dynorphin (Dyn). AVP acts on V1a receptors to increase intracellular $[Ca^{2+}]$ and to modulate neuronal activity. Dynorphin acts on κ receptors (κR), but the exact second messengers and/or channels modulated are not known. However, dynorphin decreases the depolarizing afterpotential and inhibits burst length. Abbreviations: Gaba, γ-aminobutyric acid; GabaR, γ-aminobutyric acid receptor; Glut, glutamate; GluR, glutamate receptor; V1aR, V1a subclass of AVP receptor.

AVP release. Additional osmosensitive elements are located along the lamina terminalis of the hypothalamus, in the OVLT and median preoptic nucleus. Direct osmoreceptivity has been demonstrated in excitatory OVLT neurons projecting to the SON. Activation of these neurons increases the frequency of glutamate-mediated excitatory postsynaptic potentials and contributes strongly to SON activation. Indeed, damage to the ventral wall of the lamina terminalis strongly impairs osmoregulation and can be associated with chronic hypernatremia in humans. Astrocytes invested within the SON are also osmosensitive and release the inhibitory amino acid taurine during hypotonicity. Thus, intrinsic SON and glial cell osmoreceptivity, as well as the activity of osmosensitivity afferent inputs, all participate in the fully integrated response of AVP neurons (Fig. 3).

Dehydration incurs hypovolemia and hypertonicity. Plasma volume deficits as low as 5%, induced with hemorrhage or isotonic fluid withdrawal, stimulate AVP release in the absence of any change in osmolality (Fig. 3). For AVP release, purely volemic stimuli are transduced in the thoracic vasculature, primarily by atrial stretch receptors. Unloading these receptors results in strong neuronal activation of the SON via brain stem nuclei such as the nucleus of the solitary tract, the ventrolateral medulla, and the parabrachial nucleus. Osmotic and volemic signals clearly interact, because small changes in blood volume result in a stronger osmotic response. Conversely, osmotic dilution will blunt the response to hypovolemia. The integration of these signals likely occurs in the hypothalamus, if not the SON. Hypovolemia also triggers the release of renin, which leads to a pressor response from angiotensin II (AII). By stimulating AII receptors in the subfornical organ, AII can augment AVP release via a strong projection from this organ to the SON.

Hypotension often accompanies hypovolemia and it can be difficult to separate the stimuli. Hypotensive stimuli are primarily transduced by arterial receptors in the carotid sinus and aortic arch and reach the SON via related, but not identical, brain stem neurons, as for hypovolemia. In some animal models, hypovolemia induces AVP release only when there is an associated drop in arterial pressure, and this response is dependent on nerve activity from sinoaortic receptors. Nevertheless, considerable data suggest that volume and pressure signals are separately transduced, if often integrated. In humans, the release of 1–2 pmol of AVP can be triggered by a reduction in arterial pressure by ~5%, and further reductions are exponentially related to AVP release, resulting in a large release (~100 pmol) with reductions near 40%. Receptor activation need not depend on total blood or water loss, however, because shifts in regional blood pools induced by temperature or body position are effective at altering AVP release. Like volume stimuli, hypotension also enhances the response to increased osmolality (Fig. 3).

Although V1a receptors with high affinity are densely distributed on hepatocytes, the physiological role of AVP in the regulation of blood glucose is questionable. Pronounced hypoglycemia is a trigger for AVP release, but the levels of AVP required to increase plasma glucose are thought to be high for day-to-day regulation.

## V. ELECTROPHYSIOLOGY OF AVP NEURONS

The pioneering studies of Harris and Douglas showed clearly that $Ca^{2+}$-dependent stimulus-secretion coupling, much like that observed at the neuromuscular junction, characterizes neurohypophyseal hormone release. It is now widely appreciated that release of AVP from the neural lobe is a function of both the pattern and the rate of action potential activity originating in SON and PVN neurons. Action potentials depolarize the axon terminals and increase $Ca^{2+}$ influx, which triggers the exocytosis of AVP-containing vesicles.

Hypotension, hypovolemia, and hyperosmolality all trigger a stereotypic electrical response from AVP neurons. This response consists of a phasic, bursting pattern of activity (Fig. 4). In normal animals, very few neurons exhibit this pattern. With even moderate dehydration, the majority of AVP neurons adopt this pattern. Stimulation of phasic neurons increases the firing rate within bursts and can increase burst length. Phasic activity is clearly the most efficient means of releasing AVP (Fig. 5). Studies directly using the action potential distribution of bursts to stimulate the axons electrically in the neural lobe have shown that this pattern is superior to continuous trains of stimuli at the same mean frequency for releasing AVP. The maximally efficient frequency is ~13 Hz. Efficiency is related both to the high frequency of discharge at the beginning of each burst (facilitation) and to the recovery from fatigue during interburst intervals. Recovery during these silent intervals maximizes $Ca^{2+}$ uptake into the terminals for successive bursts.

Although phasic activity is instigated and controlled by the peripheral stimuli known to release AVP, the mechanisms for this activity are intrinsic properties of the AVP neuron and can be observed in synaptically uncoupled neurons. Phasic activity is voltage dependent, with a threshold from $-60$ to $-50$ mV. Thus, peripheral stimuli must depolarize the neuron to this range before the activity can commence, regardless of whether isolated action potentials occur. With depolarization, action potentials open voltage-gated $Ca^{2+}$ channels and induce a $Ca^{2+}$-dependent depolarizing afterpotential (DAP) (Fig. 4). The DAP summates with repetitive firing and forms a plateau potential on which a long (10- to 60-s) discharge is maintained. The current underlying the DAP appears to result from the reduction in a resting $K^+$ conductance, although inward cationic currents participate. SON neurons contain many other conductances that could shape the activity of the burst, the best studied of which are low- and high-threshold $Ca^{2+}$ currents, a $Ca^{2+}$-dependent $K^+$ current that gates the firing rate, voltage-gated $K^+$ currents that influence the firing rate and spike width, and voltage-gated, nonselective cation currents, such as $I_H$.

Recent data of great interest concern an autoinhibitory feedback mechanism controlling burst length. AVP neurons also synthesize the opiate peptide dynorphin and package it within AVP dense core vesicles. Both dynorphin and AVP are released locally during neuronal activity. Dynorphin acts on κ opioid receptors on AVP neurons and serves to inhibit phasic bursting. At least one mechanism for this inhibitory effect is a direct inhibition of the DAP. Thus, accumulative release of dynorphin during spike activity may eventually terminate the burst.

The V1a (and possibly V1b or related variant) receptors are located on the membrane of AVP neurons, just as OT receptors have been found on OT neurons. Activation of these receptors increases $[Ca^{2+}]_i$ and also modulates AVP neuronal activity. The precise effect (i.e., inhibitory or excitatory) varies with the ongoing level of activity, so as to reinforce the most efficient firing rate for AVP release.

## VI. SUMMARY

Arginine vasopressin released from the posterior pituitary is synthesized within a preprohormone complex by large neurons in SON and PVN of the

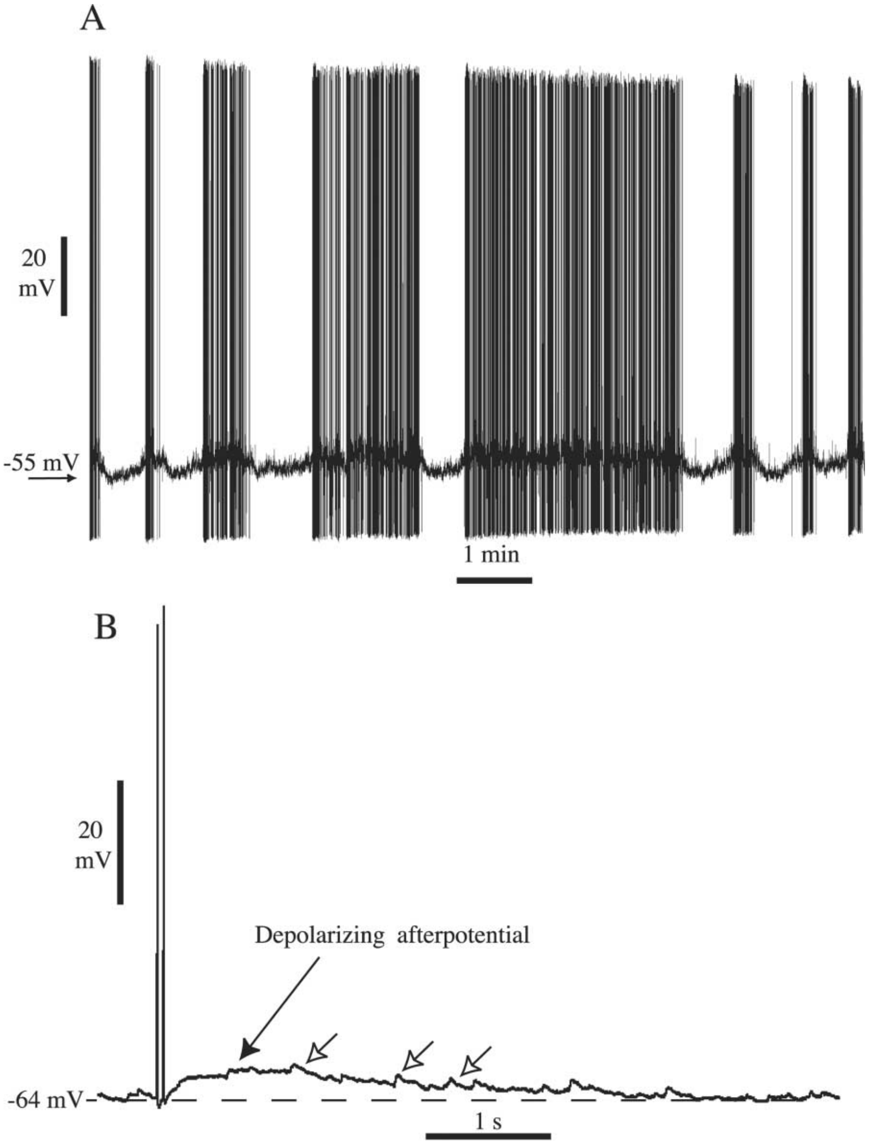

**FIGURE 4** (A) Example of a phasically bursting AVP neuron from a whole-cell recording in a hypothalamic slice from an adult rat. Note the periods of action potential activity associated with membrane depolarizations, interrupted by silent periods when the membrane is hyperpolarized. (Recording courtesy of Chunyan Li). (B) The depolarizing afterpotential implicated in phasic activity is shown following two action potentials evoked with current injection. At this more hyperpolarized potential, a depolarizing afterpotential can be evoked, but the resulting depolarization does not reach spike threshold. Note the excitatory synaptic potentials (open arrowheads).

mammalian hypothalamus. After axonal transport to the neurohypophysis, the hormone is released into the peripheral circulation by stimuli such as dehydration, hypovolemia, and hypotension. At least three cloned AVP receptors are present. V1a receptors are found primarily on vascular smooth muscle and act to transduce muscle contraction. V1b receptors are found on pituitary corticotrophs and their activation leads to ACTH release. V2 receptors are found in the kidney, where they mediate antidiuresis. AVP neurons are osmoreceptive.

## Glossary

**anterior pituitary lobe** Also called the adenohypophysis, this region contains numerous hormone-secreting cells under humoral control from hypothalamic inhibitory and excitatory factors.

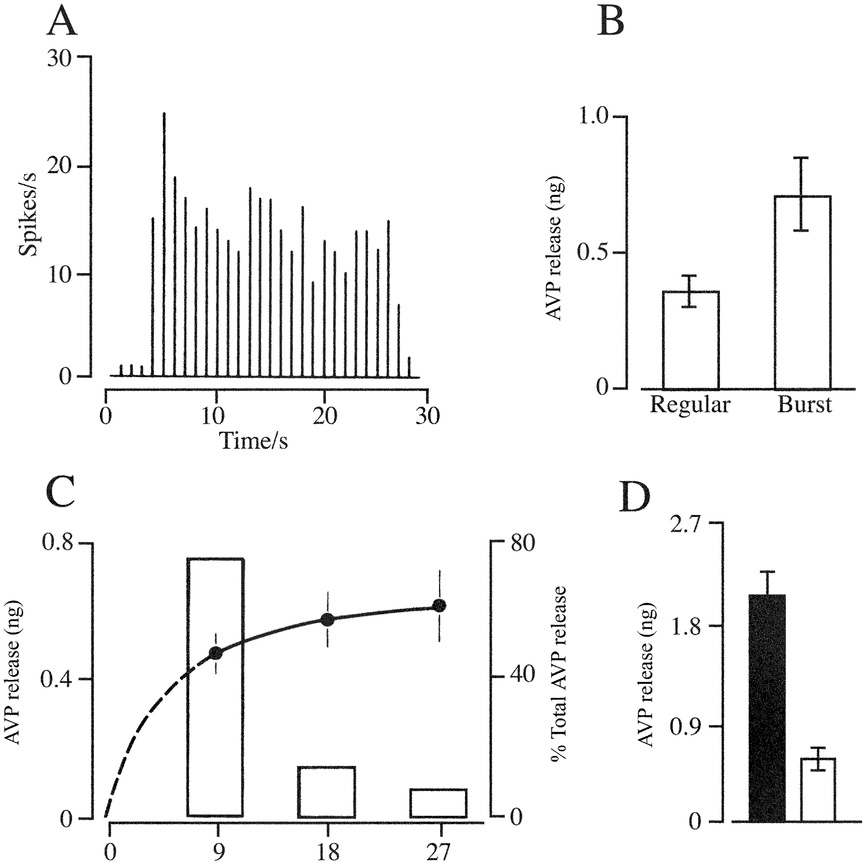

FIGURE 5 The relationship between phasic bursting activity and arginine vasopressin (AVP) release in isolated neural lobes. (A) Rate meter of a burst recorded in an AVP neuron. This burst was used to program the electrical stimulation of isolated neural lobes. The burst contained 348 spikes, lasted 26.7 s, and had a mean firing rate of 13 Hz. Note that firing rate is higher at the beginning of the burst. (B) Total AVP release evoked in isolated neural lobes is much greater with the burst as compared to when lobes are stimulated at the same frequency, with the same number of pulses, in a regular manner (i.e., the interpulse interval was constant). (C) Groups of neural lobes were stimulated with the AVP burst for the first 9 s, the first 18 s, or the complete burst. The black circles show the AVP release from each group. The bars show the amount of AVP attributable to each 9-s period as a percentage of the total amount released. Release is high in the beginning of the burst, when frequency is higher, and low in the final 18 s of the burst. The efficiency is likewise highest in the first 9 s, when each pulse evoked an average of 3.3 pg AVP, compared with 0.7 and 0.6 pg AVP, respectively, in the second and third 9-s periods. (D) Neural lobes were stimulated with four successive bursts that were either separated by 21-s silent intervals (filled bar) or were administered without silent periods (open bar). Release is higher when recovery from fatigue is allowed during the silent periods. Modified from Cazalis *et al.* (1985).

**median eminence** Collection of neurosecretory axons along middle, ventral, and posterior portions of the hypothalamus; connects the brain with the pituitary gland.

**neurohypophysis** Posterior (neural) and intermediate lobes of the mammalian pituitary gland.

**oxytocin** Polypeptide hormone closely related to vasopressin. Composed of 9 amino acids, oxytocin is synthesized in and released from neurosecretory cells in the hypothalamus of mammals; promotes uterine contractions, salt excretion, and milk ejection.

**posterior (neural) pituitary lobe** Houses vasopressin- and oxytocin-containing axon terminals, fenestrated capillaries, and a large population of astrocyte-like cells, the pituicytes.

**supraoptic and paraventricular nuclei** Clusters of neurons in the mammalian hypothalamus with axons that project to the neurohypophysis; synthesize vasopressin and oxytocin.

**vasopressin** Polypeptide hormone composed of 9 amino acids. Synthesized and released from neurosecretory cells in the hypothalamus of mammals, vasopressin increases blood pressure and promotes water reabsorption from the kidneys.

## See Also the Following Articles

**Adrenocorticotropic Hormone (ACTH) and Other Proopiomelanocortin (POMC) Peptides • Neuropeptides**

**and Control of the Anterior Pituitary • Oxytocin • Oxytocin/Vasopressin Receptor Signaling**

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# VEGF

See *Vascular Endothelial Growth Factor*

# VIP

See *Vasoactive Intestinal Peptide*

# Vitamin D

Anthony W. Norman
*University of California, Riverside*

I. VITAMIN OR HORMONE?
II. CHEMISTRY
III. PHOTOBIOLOGY
IV. VITAMIN D ENDOCRINE SYSTEM
V. NUTRITIONAL REQUIREMENTS
VI. DISEASE STATES
VII. SUMMARY

**Vitamin D is essential for life in higher animals. Classically, vitamin D has been shown to be one of the most important biological regulators of calcium homeostasis. This important biological effect is only achieved as a consequence of the conversion of vitamin D into a family of daughter metabolites, including the two key kidney-produced metabolites 1α,25(OH)$_2$-vitamin D$_3$ [1α,25(OH)$_2$D$_3$] and 24R,25(OH)$_2$-vitamin D$_3$ [24R,25(OH)$_2$D$_3$]. 1α,25(OH)$_2$D$_3$, is a steroid hormone and there is increasing evidence that 24R,25(OH)$_2$D$_3$ is also a steroid hormone.**

## I. VITAMIN OR HORMONE?

The first scientific description of the bone disease rickets, which is the hallmark of vitamin D deficiency, was provided in written scientific treatises as early as 1645. The major breakthrough in understanding the causative factors of rickets was the development of nutrition as an experimental science and the appreciation of the existence of vitamins. Considering the fact that the biologically active form of vitamin D is now known to be a steroid hormone, it is somewhat ironic that vitamin D, through a historical accident, became classified as a vitamin. In 1919–1920, Sir Edward Mellanby, while working with dogs raised exclusively indoors (in the absence of sunlight or ultraviolet light), devised a diet that allowed him to unequivocally establish that rickets was caused by a deficiency of a trace lipid component present in the dog's diet. In 1921, he wrote, "The action of fats in rickets is due to a vitamin or accessory food factor which they contain, probably identical with the fat-soluble vitamin." Furthermore, he established that cod liver oil was an excellent anti-rachitic agent. This ultimately led to the anti-rachitic factor being classified as a vitamin or as an essential dietary trace component that prevents rickets. However, in 1924 it was discovered that a precursor of vitamin D that could be converted into vitamin D by exposure to sunlight or ultraviolet light was present in skin. Thus, the substance known as vitamin D is correctly termed "a vitamin" only when there is a deficiency of sunlight exposure.

The modern era of vitamin D began in 1965–1970 with the discovery and chemical characterization of a metabolite of vitamin D, namely, $1\alpha,25(OH)_2$-vitamin $D_3$ [$1\alpha,25(OH)_2D_3$] and its nuclear receptor, VDR. It is now accepted that vitamin D is a precursor of $1\alpha,25(OH)_2D_3$, which generates biological responses as a steroid hormone.

## II. CHEMISTRY

Vitamin $D_3$ is the naturally occurring form of vitamin D and is produced from 7-dehydro-cholesterol. Vitamin $D_2$ is a synthetic form of vitamin D that is produced by irradiation of the yeast steroid, ergosterol. The structures of vitamin $D_3$ (cholecalciferol) and its provitamin 7-dehydrocholesterol are presented in Fig. 1. Vitamin D is a generic term and indicates a molecule of the general structure shown for rings A, B, C, and D with differing side chain structures. The A-, B-, C-, and D-ring structure is derived from the cyclopentanoperhydrophenanthrene ring structure for steroids. Technically, the steroid vitamin D is classified as a seco-steroid. Seco-steroids are those in which one of the rings has been broken; in vitamin D, the 9,10 carbon–carbon bond of ring B is broken, and it is indicated by the inclusion of "9,10-seco" in the official nomenclature. Vitamin D (synonym calciferol) is named according to the revised rules of the International Union of Pure and Applied Chemists (IUPAC). Because vitamin D is derived from a steroid, the structure retains its numbering from the parent compound cholesterol (see Fig. 1). Asymmetric centers are designated by using the R,S notation; the configuration of the double bonds is indicated as E for "eingang" or *trans* and Z for "zuzammen" or *cis*. Thus, the official name of vitamin $D_3$ is 9,10-seco(5Z,7E)-5,7,10(19)cholestatriene-3β-ol. Vitamin $D_3$ is currently the form of calciferol that is used for food supplementation, particularly of milk, in the United States. Vitamin $D_2$ differs from $D_3$ by virtue of the presence of a 22-ene and 24-methyl group in the side chain. Historically, vitamin $D_2$ was used in the years 1940–1960 as a food supplement to supply vitamin D activity. The official name of vitamin $D_2$ is 9,10-seco(5Z,7E)-5,7,10(19),22-ergostatetraene-3β-ol.

## III. PHOTOBIOLOGY

Vitamin $D_3$ can be produced photochemically by the action of sunlight or ultraviolet light on the precursor sterol 7-dehydrocholesterol, which is present in the epidermis or skin of most higher animals (see Fig. 2). The chief structural prerequisite of a provitamin D is that it be a sterol with a Δ5-7 diene double bond system in ring B (see Fig. 1). The conjugated double bond system in this specific location of the molecule allows the absorption of light quanta (energy) at certain wavelengths in the ultraviolet (uv) range; this can readily be provided in most geographical locations by natural sunlight. This initiates a complex series of transformations (partially summarized in Fig. 2) that ultimately results in the transformation into vitamin $D_3$. Thus, it is important to appreciate that vitamin $D_3$ can be endogenously produced and that as long as the animal (or human) has access on a regular basis to sunlight there is no dietary requirement for this vitamin.

## IV. VITAMIN D ENDOCRINE SYSTEM

Vitamin $D_3$ is not known to have any intrinsic biological activity itself. It is only after vitamin $D_3$ is metabolized, first into 25(OH)-vitamin $D_3$ [25-$(OH)D_3$] in the liver and then into $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ by the kidney, that biologically active molecules are produced. *In toto*, some 37 vitamin $D_3$ metabolites have been isolated and chemically characterized. Fig. 3 illustrates the concept of the vitamin D endocrine system. The elements of the vitamin D endocrine system include the following: (1) in the skin, photoconversion of 7-dehydrocholesterol to vitamin $D_3$ or dietary intake of vitamin $D_3$; (2) conversion vitamin $D_3$ by the liver to $25(OH)D_3$, which is the major form of vitamin D

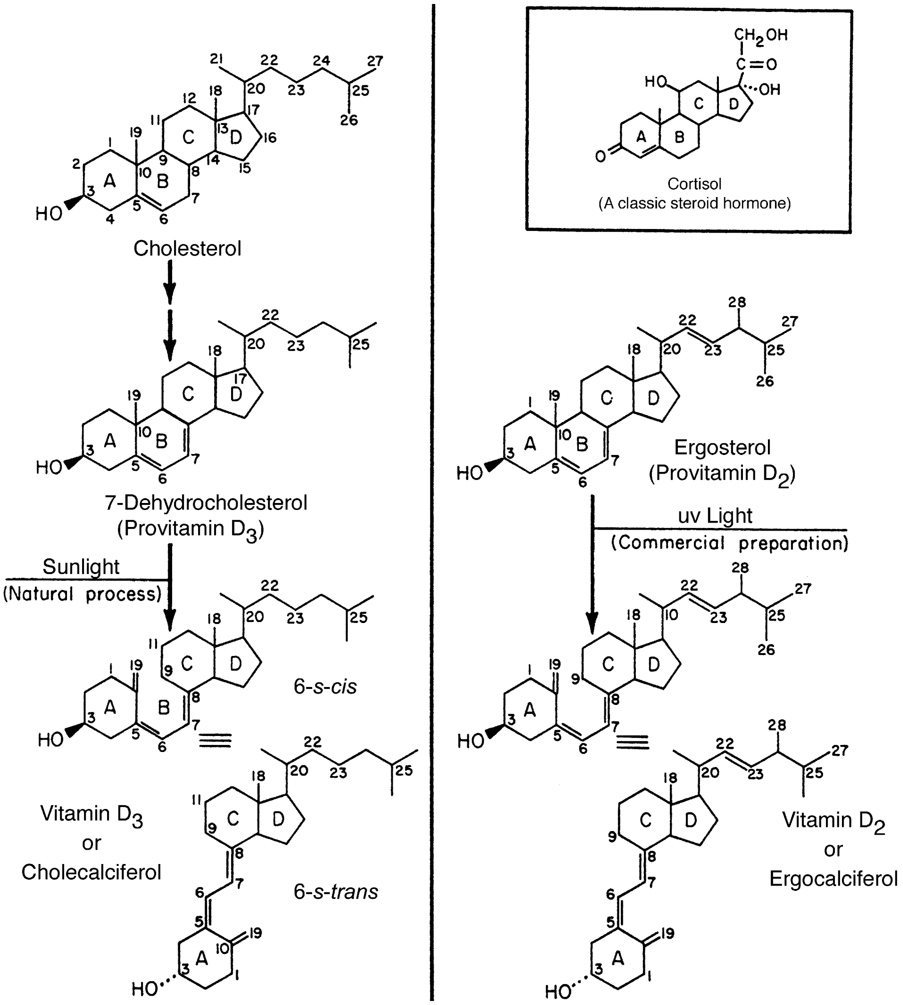

Cholesterol
7-Dehydrocholesterol
(Provitamin D3)
Sunlight
(Natural process)
6-s-cis
Vitamin D3
or
Cholecalciferol
6-s-trans
Cortisol
(A classic steroid hormone)
Ergosterol
(Provitamin D2)
uv Light
(Commercial preparation)
Vitamin D2
or
Ergocalciferol

FIGURE 1 Structural relationship of vitamin $D_3$ (cholecalciferol) and vitamin $D_2$ (ergocalciferol) with their respective provitamins (7-dehydrocholesterol and ergosterol), cholesterol, and a classic steroid hormone, cortisol (see inset box). The two structural representations presented at the bottom for both vitamin $D_3$ and vitamin $D_2$ result from the conformational flexibility in the B ring that allows 180° rotation about the 6,7 single carbon bond. This rotation generates a population of conformers that are represented by the 6-*s-cis* and the 6-*s-trans* orientations indicated in the figure. Vitamin $D_3$ is the naturally occurring form of the vitamin; it is produced from 7-dehydrocholesterol, which is present in the skin, by the action of sunlight or ultraviolet light. Vitamin $D_2$ is produced commercially by the irradiation of the sterol ergosterol with ultraviolet light.

circulating in the blood compartment; (3) conversion of $25(OH)D_3$ by the kidney (functioning as an endocrine gland) to produce the two principal dihydroxylated metabolites, $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$; (4) systemic transport of the dihydroxylated metabolites $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ to distal target organs; (5) binding of the steroid hormone $1,25(OH)_2D_3$ to either a nuclear receptor or a membrane receptor at the target organs followed by the subsequent generation of appropriate biological responses. An additional key component in the operation of the vitamin D endocrine system is the plasma vitamin D-binding protein (DBP), which carries vitamin $D_3$ and all its metabolites to their various target organs.

The three enzymes responsible for the conversion of vitamin $D_3$ into its two key daughter metabolites include the hepatic vitamin $D_3$-25-hydroxylase and the two kidney enzymes, $25(OH)D_3$-$1\alpha$-hydroxylase and $25(OH)D_3$-24R-hydroxylase. All three enzymes have been demonstrated to be cytochrome P450 mixed-function oxidases. Both renal enzymes are localized in mitochondria of the proximal tubules of the kidney. The $25(OH)D_3$-$1\alpha$-hydroxylase has been

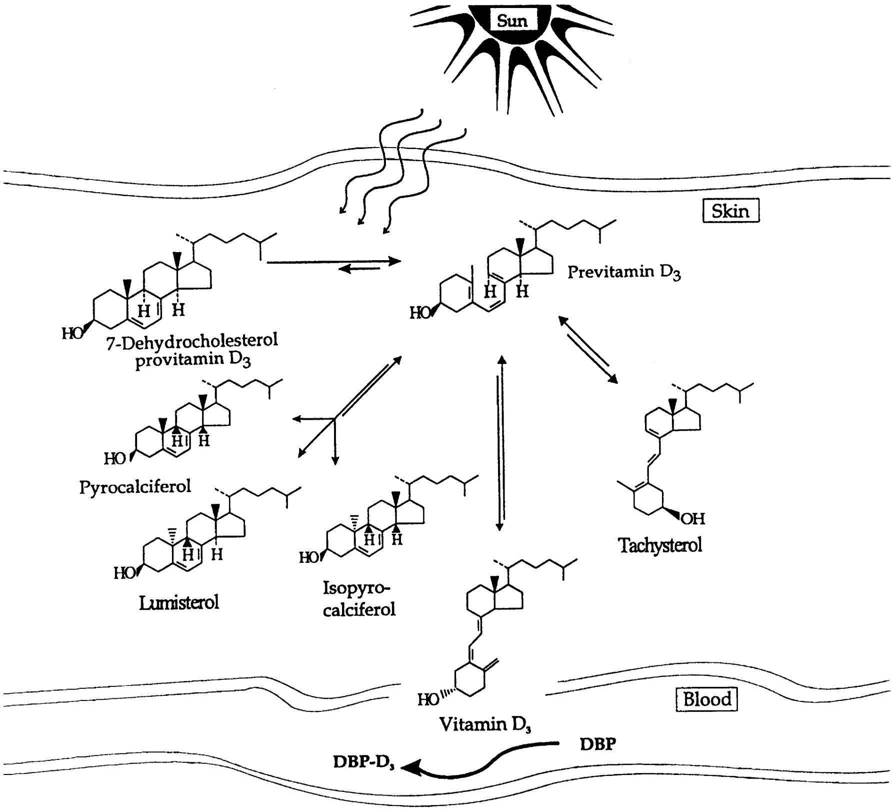


FIGURE 2 Photochemical pathway of production of vitamin $D_3$ (calciferol) from 7-dehydrocholesterol. The starting point is the irradiation of a provitamin D, which contains the mandatory $\Delta^{5,7}$-conjugated double bonds; in the skin, this is 7-dehydrocholesterol. After absorption of a quantum of light from sunlight (ultraviolet B), the activated molecule can return to the ground state and generate at least six distinct products. The four steroids that do not have a broken 9,10 carbon bond (provitamin D, lumisterol, pyrocalciferol, and isopyrocalciferol) represent the four diastereomers with either an α- or a β-orientation of the methyl group on carbon 10 and the hydrogen on carbon 9. The three seco-steroid products, vitamin $D_3$, previtamin D, and tachysterol, have the three conjugated double bonds at different positions. In the skin, the principal product is previtamin D, which then undergoes a 1,7-sigmatropic hydrogen transfer from C-19 to C-9, yielding the final vitamin D. Vitamin D can be drawn as either a 6-*s-trans* representation (this figure) or a 6-*s-cis* representation (Fig. 1), depending upon the state of rotation about the 6,7 single bond. The resulting vitamin $D_3$, which is formed in the skin, is removed by binding to the plasma transport protein, the vitamin D-binding protein (DBP), present in the capillary bed of the dermis. The DBP-$D_3$ then enters the general circulatory system.

cloned and the specific site of mutations that result in the appearance in patients of vitamin D-resistant rickets type I (VDRR-I) has been identified. These patients lack the ability to produce 1α,25$(OH)_2D_3$ and thus express the phenotype of vitamin D deficiency. In addition, 25(OH)$D_3$-24R-hydrodroxylase and vitamin $D_3$-25-hydroxylase have been cloned.

The most important point of regulation of the vitamin D endocrine system occurs through the stringent control of the activity of the renal 25(OH)$D_3$-1α-hydroxylase. In this way, the production of the hormone 1α,25$(OH)_2D_3$ can be modulated according to the calcium needs and other endocrine needs of the organism. The chief regulatory factors are 1α,25$(OH)_2D_3$ itself, parathyroid hormone (PTH), and the serum concentrations of calcium and phosphate. The secretion of PTH is inversely proportional to the serum $Ca^{2+}$ concentration and increased secretion of PTH will result in stimulation of the renal 25(OH)$D_3$-1α-hydroxylase and a concomitant increase in the serum concentration of 1α,25$(OH)_2D_3$. Probably the most important determinant of 1α-hydroxylase is the vitamin D status of the animal. When the circulating concentration of 1α,25$(OH)_2D_3$ is low, a lowered serum $Ca^{2+}$ results and production of 1α,25$(OH)_2D_3$ by the kidney is high; when the circulating concentration of 1α,25$(OH)_2D_3$ is high, the output of 1α,25$(OH)_2D_3$ by the kidney is sharply reduced.

## A. Mode of Action of 1α,25(OH)$_2$

$1\alpha,25(OH)_2D_3$ produces a wide spectrum of biological responses through interaction with both its nuclear receptor to regulate gene transcription and a second receptor associated with the cellular membrane that mediates the generation of a variety of rapid signal transduction processes (see Fig. 2).

### 1. *Genomic Responses of $1\alpha,25(OH)_2D_3$*

The genomic responses to $1\alpha,25(OH)_2D_3$ are a consequence of the stereospecific interaction of this steroid hormone with its nuclear vitamin D receptor, the VDR. The VDR is a protein of 50 kDa that binds $1\alpha,25(OH)_2D_3$ with high affinity; the $K_d \approx 0.5$ nM. The VDR is not able to bind the parent vitamin D, whereas $25(OH)D_3$ and $1\alpha$ $(OH)D_3$ only bind 0.1–0.3% as well as $1\alpha,25(OH)_2D_3$. The primary amino acid sequence of the VDR, like all nuclear receptors for steroid hormones, is divided into five functional domains: these include regions (from the N-terminal to the COOH-terminal) involved in nuclear localization, DNA binding, heterodimerization with other nuclear proteins, ligand binding, and transcriptional activation.

The process of nuclear receptor-mediated regulation of gene transcription is exquisitely dependent upon the complementary relationship between the unoccupied receptor and its cognate ligand. Thus, the unoccupied receptor is largely unable to engage in a productive fashion with the transcriptional machinery to effect meaningful regulation of gene transcription. It is only after the ligand-receptor complex has formed, resulting in conformational changes in the receptor protein, that a functional receptor protein is generated. Thus, acquisition of a detailed understanding of the complementarity of the ligand shape with that of the interior surface of the nuclear VDR receptor ligand-binding domain is believed to be the key to understanding not only the structural basis of receptor action and its formation of heterodimers and interactions with co-activators, but also to designing new drug forms of the various hormones, including $1\alpha,25(OH)_2D_3$.

The protein receptors for all steroid hormones (estrogen, progesterone, testosterone, cortisol, and aldosterone) and the protein receptors for $1\alpha,25(OH)_2D_3$, retinoic acid, and thyroid hormone are all members of the same gene superfamily; accordingly, there is a high level of conservation in their amino acid sequences, particularly in their DNA- and ligand-binding domains. The X-ray crystallographic structures of the ligand-binding domains (LBDs) of the thyroid hormone receptor, the retinoic acid receptor, the estrogen receptor, and the progesterone receptor have all been determined with their respective ligands bound. Also, the crystal structure of the nuclear receptor for vitamin D bound to its natural ligand $1\alpha,25(OH)_2D_3$ has been determined at a 1.8 Å resolution. The VDR LBD structure, as well as that of the other nuclear receptors, consists of 12 α-helices that are arranged to create a three-layer sandwich that completely encompasses the ligand $1\alpha,25(OH)_2D_3$ in a hydrophobic core. The secondary and tertiary structural features of this family of receptor proteins were found to be remarkably similar.

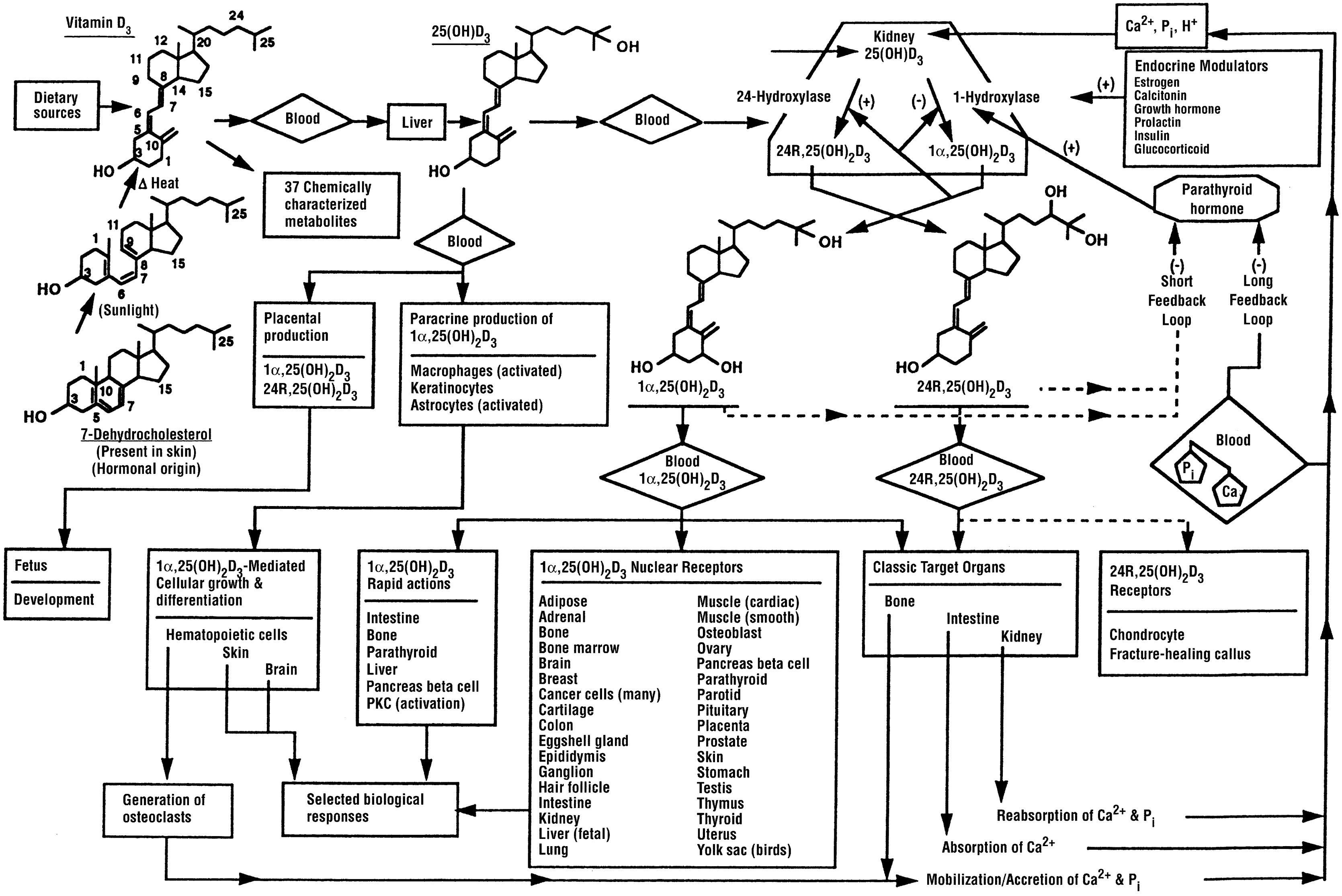
An animal model in which the gene for the VDR has been deleted (termed a gene knockout or KO) has been engineered by targeted disruption of both the first and the second zinc-fingers of the DNA-binding domain of the VDR. This VDR KO displays the phenotype of vitamin D-dependent rickets type II (VDDR-II). Despite the widespread tissue distribution of the VDR (see Fig. 2), the resultant animals were phenotypically normal at birth, indicating that there is biological redundancy with respect to most of the functions of this receptor. The most surprising observation was that all of the VDR KO mice developed alopecia (hair loss) by 7 weeks. In addition, the male and female VDR KO mice were infertile.

### 2. *Rapid Responses of $1\alpha,25(OH)_2D_3$*

The "rapid" responses mediated by $1\alpha,25(OH)_2D_3$ (see Fig. 3) are postulated to be mediated through interaction of $1\alpha,25(OH)_2D_3$ with a second protein receptor located on the external membrane of the cell. Rapid responses stimulated by $1\alpha,25(OH)_2D_3$ include the following: transcaltachia [the rapid stimulation by $1\alpha,25(OH)_2D_3$ of intestinal $Ca^{2+}$ absorption], the opening of voltage-gated $Ca^{2+}$ and $Cl^-$ channels, rapid uptake of $^{45}Ca^{2+}$ into (ROS, rat osteosarcoma 17/2.8) osteoblast cells, enhancement of the concentration of phospholipid second messengers in the intestine, activation of protein kinase C, tyrosine kinases, and mitogen-activated protein kinase.

### 3. *$24R,25(OH)_2D_3$ Biological Properties*

In comparison to $1\alpha,25(OH)_2D_3$, the biological actions of $24R,25(OH)_2D_3$ have been relatively less studied. One key question that has attracted attention is whether $1\alpha,25(OH)_2D_3$ acting alone can generate *all* the biological responses that are attributed to the parent vitamin $D_3$ or whether a second vitamin $D_3$ metabolite may be required for some responses.


Vitamin $D_3$
Dietary sources
Δ Heat
(Sunlight)
7-Dehydrocholesterol
(Present in skin)
(Hormonal origin)
Blood
Liver
37 Chemically characterized metabolites
$25(OH)D_3$
Blood
Kidney
$25(OH)D_3$
24-Hydroxylase
1-Hydroxylase
(+)
(-)
$24R,25(OH)_2D_3$
$1\alpha,25(OH)_2D_3$
$Ca^{2+}$, $P_i$, $H^+$
Endocrine Modulators
Estrogen
Calcitonin
Growth hormone
Prolactin
Insulin
Glucocorticoid
(+)
(+)
Parathyroid hormone
(-) Short Feedback Loop
(-) Long Feedback Loop
Blood
$P_i$
Ca
Blood
Placental production
$1\alpha,25(OH)_2D_3$
$24R,25(OH)_2D_3$
Paracrine production of $1\alpha,25(OH)_2D_3$
Macrophages (activated)
Keratinocytes
Astrocytes (activated)
$1\alpha,25(OH)_2D_3$
$24R,25(OH)_2D_3$
Blood $1\alpha,25(OH)_2D_3$
Blood $24R,25(OH)_2D_3$
Fetus
Development
$1\alpha,25(OH)_2D_3$-Mediated Cellular growth & differentiation
Hematopoietic cells
Skin
Brain
$1\alpha,25(OH)_2D_3$ Rapid actions
Intestine
Bone
Parathyroid
Liver
Pancreas beta cell
PKC (activation)
$1\alpha,25(OH)_2D_3$ Nuclear Receptors
Adipose
Adrenal
Bone
Bone marrow
Brain
Breast
Cancer cells (many)
Cartilage
Colon
Eggshell gland
Epididymis
Ganglion
Hair follicle
Intestine
Kidney
Liver (fetal)
Lung
Muscle (cardiac)
Muscle (smooth)
Osteoblast
Ovary
Pancreas beta cell
Parathyroid
Parotid
Pituitary
Placenta
Prostate
Skin
Stomach
Testis
Thymus
Thyroid
Uterus
Yolk sac (birds)
Classic Target Organs
Bone
Intestine
Kidney
$24R,25(OH)_2D_3$ Receptors
Chondrocyte
Fracture-healing callus
Generation of osteoclasts
Selected biological responses
Reabsorption of $Ca^{2+}$ & $P_i$
Absorption of $Ca^{2+}$
Mobilization/Accretion of $Ca^{2+}$ & $P_i$

Evidence supports the view that the combined presence of both $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ is necessary to generate the complete spectrum of biological responses attributable to the parent vitamin $D_3$. Current research on $24R,25(OH)_2D_3$ focuses on elucidating its actions in cartilage and bone cells.

## V. NUTRITIONAL REQUIREMENTS

### A. Recommended Dietary Allowance (RDA)

The World Health Organization has responsibility for defining the "International Unit" of vitamin $D_3$. Their most recent definition, provided in 1950, stated that "the International Unit of vitamin D recommended for adoption is the vitamin D activity of 0.025 μg of the international standard preparation of crystalline vitamin $D_3$." Thus, 1.0 IU of vitamin $D_3$ is 0.025 μg, which is equivalent to 65.0 pmol. With the discovery of the conversion of vitamin $D_3$ to other active seco-steroids, particularly $1\alpha,25(OH)_2D_3$, it was recommended that 1.0 Unit of $1\alpha,25(OH)_2D_3$ be set equivalent in molar terms to that of the parent vitamin $D_3$. Thus, 1.0 Unit of $1\alpha,25(OH)_2D_3$ has been operationally defined to be equivalent to 65 pmol.

The vitamin D requirement for healthy adults has never been precisely defined. Since vitamin $D_3$ is produced in the skin after exposure to sunlight, the human does not have a requirement for vitamin D when sufficient sunlight is available. The tendencies of humans to wear clothes, to live in cities where tall buildings block adequate sunlight from reaching the ground, to live indoors, to use synthetic sunscreens that block ultraviolet rays, and to live in geographical regions of the world that do not receive adequate sunlight all contribute to the inability of the skin to biosynthesize sufficient amounts of vitamin $D_3$. Thus, vitamin D does become an important essential nutritional factor in the absence of sunlight. It is known that a substantial proportion of the U.S. population is exposed to suboptimal levels of sunlight. This is particularly true during the winter months. Under these conditions, vitamin D becomes a true vitamin, which dictates that it must be supplied in the diet on a regular basis.

The current "adequate intake" allowance of vitamin D recommended in 1998 by the United States Food and Nutrition Board of the Institute of Medicine is 200 IU/day (5 μg/day) for infants, children, and adult males and females up to age 51. For adults ages 51–70, the adequate indicated level is set at 400 IU/day (10 μg/day). For adults >70 years of age, the adequate indicated level is set at 600 IU (15 μg/day). The adequate allowance for pregnancy and lactation is set at 200 IU/day (5 μg/day). These recommendations are all summarized in a 1998 publication from the Food and Nutrition Board of the Institute of Medicine.

In the United States, adequate amounts of vitamin D can readily be obtained from the diet and/or from casual exposure to sunlight. The uv exposure can be as little as three times per week exposure of the face and hands to ambient sunlight for 20 min. However, in some parts of the world where food is not routinely fortified and sunlight is often limited during some periods of the year, obtaining adequate amounts of vitamin D becomes more of a problem. As a result, the incidence of the bone disease rickets in these countries is higher than in the United States.

#### 1. *Food Sources of Vitamin D*

Animal products constitute the bulk source of vitamin D that occurs naturally in unfortified foods. Salt water fish, such as herring, salmon, and sardines, and fish liver oils are good sources of vitamin $D_3$. Small quantities of vitamin $D_3$ are also derived from eggs, veal, beef, butter, and vegetable oils, whereas plants, fruits, and nuts are extremely poor sources of vitamin D. In the United States, fortification of foods such as milk (both fresh and evaporated), margarine and butter, cereals, and chocolate mixes help in meeting the RDA recommendations. Because only fluid milk is fortified with vitamin D, other dairy products (cheese, yogurt, etc.) do not provide the vitamin.

### B. Excess and Toxicity

#### 1. *Vitamin D*

Excessive amounts of vitamin D are not normally available from usual dietary sources and thus reports

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**FIGURE 3** Summary of the vitamin D endocrine system. The kidney is the principal endocrine gland that produces in a regulated fashion small amounts of both $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$. During pregnancy, these same two metabolites are also produced by the placenta. Target organs for the steroid hormone $1\alpha,25(OH)_2D_3$ by definition contain the nuclear receptor $1\alpha,25(OH)_2D_3$ (VDR). Also, $1\alpha,25(OH)_2D_3$ produces selected biological responses via a rapid signal transduction process. The biological roles of $24R,25(OH)_2D_3$ are believed to occur in the bone and cartilage.

of vitamin D intoxication are rare. However, there is always the possibility that vitamin D intoxication may occur in individuals who are taking excessive amounts of supplemental vitamins. In 1993, there was one report of vitamin D intoxication occurring from drinking milk that had been fortified with inappropriately high levels of vitamin $D_3$. Symptoms of vitamin D intoxication include hypercalcemia, hypercalciuria, anorexia, nausea, vomiting, thirst, polyuria, muscular weakness, joint pains, diffuse demineralization of bones, and general disorientation. If allowed to go unchecked, death will eventually occur. The extent of toxicity has been shown in some instances to be related to the level of dietary intake of calcium.

The biological basis for intoxication resulting from the inappropriate intake of the parent vitamin $D_3$ is believed to occur from the unrestrained conversion by the liver of the vitamin $D_3$ to $25(OH)D_3$; this is a largely unregulated metabolic step. The vitamin D intoxication is thought to occur as a result of high plasma levels of 25(OH)D rather than high plasma levels of $1\alpha,25(OH)_2D_3$. Patients suffering from hypervitaminosis D have been shown to exhibit a 15-fold increase in plasma 25(OH)D concentration compared to normal individuals; however, their $1\alpha,25(OH)_2D$ levels are not substantially altered. It has also been shown that large concentrations of $25(OH)D_3$ can mimic the actions of $1\alpha,25(OH)_2D_3$ at the level of the VDR, which can lead to a massive stimulation of intestinal $Ca^{2+}$ absorption and bone $Ca^{2+}$ resorption and ultimately the occurrence of soft tissue calcification and kidney stones. The use of pamidronate, a bisphosphonate inhibitor of bone resorption, has been proposed to reduce the hypercalcemia secondary to acute vitamin D intoxication.

## VI. DISEASE STATES

Figure 4 describes human disease states related to vitamin D and the vitamin D endocrine system. Conceptually, human clinical disorders related to vitamin D can be considered as those arising because of the following: (1) altered availability of vitamin D; (2) altered conversion of vitamin $D_3$ to $25(OH)D_3$; (3) altered conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ and/or $24R,25(OH)_2D_3$; (4) variations in end organ responsiveness to $1\alpha,25(OH)_2D_3$ or possibly $24R,25(OH)_2D_3$; and (5) other conditions of uncertain relation to vitamin D. Thus, the clinician/nutritionist/biochemist is faced with a problem, in a diagnostic sense, of identifying parameters of hypersensitivity, antagonism, or resistance (including genetic aberrations) to vitamin D or one of the other of its metabolites as well as identifying perturbations of metabolism that result in problems in production and/or delivery of the hormonally active form, $1\alpha,25(OH)_2D_3$.

### A. Vitamin D Deficiency and Rickets

The classic deficiency state resulting from a dietary absence of vitamin D or lack of ultraviolet (sunlight)

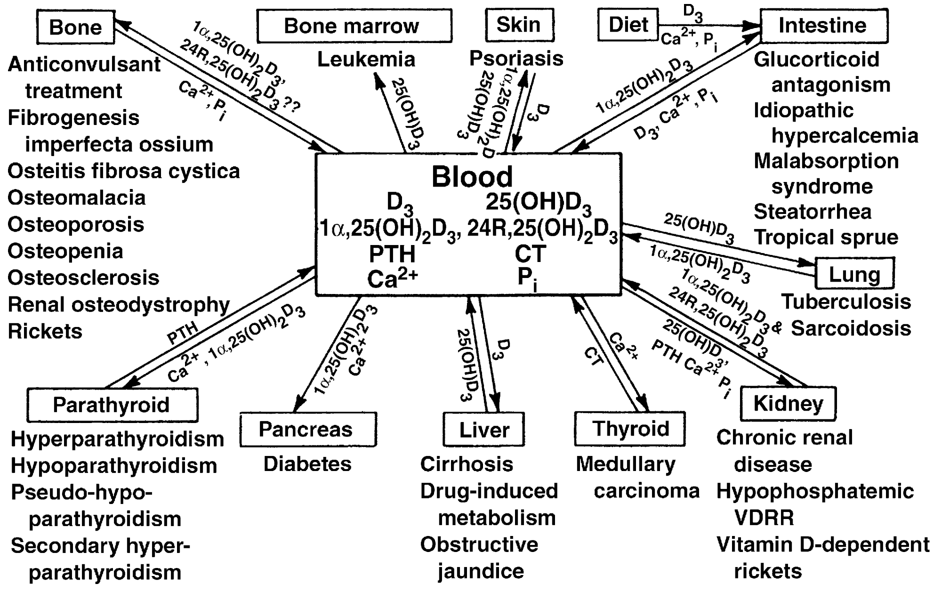
Bone
Anticonvulsant treatment
Fibrogenesis imperfecta ossium
Osteitis fibrosa cystica
Osteomalacia
Osteoporosis
Osteopenia
Osteosclerosis
Renal osteodystrophy
Rickets
1α,25(OH)2D3, 24R,25(OH)2D3 ??
Ca2+, Pi
Bone marrow
Leukemia
25(OH)D3
Skin
Psoriasis
1α,25(OH)2D3
25(OH)D3
D3
Diet
D3
Ca2+, Pi
Intestine
Glucorticoid antagonism
Idiopathic hypercalcemia
Malabsorption syndrome
Steatorrhea
Tropical sprue
1α,25(OH)2D3
D3, Ca2+, Pi
Blood
D3
25(OH)D3
1α,25(OH)2D3, 24R,25(OH)2D3
PTH
CT
Ca2+
Pi
25(OH)D3
Lung
Tuberculosis & Sarcoidosis
1α,25(OH)2D3
1α,25(OH)2D3
24R,25(OH)2D3
25(OH)D3, PTH Ca2+, Pi
Kidney
Chronic renal disease
Hypophosphatemic VDRR
Vitamin D-dependent rickets
PTH
Ca2+, 1α,25(OH)2D3
Parathyroid
Hyperparathyroidism
Hypoparathyroidism
Pseudo-hypoparathyroidism
Secondary hyperparathyroidism
1α,25(OH)2D3
Ca2+
Pancreas
Diabetes
25(OH)D3
D3
Liver
Cirrhosis
Drug-induced metabolism
Obstructive jaundice
CT
Ca2+
Thyroid
Medullary carcinoma

FIGURE 4 Human disease states related to vitamin D and its endocrine system.

exposure is the bone disease rickets (in children) or osteomalacia (in adults). The clinical features of rickets and osteolmalacia depend on the age of onset. The classical skeletal disorder of rickets includes deformity of the bones, especially the knees, wrists, and ankles, as well as associated changes in the costochondrial joint functions, which have been termed by some as the rachitic rosary. If rickets develops in the first 6 months, infants may suffer from convulsions or develop tetany due to a low blood calcium level (usually $<7$ mg/100 ml; normal blood calcium levels are 9–10.5 mg/100 ml) but may have only minor skeletal changes. After 6 months, bone pain as well as tetany is likely to be present. Since osteomalacia occurs after growth and development of the skeleton are complete (i.e., the adult stage of life), its main symptoms are muscular weakness and bone pain with little bone deformity.

A characteristic feature of bone osteomalacia and rickets is the failure of the organic matrix of bone (osteoid) to calcify. This leads to the appearance of excessive quantities of uncalcified osteoid. In addition, there is often a high serum level of alkaline phosphatase, a fact that is often used to assist in the clinical diagnosis of osteomalacia. Also, low serum levels of $25(OH)D_3$ have been found to be diagnostic for the presence of rickets or osteomalacia. The normal serum level of $25(OH)D_3$ is 25–35 ng/ml. When the serum $25(OH)D_3$ level is below 5 ng/ml, the individual is classified as being vitamin D-deficient. When the serum $25(OH)D_3$ level is below 10 ng/ml, the individual is "at risk" for development of vitamin D deficiency.

The nutritional availability of vitamin D is particularly important both in the newborn and young child and in the elderly. Thus, circumstances of deprivation of sunlight through seasonal variation (winter) or skin pigmentation in Africans or African Americans or certain cultural groups, including Muslims, associated with clothing that covers the entire body and face, can lead to the onset of clinical rickets or osteomalacia, characterized by low serum $25(OH)D_3$ levels. Accordingly, the nursing infant can be at risk for rickets if her/his mother is vitamin D-deficient.

## VII. SUMMARY

Current evidence substantiates the concept that the classical biological actions of the nutritionally important fat-soluble vitamin D in mediating calcium homeostasis are supported by a complex vitamin D endocrine system that coordinates the conversion of vitamin $D_3$ into $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$. It is now clear that the vitamin D endocrine system embraces many more target tissues than simply the intestine, bone, and kidney. Notable additions to this list include the pancreas, pituitary, breast tissue, placenta, hematopoietic cells, skin, and cancer cells of various origins. Key advances in understanding the mode of action of $1\alpha,25(OH)_2D_3$ have been made by a thorough study of nuclear receptors as well as emerging studies describing a membrane receptor for this steroid hormone. Integral to these observations are efforts to define the signal transduction systems that are subservient to the nuclear and membrane receptors for $1\alpha,25(OH)_2D_3$ and to obtain a thorough study of the tissue distribution and subcellular localization of the gene products induced by this steroid hormone. There are clinical applications for $1\alpha,25(OH)_2D_3$ or related analogues for treatment of the bone diseases of renal osteodystrophy and osteoporosis, psoriasis, and hypoparathyroidism. Other clinical targets for $1\alpha,25(OH)_2D_3$ currently under investigation include its use in leukemia and breast, prostate, and colon cancer as well as its use as an immunosuppressive agent.

## Glossary

**endocrine system** The integrated interaction between an endocrine gland (source of a hormone) and target cells (location of the hormone's receptor) to generate in a regulated fashion the selective biological responses for that system.

**hormone** Any of several chemical classes of compounds produced in regulated quantities by an endocrine gland that acts as a chemical messenger, usually delivered through the circulatory system to a target cell, which by definition possesses a receptor for that hormone so that a specific biological response may be generated.

**photochemical reaction** A chemical reaction mediated or enhanced by some wavelength of light.

**receptor** A protein molecule that binds very specifically to its cognate hormone to generate a receptor–hormone complex that initiates a cellular signal transduction process resulting in a biological response.

**Recommended Dietary Allowance (RDA)** Recommendations for the amounts of various dietary nutrients (e.g., vitamins and minerals) required to maintain human health.

**rickets** A bone disease in infants and children characterized by a reduction of bone calcium content that results from a dietary deficiency of vitamin D or lack of adequate exposure to sunlight.

**steroid** A member of the lipid class of compounds that is composed of a four-ring structure, the cyclopentanoperhydro-phenanthrene nucleus, and is the basic structural component of steroid hormone families such as estrogens, progestogens, androgens, mineralocorticoids, and glucocorticoids.

**vitamin** Any of a group of essential organic substances provided in trace amounts in food components to effect the normal function of a cellular metabolic process.

## See Also the Following Articles

**Steroid Nomenclature • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D: Biological Effects of 1,25(OH)$_2$D$_3$ in Bone • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Metabolism • Vitamin D: Nuclear Receptor for 1,25(OH)$_2$D$_3$**

## Further Reading

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# Vitamin D and Cartilage

Barbara D. Boyan[*,**], Victor L. Sylvia[*], David D. Dean[*], and Zvi Schwartz[*,**,†]

[*]*University of Texas Health Science Center at San Antonio* • [**] *Georgia Institute of Technology and Emory University, Atlanta* • [†]*Hebrew University Hadassah, Jerusalem*

I. INTRODUCTION
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**Two metabolites of vitamin D, 1α,25-dihydroxy vitamin D$_3$ (1α,25(OH)$_2$D$_3$) and 24R,25-dihydroxy vitamin D$_3$ (24R,25(OH)$_2$D$_3$) regulate growth plate physiology. The effects of each metabolite are limited to different subsets of cells in specific zones of the growth plate; different mechanisms of action are used by the metabolites to exert their effects in their target cells.**

## I. INTRODUCTION

Cartilage is one of the primary target tissues for vitamin D action. During embryonic development, a cartilage anlage provides the form of the skeleton. Mineralization of the cartilage must occur before it can serve as a structural support for bone formation, ultimately being replaced by bone marrow. At the ends of the long bones of the skeleton, at cranial sutures, at the scapula, and at the mandibular condyle, the embryonic cartilage forms a specialized tissue called the growth plate. This tissue remains in postfetal life and provides the mechanism for continued bone growth.

Cartilage cells in the growth plate proceed through a lineage cascade that culminates in the calcification of the tissue (Fig. 1). Chondrocytes in the resting zone, also called the reserve zone, of the growth plate are surrounded by an extracellular matrix that is rich in type II collagen and sulfated

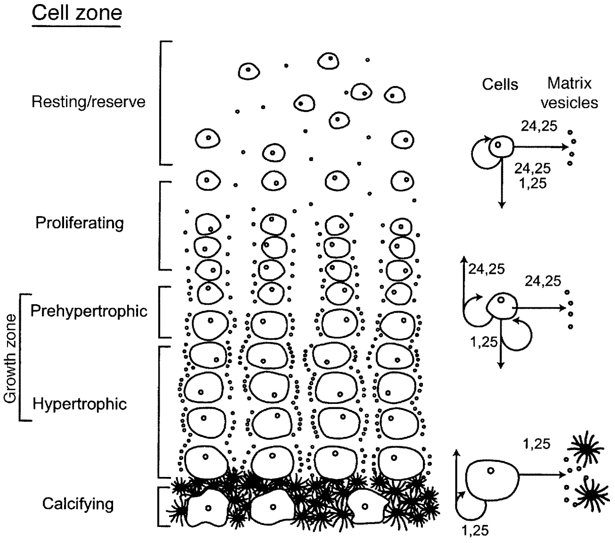

FIGURE 1 Growth plate cartilage. Cells progress through their lineage in a linear fashion in time and space. Vitamin D metabolites $1,25(OH)_2D_3$ (1,25) and $24,25(OH)_2D_3$ (24,25) produced by the chondrocytes act back on the cells in an autocrine manner or on adjacent chondrocytes in a paracrine manner. In addition, they act on matrix vesicles in the extracellular matrix.

glycosaminoglycans in the form of proteoglycan aggregates. This region of the growth plate cushions the tissue from mechanical load. Upon signals that are not yet understood, chondrocytes at the base of the resting zone align in columns and undergo a set number of divisions. Once proliferation is complete, the phenotype of the cartilage cells is altered. The cells are described as being in the zone of maturation or the prehypertrophic cell zone. There are many features of this zone that are not well understood but it appears to be a staging ground for the rapid increase in cell size that characterizes the zone of hypertrophy. This increase in size is primarily in the longitudinal direction and is accompanied by rapid turnover of extracellular matrix, particularly the breakdown of type II collagen and proteoglycan aggregate. In addition, the cells produce increased numbers of extracellular matrix vesicles that contain matrix-processing enzymes and serve as focal points for initial calcification of the tissue. Many of the chondrocytes in this zone are apoptotic. At the base of the hypertrophic cell zone, calcification of the cartilage is complete. This is accompanied by vascular invasion and migration of mesenchymal cells that ultimately differentiate into osteoblasts and marrow stromal cells, resulting in bone and bone marrow formation. A similar sequence of events is recapitulated during fracture repair and during the process of osteoinduction.

In the absence of vitamin D, mineralization of the growth plate fails to occur. As a consequence, the hypertrophic cell zone increases in length since proliferation continues at its normal rate. Because the hypertrophic zone is weak structurally, it cannot withstand weight-bearing loads. As a result, a condition known as rickets develops, in which there is characteristic bowing of the long bones.

In experimental animals, rickets can be cured rapidly by injection with either 25-hydroxy vitasmin $D_3$ ($25(OH)D_3$) or metabolites of vitamin D that are hydroxylated on the 1-carbon, such as $1\alpha,25(OH)_2D_3$. This effect is due to the rapid release of $Ca^{2+}$ into the extracellular matrix of the cells. Other studies have shown that rickets in rats that are deficient in vitamin D as well as phosphate can be cured by systemic injection of $24R,25(OH)_2D_3$ or local injection of this metabolite into the bone. Since this metabolite is not associated with $Ca^{2+}$ transport, these observations suggest either that the $24R,25(OH)_2D_3$ is further metabolized to 1,24,25-trihydroxy vitamin $D_3$ ($1,24,25(OH)_3D_3$) in the tissue or that $24R,25(OH)_2D_3$ also regulates growth plate chondrocytes directly, affecting other aspects of cell physiology that contribute to the development of the rachitic syndrome.

Vitamin D is also an important regulator of growth plate physiology in vitamin D replete animals. When vitamin D replete adolescent rats are given radiolabeled $25(OH)D_3$, both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ are concentrated in the growth plate to a greater extent than in kidney or serum. This supports the hypothesis that these vitamin D metabolites have functions in the growth plate in addition to calcium ion transport. There are numerous studies that indicate this to be the case and these are reviewed below.

The sensitivity of growth plate cartilage to vitamin D and the fact that this tissue contains a single cell type that transits its lineage cascade in a linear manner that can be visualized under a dissecting microscope have made this tissue an excellent model for studying the mechanisms of action of this hormone. The development of techniques for the culture of growth plate chondrocytes has enabled investigators to identify specific subpopulations of cells that respond to $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$. These studies have also led to rapid advances in our understanding of the mechanisms involved.

## II. ROLE OF $1\alpha,25(OH)_2D_3$

Growth plate chondrocytes contain nuclear receptors for $1\alpha,25(OH)_2D_3$ and respond to this vitamin D metabolite via traditional nuclear receptor-mediated mechanisms. In addition to the rapid release of $Ca^{2+}$ ions after treatment with $1\alpha,25(OH)_2D_3$, there are a number of other changes that occur in the growth plate in response to this metabolite. $1\alpha,25(OH)_2D_3$ causes an increase in alkaline phosphatase activity in the hypertrophic zone of the growth plate, as well as an increase in phospholipase $A_2$ activity. This results in the increased production of lysophospholipids. Other aspects of phospholipid metabolism are affected as well, including the specific breakdown of phospholipids not associated with the nucleation of hydroxyapatite crystals in the extracellular matrix. Moreover, the activity of various matrix-processing enzymes is increased. These observations indicate that the primary functions of $1\alpha,25(OH)_2D_3$ are to prepare the matrix for calcification and to ensure that the mineral ions necessary for calcium phosphate formation are present in sufficient quantity. This hypothesis is supported by the fact that many of the effects described above involve matrix vesicles, which are extracellular organelles produced by the chondrocytes that contain matrix-processing enzymes and serve as foci for initial mineral formation.

Studies examining the effects of $1\alpha,25(OH)_2D_3$ on growth plate chondrocytes in culture show that this metabolite inhibits the proliferation of both resting zone and growth zone (prehypertrophic and upper hypertrophic cell zones) cells. However, the effects of this metabolite on differentiation are limited to cells from the growth zone. In confluent cultures of rat costochondral growth plate chondrocytes grown in the presence of serum, $1\alpha,25(OH)_2D_3$ increases the production of prostaglandin E1 (PGE1) and PGE2, stimulates the activity of matrix vesicle alkaline phosphatase and phospholipase $A_2$, and increases the activity of matrix metalloproteinases in these extracellular organelles. In contrast, cultures of chick epiphyseal growth plate chondrocytes grown under serum-free conditions respond to $1\alpha,25(OH)_2D_3$ with a decrease in cellular alkaline phosphatase activity. Whether these differences are due to the species, the presence of serum, or the tissue from which the cells were derived, cells versus matrix vesicles, or the experimental design is not known.

$1\alpha,25(OH)_2D_3$ also modulates growth plate physiology indirectly by regulating the storage and activation of transforming growth factor-β (TGF-β) in the extracellular matrix of prehypertrophic and hypertrophic chondrocytes (Fig. 2). Synthesis of latent TGF-β-binding protein by growth zone chondrocytes is regulated by $1\alpha,25(OH)_2D_3$ both *in vivo* and *in vitro*. $1\alpha,25(OH)_2D_3$ also acts directly on matrix vesicle membranes, increasing phospholipase $A_2$ activity and releasing enzymes that can activate latent TGF-β1. It is now known that $1\alpha,25(OH)_2D_3$ is involved in the release of active stromelysin-1, also called matrix metalloproteinase-3, from matrix vesicles and that this enzyme is responsible for the release of latent TGF-β1 from the matrix and its subsequent activation. Interestingly, TGF-β1 regulates the metabolism of $25(OH)D_3$ in the growth plate, thereby completing the regulatory feedback loop.

## III. ROLE OF $24R,25(OH)_2D_3$

A role for $24R,25(OH)_2D_3$ in the growth plate has been controversial. Rickets can be treated by direct injection of this metabolite into the growth plate, but the possibility exists that this is due to conversion of $24R,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$. In experiments using difluorinated analogues of $24,25(OH)_2D_3$ that prevented further metabolism to $1,24,25(OH)_3D_3$, rapid healing of rickets did not occur, leading to the assumption that $24R,25(OH)_2D_3$ had no active function in the tissue. However, there were a number of studies using rat mandibular condyle organ cultures as an experimental model that argued to the contrary. In addition, the effects of systemically administered $24R,25(OH)_2D_3$ on matrix-processing enzymes in rachitic rat epiphyseal growth plates are very different from the effects of $1\alpha,25(OH)_2D_3$, supporting the contention that they are not due to further metabolism of the metabolite on the 1-carbon. Moreover, specific binding of radiolabeled $24,25(OH)_2D_3$ was found in the growth plate by autoradiography, indicating that receptors for this metabolite are present. Although a nuclear receptor for this vitamin D metabolite has not been purified to homogeneity, recent studies indicate that membrane receptors specific for $24R,25(OH)_2D_3$ exist.

Cell culture studies confirm that $24R,25(OH)_2D_3$ directly affects rat costochondral growth plate chondrocytes, but the response to this metabolite is primarily seen in cells from the resting zone. $24R,25(OH)_2D_3$ stimulates matrix vesicle alkaline phosphatase activity, but it inhibits phospholipase $A_2$ activity and decreases the production of PGE1 and PGE2. The activity of matrix-processing enzymes in matrix vesicles is also decreased. $24R,25(OH)_2D_3$ stimulates the synthesis of latent TGF-β-binding protein by resting zone cells both *in vivo* and *in vitro*,

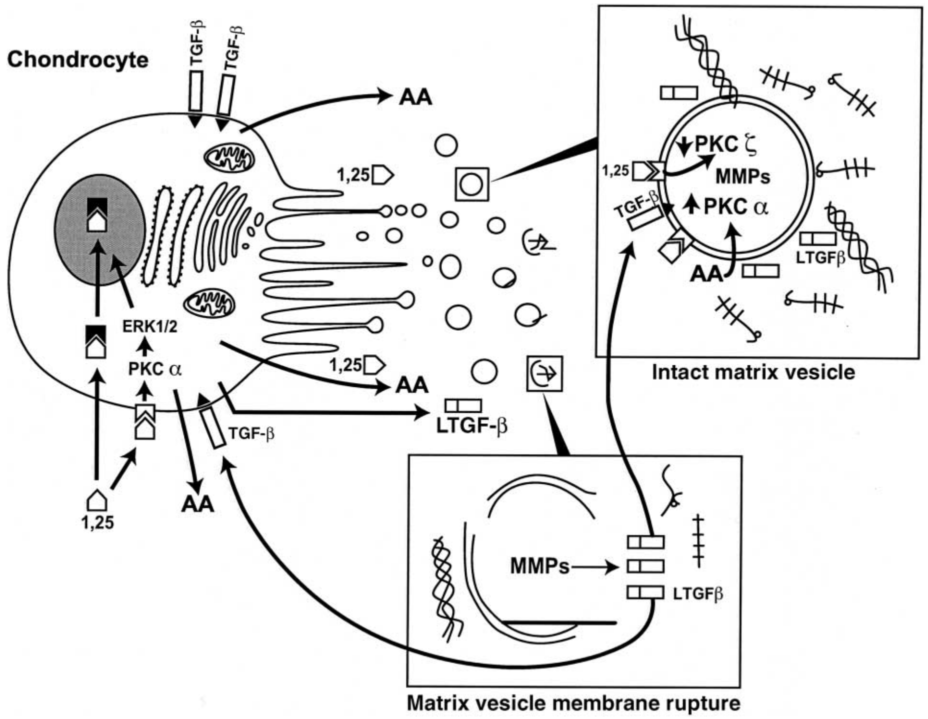

**FIGURE 2** Growth plate chondrocytes secrete $1,25(OH)_2D_3$ (1,25) into the extracellular matrix, where it acts directly on matrix vesicles via membrane-associated receptors (1,25-mVDR), decreasing protein kinase C ζ (PKC ζ) activity. Alkaline phosphatase and phospholipase $A_2$ activities are also increased, leading to increased local phosphate concentration, calcium phosphate crystal formation (line on inner surface of matrix vesicle membrane), and destabilization of the membrane. The mineral within the matrix vesicle provides calcification initiation sites. Matrix-processing enzymes are released into the matrix, causing proteoglycan degradation and facilitating further calcification. Matrix vesicle stromelysin-1 releases and activates latent TGF-β1 stored in the matrix. TGF-β1 can then act back on the cell. Secreted $1,25(OH)_2D_3$ also binds to the 1,25-mVDR on the cell membrane, causing activation of PKC and MAP kinase, ultimately leading to new gene expression. $1,25(OH)_2D_3$ binds to the 1,25-nVDR as well, also contributing to genomic regulation of the cells. Phospholipase $A_2$ is activated by $1,25(OH)_2D_3$, releasing arachidonic acid, which can participate in the cell response and act on matrix vesicle PKC as well.

but this metabolite is not involved in the activation of latent TGF-β. These observations all suggest that $24R,25(OH)_2D_3$ modulates the differentiation of the cells, but down-regulates matrix turnover that leads to calcification of the matrix. This is supported by the fact that $24R,25(OH)_2D_3$ causes uptake rather than release of $Ca^{2+}$.

However, these results do not explain how injection of $24R,25(OH)_2D_3$ into a rachitic growth plate can lead to repair of a rachitic lesion. It is now known that $24R,25(OH)_2D_3$ induces resting zone cells to acquire a growth zone phenotype. Short-term exposure to $24R,25(OH)_2D_3$ increases the proliferation of resting zone cells, and this is accompanied by an increase in the levels of stathmin. Prolonged exposure to $24R,25(OH)_2D_3$ causes a decrease in proliferation. Responsiveness to the metabolite is also reduced, and the cells become responsive to $1\alpha,25(OH)_2D_3$. In addition, $24R,25(OH)_2D_3$ regulates the expression and activity of 1-hydroxylase, leading to production of $1,25(OH)_2D_3$ and potentially to production of $1,24,25(OH)_3D_3$.

## IV. METABOLISM OF $25(OH)D_3$

The fact that growth plate cartilage has the ability to metabolize $25(OH)D_3$ has been known for some time. Recent studies using the rat costochondral cartilage cell model have enabled us to examine the mechanisms involved. Resting zone and growth zone chondrocytes constitutively produce $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ at levels that are comparable to kidney cells, with production of $1,25(OH)_2D_3$ being greater than production of $24,25(OH)_2D_3$. This correlates with mRNA levels for 1-hydroxylase

(Cyp27B1) and 24-hydroxylase (Cyp24), as well as with constitutive activities of these enzymes. The metabolism of $25(OH)D_3$ is regulated in a cell-specific manner. In resting zone cells, the production of $1,25(OH)_2D_3$ is regulated by TGF-β1 within 1 h and by dexamethasone within 24 h. Production of $24,25(OH)_2D_3$ is regulated by TGF-β1 within 24 h, suggesting that it may be due to the earlier increase in $1,25(OH)_2D_3$. This is in fact the case. TGF-β1 stimulates an increase in 24-hydroxylase gene expression and activity, whereas expression of 1-hydroxylase is decreased. In contrast to its effects on $25(OH)D_3$ metabolism in resting zone cells, TGF-β1 has no effect on the production of either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ in growth zone cell cultures. $24R,25(OH)_2D_3$ decreases 24-hydroxylase and 1-hydroxylase activity in resting zone cells, but affects neither hydroxylase in growth zone cells. $1\alpha,25(OH)_2D_3$ increases 24-hydroxylase activity and $24,25(OH)_2D_3$ production in growth zone cells but has no effect on 1-hydroxylase activity.

These complex interrelationships indicate that the secreted hormones can act back on the cells that produce them. The 24-hydroxylase gene Cyp24 has a $1\alpha,25(OH)_2D_3$-response element in its promoter, but to date there have been no reports of a $1\alpha,25(OH)_2D_3$-response element in the promoter of the Cyp27B1 gene. Recent studies indicate that both promoters contain activator protein 1 (AP-1) sites, and AP-1 is sensitive to mitogen-activated protein (MAP) kinase, which is activated by $24R,25(OH)_2D_3$ in resting zone cells and by $1\alpha,25(OH)_2D_3$ in growth zone cells. These observations suggest that the secreted metabolites may modulate their own metabolism through mechanisms that are independent of the nuclear vitamin D receptor (1,25-nVDR) or that augment the actions of the 1,25-nVDR.

## V. MEMBRANE-MEDIATED ACTIONS

Rat costochondral growth plate chondrocytes produce $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ at levels that correspond to those at which biological effects are observed in cell culture, $10^{-8}$ and $10^{-7}$ M, respectively. This also corresponds to the amount of radiolabeled $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ found in the growth plates of vitamin D replete rats treated with radiolabeled $25(OH)D_3$. These observations indicate that locally produced metabolites are important in the physiology of the cells and suggest that they act in an autocrine or paracrine manner. In fact, $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ act directly on the cells via mechanisms involving activation of protein kinase C α (PKC α) by specific membrane-associated receptors (1,25-mVDR; 24, 25-mVDR). Both resting zone cells and growth zone cells possess both types of membrane receptors, yet the effects of $1\alpha,25(OH)_2D_3$ are limited to growth zone cells and the effects of $24R,25(OH)_2D_3$ are limited to resting zone cells. It is now known that distinctly different mechanisms are responsible for the action of the metabolites in their target cells.

$1\alpha,25(OH)_2D_3$ causes a rapid increase in arachidonic acid release via cytosolic phospholipase $A_2$. The arachidonic acid acts as a co-factor, activating PKC α directly. It also serves as a substrate for constitutive cyclooxygenase 1 (COX-1), resulting in increased prostaglandin production. Inhibition of COX-1 inhibits the action of $1\alpha,25(OH)_2D_3$ on PKC as well as the membrane-mediated effects of $1\alpha,25\ (OH)_2D_3$ on the physiology of the cell. PGE2 produced as a consequence of $1\alpha,25(OH)_2D_3$ action on growth zone cells acts on its own prostaglandin E type 1 (EP1) receptors, increasing PKC through a protein kinase A-dependent mechanism. $1\alpha,25(OH)_2D_3$ also activates phosphatidylinositol-specific phospholipase C-β, leading to the formation of diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$). Diacylglycerol binds to cytosolic PKC α and activates the translocation of the enzyme to the plasma membrane, thereby increasing the plasma membrane enzyme activity. The released $IP_3$ activates the release of $Ca^{2+}$ from the endoplasmic reticulum, which is also a co-factor for PKC α. These effects of $1\alpha,25(OH)_2D_3$ on PKC are mediated by the 1,25-mVDR.

$24R,25(OH)_2D_3$ also activates PKC α, but it can do so only in resting zone cells. $24R,25(OH)_2D_3$ causes a rapid decrease in arachidonic acid release, followed by an increase in release after 15 min. Production of prostaglandin is also reduced. The action of PGE2 on its EP1 receptor inhibits PKC activity in these cells, so reduction of PGE2 has the effect of reducing the inhibition, in essence contributing to the stimulatory effect of $24R,25(OH)_2D_3$ on the enzyme. $24R,25(OH)_2D_3$ also causes an increase in diacylglycerol production but there is no corresponding increase in $IP_3$. Instead, the diacylglycerol is a result of the activation of phospholipase D. Moreover, translocation of PKC α to the plasma membrane does not occur.

Many of the physiological effects of $1\alpha,25(OH)_2D_3$ on growth zone cells and of $24R,25(OH)_2D_3$ on resting zone cells are mediated, at least in part, by the activation of PKC α. Some of these effects do not require new gene expression, but other effects clearly have a genomic component. For $1\alpha,25(OH)_2D_3$, the 1,25-nVDR is likely to play a role. However, an equivalent receptor for $24R,25(OH)_2D_3$ has not been isolated, raising the possibility that any genomic response may be mediated through other mechanisms, such as the 24,25-mVDR. In other systems, MAP kinase has been shown to mediate the downstream effects of the PKC $\alpha$-dependent signaling pathway. This appears to be the case in cartilage as well. $1\alpha,25(OH)_2D_3$ activates the extracellular signal-related kinase 1/2 (ERK1/2) family of MAP kinases at the same time that peak activation of PKC $\alpha$ is observed. Similarly, $24R,25(OH)_2D_3$ activates ERK1/2 MAP kinase at the time that peak increases in PKC $\alpha$ occur in resting zone cells.

These experiments explain how $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ exert their membrane-mediated effects in the chondrocytes but they do not explain how secreted metabolites can elicit one set of responses in the cells but another set of responses in matrix vesicles. Matrix vesicles are produced by resting zone and growth zone chondrocytes under genetic control. $1\alpha,25(OH)_2D_3$ increases PKC activity in matrix vesicles produced by growth zone cells by increasing the synthesis of protein kinase $\zeta$ (PKC $\zeta$). Similarly, $24R,25(OH)_2D_3$ increases PKC activity in matrix vesicles produced by resting zone cells by increasing the synthesis of PKC $\zeta$. Thus, one mechanism for discriminating the effects of the vitamin D metabolites on matrix vesicles versus cells is by differential segregation of PKC isoforms; PKC $\alpha$ predominates in the cell and PKC $\zeta$ predominates in the matrix vesicle. In addition, when matrix vesicles are incubated directly with the vitamin D metabolites, the responsive isoform is PKC $\zeta$ rather than PKC $\alpha$, and activity is decreased rather than increased. These effects are mediated by the membrane receptors for each metabolite and are by definition nongenomic because matrix vesicles lack DNA and RNA. In addition to the direct regulation of PKC $\zeta$ by $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, matrix vesicle PKC is regulated by arachidonic acid, causing an increase in enzyme activity. Since arachidonic acid is a co-factor for PKC $\alpha$, which is also present in the matrix vesicles, it is likely that this is the affected isoform. Arachidonic acid is released by growth zone chondrocytes as a consequence of the action of $1\alpha,25(OH)_2D_3$ on phospholipase $A_2$, providing another membrane-mediated mechanism by which the cell can transmit signals to the extracellular organelles and regulate their activity nongenomically.

## VI. SUMMARY

This article has described how two metabolites of vitamin D, $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, regulate the physiology of growth plate cartilage. Each metabolite exerts its effects primarily on a subset of the cells as the chondrocytes mature through the endochondral lineage cascade. Some of the responses are mediated through traditional 1,25-nVDR mechanisms, resulting in new gene expression and protein synthesis. Other responses involve the action of membrane-associated receptors for each metabolite, and these too can result in gene expression through the PKC $\alpha$ signaling pathway and MAP kinase activation. $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ also exert direct effects on extracellular organelles via their respective membrane receptors, thereby modulating matrix maturation, growth factor activation, and calcification.

## Glossary

| | |
|---|---|
| **chondrocyte** | Cartilage cell. |
| **matrix vesicles** | Extracellular organelles produced by chondrocytes that are sites for initiation of calcification and also contain matrix-processing enzymes. |
| **1,25-nVDR** | Nuclear receptor for $1\alpha,25(OH)_2D_3$. |
| **1,25-mVDR** | Membrane-associated receptor for $1\alpha,25(OH)_2D_3$. |
| **24,25-mVDR** | Membrane-associated receptor for $24R,25(OH)_2D_3$. |

## See Also the Following Articles

**Vitamin D • Vitamin D and Human Nutrition • Vitamin D-Binding Protein • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Metabolism**

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# Vitamin D and Human Nutrition

Norman H. Bell
*Medical University of South Carolina*

I. INTRODUCTION
II. RICKETS
III. OSTEOMALACIA
IV. OSTEOPOROSIS
V. TREATMENT
VI. SUMMARY

**Vitamin D is an essential fat-soluble vitamin that is acquired from dietary sources or by dermal synthesis in response to exposure to sunlight. Nutritional deficiency of vitamin D causes rickets in infants and children and osteomalacia in adults; it may also be a contributing factor in the development of osteoporosis and fractures in the elderly. Treatment with vitamin D can correct and prevent these bone diseases.**

## I. INTRODUCTION

Vitamin D, an essential fat-soluble vitamin, is derived from the diet as vitamin $D_2$ (irradiated ergosterol) or by dermal synthesis from 7-dehydrocholesterol as vitamin $D_3$ in response to solar ultraviolet light and body heat. Vitamin D itself is biologically inactive and must undergo hydroxylation in the liver to form 25-hydroxyvitamin D [25(OH)D] and in the kidney to form 1,25-dihydroxyvitamin D $[1,25(OH)_2D]$, by vitamin D 25-hydroxylase and 25(OH)D-1α-hydroxylase, respectively. The metabolites of vitamin $D_2$ and $D_3$ have similar biologic activity, although those of vitamin $D_2$ are less toxic. 24-Hydroxylase is the rate-limiting enzyme for degradation of 25(OH)D and $1,25(OH)_2D$ and is genomically induced by $1,25(OH)_2D$.

Nutritional deficiency of vitamin D leads to rickets in infants and children and to osteomalacia in adults and can be a contributing factor to the development of osteoporosis and fractures in older adults. These bone diseases can be corrected and prevented by treatment with vitamin D. Dark-skinned individuals who reside in areas of limited sunlight exposure are at particular risk for developing vitamin D deficiency and its consequences. This occurs because melanin pigment in skin absorbs the photons of light energy and prevents activation of 7-dehydrocholesterol to eventually form vitamin $D_3$. Vitamin D deficiency is prevalent worldwide, especially in underdeveloped countries where foods are not fortified.

## II. RICKETS

Deficiency of vitamin D is the major cause of rickets in infants and children and results from inadequate exposure to sunlight or intake of dietary vitamin D. Except for oily fishes and fortified foods, the normal diet provides little in the way of vitamin D so that adequate dermal exposure to sunlight or supplementation of vitamin D is essential to prevent rickets. In human milk, the concentrations of vitamin D and 25(OH)D are not adequate to meet daily requirements. Thus, rickets occurs in breast-fed infants who are not given vitamin D supplements, in infants before they are able to walk and be outdoors, in children living at extremes of latitude, and in children who have diminished exposure of skin as a consequence of excess clothing and lack of exposure to sunlight. African American infants with dark skin are at particular risk. In underdeveloped countries in the Middle East, for example, vitamin D deficiency is prevalent in Arab adults and children who reside near the equator because of avoidance of sunlight, wearing of clothes and veils that cover the body, lack of vitamin D supplements, and consumption of nonfortified foods and diets that are low in calcium or high in inhibitors of calcium absorption. Vitamin D deficiency also occurs in vegetarians and those who consume macrobiotic diets, regardless of where they live.

Deficiency of vitamin D leads to decrease in serum 25(OH)D and $1{,}25(OH)_2D$ and the intestinal absorption of calcium that in turn causes hypocalcemia, secondary hyperparathyroidism, and hypophosphatemia and results in diminished mineralization of the skeleton (Fig. 1). A decrease in serum 25(OH)D is the hallmark of vitamin D deficiency.

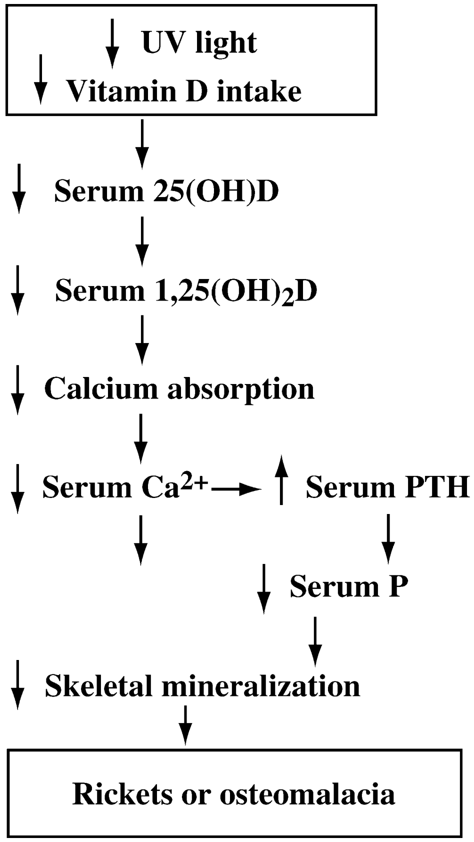

FIGURE 1 Pathogenesis of rickets and osteomalacia.

Children with rickets may have muscle weakness, stridor (a harsh, high-pitched respiratory sound), a waddling gait, impaired growth, and fractures and may have cataracts, seizures, tetany sometimes associated with carpopedal spasm, and a positive Chvostek sign as a consequence of hypocalcemia and tetany. Dental problems include delayed tooth eruption, defects in tooth structure, and increased incidence of caries. Patients are prone to develop pneumonia. It is not clear whether this is caused by immune deficiency as a consequence of vitamin D deficiency or myopathy resulting in poor clearing of bronchopulmonary secretions. Myopathy may include left ventricular myocardial dysfunction. These abnormalities are all corrected by vitamin D and adequate calcium intake.

The rate of growth of different bones varies with age. Since skeletal deformities develop at sites of rapid growth, skeletal changes of rickets vary with age and may indicate the age of onset of the disease. With rapid growth in neonates, the skull is particularly affected. Softening of the cranium or craniotabes may occur with parietal flattening, frontal bossing, and widened sutures. Rapid growth of the arms and rib cage in early childhood is associated with widening of the forearm at the wrist and thickening of the costochondral junctions that results in the rachitic rosary. There may be forward projection of the breastbone or “pigeon chest” and scoliosis or kyphosis of the spine. Indentations of the lower ribs at the site of attachment of the diaphragm may occur and are called Harrison’s groove. With rapid growth of long bones, bowing of the lower extremities, genu varus (bowleg) or valgus (knock-knee), may result because of weight-bearing. Deformities of the pelvis may also occur and may severely compromise weight-bearing. Secondary hyperparathyroidism may cause resorption of the phalanges and distal ends of long bones, such as the clavicles and humeri.

Biochemical findings include low serum 25(OH)D, calcium, and phosphorus and elevated serum immunoreactive parathyroid hormone (PTH) and alkaline phosphatase. Serum 1,25$(OH)_2$D may be low, normal, or increased depending on the degree of 25(OH)D deficiency and secondary hyperparathyroidism. When present, increases in serum 1,25$(OH)_2$D induce 24-hydroxylase, enhance degradation of 25(OH)D and 1,25$(OH)_2$D, and hasten the development of vitamin D deficiency.

## III. OSTEOMALACIA

Osteomalacia caused by nutritional deficiency of vitamin D occurs primarily in Asian Indians and Pakistanis, more commonly in those who live far from the equator and have limited exposure to sunlight. Inadequate intake of vitamin D, increased skin pigmentation, practice of purdah by women in which veils are worn during pregnancy, parturition, and lactation, and consumption of a vegetarian diet are contributing factors.

Patients with osteomalacia may be asymptomatic or have muscle weakness and diffuse skeletal pain in the lower back, hip, or sites of fractures. Decreased skeletal density is the most common X-ray finding. Looser zones or pseudo-fractures may occur and are typically bilateral and symmetrical. Common sites are axillary margins of the scapulae, lower ribs, superior and inferior pubic rami, inner margins and neck of the proximal femora, and posterior margins of the proximal ulnae. Bone resorption caused by secondary hyperparathyroidism is sometimes the most prominent radiographic finding.

In patients with osteomalacia, bone density determined by dual-photon absorptiometry is reduced and bone scans show increased uptake of technetium-99m pyrophosphate by long bones and wrists and prominence of the calvaria and mandible. Beading of the costochondral junctions may occur and increased tracer uptake by the sternum and its margins produces the so-called "tie sternum." Pseudo-fractures appear as hot spots.

## IV. OSTEOPOROSIS

Vitamin D deficiency is often present in older men and women. Decreased dermal production of 7-dehydrocholesterol, the precursor of vitamin $D_3$, decreased exposure intake of vitamin D, decreased exposure to sunlight, down-regulation of the vitamin D receptor in the small intestine, and decreased production of 25(OH)D and 1,25$(OH)_2$D are contributing factors. Older individuals may be house-bound as well. In addition to vitamin D, calcium intake is often inadequate in older men and women.

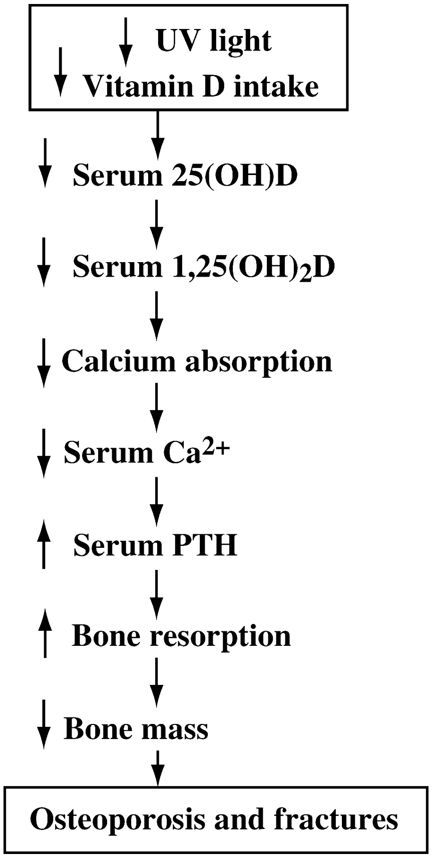

FIGURE 2 Pathogenesis of osteoporosis.

Deficiency of vitamin D leads to decrease in serum 25(OH)D and 1,25$(OH)_2$D, the intestinal absorption of calcium that in turn causes hypocalcemia, secondary hyperparathyroidism, increased bone resorption, bone loss, osteoporosis, and fractures (Fig. 2).

## V. TREATMENT

The most recent recommended daily intakes of vitamin D and calcium by the National Academy of Science are listed in Table 1; values for vitamin D range from 200 IU per day in children to 600 IU per day in older individuals. The normal range for serum 25(OH)D is between 10 and 60 ng/ml. However, an increase in circulating PTH is the most sensitive

TABLE 1 Recommendations for Daily Intake of Calcium and Vitamin D

| Age (years) | Calcium (mg) | Vitamin D (IU)[a] |
|---|---|---|
| 4–8 | 800 | 200 |
| 9–18 | 1300 | 200 |
| 19–50 | 1000 | 200 |
| 51–65 | 1200 | 400 |
| >65 | 1200 | 600 |

[a] 1 IU equals 15 ng.

indicator of vitamin D deficiency, and the lowest value for serum 25(OH)D at which there is a plateau of circulating immunoreactive PTH ranges from approximately 30 to 40 ng/ml. In a recent study, serum 25(OH)D (28 to 50 ng/ml) and serum and urinary calcium remained in the normal range in normal men and women given 4000 IU per day for 2 to 5 months. Furthermore, a comprehensive review of the literature indicated that serum 25(OH)D does not begin to increase abnormally and cause hypercalcemia until a daily dose of more than 10,000 IU is administered. Thus, there is a wide margin of safety between therapeutic and toxic doses of vitamin D.

In patients with rickets or osteomalacia, the goals of treatment are to correct and prevent the effects of hypocalcemia, cataracts, seizures, and skeletal effects of secondary hyperparathyroidism, to prevent and correct the skeletal deformities of rickets and osteomalacia, to prevent hypercalcemia, hypercalciuria, and their consequences, and to produce normal growth and development of the skeleton in children.

Vitamin D deficiency is treated with vitamin D. Although 400 IU per day of the vitamin is an adequate maintenance dose, the initial dose should be larger, perhaps 1200 IU per day.

Stoss therapy with an intramuscular dose of 150,000 IU of vitamin D or more is recommended by some. Excess vitamin D produces intoxication that is associated with hypercalcemia and hypercalciuria; it is caused by increased circulating 25(OH)D and is produced by daily doses in excess of 10,000 IU. Serum 1,25$(OH)_2$D is usually in the normal range. Available evidence indicates that hypercalcemia does not occur at doses of 10,000 IU per day of vitamin D or less. Vitamin D intoxication is treated by discontinuing the vitamin D, by forcing fluids, and, if necessary, by treatment with dexamethasone or hydrocortisone.

Although it is well documented that elderly subjects may be deficient in vitamin D, that vitamin D deficiency can lead to secondary hyperparathyroidism, increased bone resorption, and increased bone loss, and that the incidence of fractures can be reduced by treatment with vitamin D and calcium, results of studies in which 25$(OH)D_3$ and calcium were given separately to men and women show that calcium is more effective than 25$(OH)D_3$ at increasing bone mineral density. The recommended intake for calcium in individuals over the age of 65 years is 1200 mg per day.

## VI. SUMMARY

Vitamin D deficiency is prevalent especially in underdeveloped countries where foods are not fortified with vitamin D. Breast-fed infants, particularly African American infants, are at risk for rickets if they are not given vitamin D or exposed to sunlight. Asian Indians and Pakistanis are at risk for developing rickets and osteomalacia especially when they have limited access to sunlight and vitamin D. Aging men and women are prone to vitamin D deficiency, osteoporosis, and fractures and can be treated with calcium and vitamin D. Recent evidence indicates that daily doses of vitamin D as high as 4000 IU are well tolerated and do not increase serum 25(OH)D or serum or urinary calcium abnormally.

## Glossary

**carpopedal spasm** Spasm of the wrist and foot produced by hypocalcemia and tetany.

**Chvostek's sign** A spasm of the facial muscles in response to tapping of the facial nerve that is caused by hypocalcemia and tetany.

**craniotabes** Caused by softening of the cranium and associated with parietal flattening of the sides of the skull, frontal bossing or exaggeration of the curvature of the forehead, and widened sutures or sites of attachment of bones to one another.

**kyphosis** Enhanced curvature of the spine as seen from the side, or hunchback.

**osteomalacia** A bone disease that occurs in adults as a result of vitamin D deficiency and that is caused by abnormal mineralization of the skeleton and weak bone that is prone to bend and fracture.

**osteoporosis** A bone disease that is characterized by decreased bone mass and alterations in microarchitecture, resulting in increased skeletal fragility and risk of fracture.

**pigeon chest** The forward projection of the breastbone that occurs with rickets.

**pseudo-fracture** A thickening of periosteum or bone surface and formation of new bone over what appears to be a fracture on X ray. It is usually found at the site of a pulsating artery and may result from softening of bone and arterial pulsation.

**rachitic rosary** Caused by thickening of the costochondral junctions of the ribs.

**rickets** A bone disease that occurs in children as a result of vitamin D deficiency and is caused by abnormal mineralization of the skeleton and weak bone that is prone to bend and fracture. Rapid growth of the arms and rib cage in early childhood is associated with widening of the forearm at the wrist and scoliosis or kyphosis of the spine. Indentations of the lower ribs at the site of attachment of the diaphragm may occur and are called Harrison's groove.

**scoliosis** A lateral curvature of the spine.

## See Also the Following Articles

**Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Vitamin D • Vitamin D and Cartilage • Vitamin D: Biological Effects of 1,25(OH)$_2$D$_3$ in Bone • Vitamin D: Biological Effects of 1,25(OH)$_2$D$_3$ in the Intestine and Kidney • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D Metabolism**

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# Vitamin D-Binding Protein

Ivy Hurwitz and Nancy E. Cooke
*University of Pennsylvania*

I. INTRODUCTION
II. REGULATION AND EXPRESSION OF DBP
III. DBP POLYMORPHISMS
IV. DBP FUNCTIONS
V. SUMMARY

**Vitamin D-binding protein (DBP) plays a major role in the binding, solubilization, and serum transport of the principal vitamin D metabolites 25-hydroxyvitamin D$_3$, the major circulating metabolite, and 1,25-dihydroxyvitamin D$_3$, the more biologically active metabolite. However, circulating DBP concentrations are approximately 20-fold higher than that of total vitamin D metabolites. This large excess is unusual among other serum carrier proteins, suggesting other roles for this protein.**

## I. INTRODUCTION

Vitamin D-binding protein (DBP), also referred to as group-specific component of serum or Gc-globulin, was initially identified by its polymorphic migration pattern on serum electrophoresis. Although its function remained relatively unknown at that time, its highly polymorphic nature allowed DBP to play a to fracture. In the case of rickets, a number of additional skeletal abnormalities are found, including bowing of the long bones, joint pain and swelling, bone pain, and abnormalities of the teeth. In severe vitamin D insufficiency, infants with rickets may show delayed development and even respiratory failure if thoracic cage development is impaired sufficiently to restrict breathing.

## II. NORMAL VITAMIN D PHYSIOLOGY

### A. Synthesis

Calcium is absorbed in the upper small intestine under the control of the active vitamin D hormone, 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$], also known as calcitriol (in this article these terms are used interchangeably). There are two forms of vitamin D: vitamin $D_2$ (ergocalciferol), of plant origin, and vitamin $D_3$ (cholecalciferol), of animal origin. Vitamin $D_3$ can be synthesized in the skin with the aid of sunlight or it is ingested in the diet. The distribution of vitamin $D_3$ in food is quite restricted, being substantial mainly in oily fish. Because dietary vitamin D is so limited, in some countries various foods (milk, cereals, etc.) have been fortified with either vitamin $D_2$ or vitamin $D_3$ in order to avoid widespread vitamin D deficiency. Even in countries with standard vitamin D fortification programs, however, nutritional sources of vitamin D may be limited and exposure to sunlight is critical for adequate endogenous production of vitamin $D_3$.

As outlined in Fig. 1, vitamin D is ingested in the diet or is endogenously manufactured following exposure to ultraviolet rays of sunlight, which causes conversion of the precursor 7-dehydrocholesterol to vitamin $D_3$ in the skin. Vitamin $D_3$ is subsequently converted to the active hormone by a two-step process, first by 25-hydroxylation in the liver to 25-hydroxyvitamin $D_3$ and then by 1-hydroxylation in the kidney to 1,25-dihydroxyvitamin $D_3$. 1,25-Dihydroxyvitamin $D_3$ then acts via the intranuclear vitamin D receptor (VDR) to regulate target genes, in a pathway similar to those of other steroid hormones. The target genes are involved in regulation of calcium and phosphate delivery to the bone-forming sites to mineralize osteoid.

Any step in the pathway of calcitriol synthesis or action that is defective can result in rickets/osteomalacia. The usual causes of rickets/osteomalacia are shown in Fig. 1 and are detailed in Table 1. The most common cause of rickets/osteomalacia worldwide is nutritional vitamin D deficiency, combined with insufficient synthesis in the skin. This can be caused by inadequate dietary consumption of vitamin D and/or by malabsorption of vitamin D in the gastrointestinal tract, coupled with inadequate sunlight exposure. Because vitamin D supplementation

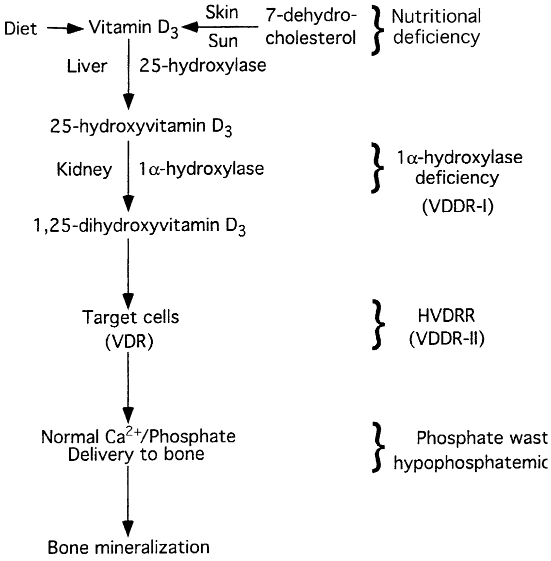

**FIGURE 1** Calcitriol synthesis and action. Defects at various points in the synthetic pathway of calcitriol (1,25-dihydroxyvitamin $D_3$) cause osteomalacia or rickets due to interference with normal mineralization of bone. VDR, Vitamin D receptor; VDDR-I/II, vitamin D-dependent rickets types I and II; HVDRR, hereditary 1,25-dihydroxyvitamin $D_3$-resistant rickets.

**TABLE 1 Etiology of Rickets or Osteomalacia**

- Nutritional: vitamin D deficiency
  - Deficient synthesis due to inadequate sunlight exposure
  - Dietary deficiency
- Gastrointestinal disorders
  - Malabsorption syndrome
  - Hepato-biliary disease
  - Pancreatic disease
- Renal insufficiency
- Tumor-induced osteomalacia (TIO)
- Hereditary causes
  - X-Linked hypophosphatemic rickets (XLH)
  - 1α-Hydroxylase deficiency (vitamin D-dependent rickets, type I)
  - Hereditary vitamin D-resistant rickets (HVDRR, vitamin D-dependent rickets, type II)
  - Autosomal dominant hypophosphatemic rickets (ADHR)
- Miscellaneous causes
  - Acidosis
  - Phosphate depletion
  - Renal tubular disorders

of milk or other dietary products reduces nutritional causes of rickets/osteomalacia in the United States and in other developed countries, other etiologies are becoming more important. Other causes of rickets/osteomalacia include the inability to synthesize calcitriol due to renal failure or due to a genetic defect in 1α-hydroxylase, the key enzyme involved in calcitriol synthesis. Abnormalities that cause excess loss of phosphate include genetic defects or tumors that produce phosphaturia. Finally, mutations in the vitamin D receptor, the protein that mediates calcitriol actions in the intestine and other target tissues, can cause rickets in children.

## B. Regulation of Calcium and Phosphate Homeostasis

Control of calcium levels in the serum is tightly regulated by the interplay of calcitriol and parathyroid hormone (PTH). As depicted in Fig. 2, serum calcium concentration is monitored by the calcium-sensing receptor present in the parathyroid glands and possibly also in the kidney and other sites. PTH synthesis is negatively regulated by calcium and by calcitriol. When the calcium concentration falls, the PTH level rises in an attempt to restore serum calcium to normal. The actions of PTH on the skeleton increase bone resorption whereas the actions of PTH on the kidney reduce the level of calcium excretion. Both processes help to maintain serum calcium within the normal range. Importantly, the actions of PTH on the kidney also result in increased activation of the 1α-hydroxylase enzyme, which augments conversion of circulating 25-hydroxyvitamin $D_3$ to the active hormone calcitriol. The increased concentration of calcitriol activates intestinal cells to increase calcium absorption along the length of the intestine. These combined actions restore the circulating calcium concentration to normal. Elevated levels of PTH return to normal in response both to normalization of serum calcium and to feedback inhibition of PTH production by a direct action of calcitriol on the parathyroid glands.

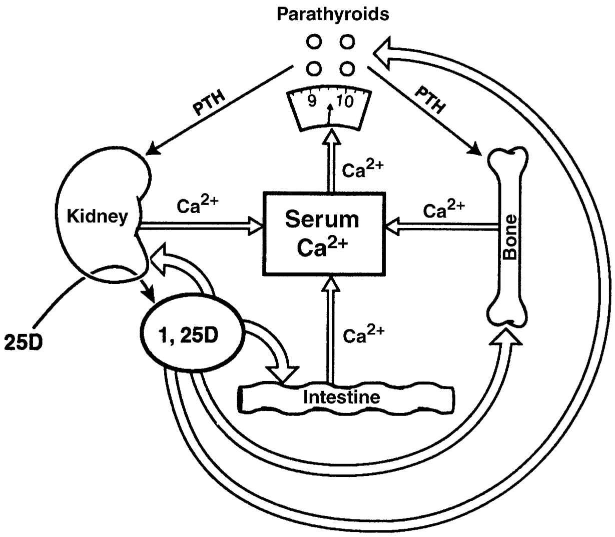

**FIGURE 2** Regulation of serum $Ca^{2+}$ levels. The control of calcium levels in the blood is tightly regulated by vitamin D and parathyroid hormone (PTH). Gastrointestinal absorption of calcium from the diet is controlled by the activity of 1,25-dihydroxyvitamin $D_3$ (1,25D). The active form of vitamin D is synthesized in the kidney by 1α-hydroxylation of circulating 25-hydroxyvitamin $D_3$ (25D). 1,25-Dihydroxyvitamin $D_3$ also acts on bone to resorb calcium and on the parathyroid glands to suppress PTH. The calcium-sensing receptor in the parathyroids detects the concentration of serum calcium. When calcium levels begin to fall, PTH secretion is increased, which acts on the kidney to stimulate 1α-hydroxylase activity and increase 1,25-dihydroxyvitamin $D_3$ synthesis. PTH also acts on bone and kidney to elevate serum calcium levels. Reproduced from Feldman *et al.* (2001), with permission from Elsevier Science.

Control of phosphate levels is in part controlled by vitamin D but, importantly, is also regulated by other systems that are incompletely understood. Calcitriol increases both calcium and phosphate absorption from the intestine. PTH, although it increases calcium reabsorption in the kidney, inhibits phosphate reabsorption, causing phosphaturia. Renal phosphate transport is controlled by sodium phosphate cotransporter type IIa (NPT2), which is responsible for the bulk of phosphate reabsorption in the proximal tubule of the kidney. It is postulated that there is a phosphate-regulating hormone, phosphatonin, that regulates NPT2. Recent data suggest that fibroblast growth factor 23 (FGF 23) may be the phosphate-regulating molecule. Abnormalities of phosphatonin balance contribute to several causes of rickets/osteomalacia (see Section IV,A).

## C. The Vitamin D Receptor

Although called a vitamin, 1,25-dihydroxyvitamin $D_3$ is actually a member of the steroid hormone family. 1,25-Dihydroxyvitamin $D_3$ regulates calcium metabolism and promotes other physiologic actions through the vitamin D receptor, a member of the steroid/thyroid/retinoid receptor gene superfamily. These receptors regulate gene transcription by acting as ligand-activated transcription factors (see Fig. 3). Members of this receptor family share a modular structure comprising an N-terminal transactivation domain of variable length, a DNA-binding domain

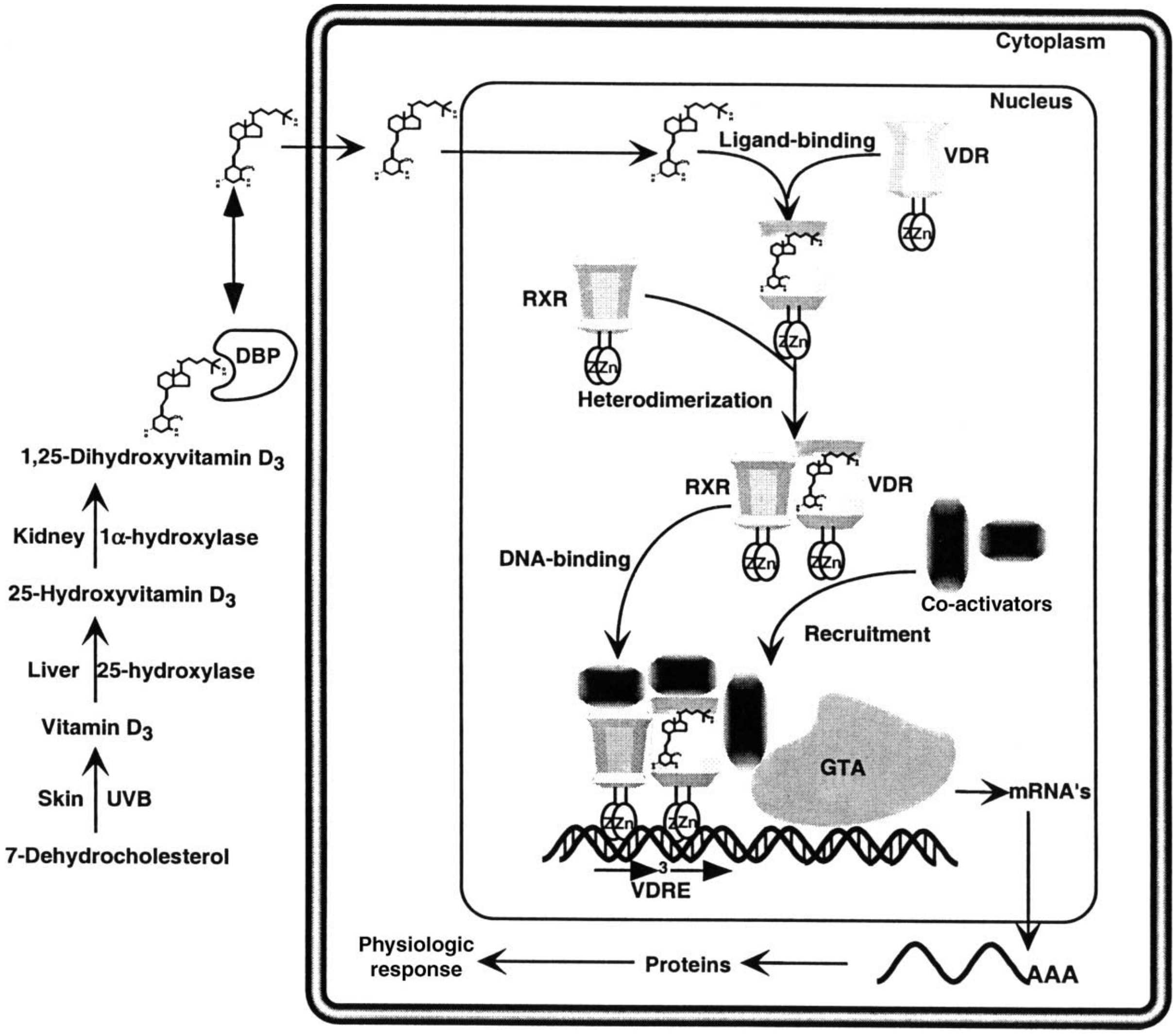
Cytoplasm
Nucleus
Ligand-binding
VDR
Zn
RXR
Heterodimerization
RXR
VDR
DNA-binding
Co-activators
Recruitment
GTA
mRNA's
3
VDRE
Physiologic response
Proteins
AAA
DBP
1,25-Dihydroxyvitamin D3
Kidney
1α-hydroxylase
25-Hydroxyvitamin D3
Liver
25-hydroxylase
Vitamin D3
Skin
UVB
7-Dehydrocholesterol

**FIGURE 3** Mechanism of 1,25-dihydroxyvitamin $D_3$ action on target cells. After conversion of 7-dehydrocholesterol in the skin by ultraviolet B (UVB) light, the active form of vitamin D, 1,25-dihydroxyvitamin $D_3$, is synthesized by sequential hydroxylation steps in the liver and kidney. Once in the circulation, some of 1,25-dihydroxyvitamin $D_3$ is bound by the vitamin D-binding protein (DBP), and a small amount remains free. The free 1,25-dihydroxyvitamin $D_3$ enters the cell and binds to the vitamin D receptor (VDR). The occupied VDR heterodimerizes with the retinoid X receptor (RXR) and binds to vitamin D response elements (VDREs) on target genes. Binding to DNA is carried out by the two zinc (Zn) finger DNA-binding domains of the VDR. Co-activators are recruited to the VDR-RXR complex, allowing the general transcription apparatus (GTA) to initiate gene transcription. Reproduced from Malloy, P. J., Pike, J. W., and Feldman, D. (1999). The vitamin D receptor and the syndrome of hereditary 1, 25-dihydroxyvitamin D-resistant rickets. *Endocr. Rev.* **20**, 156–188. Copyright The Endocrine Society.

(DBD) that enables interaction of the receptor proteins with hormone response elements in promoter regions of target genes, and a C-terminal ligand-binding domain (LBD). In addition, a highly conserved region at the C-terminus of the LBD, defined as the activation function-2 domain (AF-2), regulates transcription. Like other steroid receptors, the VDR LBD binds specifically to its cognate ligand, calcitriol, triggering a series of molecular events leading to the activation of vitamin D-responsive genes. Regulation of specific gene transcription by the VDR requires its binding as a heterodimer with the retinoid X receptor (RXR). RXR heterodimerizes with a number of other receptors in the steroid/thyroid/retinoid gene superfamily, including thyroid receptor, retinoic acid receptor, and the peroxisome proliferator-activating receptor. Initiation of gene transcription also involves the recruitment of co-activator proteins that act as bridging factors linking the VDR to the preinitiation complexes and RNA polymerase II. Co-activators include a family of closely related p160 proteins such as SRC-1/NcoA1, TIF2/GRIP1/NcoA2/SRC-2, and pCIP/Rac3/ACTR/AIB1/SRC-3. Other co-activators include a group of proteins collectively called vitamin D receptor-interacting proteins (DRIPs). These co-activator proteins associate with nuclear receptors in a ligand-dependent manner to enhance transactivation of target genes.

### D. Mechanism of Action

How do cells respond to calcitriol? A simplified model of calcitriol action is shown in Fig. 3. After the hormone is produced and secreted by the kidney, it is transported in the blood either bound to vitamin D-binding protein (DBP) or in the free state. The free hormone is lipid-soluble and readily gains entry into cells by permeating the lipid bilayer of the cell membrane. Cells that respond to calcitriol have VDRs located in the cell nuclear compartment, where they are loosely associated with the RXR. Once inside the cell, calcitriol encounters the VDR and binds in the ligand-binding pocket in the LBD. Calcitriol binding causes the VDR to bind more tightly to the RXR and to interact with vitamin D response elements (VDREs) on target genes. When calcitriol occupies the ligand-binding pocket, helix H12 in the VDR LBD swings into position, locking the hormone inside the binding domain and at the same time forming a new surface interface for interaction with co-activators. The co-activators that are recruited by the VDR, such as steroid receptor co-activator-1 (SRC-1), are then able to remodel the chromatin so that assembly of the transcriptional apparatus can take place. The VDR can then recruit the DRIP complex and begin activating gene transcription.

## III. VITAMIN D DEFICIENCY, RICKETS AND OSTEOMALACIA

### A. Nutritional Deficiency

Vitamin D deficiency causes a constellation of metabolic abnormalities that combine to cause osteomalacia or rickets. The insufficiency of vitamin D, if severe, can result in diminished intestinal calcium absorption and hypocalcemia. The calcium-sensing receptor present in the parathyroid gland detects the low serum calcium level. This leads to an increase in PTH synthesis and results in secondary hyperparathyroidism. The elevated levels of PTH allow correction of the hypocalcemia by three actions on bone and kidney. One action is aimed at stimulating renal 1α-hydroxylase activity to cause increased renal production of calcitriol. However, for increased production of calcitriol, this step requires adequate vitamin $D_3$ and 25-hydroxyvitamin $D_3$ substrate, which is inadequate in vitamin D deficiency. A second PTH action on bone is to increase bone resorption, thus increasing calcium and phosphate flux from bone to serum. A third PTH action is to increase renal calcium reabsorption and decrease calciuria. However, PTH, while decreasing calciuria, increases phosphaturia, thus reducing the availability of this mineral component for bone mineralization. The overall result of vitamin D deficiency is hypocalcemia, secondary hyperparathyroidism, and defective mineralization of osteoid, leading to the development of osteomalacia.

It is clear that adequate calcium and vitamin $D_3$ are required for bone health. The vitamin $D_3$ requirement is estimated to be 400 international units (IU) per day, although elderly subjects may require 600 IU or more. Most infants and children in the United States have adequate vitamin D intake because of vitamin D supplementation of milk. During adolescence, when consumption of milk and dairy products diminishes, decreased dietary vitamin D may adversely affect calcium absorption and thereby lead to impaired skeletal health. Vitamin D insufficiency in the elderly population greatly increases their risk for both osteoporosis and osteomalacia. This problem is more severe in countries that do not supplement milk or other foods with vitamin D and where sunlight exposure may be inadequate.

Except for oily fish, most dietary components lack substantial levels of vitamin $D_3$. In the United States, milk is ostensibly fortified with 400 IU of vitamin $D_3$ per quart, although amounts vary. For the many individuals who do not include in their diet adequate amounts of milk or dairy products, exposure to ultraviolet radiation from sunlight is necessary to achieve normal vitamin D status. Endogenous vitamin D production occurs in the skin, which contains a vitamin $D_3$ precursor, 7-dehydrocholesterol; this compound undergoes photolysis on exposure to solar irradiation and is converted to vitamin D. Thus, inadequate dietary intake and inadequate sun exposure lead to vitamin D insufficiency. This problem is exacerbated by several modern trends, including avoidance of dairy products to control weight or high cholesterol and avoidance of sunlight to reduce the risk of skin cancer. Also, many elderly individuals have limited mobility and do not have the opportunity for adequate sunlight exposure. These circumstances have led to a substantial increase in the frequency of osteomalacic bone disease and fractures in the elderly, especially among the nursing home population. In wintertime, the problem of reduced sunlight further diminishes vitamin $D_3$ synthesis and worsens the tendency toward vitamin $D_3$ insufficiency. Races of people with dark skin have further difficulty with adequate vitamin $D_3$ synthesis, because the melanin in the skin is a natural sunscreen that reduces the penetration of ultraviolet rays into the layers of the dermis where vitamin $D_3$ is formed.

Deficient dietary calcium intake may also cause osteomalacia or exacerbate the problem of vitamin D insufficiency. There exists a spectrum of etiologies ranging from pure vitamin D deficiency with normal calcium to adequate vitamin D sufficiency with inadequate calcium. Worldwide, many cases of osteomalacia are due to vitamin D insufficiency combined with relative calcium insufficiency. Certain dietary habits common in selected populations may exacerbate vitamin D insufficiency by inhibiting vitamin D absorption or by increasing the metabolic clearance of 25-hydroxyvitamin $D_3$. The consumption of chapattis, an East Asian bread made from wheat flour with high phytate levels, impairs both calcium and vitamin D absorption. Thus, inadequate sunlight exposure and dietary habits combine in a variety of situations—e.g., because of customs, life-style choices (vegetarianism, high-fiber diets), or economic, age-related, and geographic circumstance (living at high altitude, calcium-deficient diets)—to exacerbate vitamin D insufficiency, thus determining the risk and severity of osteomalacic bone disease.

## B. Gastrointestinal Problems and Malabsorption of Vitamin D

In parts of the world where there are no programs to fortify foodstuffs with vitamin D, the diet of much of the population is estimated to contribute only one-quarter to one-third of the daily requirement of vitamin D. In such populations, therefore, gastrointestinal malabsorption may worsen the vitamin D insufficiency, but is not often the sole cause of osteomalacia. Some gastrointestinal problems are associated with increased metabolic clearance of vitamin D metabolites, compounding the malabsorption problem. Gastrointestinal diseases associated with osteomalacia include celiac disease (gluten enteropathy), cirrhosis, biliary obstruction, pancreatic insufficiency, inflammatory bowel disease, and post-gastrectomy or jejuno-ileal bypass surgery. Patients receiving total parenteral nutrition (TPN), usually because of chronic bowel disease, develop osteomalacia due to inadequate mineral or vitamin D supplementation. Anticonvulsant therapy increases the metabolic clearance of vitamin D metabolites, requiring vitamin D supplementation.

## C. Renal Osteodystrophy

Renal failure is associated with complex abnormalities of vitamin D and calcium metabolism. Phosphate retention due to inadequate kidney function causes hypocalcemia by complexing calcium and inhibiting renal 1α-hydroxylase activity, and therefore diminishing calcitriol synthesis. Also, as kidneys shrink and renal functional tissue declines, 1α-hydroxylase activity is further diminished, leading to deficiency of calcitriol production. These changes also lead to secondary hyperparathyroidism, compounding the bone abnormality. The constellation of hypocalcemia, secondary hyperparathyroidism, and calcitriol deficiency causes osteomalacia and renal osteodystrophy. Recognition of this sequence of events has led to important changes in the medical management of renal failure in an attempt to prevent the development of renal osteodystrophy. Two major therapeutic measures include the use of phosphate binders to minimize and/or prevent the development of elevated phosphate concentration and its consequent hypocalcemia, and supplementation with calcitriol to avoid deficiency of active vitamin D.

## D. Tumor-Induced Osteomalacia

Some small mesenchymal tumors (hemangiopericytomas, fibromas, angiosarcomas, etc.) can cause phosphaturia and hypophosphatemia, leading to osteomalacia. The syndrome is known as tumor-induced osteomalacia (TIO), or oncogenic osteomalacia. The mechanism involves the synthesis and secretion of excessive amounts of a phosphaturic factor. Currently, the major candidate for this role is FGF 23. How FGF 23 causes phosphaturia is still not completely understood. However, recent findings from the study of X-linked hypophosphatemia and autosomal dominant hypophosphatemic rickets have shed light on TIO and on the mechanisms for all three phosphaturic entities. The tumors causing TIO originally were thought to be benign and of mesenchymal origin, but malignant tumors have also been reported to cause the syndrome. Often the tumors are small and difficult to locate. The levels of calcitriol are inappropriately low (hypophosphatemia should stimulate calcitriol production) and so the tumor product is also thought to interfere with renal 1α-hydroxylation. The osteomalacia responds to treatment with large phosphate supplements, which restore phosphate levels to normal. The syndrome can be cured by successfully removing the tumor.

## IV. HEREDITARY RICKETS

A comparison of hereditary causes of rickets is shown in Table 2.

### A. Hypophosphatemic Rickets

#### 1. *X-Linked Hypophosphatemic Rickets*

X-Linked hypophosphatemia (XLH), an X-linked dominant disorder caused by renal phosphate wasting, results in severe skeletal abnormalities and growth retardation. The primary mechanism, defective phosphate reabsorption in the renal proximal tubule, impairs phosphate reabsorption. The clinical presentation is usually not apparent until 6–12 months of age and ranges from mild abnormalities of the bones to severe rickets and osteomalacia. Children exhibit rachitic bone deformities, including enlargement of the wrists and knees and bowing of the lower extremities. Defects in tooth development and premature cranial synostoses may also be present. Low or inappropriately normal circulating levels of calcitriol are found despite the hypophosphatemia. The low serum phosphate normally causes an increase in 1α-hydroxylase activity and enhanced calcitriol production, suggesting that XLH may also result in abnormal regulation of 1α-hydroxylase.

The gene causing XLH has been cloned; it is a phosphate-regulating gene with homologies to endopeptidases and is located on the X-chromosome, thus it has been named *PHEX*. The *PHEX* gene is homologous to a family of endopeptidases that includes endothelin-converting enzyme-1 and neutral endopeptidase. The *PHEX* gene encodes a 749-amino-acid membrane-bound protein that is expressed in bone, adult ovary, lung, and fetal liver. A number of genetic defects in the *PHEX* gene have been found in patients with XLH. Because many of the mutations are inactivating mutations, the X-linked dominant expression of the disorder is likely the result of the loss of a single functioning allele (haploinsufficiency) coded by the X-chromosome rather than the result of a dominant negative effect.

#### 2. *Autosomal Dominant Hypophosphatemic Rickets*

Autosomal dominant hypophosphatemic rickets (ADHR), an autosomal dominant disorder caused by phosphate wasting, has findings commonly seen in other phosphate wasting disorders. Patients have short stature, bone pain, rickets, and osteomalacia. The patients also have inappropriately normal serum calcitriol concentrations.

The gene causing ADHR has been cloned; recent data have suggested that fibroblast growth factor 23 is the phosphate-regulating molecule, thus the ADHR gene has been named *FGF23*. The *FGF23* gene encodes a member of the fibroblast growth factor family, a 251-amino-acid protein that is expressed in heart, lymph node, thymus, and liver. Three unique mutations have been found in the *FGF23* gene in patients with ADHR. These mutations affect two arginine residues located in a consensus proteolytic cleavage site. The mutations prevent degradation of *FGF-23* and thus result in enhanced or prolonged action leading to phosphate wasting and the syndrome of ADHR.

**TABLE 2 Comparison of Genetic Causes of Rickets**[a]

| Component | 1α-Hydroxylase deficiency | HVDRR | XLH | ADHR |
|---|---|---|---|---|
| Gene | *CYP27B1* | *VDR* | *PHEX* | *FGF23* |
| $1,25(OH)_2D_3$ | Low | High | (Normal) | (Normal) |
| PTH | High | High | Normal | Normal |
| Calcium | Low | Low | Normal | Normal |
| Phosphate | Low | Low | Low | Low |
| Alopecia | No | Yes | No | No |

[a] 1α-Hydroxylase deficiency, causes vitamin D-dependent rickets, type I; HVDRR, hereditary vitamin D-resistant rickets; XLH, X-linked hypophosphatemic rickets; ADHR, autosomal dominant hypophosphatemic rickets; (normal) indicates inappropriately normal relative to decreased serum phosphate concentration.

### *3. Mechanism of Phosphate Loss in XLH, ADHR, and TIO*

How are *PHEX* and *FGF23* involved in the pathophysiology of XLH, ADHR, and TIO? One current hypothesis is that under conditions of normal phosphate regulation the *PHEX*-encoded enzyme regulates the bioavailability of the *FGF23*-encoded protein, the putative phosphatonin molecule. As *FGF23* product is secreted from cells, some of it is degraded to inactive metabolites by the membrane-bound *PHEX* endopeptidase. The remaining active *FGF23* product enters the circulation and interacts with a receptor protein on the renal tubule cells. Binding transmits a signal to down-regulate the activity of the sodium-dependent phosphate cotransporter (NPT2) in the kidney and to decrease phosphate reabsorption. In XLH patients, the mutant *PHEX* protein or lack thereof is unable to degrade the *FGF23* protein. This leads to excess amounts of *FGF23* protein in the circulation. As a result, the signal to down-regulate NPT2 activity is magnified, leading to renal phosphate wasting. In ADHR, on the other hand, mutations in *FGF23* prevent the proteolytic processing step by the *PHEX* product and therefore there is an overabundance of active *FGF23* protein, which then leads to down-regulation of NPT2 activity, which causes phosphate wasting. In TIO, tumors overexpress *FGF23* protein, and elevated secretion by the tumors also leads to phosphate wasting and osteomalacia in this condition.

## B. 1α-Hydroxylase Deficiency

This disease was originally known as vitamin D-dependent rickets type I (VDDR-I). Other names include pseudo-vitamin D deficiency type I and pseudo-vitamin D deficiency rickets (PDDR). We prefer to refer to the entity as 1α-hydroxylase deficiency because it has been shown to be caused by mutations in the cytochrome P450 enzyme, 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase). The human 1α-hydroxylase gene (*CYP27B1*) is located on chromosome 12. A number of mutations that disrupt 1α-hydroxylase activity are found scattered throughout the entire region of the *CYP27B1* gene. 1α-Hydroxylase deficiency is a rare autosomal recessive disease that is manifested at an early age. Patients exhibit hypocalcemia, elevated levels of PTH and alkaline phosphatase, and low levels of urine calcium. Affected children present with hypotonia, muscle weakness, growth failure, and rickets. Tetany and convulsions may occur with severe hypocalcemia. Patients have normal serum concentrations of 25-hydroxyvitamin $D_3$ but low levels of $1{,}25(OH)_2D_3$ due to the defective synthesis of $1{,}25(OH)_2D_3$. PTH infusion does not increase circulating $1{,}25(OH)_2D_3$ levels, consistent with a defect in 1α-hydroxylase activity. Very large doses of vitamin $D_3$ or 25-hydroxyvitamin $D_3$ are required for adequate treatment of 1α-hydroxylase deficiency; often, daily vitamin D doses of 20,000 to over 100,000 IU are needed. On the other hand, modest doses of $1{,}25(OH)_2D_3$ (0.25–2 μg/day) tend to be sufficient to restore calcium to normal and to heal the rickets. Because mutations in 1α-hydroxylase block $1{,}25(OH)_2D_3$ synthesis, this latter treatment bypasses the defect and reverses the abnormalities caused by the disease.

## C. Hereditary 1,25-Dihydroxyvitamin $D_3$-Resistant Rickets

Hereditary 1,25-dihydroxyvitamin $D_3$-resistant rickets (HVDRR) is known as vitamin D-dependent rickets type II (VDDR-II), or pseudo-vitamin D deficiency type II. This rare genetic disease arises as a result of mutations in the VDR; HVDRR is an autosomal recessive disease characterized by early-onset rickets, hypocalcemia, secondary hyperparathyroidism, and normal or elevated serum $1{,}25(OH)_2D$ levels. Parents of children with HVDRR usually have a history of consanguinity and have no evidence of bone disease. In many cases, the affected child exhibits total body alopecia, lacking all body hair, including eyelids and eyelashes. The alopecia, which usually occurs within the first year after birth, provides evidence for the HVDRR syndrome.

Several missense mutations have been identified in the VDR DBD. These usually occur in highly conserved amino acids. In a few families, nonsense mutations that result in premature termination of the VDR have also been found. Some premature termination signals result from mutations that cause exon skipping or affect RNA splicing. A number of missense mutations in the VDR LBD have also been identified. In one case, an arginine at codon 274 was mutated to leucine, which affected $1{,}25(OH)_2D_3$ binding. Arginine 274 is the contact point for the 1-hydroxyl group of $1{,}25(OH)_2D_3$ in the ligand-binding pocket of the VDR. In a second case, histidine was mutated to glutamine at codon 305, which caused a 5- to 10-fold reduction in $1{,}25(OH)_2D_3$ binding and a similar reduction in gene transactivation. Histidine 305 is the residue that makes contact with the 25-hydroxyl group of $1{,}25(OH)_2D_3$. Some mutations also disrupt RXR heterodimeriztion with the VDR. An arginine to cysteine mutation at codon 391 interfered with RXR binding but did not affect $1,25(OH)_2D_3$ binding, whereas another mutation, phenylalanine to cysteine at codon 251, affected both $1,25(OH)_2D_3$ binding and RXR heterodimeriztion. Interestingly, there has been one case of HVDRR in which no mutations were found in the VDR. The patient exhibited all the signs of HVDRR, including alopecia. This case highlights the fact that proteins other than VDR are involved in $1,25(OH)_2D_3$ signaling and that defects in these proteins may also cause the HVDRR syndrome. Genetic defects in co-activators may also be the molecular basis for other steroid hormone-resistant syndromes.

Some children with HVDRR respond to large doses of calcitriol, which can overcome the VDR ligand-binding defects, whereas others respond to large doses of calcium and calcitriol. However, in cases in which the VDR is completely inactivated, the patients are unresponsive to large doses of vitamin D derivatives or oral calcium supplements. Successful treatment of these children has been achieved by chronic intravenous administration of calcium infusions, given nightly over a period of many months. This treatment bypasses the intestinal defect in calcium absorption and over time is able to correct the hypocalcemia. The treatment eventually results in normalization of serum calcium levels, correction of secondary hyperparathyroidism, and normal mineralization of bone and healing of rickets, as evidenced on X-rays. Clinical improvement can be sustained if adequate serum calcium and phosphorus concentrations are maintained. Although intravenous calcium may have been required to render the child normocalcemic, the benefits can sometimes be maintained thereafter by oral calcium. However, alopecia, if present, does not improve as a consequence of the treatment.

## V. SUMMARY

Many forms of rickets/osteomalacia are due to genetic defects in the synthesis or action of calcitriol or to conditions that cause phosphate loss. Recent improvements in our understanding of the molecular mechanisms by which these defects cause rickets or osteomalacia have led to improved diagnostic and treatment strategies. However, the commonest cause of rickets/osteomalacia worldwide is deficiency of vitamin D. Fortification of dietary foodstuffs or provision of vitamin $D_3$ supplements to individuals at risk would greatly reduce the impact of this devastating disease of the skeleton.

## Glossary

**$1\alpha$-hydroxylase** Cytochrome P450 enzyme that converts 25-hydroxyvitamin $D_3$ to 1,25-dihydroxyvitamin $D_3$ by catalyzing the addition of a hydroxyl group at the C-1 position.

**osteomalacia** Bone disease in which mineralization of the collagen matrix of bone is defective, causing softening of the bones and susceptibility to fracture.

**osteoporosis** Bone disease causing increased susceptibility to fracture; although the bones have a normal content of mineral and matrix, bone loss causes porosity and fragility.

**PHEX** Gene on the X chromosome that encodes a membrane-bound protein with endopeptidase activity; mutations have been found in the disease X-linked hypophosphatemia.

**rickets** Osteomalacic disease in a growing child; caused by inadequate mineralization of bone and cartilage, resulting in bowing of the weight-bearing bones of the extremities, joint swelling, pain in bones and joints, and other skeletal problems due to softening of the bones.

**vitamin D** receptor Member of the steroid/retinoid/thyroid superfamily of nuclear receptors; mediates 1,25-dihydroxyvitamin $D_3$ actions to regulate gene expression in target cells.

## See Also the Following Articles

**Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Vitamin D • Vitamin D and Human Nutrition • Vitamin D-Binding Protein • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in the Intestine and Kidney • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Metabolism • Vitamin D: Nuclear Receptor for $1,25(OH)_2D_3$**

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# Vitamin D-Dependent Calbindins (CaBP)

Angela Porta, Puneet Dhawan,
Kristen Gengaro, Yan Liu, Xiaorong Peng, and
Sylvia Christakos
*University of Medicine and Dentistry of New Jersey*

I. INTRODUCTION
II. LOCALIZATION AND FUNCTIONAL SIGNIFICANCE
III. REGULATION OF CALBINDIN GENE EXPRESSION
IV. SUMMARY

**Calbindin-$D_{9k}$ and calbindin-$D_{28k}$ are intracellular calcium-binding proteins that are present in many different tissues whose production is stimulated by the steroid hormone $1\alpha,25(OH)_2D_3$. The calbindins serve different functions and are regulated by several steroids as well as by factors that affect signal transduction pathways. Understanding their functions and activities may lead to therapeutic intervention in disorders of calcium metabolism and bone development.**

## I. INTRODUCTION

Increased synthesis in the intestine and kidney of the calcium-binding protein, calbindin, the first identified molecular target of vitamin D action, is one of the most pronounced effects of vitamin D known. There are two major subclasses of calbindin: calbindin-$D_{9k}$ (a 9000-Da protein that is present in highest concentrations in mammalian intestine and in mouse and neonatal rat kidney, placenta, and uterus) and calbindin-$D_{28k}$ (a 28,000-Da protein that is highly conserved in evolution and is present in highest concentrations in avian intestine and mammalian and avian kidney, brain, and pancreas). There is no amino acid sequence homology between calbindin-$D_{9k}$ and calbindin-$D_{28k}$. The calbindins belong to a family of intracellular proteins that bind calcium with high affinity. Other members of this family include calmodulin, parvalbumin, troponin C, calretinin, calcineurin, the myosin light chains, and S100α and S100β. A characteristic of all of these proteins is the EF-hand structural motif. The EF-hand domain is an octahedral calcium-binding structure formed by a helix–loop–helix conformation of the polypeptide chain. The loop contains the side chain oxygens necessary for binding the calcium cation, and within one protein, amino acid linker sequences connect multiple EF hands. Calbindin-$D_{28k}$ contains six EF hands but only four are functional and bind calcium. Calbindin-$D_{9k}$ contains two EF hands and both bind calcium. Studies related to the distribution of calbindin and its cellular colocalization with the vitamin D receptor have resulted in key advances in our understanding of the diversity of the vitamin D endocrine system (Table 1). The focus here is on the localization, proposed functional significance, and regulation of these calcium-binding proteins. Insights obtained by studying these proteins allow an understanding of the multiple actions of the vitamin D endocrine system.

(e.g., psoriasis), and inducer of cell differentiation (e.g., cancer).

This article discusses the effect of $1,25(OH)_2D_3$ in three important tissues wherein differentiation is essential, namely, in cancer tissues, skin, and tissues of the immune system.

## II. CANCER

A wide variety of malignant cells and tissues possess nuclear receptors for $1,25(OH)_2D_3$. Several laboratories have demonstrated that immature mouse myeloid cells, upon treatment with $1,25(OH)_2D_3$, differentiate toward more mature macrophage-like cells that are characterized by a decreased growth rate. The same effect was later observed in human leukemia cell lines such as HL-60 and U937. These cells acquire the morphology of monocytes, are able to perform phagocytosis, and possess differentiation-specific characteristics [such as expression of nonspecific esterases, production of superoxides, and expression of surface markers (e.g., CD14, CD11b)]. The anti-proliferative and prodifferentiating effects of $1,25(OH)_2D_3$ are not limited to leukemia cells but are also seen in a wide variety of other cancer cells possessing the vitamin $D_3$ receptor such as melanoma, breast cancer, colon cancer, and prostate cancer.

The molecular mechanisms responsible for cell growth arrest and terminal differentiation are still unclear. It has been demonstrated that $1,25(OH)_2D_3$-treated cancer cells accumulate in the G1 phase of the cell cycle. The regulation of cell cycle genes could be a possible explanation for the anti-proliferative effects of $1,25(OH)_2D_3$. Table 1 gives an overview of genes regulated by $1,25(OH)_2D_3$ at the transcriptional level in cancer cells. $1,25(OH)_2D_3$ increases the expression level of the cell cycle inhibitory proteins p21 and p27 in a wide variety of cancer cell lines such as leukemia, prostate cancer, and breast cancer cells. The promoter region of p21 even contains a vitamin D-response element (VDRE), suggesting that p21 is a direct target of $1,25(OH)_2D_3$. However, treatment of MCF-7 breast cancer cells with $1,25(OH)_2D_3$ and antibodies neutralizing transforming growth factor-β (TGF-β) completely abrogates $1,25(OH)_2D_3$-induced up-regulation, whereas the growth inhibitory effect was only partially reversed. Moreover, a recent report questions the inhibitory function of p21 in $1,25(OH)_2D_3$-induced growth reduction as an *in vitro* and *in vivo* down-regulation of p21 was observed in squamous cell carcinoma after treatment with $1,25(OH)_2D$. The cyclin-dependent kinase (cdk) inhibitors p15 and p18 are up-regulated in myeloid U937 cells following treatment with $1,25(OH)_2D_3$. Cell cycle arrest and differentiation in these cells are preceded by a proliferative burst and a transient increase in cyclin D1, A, and E protein levels. The activity of cdk2 and cdk6, involved in the transition from G1 to S, is reduced following treatment with $1,25(OH)_2D_3$ and may contribute to the observed growth inhibitory effect.

Incubation of HL-60 cells with $1,25(OH)_2D_3$ increases cdk5 activity, which is thought to facilitate the G1 to S phase transition in cells approaching replicative quiescence and to enhance concomitant monocytic differentiation. In addition to cell cycle genes, other genes such as proto-oncogenes and tumor suppressor genes are also regulated by $1,25(OH)_2D_3$ (Table 1). It is not always clear whether this modulation represents a direct effect of $1,25(OH)_2D_3$ or is

**TABLE 1 Genes Regulated by $1,25(OH)_2D_3$ at the Transcriptional Level in Cancer Cells**

| | | |
|---|---|---|
| *Angiogenesis* | *Apoptosis regulatory genes* | *Receptors* |
| VEGF ↓ | p53 ↑ | VDR ↑ |
| | Clusterin ↑ | AR ↑ |
| | | ER ↓ |
| *Growth factors* | *Proto-oncogenes and tumor suppressor genes* | *Cell cycle regulatory genes* |
| TGF-β ↑ | c-myc ↓ | Cyclin A ↓ |
| IGF-II ↓ | c-fos ↑ | Cyclin E ↓ |
| IGF-BP3 ↑ | c-fms ↑ | Cyclin D1 ↓ |
| IGF-BP5 ↑ | c-jun ↑ | p21 ↑ |
| PTHrP ↓ | E-cadherin ↑ | P27 ↑ |
| IL-1β ↓ | BRCA1 ↑ | |
| IL-6 ↑ | | |

*Note:* AR, androgen receptor; BP, binding protein; BRCA1, breast cancer susceptibility gene; ER, estrogen receptor; IGF, insulin-like growth factor; IL, interleukin; PTHrP, parathyroid hormone-related peptide; TGF-β, transforming growth factor-β; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor.

TABLE 2 Anti-Tumor Effects of $1,25(OH)_2D_3$ in Different Animal Models of Cancer

| Administration | Tumor model | Anti-tumor effect |
|---|---|---|
| Oral | Colon cancer | Reduction of tumor incidence |
| Intraperitoneal | Head and neck squamous cell carcinoma | Suppression of tumor growth |
| Intraperitoneal | Lymphoma | Increase in survival time |
| Intraperitoneal | Liver cancer | Suppression of tumor growth |
| Intravesical | Bladder cancer | Reduction of tumor incidence |
| Hepatic arterial infusion | Liver cancer | Suppression of tumor growth |

*Note:* Data are from studies published in 2000–2001.

rather a consequence of its overall effect on proliferation and differentiation. Suppression of c-*myc* transcription in HL-60 cells following treatment with $1,25(OH)_2D_3$ is thought to be mediated by binding of the nuclear phosphoprotein HOXB4 to the promoter region of the gene. A rapid up-regulation of the proto-oncogene c-*fos* has been reported in several cell lines treated with $1,25(OH)_2D_3$. This up-regulation is thought to be a direct effect of $1,25(OH)_2D_3$ mediated by a VDRE in the promoter region of the gene. Exposure of HL-60 cells to $1,25(OH)_2D_3$ induces the expression of c-*fms*, which is the receptor for macrophage colony-stimulating factor, concomitantly with the induction of the monocytic phenotype.

Enhanced expression of some tumor suppressor genes was reported in breast cancer cells after incubation with $1,25(OH)_2D_3$. The cell surface adhesion molecule E-cadherin and the breast cancer susceptibility gene BRCA1 are up-regulated following treatment with $1,25(OH)_2D_3$. Recently, it was demonstrated that $1,25(OH)_2D_3$ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling.

Another mechanism by which $1,25(OH)_2D_3$ and its analogues inhibit cell growth is the induction of programmed cell death (apoptosis) in various cancer cells including breast, colon, and prostate cancer cells. A whole set of genes involved in the cascade of apoptosis are influenced by $1,25(OH)_2D_3$. However, some contradictory reports have been published on the role of apoptotic cell death in the growth inhibition caused by this compound.

The *in vitro* studies showing the anti-proliferative and prodifferentiating effects of $1,25(OH)_2D_3$ on cancer cells have been confirmed *in vivo* in different animal models (Table 2). Indeed, the tumor incidence or tumor growth was reduced in different cancers such as colon, liver, or bladder cancer. To overcome the calcemic side effects of $1,25(OH)_2D_3$ (hypercalciuria, hypercalcemia, and increased bone resorption), attempts were made to generate chemically modified $1,25(OH)_2D_3$ molecules (analogues) that retain the beneficial anti-proliferative and prodifferentiating effects but not the unwanted calcemic side effects. Some of these potent analogues with beneficial effects on tumor growth in animals are now being further investigated in clinical trials (Table 3). These drugs can be used not only as monotherapy but also as adjuvant therapy in combination with chemotherapeutics or hormone therapy. The combined agents may act in concert and produce additional or synergistic effects on cell proliferation and differentiation but also allow dose reduction, thereby decreasing adverse side effects.

## III. SKIN

The skin occupies a central position in the vitamin D endocrine system: it is not only the site of synthesis of vitamin $D_3$, as cutaneous cells are also capable of converting vitamin $D_3$ to its active form, $1,25(OH)_2D_3$. Moreover, they contain the VDR, rendering them responsive to the actions of $1,25(OH)_2D_3$. The exact physiological function of vitamin D in the skin is still elusive. However, humans or mice with a dysfunctional VDR display a postnatal alopecia, which cannot be cured by normalization of calcium levels by dietary means. These findings firmly indicate that the VDR is directly implicated in hair follicle biology. In this context, recent mechanistic studies refer to a ligand-independent role for VDR–retinoic acid X receptor dimers in anagen initiation in the epithelial (keratinocyte) component of the hair follicle apparatus.

Whereas almost every cutaneous cell type contains the VDR (Table 4), the epidermal keratinocyte is generally considered the main vitamin D target cell in the skin. $1,25(OH)_2D_3$ at pharmacological concentrations is a potent inducer of keratinocyte growth arrest and differentiation. These properties were the basis for the successful application of $1,25(OH)_2D_3$ analogues in the treatment of hyperproliferative skin

**TABLE 3 Clinical Trials with $1,25(OH)_2D_3$ and Analogues**

| Compound | Administration | Tumor | Effects |
|---|---|---|---|
| $1,25(OH)_2D_3$ | Oral | Hormone refractory metastatic prostate cancer | No toxicity; no reduction of tumor mass |
| $1,25(OH)_2D_3$ | Oral | Myelodysplastic syndrome | Clinical response in 70% of patients; no toxicity |
| $1,25(OH)_2D_3$ | Oral | Myelodysplastic syndrome | No clinical effect |
| Calcipotriol | Topical | Breast cancer | Reduction of tumor diameter in 20% of patients |
| EB 1089 | Oral | Advanced breast and colon cancer | Stabilization of the disease in 17% of patients |
| $1,25(OH)_2D_3$ + docetaxel | Oral | Androgen-independent prostate cancer | Reduction in PSA; limited or no toxicity |
| $1\alpha(OH)D_3$ + surgery + chemotherapy + radiotherapy | Oral | Glioblastomas and anaplastic astrocytomas | Progressive and durable tumor regression in 20% of patients; no toxicity |

*Note:* EB 1089, 22,24-diene-24,26,27-trihomo-$1\alpha,25(OH)_2D_3$.

diseases such as psoriasis and certain forms of ichthyosis. The $1,25(OH)_2D_3$-dependent growth arrest and differentiation in keratinocytes are likely to be based on a similar mechanism of action as described for cancer cells: $1,25(OH)_2D_3$-treated keratinocytes fail to progress from the $G_1$ to the S phase of the cell cycle due to induction of the negative cell cycle regulators p21 and p27 and suppression of the proto-oncogene c-myc. Induction of autocrine or paracrine growth regulators such as transforming growth factor-β, tumor necrosis factor α (TNFα), and parathyroid hormone-related peptide may also contribute to the anti-mitotic $1,25(OH)_2D_3$ effect in epidermal cells. $1,25(OH)_2D_3$-dependent keratinocyte differentiation is accompanied by induction of cornified envelope precursors such as involucrin and its cross-linking enzyme transglutaminase type I. There is also a stimulatory effect on stratification by translocation of E-cadherin to assembling adherens junctions, which will further enhance the prodifferentiative actions of $1,25(OH)_2D_3$.

Low concentrations of $1,25(OH)_2D_3$ exert mitogenic activity rather than a growth-inhibiting effect on keratinocytes, especially in cells that are committed to differentiate. Increased proliferation is also observed following application of $1,25(OH)_2D_3$ compounds to normal mouse or human skin. In contrast, hyperproliferative epidermis (in psoriasis or induced by application of mitogens) responds to $1,25(OH)_2D_3$ derivatives with growth arrest. These data indicate that $1,25(OH)_2D_3$ exerts a normalizing rather than an unidirectional anti-proliferative effect on epidermal growth.

Skin fibroblasts also respond to $1,25(OH)_2D_3$ treatment with normalization of their growth rate and collagen production. Therefore, $1,25(OH)_2D_3$ compounds can be therapeutically used for diseases with excessive fibroblast activity (scleroderma) or insufficient fibroblast activity (skin atrophy). $1,25(OH)_2D_3$ stimulates the production of melanin in melanocytes, a feature that can be applied in the treatment of vitiligo. Despite the obvious role for the VDR in hair biology, no application of $1,25(OH)_2D_3$ analogues in hair disorders is yet available; however, animal models open promising perspectives for the prevention of chemotherapy-induced alopecia.

**TABLE 4 Vitamin D Target Cells in the Skin with Effects of $1,25(OH)_2D_3$ and Clinical Application of $1,25(OH)_2D_3$ Analogues**

| Target cell | Effect of $1,25(OH)_2D_3$ | Clinical use |
|---|---|---|
| Keratinocyte | Regulation of growth and differentiation | Psoriasis; hyperproliferative ichthyosis |
| Fibroblast | Regulation of growth and collagen production | Scleroderma; skin atrophy |
| Hair follicle cell | Hair cycle regulation | Alopecia (chemotheraphy)? |
| Melanocyte | Stimulation of melanogenesis | Vitiligo |

## IV. IMMUNE SYSTEM

The detection of VDRs in almost all cells of the immune system, especially antigen-presenting cells (macrophages and dendritic cells) and activated T lymphocytes, led to the investigation of a potential for $1,25(OH)_2D_3$ as an immunomodulator. Application of the molecule *in vitro* and *in vivo* has led to interesting observations, confirming a role for $1,25(OH)_2D_3$ and its analogues in the immune system. Not only is the VDR present in all cells of the immune system, activated macrophages are able to synthesize and secrete $1,25(OH)_2D_3$. These cells indeed express the enzyme $25(OH)D_3$-1α-hydroxylase, as could recently be demonstrated at the molecular level by reverse transcription-polymerase chain reaction (RT-PCR) in activated macrophages. Although cloning and sequencing of the mRNA clearly demonstrated this enzyme to be identical to the known renal form, its regulation seems to be under completely different control. The macrophage enzyme mainly runs through immune signals, with interferon-γ (IFN-γ) being a powerful stimulator. In macrophages, no clear down-regulation of the enzyme by $1,25(OH)_2D_3$ could be observed, explaining the hypercalcemia occurring in situations of macrophage overactivation such as tuberculosis or sarcoidosis. The secretion of classical macrophage products such as cytokines [interleukin-1 (IL-1), TNFα, and IL-12] precedes the transcription of the enzyme and as a consequence the secretion of $1,25(OH)_2D_3$. Therefore, its timing is compatible with that of a suppressive signal, allowing first an activation and further recruitment of the other members of the immune system, followed by inhibiting signals, such as prostaglandin E2 (PGE2), to limit the extent of the reaction (Fig. 1).

As a true immunomodulator, $1,25(OH)_2D_3$ not only interacts with T cells but also targets the central cell in the immune cascade, the antigen-presenting cell.

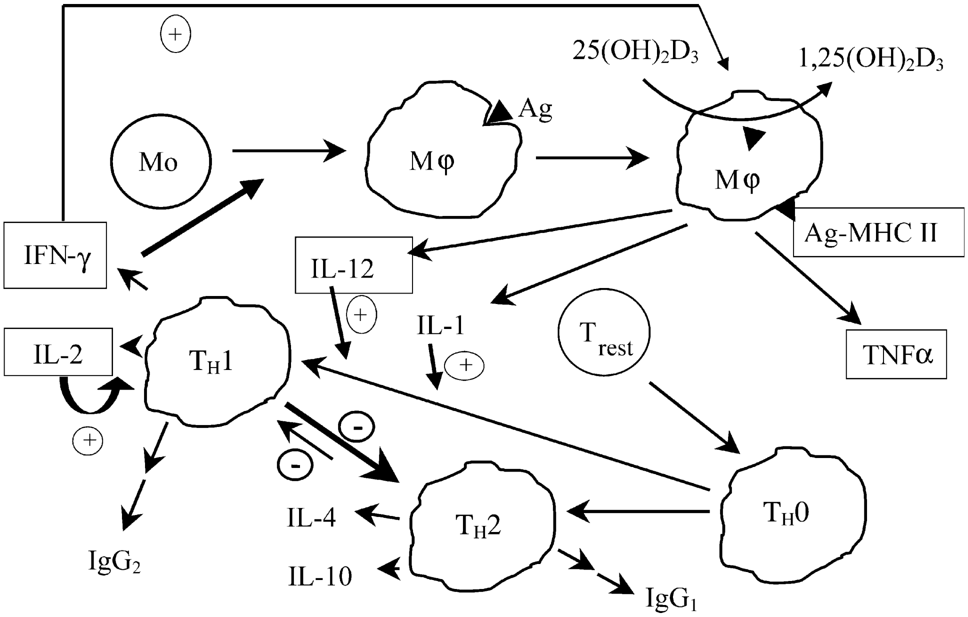

**FIGURE 1** Regulation of the immune response by $1,25(OH)_2D_3$. Upon contact with an antigen (Ag), e.g., a bacterial component, blood monocytes (Mo) or dendritic cells will become activated (Mφ) and present this antigen in the context of MHC class II molecules to the rest of the immune system. Moreover, by secretion of chemokines and activating cytokines [e.g., interleukin-12 (IL-12), tumor necrosis factor α (TNFα)], these activated cells will recruit T lymphocytes (CD4 cells) and activate them, depending on the cytokine environment, into T helper 1 ($T_H1$) or $T_H2$ cells. $T_H1$ cells, which produce mainly interferon-γ (IFN-γ) and IL-2, will further activate the macrophage and close a positive feedback loop, leading to amplification of the immune response. $1,25(OH)_2D_3$ and its analogues can modulate the immune response via several mechanisms. $1,25(OH)_2D_3$ inhibits IL-12 production and down-regulates co-stimulatory molecule expression by dendritic cells, thus inhibiting the development of $T_H1$ cells along the $T_H1$ pathway and favoring the induction of $CD4^+CD25^+$ regulatory T cells. $1,25(OH)_2D_3$ also exerts direct effects on T cells by inhibiting IL-2 and IFN-γ production and by inducing, in particular, $T_H2$ cells in target tissues. In addition, $1,25(OH)_2D_3$ inhibits the recruitment of $T_H1$ cells to sites of inflammation. Macrophages (Mφ) can synthesize $1,25(OH)_2D_3$ and this may also contribute to the regulation of the immune response.

Here, $1,25(OH)_2D_3$ stimulates the differentiation of monocytes toward "good" phagocytosis and killing of bacteria. Differentiation of monocytes into dendritic cells and further differentiation of these cells from immature to mature dendritic cells is almost completely inhibited by $1,25(OH)_2D_3$. Incubation of monocytes with $1,25(OH)_2D_3$ *in vitro* results in a cell type totally different from classical dendritic cells, but also different from monocytes. CD14 is highly expressed on the surface, but human leukocyte antigen class II proteins and B7.2 proteins are down-regulated. Antigen-presenting cells are not able to promote an immune response, since they are not able to properly stimulate T cells to proliferation or cytokine secretion. On the other hand, these cells have also not differentiated into macrophages, since they are unable to perform chemotaxis or phagocytosis of bacteria. Also, the crucial signals secreted by antigen-presenting cells for the recruitment and activation of T cells are directly influenced by $1,25(OH)_2D_3$. A key cytokine in the immune system, IL-12 is clearly inhibited by $1,25(OH)_2D_3$ and its analogues. This monocyte-produced substance is the major determinant of the direction in which the immune system will be activated. IL-12 stimulates the development of CD4 T helper 1 ($T_H1$) cells and inhibits the development of CD4 $T_H2$ lymphocytes. $T_H1$ lymphocytes secrete mainly IL-2 and IFN-γ and are considered to be the most important cells in graft rejection and autoimmunity. $T_H2$ cells secrete IL-4, IL-5, and IL-10 and are considered to be regulator cells. The observation of clear inhibition of IL-12 by $1,25(OH)_2D_3$ and its analogues (*in vitro* by enzyme-linked immunosorbent assay or by intracellular fluorescence-activated cell sorting (FACS) analysis or *in vivo* by RT-PCR) is essential in understanding the observed effects of these substances *in vitro* on T-cell proliferation and cytokine production and *in vivo* on graft survival and autoimmunity prevention (Table 5). By inhibiting IL-12 secretion, $1,25(OH)_2D_3$ directly interferes with the heart of the immune cascade and shifts the reaction toward a $T_H2$ profile. $1,25(OH)_2D_3$ also influences the secretion of other cytokines secreted by monocyte-derived cells: the suppressive PGE2 is stimulated and the monocyte recruiter granulocyte/macrophage colony-stimulating factor is suppressed. Several T-cell cytokines, especially the $T_H1$ type, are also direct targets for $1,25(OH)_2D_3$ and its analogues. $1,25(OH)_2D_3$-mediated inhibition of IL-2 secretion occurs through impairment of nuclear factor of activated T cells (NFAT) complex formation, since the receptor complex itself binds to the distal NFAT-binding site in the human IL-2 promoter. Another key T-cell cytokine that by itself further stimulates antigen presentation, IFN-γ, is directly (via a VDRE) down-regulated by $1,25(OH)_2D_3$. Moreover, progressive deletion analysis of the IFN-γ promoter revealed that negative regulation by $1,25(OH)_2D_3$ is also present at the level of an upstream region containing an enhancer element. Finally, it was recently demonstrated that $1,25(OH)_2D_3$ also directly stimulates IL-4 production by $T_H2$ cells. The combination of these effects on dendritic cell phenotype and cytokine secretion, with the direct effects on T-cell cytokine profile, results in the differentiation of T cells toward a $T_H2$ profile. This profile is beneficial in achieving prolonged organ graft survival and in prevention of organ-specific autoimmune diseases such as multiple sclerosis or type 1 diabetes.

TABLE 5 **Beneficial Effects of $1,25(OH)_2D_3$ and its Analogues in Animal Models of Autoimmunity and Transplantation**

| |
|---|
| **Autoimmunity** |
| Autoimmune diabetes |
| Chemically induced diabetes mellitus |
| Collagen-induced arthritis |
| Experimental allergic encephalomyelitis |
| Experimental autoimmune thyroiditis |
| Heyman nephritis |
| Lupus nephritis |
| Mercuric chloride-induced glomerulonephritis |
| **Transplantation** |
| Aorta |
| Bone marrow |
| Heart |
| Kidney |
| Liver |
| Pancreatic islets |
| Skin |
| Small bowel |

The fact that $1,25(OH)_2D_3$ and its analogues influence the immune system, not by pure immunosuppression, but by immunomodulation through induction of immune shifts and regulator cells, makes these products very appealing for clinical use, especially in the treatment and prevention of autoimmune diseases (Table 5). In autoimmune diabetes in the non-obese diabetic (NOD) mouse, up-regulation of regulator cells and a shift from $T_H1$ toward $T_H2$ locally in the pancreas and islet grafts of treated mice could be observed, and other effects on the immune system have also been described, the most important being a restoration of defective apoptosis sensitivity in lymphocytes, leading to better elimination of potentially dangerous autoimmune effector cells.

This increase in immunocyte apoptosis in NOD mice by $1,25(OH)_2D_3$ and its analogues has been reported to occur following different apoptosis-inducing signals and could explain why an early short-term treatment with these products, before the onset of autoimmunity, can lead to long-term protection and a restoration of tolerance.

Finally, clear additive and even synergistic effects were observed between $1,25(OH)_2D_3$ or its analogues and other more classical immunomodulators such as cyclosporin A and sirolimus (rapamycin). These effects were observed *in vitro* and could be confirmed *in vivo* in models of autoimmunity (diabetes and experimental allergic encephalomyelitis) and in graft destruction (Table 5).

In conclusion, these data suggest a physiological role for $1,25(OH)_2D_3$ in the immune system as an inhibiting signal secreted by activated macrophages and received by activated T cells, thus limiting the immune reaction (Fig. 1). Analysis of the VDR knockout mouse model, however, confirms that all immune effects of $1,25(OH)_2D_3$ are mediated through the VDR but demonstrate that $1,25(OH)_2D_3$ is possibly a redundant signal in the immune system.

## V. SUMMARY

The combined presence of $25(OH)D_3$-1α-hydroxylase and the VDR in several nonclassical tissues introduced the concept of a paracrine role for $1,25(OH)_2D_3$ outside that of calcium and bone metabolism. Moreover, the fact that $1,25(OH)_2D_3$ was found to be capable of regulating cell differentiation and proliferation of normal (keratinocytes and immune cells) and malignant cells (leukemia, breast, prostate, and colon cancer cells) gave new perspectives on this molecule. The efforts made by companies and laboratories worldwide to develop analogues of $1,25(OH)_2D_3$ with reduced calcemic effects opened therapeutic applications for these compounds as inhibitors of cell proliferation (e.g., treatment of psoriasis or cancer), as inducers of cell differentiation (e.g., treatment of cancer), and as immune modulators (e.g., treatment of autoimmune diseases or prevention of graft rejection). Moreover, the synergistic effects of $1,25(OH)_2D_3$ analogues with retinoids, anti-estrogens, conventional chemotherapeutics, and classical immunomodulators may result in better response rates for the treatment of cancer and immune disorders. However, more research and clinical trials are needed to select the best $1,25(OH)_2D_3$ analogue for each application.

## Glossary

| | |
|---|---|
| **alopecia** | Hair loss that can occur in patches (alopecia areata) or globally. Causes range from hormones to autoimmunity. |
| **anagen** | The growth phase of the growth cycle of mature hair follicles; anagen is followed by regression (catagen), rest (telogen), and shedding (exogen). |
| **apoptosis** | Programmed cell death; cells undergoing apoptosis are cleared from the system silently without the initiation of inflammation. |
| **autoimmune diseases** | Disorders initiated by the activation of autoreactive T cells that result in the destruction of tissues and cells by the body's own immune system. |
| **cornified envelope** | Proteinaceous reinforced structure beneath the plasma membrane in differentiated keratinocytes consisting of cross-linked precursor proteins. |
| **epidermis** | Outer epithelial layer of the skin consisting mainly of cells called keratinocytes. |
| **ichthyosis** | Skin disease in which the skin is very dry and exhibits fish-like scales. |
| **$1,25(OH)_2D_3$** | The dihydroxylated, biologically active form of vitamin D. It is a central hormone in calcium homeostasis and bone metabolism, but has also a number of other functions and notably powerful immunomodulatory properties. |
| **vitamin D receptor (VDR)** | A member of the superfamily of nuclear receptors for steroid hormones, thyroid hormone, and retinoic acid. The VDR functions as a $1,25(OH)_2D_3$-activated transcription factor that ultimately influences the rate of RNA polymerase II-mediated transcription. VDRs are present not only in cells typically involved in calcium and bone metabolism, but also in other cell types, such as cells of the immune system. |
| **vitamin D** | A vitamin/hormone with a central role in calcium and bone metabolism; it can be absorbed from the diet or can be synthesized by the skin via stimulation by ultraviolet light. Deficiency of vitamin D leads to a disorder known as rickets or osteomalacia. |
| **vitamin D analogues** | Chemically modified molecules derived from $1,25(OH)_2D_3$. Modifications have been made throughout the molecule, to obtain analogues with the desired properties. More than 1000 different vitamin D analogues have been synthesized worldwide. |

## See Also the Following Articles

**Adrenocorticosteroids and Cancer • Apoptosis • Cancer Cells and Progrowth/Prosurvival Signaling • Glucocorticoids and Autoimmune Diseases • Sex Hormones and the Immune System • Vitamin D • Vitamin D Metabolism • Vitamin D: Nuclear Receptor for $1,25(OH)_2D_3$ • Vitamin D Receptors and Actions in Nonclassical Target Tissues**

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# Vitamin D Metabolism

HELEN L. HENRY
*University of California, Riverside*

I. INTRODUCTION
II. VITAMIN $D_3$-25-HYDROXYLASE
III. 25(OH)$D_3$-1α-HYDROXYLASE
IV. 25(OH)$D_3$/1α,25(OH)$_2D_3$-24R-HYDROXYLASE
V. REGULATION OF VITAMIN D METABOLISM
VI. OTHER PATHWAYS OF VITAMIN D METABOLISM

**Vitamin $D_3$ is produced in the skin through the action of ultraviolet light and is subsequently hydroxylated in the liver and then in the kidney to form 1α,25-dihydroxyvitamin $D_3$, the biologically active form of vitamin D. The biochemical reactions that occur in vitamin D metabolism, the enzymes that catalyze these reactions, and the genes that encode these enzymes are discussed in this article. In addition, the factors that regulate the pathways of vitamin D metabolism are examined.**

## I. INTRODUCTION

Ultraviolet light and heat bring about the conversion of 7-dehydrocholesterol to vitamin $D_3$ in the skin of vertebrates on exposure to sunlight (Fig. 1). In the liver, vitamin $D_3$ is hydroxylated at carbon 25 on the side chain to form 25-hydroxyvitamin $D_3$ [25(OH)$D_3$], the major circulating vitamin $D_3$ metabolite. 25(OH)$D_3$ serves as the substrate for the production in the kidney of the biologically active steroid hormone, 1α,25-dihydroxyvitamin $D_3$ [1α,25-(OH)$_2D_3$] and of 24R,25-dihydroxyvitamin $D_3$ [24R,25(OH)$_2D_3$]. The cytochrome P450-dependent enzymes that catalyze these three reactions are

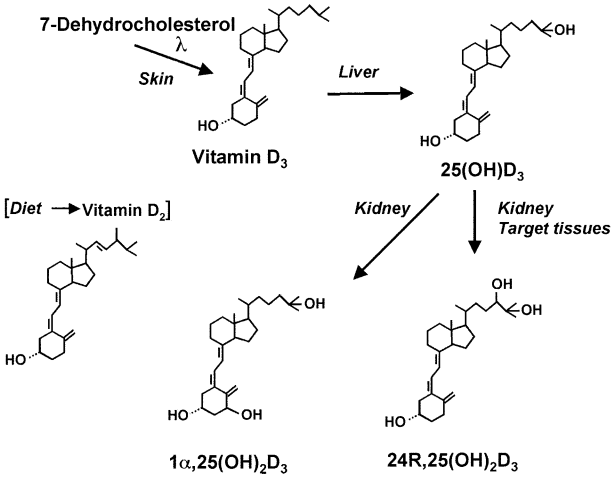

FIGURE 1 Metabolism of vitamin D. Following its production in the skin through the action of ultraviolet light, vitamin $D_3$ is hydroxylated in the liver by 25-hydroxylase (CYP27A) and then in the kidney by 25(OH)$D_3$-1α-hydroxylase (CYP27B) to form 1α,25(OH)$_2D_3$, the steroid hormonally active form of vitamin D. 25(OH)$D_3$ is also hydroxylated in the kidney and in 1α,25(OH)$_2D_3$ target tissues at carbon 24 by the 24R-hydroxylase (CYP24). Vitamin $D_2$, which is derived from and differs only in the side chain from vitamin $D_3$, which is derived from ergosterol, can undergo the same activation steps to 1α,25(OH)$_2D_2$.

the vitamin D3-25-hydroxylase (CYP27A1), 25(OH)-$D_3$-1α-hydroxylase (CYP27B1), and 25(OH)$D_3$-24R-hydroxylase (CYP24), each of which is discussed in more detail below.

The side chain of both 25(OH)$D_3$ and 1α,25$(OH)_2D_3$ is subject to further metabolism, as shown in Fig. 2. Following 24R-hydroxylation, the same cytochrome P450 catalyzes further hydroxylation, oxidation, and cleavage of four carbons from the side chain; another pathway leads to the formation of 26,23-lactone derivative.

Vitamin $D_2$ (Fig. 1) is derived from the plant sterol ergosterol and is used as a dietary supplement for humans and domesticated animals. In humans, it is considered to be metabolized to 1α,25$(OH)_2D_2$ in a manner that is qualitatively and quantitatively similar to that of vitamin $D_3$. However, the presence of the double bond between carbons 22 and 23 and the methyl group attached to carbon 24 impedes the side chain metabolism of the vitamin D metabolites derived from ergosterol.

The enzymes that catalyze the transformations of vitamin D and its metabolites are cytochrome P450-dependent mixed-function oxidases or steroid hydroxylases. In general, these enzymes depend on a source of electrons to reduce molecular oxygen and either one (for microsomal enzymes) or two (for mitochondrial enzymes) accessory proteins to transport the electrons to the specific cytochrome P450 responsible for the stereospecific hydroxylation of the substrate.

## II. VITAMIN $D_3$-25-HYDROXYLASE

The most abundant circulating form (15–60 ng/ml) of vitamin $D_3$ is the 25-hydroxylated derivative, 25(OH)$D_3$, which is formed in the liver by the mixed-function oxidase, vitamin $D_3$-25-hydroxylase. Although other tissues, such as the kidney and intestine, are capable of catalyzing this reaction, the liver makes by far the largest and physiologically most significant contribution to the circulating levels of the prohormone, 25(OH)$D_3$.

The experimental evidence for the subcellular localization of 25-hydroxylase activity depends on the species under investigation. For example, in rats and rabbits, activity has been reported in both microsomes and mitochondria and both subcellular forms have been purified to homogeneity. In humans, only a mitochondrial form of 25-hydroxylase activity has been demonstrated; on this basis, as well as the fact that in rats microsomes contain fivefold less activity than do mitochondria, some authors have questioned the physiological significance of the microsomal 25-hydroxylase activity and most recent investigations have focused on the mitochondrial enzyme.

The mammalian mitochondrial vitamin $D_3$-25-hydroxylase that has been cloned is CYP27A1, which also catalyzes the hydroxylation of carbons 26 and 27 of cholesterol, during steps in the formation of bile acids. CYP27A1 cDNA expressed in *Escherichia coli* hydroxylates vitamin $D_3$ at several positions on the side chain in addition to carbon 25 and carbon

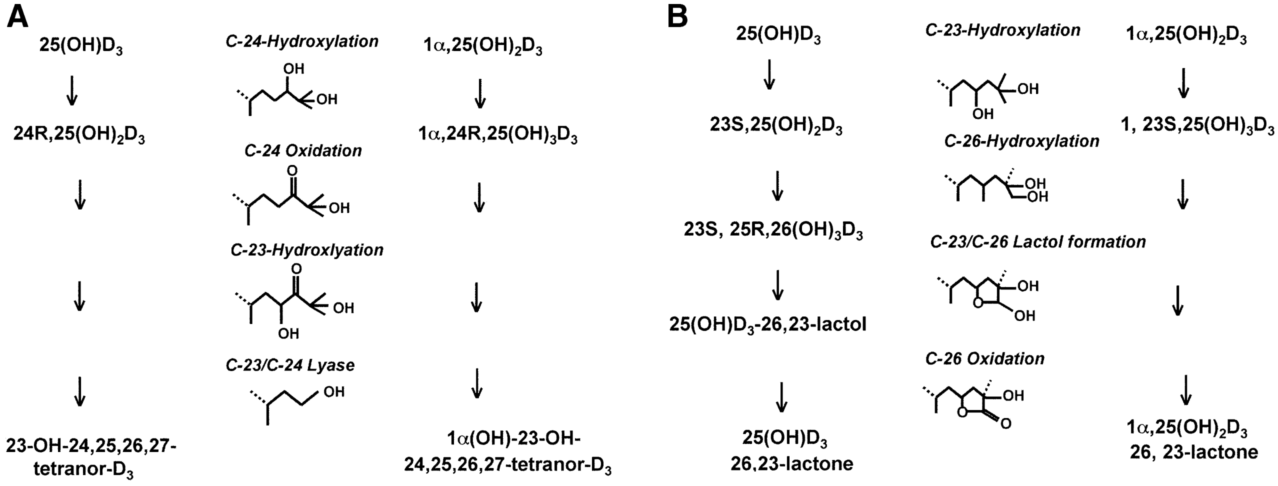
A
25(OH)$D_3$
C-24-Hydroxylation
OH
OH
1α,25$(OH)_2D_3$
24R,25$(OH)_2D_3$
C-24 Oxidation
1α,24R,25$(OH)_3D_3$
O
OH
C-23-Hydroxlyation
O
OH
OH
C-23/C-24 Lyase
OH
23-OH-24,25,26,27-tetranor-$D_3$
1α(OH)-23-OH-24,25,26,27-tetranor-$D_3$
B
25(OH)$D_3$
C-23-Hydroxylation
OH
OH
1α,25$(OH)_2D_3$
23S,25$(OH)_2D_3$
C-26-Hydroxylation
1, 23S,25$(OH)_3D_3$
OH
OH
23S, 25R,26$(OH)_3D_3$
C-23/C-26 Lactol formation
OH
OH
25(OH)$D_3$-26,23-lactol
C-26 Oxidation
OH
O
25(OH)$D_3$ 26,23-lactone
1α,25$(OH)_2D_3$ 26, 23-lactone

FIGURE 2 Metabolism of the vitamin D side chain. (A) The reactions carried out by CYP24 leading to the cleavage of four carbons. In the kidney, a significant amount of 24R,25$(OH)_2D_3$ is released prior to the subsequent oxidation steps. The complete C-24 oxidation pathway occurs in most target tissues of 1α,25$(OH)_2D_3$. (B) The C-23 oxidation pathway leading to the formation of the C-23/C-26 lactone derivatives. As indicated, both pathways can operate on substrates with or without the hydroxylation at carbon 1 in the A ring.

1. Whether CYP27A is the only enzyme responsible for the formation of 25-hydroxylated vitamin D derivatives, however, is brought into question by lack of evidence that it forms $25(OH)D_2$, the predominant circulating derivative of vitamin $D_2$, as efficiently as it forms $25(OH)D_3$.

## III. $25(OH)D_3$-1α-HYDROXYLASE

It has been appreciated for three decades that the kidney is the major site of production of circulating $1\alpha,25(OH)_2D$ (20–60 pg/ml), although, as discussed below, other tissues and cell types, notably skin, placenta, and cells of hematopoietic lineage, have been shown to produce $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$. In the kidney and in other tissues in which subcellular localization has been investigated, 1α-hydroxylase activity is exclusively mitochondrial.

cDNA sequences for the rat, mouse, pig, and human enzymes show that the primary structure of the 1α-hydroxylase is most similar to that of CYP27A1; therefore, it has been designated CYP27B1. The CYP27B1 gene, which is located on mouse chromosome 10 and human chromosome 12, is composed of nine exons and appears to be present in a single copy.

Although studies in vitamin D-deficient animals suggest that 1α-hydroxylase enzymatic activity is localized in the proximal tubule of the renal nephron, recent *in situ* hybridization and immunohistochemical studies of normal human kidney indicate that the enzyme and its message are more highly expressed in the distal portions of the nephron. It is likely that differential localization of the enzyme along the nephron under different conditions of vitamin D status reflects the dual autocrine/paracrine and endocrine roles of the kidney in calcium homeostasis.

Although the belief that the kidney is the principal site of $1\alpha,25(OH)_2D_3$ production is supported by studies in uremic animals as well as by clinical experience with patients with chronic renal failure, there is also evidence for the extrarenal production of $1\alpha,25(OH)_2D_3$. The most studied of these include keratinocytes and skin *in vivo*, various placental preparations, and the cells of the hematopoietic system. 1α-Hydroxylase activity in these cell latter types is not regulated by those components of the calcium homeostatic system described below for the renal 1α-hydroxylase but rather by immune cell regulators such as interferon-γ and lipopolysaccharide. It is thought that these occurrences of extrarenal 1α-hydroxylase activity serve autocrine/paracrine functions.

## IV. $25(OH)D_3/1\alpha,25(OH)_2D_3$-24R-HYDROXYLASE

Whereas the 1α-hydroxylase has but one naturally occurring substrate, the 24R-hydroxylase has two. The same enzyme catalyzes the 24-hydroxylation and subsequent side chain modification of both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ (Fig. 2). Depending on the enzyme preparation and assay conditions used, the affinity of the enzyme for $25(OH)D_3$ is either much greater than or much less than that for $1\alpha,25(OH)_2D_3$. Given the fact that circulating levels of $25(OH)D_3$ are approximately three orders of magnitude greater than those of $1\alpha,25(OH)_2D_3$ and that the turnover number is greater for $25(OH)D_3$ than for $1\alpha,25(OH)_2D_3$, there is, in the whole animal, considerable capacity to produce 24R, $25(OH)_2D_3$.

The cDNA encoding CYP24 has been cloned from rat, mouse, pig, and human kidneys. Expression studies in *E. coli* have shown that all of the reactions depicted in Fig. 2, C-24-hydroxylation, C-24 oxidation, C-23-hydroxylation, and C-23/C-24 cleavage, are catalyzed by a single cytochrome P450.

The 24R-hydroxylase gene, which contains 12 exons, has been localized to human chromosome 20 and mouse chromosome 2. The promoter region of the rat gene contains two vitamin D-response elements that are likely involved in the induction of the 24-hydroxylase by $1\alpha,25(OH)_2D_3$ (see below).

Although first identified and characterized in the kidney, where it is notable for its regulation that is reciprocal to that of the 1α-hydroxylase, the 24R-hydroxylase is actually very widespread, occurring in all target tissues that contain the receptor for $1\alpha,25(OH)_2D_3$, VDR. In fact, the inducibility of the 24R-hydroxylase by $1\alpha,25(OH)_2D_3$ is often taken as *prima facie* evidence of a cell being a target of $1\alpha,25(OH)_2D_3$ action. Like the 1α-hydroxylase, the 24R-hydroxylase is exclusively mitochondrial.

Although there is no controversy regarding the fundamental importance of the 1α-hydroxylase in the production of a biologically important steroid hormone, $1\alpha,25(OH)_2D_3$, the biological significance of the 24R-hydroxylase is less clear-cut. It is undoubtedly important to the inactivation and catabolism of $1\alpha,25(OH)_2D_3$ in the target cells in which is it induced by this steroid through the nuclear actions of the VDR. A 24R-hydroxylase knockout mouse has been produced in which observed deficiencies in bone metabolism were attributed not to the absence of $24R,25(OH)_2D_3$ but to excess $1\alpha,25(OH)_2D_3$ resulting from its impaired catabolism.

On the other hand, the fact that substantial amounts of $24R,25(OH)_2D_3$ are produced by the kidney along with evidence that $24R,25(OH)_2D_3$ may have biological actions distinct from those attributable to $1\alpha,25(OH)_2D_3$ leaves open the possibility of an endocrine role for the kidney in the production of $24R,25(OH)_2D_3$ as well as $1\alpha,25(OH)_2D_3$.

## V. REGULATION OF VITAMIN D METABOLISM

The overall pathway leading to the production of the hormonally active $1\alpha,25(OH)_2D_3$ begins with the availability of vitamin D either from production in the skin or from the diet. If the supply of the parent vitamin is limited, deficiency of $1\alpha,25(OH)_2D_3$ will result in bone abnormalities. There is no documented physiologically significant regulation of the 25-hydroxylation of vitamin D by the liver, and in fact, circulating levels of $25(OH)D_3$ are widely used to assess vitamin D status.

### A. Kidney

The production of $1\alpha,25(OH)_2D_3$ by the kidney is tightly regulated by factors that generally have the opposite effects on the production of $24R,25(OH)_2D_3$. The most thoroughly studied of these factors, and probably the most important in normal physiological circumstances, are the prevailing levels of $1\alpha,25(OH)_2D_3$ itself and of parathyroid hormone (PTH). As depicted in Fig. 3, $1\alpha,25(OH)_2D_3$ inhibits its own synthesis and increases that of $24R,25(OH)_2D_3$. The effect of $1\alpha,25(OH)_2D_3$ on 24R-hydroxylase is primarily if not exclusively mediated through increased mRNA levels, but the mechanism by which $1\alpha,25(OH)_2D_3$ reduces the 1α-hydroxylase is not as clear.

The second important physiological regulator of the renal hydroxylation of $25(OH)D_3$ is PTH, which increases the synthesis of $1\alpha,25(OH)_2D_3$ and decreases that of $24R,25(OH)_2D_3$. Low dietary calcium has the same effects on the two hydroxylase activities, which are probably mediated through increased PTH secretion. Intracellular signaling systems involved in mediating the effects of PTH on the two hydroxylases include cyclic AMP-dependent protein kinase as well as protein kinase C. The steady-state level of mRNA encoding CYP27B (1α-hydroxylase) is increased and that for CYP24 is decreased by PTH, but whether the entire effect of the peptide hormone on the activity of the two hydroxylases can be accounted for by alterations in mRNA levels has not been established.

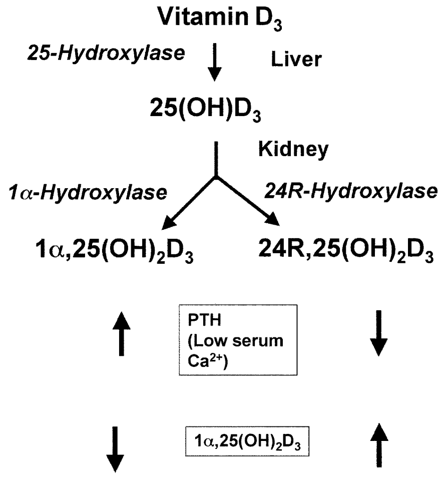

FIGURE 3 Regulation of vitamin D metabolism. The hydroxylation of vitamin D in the liver is not regulated in physiological circumstances so that the circulating levels of $25(OH)D_3$ are a reflection of vitamin D status. In the kidney, 1α-hydroxylation and 24R-hydroxylation of $25(OH)D_3$ are regulated in a reciprocal fashion by $1\alpha,25(OH)_2D_3$ and by PTH.

Changes in the levels of other hormones have been reported to influence the amounts of $1\alpha,25(OH)_2D_3$ and/or $24R,25(OH)_2D_3$ produced by the kidney. These include calcitonin, glucocorticoids, growth hormone, and sex steroids. The direction and magnitude of the effects of these hormones on 1α-hydroxylase and 24R-hydroxylase activities are variable, depending on the animal model under investigation. It is likely that although the effects of these hormones on vitamin D metabolism may be important in certain special endocrine circumstances, they are of less significance than $1\alpha,25(OH)_2D_3$ and PTH in the normal homeostatic control of the renal hydroxylation of $25(OH)D_3$.

### B. Bone and Intestine

Studies of the regulation of the mRNA for 24R-hydroxylase in bone and intestine have shown that, as in the kidney, these are increased by $1\alpha,25(OH)_2D_3$. However, as shown in Fig. 4, the effects of PTH and activation of protein kinase C on 24R-hydroxylase mRNA are tissue specific.

## VI. OTHER PATHWAYS OF VITAMIN D METABOLISM

### A. C-23 Oxidation

As shown in Fig. 2, the side chain of either $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ can undergo hydroxylation at

| | Kidney | Intestine | Bone |
|---|---|---|---|
| $1\alpha,25(OH)_2D_3$ | ↑ | ↑ | ↑ |
| + PTH | ↓ | → | ↑ |
| + ↑ PKC | ↑ | ↑ | → |
| + Low $Ca^{2+}$ | ↓ | ↑ | ↑ |

FIGURE 4 Tissue-specific regulation of 24R-hydroxylase mRNA. In all three tissues, $1\alpha,25(OH)_2D_3$ is required to induce the 24-hydroxylase from basal levels. Once induced, the 24R-hydroxylase is modulated differentially by PTH, activation of protein kinase C, and low dietary calcium in the three tissues.

carbon 23, leading to the formation of the 23(S), 25(R)-26,23-lactone derivatives. Human CYP24, expressed in *E. coli,* is capable of catalyzing all of these reactions with both substrates, but other enzymes may be involved *in vivo*. In terms of their contribution to the catabolism of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$, there is considerable species variation in the utilization of the C-23 and C-24 oxidation pathways. For example, rats and mice use the C-24 pathway almost exclusively, whereas C-23 derivatives predominate in the guinea pig.

### B. 3-Epimerization

The formation of 3-epi-$1\alpha,25(OH)_2D_3$ has been reported in human keratinocytes, bone cells, and parathyroid cells. Although metabolites of this compound have been reported to possess biological activity, the physiological importance of this pathway has not been established.

## Glossary

**cytochrome P450** In eukaryotes, a family of microsomal and mitochondrial heme-containing proteins (named for their characteristic absorption of light of 450 nm when carbon monoxide is bound) that reduce molecular oxygen, one atom of which is incorporated stereospecifically into the substrate as a hydroxyl group; those that use endogenous steroids for substrates are sometimes called steroid hydroxylases and many catalyze steps subsequent to the initial hydroxylation.

**prohormone** A precursor (either peptide or steroid) to an active hormone that is produced in significant amounts as an intermediate in the pathway of production of the active hormone.

**protein kinase C** A family of serine/threonine protein kinases that are activated by $Ca^{2+}$ and/or phospholipids in response to extracellular signals.

**VDR** The specific target tissue receptor for 1α,25dihydroxyvitamin $D_3$, the steroid hormone form of vitamin D.

**vitamin D-response element** The specific vitamin D receptor-binding DNA sequence in the promoter regions of genes whose transcription is altered by 1α,25-dihydroxyvitamin $D_3$.

## See Also the Following Articles

**Steroid Nomenclature • Vitamin D • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D-Binding Protein • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Receptors and Actions in Nonclassical Target Tissues**

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# Vitamin D: Nuclear Receptor for 1,25(OH)$_2$D$_3$

J. Wesley Pike and Nirupama K. Shevde
*University of Wisconsin*

I. INTRODUCTION
II. STRUCTURAL ORGANIZATION OF THE VDR
III. REGULATION OF GENE EXPRESSION THROUGH DNA BINDING
IV. THE VDR FUNCTIONS AS A HETERODIMER
V. ROLE OF 1,25(OH)$_2$D$_3$ IN VDR ACTIVATION
VI. VDR RECRUITMENT OF TRANSCRIPTIONAL CO-REGULATORS
VII. HEREDITARY RESISTANCE TO 1,25(OH)$_2$D$_3$
VIII. SUMMARY

**The biological actions of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] in tissues and cells are orchestrated within the nucleus through complex changes in gene expression. These changes lead to cell-specific alterations in the level of proteins that regulate the cell cycle, modulate differentiated cell function, or regulate subsequent levels of gene expression through their additional actions on genes or cells. Most, if not all, of the molecular actions of 1,25(OH)$_2$D$_3$ in the nucleus are mediated by a receptor protein termed the vitamin D receptor (VDR). This receptor is a specific member of a large gene family of transcription factors that function to mediate the actions of all the known steroid hormones, among them the estrogens and androgens, progesterone, the glucocorticoids, and thyroid hormone. As discussed in this article, the structural features of the VDR are well adapted to this functional role in regulating gene expression.**

## I. INTRODUCTION

Vitamin D$_3$, derived from 7-dehydrocholesterol through the actions of heat and ultraviolet light, undergoes further enzymatic activation first to 25-hydroxyvitamin D$_3$ in the liver and then to 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] in the kidney. This final metabolic conversion, which is highly regulated by parathyroid hormone as well as a variety of additional hormones and circulating factors, leads to what is now considered to be the active form of vitamin D$_3$. By virtue of its small size, lipophilic nature, and mechanism of action, 1,25(OH)$_2$D$_3$ is not considered to be a vitamin but rather a steroid hormone.

The classical role of 1,25(OH)$_2$D$_3$ is to regulate mineral homeostasis, achieved in part through regulatory actions on the parathyroid gland and through coordinate actions on intestine, kidney, and bone. Accordingly, the discovery of the vitamin D receptor (VDR) was initially made through investigations in those tissues. Currently, however, the VDR is found in a wide variety of cells and tissues, including those of skin, liver, pancreas, muscle, breast, prostate, adrenal, and thyroid and in cells of mesenchymal and hematopoietic origin. Although the VDR in these tissues is derived from the same chromosomal gene, its role in cellular function is pleiotropic and not necessarily involved in the control of mineral balance. Indeed, one of its most basic functions appears to be that of regulating cellular proliferation and differentiation, an activity that may be found associated with most steroid and adrenal hormones. This regulatory feature is likely a fundamental component of all biological responses to 1,25(OH)$_2$D$_3$, including those that involve mineral homeostasis.

## II. STRUCTURAL ORGANIZATION OF THE VDR

Evidence for the existence of a receptor for 1,25(OH)$_2$D$_3$ predated the discovery of the hormone itself and supported the idea that the mechanism of action of vitamin D might be similar to that of other steroids. Over the ensuing years, research revealed numerous biochemical features of the VDR. These characteristics included the ability of the receptor to bind its ligand, 1,25(OH)$_2$D$_3$, with very high affinity

**TABLE 1 Overview of Nonclassical $1,25(OH)_2D_3$ Target Tissues**

Cardiovascular tissue and muscle
- Cardiac myocytes
- Skeletal muscle
- Vascular smooth muscle

Reproductive tissues
- Chicken egg shell gland[b]
- Epididymis
- Mammary gland[b]
- Ovary
- Oviduct
- Prostate
- Placenta[b]
- Testis
- Uterus

Other endocrine organs
- Adrenals
- Pancreatic beta cells
- Parathyroid gland[a]
- Pituitary
- Salivary glands[b]
- Thyroid

Other targets
- Adipose tissue
- Central nervous system
- Choroid plexus
- Colon[b]
- Hematolymphopoietic cells
- Liver
- Lung
- Retina
- Skin
- Stomach
- Thymus
- Variety of cancers

[a] Site of a key hormonal role for $1,25(OH)_2D_3$/VDR in regulating the $Ca^{2+}$ homeostatic endocrine system.

[b] Although these tissues are not "classical" sites of plasma calcium regulation, regulation of $Ca^{2+}$ translocation is likely to be a principal function of $1,25(OH)_2D_3$/VDR therein.

DNA response element sequence in gel-shift studies. Collectively, studies using all of these approaches provide a large body of complementary data about the receptors and their putative sites of action.

## III. $1,25(OH)_2D_3$ FUNCTIONS IN NONCLASSICAL TARGETS: COMMONALITIES AND GENERALITIES

Many effects have been described for $1,25(OH)_2D_3$ actions in nonclassical targets (Table 3). Although some of these effects (e.g., effects on signal transduction pathways, $Ca^{2+}$ buffering, and growth regulation) parallel events known to occur in classical targets in the regulation of plasma $Ca^{2+}$ homeostasis, tissue-specific effects also occur, and knowledge of their existence will likely continue to grow. Moreover, in contrast to many other effects, $1,25(OH)_2D_3$ induction of the vitamin D-related calbindins—which is typical of the traditional $Ca^{2+}$-translocating tissues—does not occur in many of the nonclassical targets.

**TABLE 2 Types of Analyses Used to Define New VDR Targets**

| Approach | Example |
|---|---|
| Biochemical | $[^3H]$-$1,25(OH)_2D_3$ binding studies and Scatchard analysis<br>Sucrose density-gradient analysis<br>DNA cellulose chromatography<br>Immunoblotting |
| Histological | Autoradiography<br>Immunohisto/cytochemistry |
| Molecular | Northern analysis<br>Gel shift |

The hormonal effects of $1,25(OH)_2D_3$ in its targets involve two separate, but likely interdependent, mechanisms, termed "genomic" and "nongenomic" processes. Genomic actions are those in which the nuclear VDR regulates gene transcription at the DNA level. Interestingly, although this is the classical mechanism of action of the vitamin D endocrine system, specific details (e.g., the identity of the regulated genes) are not well delineated in most nontraditional targets. Nongenomic actions of $1,25(OH)_2D_3$ are those mediated principally through actions at/on the cellular membrane. Although several laboratories have exerted considerable effort to identify the putative membrane receptors for $1,25(OH)_2D_3$, details of the molecular structures are not yet available. In other steroid receptor systems, there may be multiple membrane receptor forms—some with identity to the nuclear receptor forms and some with totally different molecular origins. It is hoped that it will soon be known whether this interesting pattern of diversity of membrane receptor forms also holds for the vitamin D hormone. Despite both the lack of definition of the precise membrane effectors and the relatively recent focus on membrane-related events, a plethora of data exist defining apparent nongenomic signal transduction mechanisms of $1,25(OH)_2D_3$ in these systems. It is important to note that many signal transduction pathways have been implicated in the nongenomic actions of $1,25(OH)_2D_3$, sometimes occurring in different tissues but sometimes all within one tissue (e.g., skeletal muscle). Moreover, it is increasingly clear that these pathways are all intimately intertwined, and thus often affect one another in the response process.

$1,25(OH)_2D_3$ exerts important tissue- and development-specific effects on growth and differentiation. Its hormonal effects are important both in regulating normal growth processes and in clinically relevant conditions such as cancer (e.g., breast or prostate) and in tissue regeneration processes. In many tissues (e.g., cells of the hematolymphopoietic lineage),

TABLE 3 Examples of $1,25(OH)_2D_3$ Effects in Nonclassical Targets

| Mechanism | Tissue |
|---|---|
| Regulation of intracellular signaling pathways | Many |
| Proliferation/differentiation | Many |
| Protection against cellular damage (including $Ca^{2+}$ buffering) | A growing list that includes the CNS, heart, and pancreas |
| Hormone secretion | Pancreas, ovary? |
| Regulation of $1,25(OH)_2D_3$ responsiveness | |
| 24-Hydroxylase | Universal |
| VDR levels | Pancreas and skin (not heart, testis, or lung) |
| Calbindin levels | Rare |

growth inhibition correlates with specific effects to induce differentiation.

### A. Cytoprotective Effects of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ exerts important cytoprotective effects in a number of targets, especially by increasing antioxidant activity or by attenuating increases in free intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$). Thus, the hormone protects against damage from a long list of toxins and carcinogens known to act principally via release of free radicals. Buffering of $[Ca^{2+}]_i$ may occur through several mechanisms, including, for example, increased $Ca^{2+}$ channel activity or increases in $Ca^{2+}$-binding proteins such as calbindin-$D_{28k}$.

### B. Regulation of Vitamin D-Related Responses

In many tissues, $1,25(OH)_2D_3$ alters processes that contribute to the ability of the molecule to regulate its own responsiveness within the tissue. $1,25(OH)_2D_3$ treatment almost universally up-regulates 24-hydroxylase activity, which is an early step in $1,25(OH)_2D_3$ clearance pathways. In more tissue-specific patterns, such treatment also regulates VDR levels and 1α-hydroxylase activity. In the latter case, up-regulation of tissue 1α-hydroxylase activity is thought to provide a local enhancement of the hormonal action with minimal effects on plasma calcium levels. Uncontrolled elevation of this paracrine system may be responsible for the hypercalcemia associated with some diseases such as sarcoidosis. There are also emerging reports of other cellular proteins (e.g., a heat-shock 70-related protein and the $Ca^{2+}$-binding protein calreticulin) that interfere with genomic actions of the VDR, and their regulation by $1,25(OH)_2D_3$ may be a feedback mechanism to turn off $1,25(OH)_2D_3$ responsiveness.

## IV. $1,25(OH)_2D_3$ FUNCTIONS IN NONCLASSICAL TARGETS: TISSUE-SPECIFIC EFFECTS

### A. Skeletal Muscle: a Tissue with Well-Delineated Nongenomic Effects of $1,25(OH)_2D_3$

Vitamin D deficiency has long been known to be associated with a generalized muscle weakness, which can lead to muscle atrophy and which is reversed by vitamin D treatment. It now appears that there are both nongenomic and genomic mechanisms through which $1,25(OH)_2D_3$ regulates skeletal muscle function. In a long and elegant series of studies, the research team of Boland and de Boland has delineated the nongenomic mechanisms induced by $1,25(OH)_2D_3$, principally using models from chick skeletal muscle. Their studies define a system in which there is an intricate interplay between numerous intracellular signaling pathways, which likely also interact with the nuclear VDR mechanisms. In this system, $1,25(OH)_2D_3$ treatment results in rapid activation of phospholipase C, producing inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). These effectors result in an increase in $[Ca^{2+}]_i$ levels, with an initial release (1 min) from internal stores and then a sustained phase (up to 5 min) of uptake through both L-type and store-operated $Ca^{2+}$ channels. These effects parallel, but are not dependent on, increases in adenylyl cyclase and protein kinase A activities. However, the increases in $[Ca^{2+}]_i$ are dependent on activation of calmodulin, protein kinase C, and tyrosine kinase pathways. In fact, the relationship between $1,25(OH)_2D_3$ stimulation and activation of tyrosine kinases is complex in this system. $1,25(OH)_2D_3$ induces tyrosine kinase cascades, which result in increased phosphorylation of tyrosine residues in several proteins, including, in particular, phospholipase C-γ (which increases $[Ca^{2+}]_i$ from internal stores and by uptake through $Ca^{2+}$ channels), the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2, and c-myc. The $1,25(OH)_2D_3$ effect on MAPK activation is dependent on $[Ca^{2+}]_i$ and protein kinase C (PKC). In addition, $1,25(OH)_2D_3$ treatment increases Src kinase activity and decreases its tyrosine phosphorylation, and also causes increases in the levels of a VDR–Src kinase complex that is associated with elevated tyrosine phosphorylation of the VDR. The function of this tyrosine-phosphorylated VDR–Src kinase complex and its relationship to nuclear actions of the VDR have not yet been established. In rat skeletal muscle, $1,25(OH)_2D_3$ also activates phospholipase D, which results in a second phase of DAG release from phosphatidylcholine. This activation step is dependent on elevated $[Ca^{2+}]_i$ and G-protein activity, but its contributions to the $1,25(OH)_2D_3$ effects in this system have not been fully delineated.

The rapid $1,25(OH)_2D_3$ effects, via tyrosine kinase cascades, on the growth-related MAPK activity and the mitogenic effects of c-myc highlight the concept of cross-talk between the nongenomic pathways activated by $1,25(OH)_2D_3$ and nuclear actions of the VDR. The effects of $1,25(OH)_2D_3$ on growth and differentiation in this system are complex and depend on the developmental state. For example, in undifferentiated chick embryo myoblasts, there is stimulation of DNA synthesis and inhibition of myogenesis. As cultures form more differentiated myotubes, the $1,25(OH)_2D_3$ effects result in inhibition of DNA synthesis and an increase in differentiation as defined by increased synthesis of biochemical markers typical of differentiated muscle. Moreover, PKC $\alpha$ activation was associated with the proliferation phase, whereas its activity was decreased during the differentiation stage.

## B. CNS: Neuroprotective Roles of $1,25(OH)_2D_3$

Numerous sites throughout the central nervous system contain VDRs, but the effects of VDRs are not well understood in each of these sites. The cerebellum contains high levels of the vitamin D-related calbindin-$D_{28k}$, where its expression is not altered by $1,25(OH)_2D_3$/vitamin D status. Nevertheless, there is evidence for roles of $1,25(OH)_2D_3$ in the brain under a variety of conditions. For example, changes in VDR distribution patterns in the central nervous system (CNS) during development suggest possible roles in this process. In cerebral cortex, $1,25(OH)_2D_3$ treatment alters the levels of some enzyme activities (e.g., acetylcholinesterase, citrate synthase, and acyl phosphatase), but not others (e.g., cytochrome *c* oxidase and acid phosphatase).

Chronic treatment with $1,25(OH)_2D_3$ has been reported to provide neuroprotective roles in models of both aging and stroke. The rationale for the aging studies comes in part from putative links between altered calcium homeostasis and the development of Alzheimer's disease. Consistent with this hypothesis, when rats are treated chronically with $1,25(OH)_2D_3$, there is an increase in neuronal density in the hippocampus, wherein a loss of neurons is considered a reliable biomarker for aging. In the cerebral artery ligation model of stroke, $1,25(OH)_2D_3$ pretreatment decreases ischemic brain injury concomitant with elevated levels of plasma calcium. In this model, $1,25(OH)_2D_3$ treatment also increases levels of glial cell-derived neurotropic factor in the cortex, which has been associated with reduced cerebral infarction in this model. $1,25(OH)_2D_3$ pretreatment in culture models also protects dopaminergic neurons against cytotoxic effects of numerous insults, including reactive oxygen species, glutamate, specific dopaminergic toxins, or elevated $Ca^{2+}$ entry induced by $Ca^{2+}$ ionophores. Mechanistic studies have indicated that these $1,25(OH)_2D_3$ protective effects may be via direct actions on neurons, in that physiological doses of $1,25(OH)_2D_3$ can reduce the damage to hippocampal neurons *in vitro* that results from excess/inappropriate neurotransmitter release and the resulting sustained excitation. Similar doses of $1,25(OH)_2D_3$ also reduce L-type $Ca^{2+}$ channel levels by effects at the mRNA level, suggesting that the resulting decrease in $Ca^{2+}$ entry into the cell may play a role in the neuroprotective effect. Another clue to the neuroprotective role of $1,25(OH)_2D_3$ comes from a study in which $1,25(OH)_2D_3$ treatment increased the activity of $\gamma$-glutamyl transferase, an enzyme that has been implicated in scavenging reactive oxygen species, particularly in pericytes and peripheral astrocytes in rat brain.

CNS inflammatory disease can be associated with excess production of inducible nitric oxide synthase (iNOS). In mice with clinical signs of experimental allergic encephalitis, $1,25(OH)_2D_3$ treatment reduces CNS iNOS levels in a region- and cell-specific pattern and results in improvement of the clinical signs of the disease. Subsequent studies have documented loss of paralysis, decreased white matter and meningeal inflammation, and decreased CNS macrophage accumulation after $1,25(OH)_2D_3$ treatment. These results suggest that $1,25(OH)_2D_3$ provides a protective role in iNOS-associated CNS diseases. Although these $1,25(OH)_2D_3$ effects may relate more to immunomodulatory effects rather than direct neuronal actions, the beneficial effects of 1,25$(OH)_2D_3$ treatment in this experimental model of multiple sclerosis are particularly interesting in light of prior suggestions that multiple sclerosis in humans may correlate with diminished activity of the vitamin D endocrine system.

## C. Pancreas: Nongenomic and Cytoprotective Effects of 1,25$(OH)_2D_3$

There is evidence indicating that insulin secretion and synthesis are impaired in vitamin D deficiency and are restored with 1,25$(OH)_2D_3$ treatment. The defects have been shown to relate to the loss of 1,25$(OH)_2D_3$ effects on several signal transduction pathways important in effecting glucose-signaled insulin secretion in pancreatic beta cells, including those involved in regulating $[Ca^{2+}]_i$, phospholipase C, PKC, and adenylyl cyclase. These insulinotropic effects of 1,25$(OH)_2D_3$ are likely mediated through nongenomic mechanisms, as evidenced in part by their inhibition by a membrane-specific 1,25$(OH)_2D_3$ antagonist [1β,25$(OH)_2D_3$]. In addition, 1,25$(OH)_2D_3$ stimulates the production of a number of specific, but as yet unidentified, proteins in islet cells by a mechanism that seems to involve genomic pathways. These observations may also be important in clinical situations associated with transient defects in glucose signaling. For example, in patients with gestational diabetes, 1,25$(OH)_2D_3$ treatment reduces plasma glucose levels, possibly by increasing insulin sensitivity.

Unlike most of the other nonclassical 1,25$(OH)_2D_3$ targets, in addition to nuclear VDRs, pancreatic beta cells (and alpha cells in some species) contain the vitamin D-related calbindin-$D_{28k}$, and it is regulated by 1,25$(OH)_2D_3$ in the beta cells. However, studies designed to identify a role for calbindin-$D_{28k}$ in 1,25$(OH)_2D_3$ effects on insulin secretion have thus far produced negative results. Pancreatic tissue damage, induced experimentally, for example, by oxidative stress, can result in islet cell destruction by cytokine-induced pathways. In this model of beta cell destruction and diabetes onset or islet allograft rejection, 1,25$(OH)_2D_3$ exposure reduces several markers of oxidative stress and decreases immune/-cytokine activation, suggesting that the hormone can exert a protective effect against immune destruction in these systems. In another model of diabetes in the nonobese diabetic (NOD) mouse, 1,25$(OH)_2D_3$ prevents the development of diabetes by a mechanism that results in reduced resistance to apoptotic signals in NOD thymocytes and thus disrupts the autoimmune response.

## D. Cardiac Myocytes, Including the Atrial Endocrine Myocytes

There are complex effects of vitamin D deficiency, 1,25$(OH)_2D_3$ supplementation, and vitamin D or 1,25$(OH)_2D_3$ excess in the heart. The complexity derives in part from the attendant changes in parathyroid hormone (PTH) and plasma or tissue calcium. For example, low 1,25$(OH)_2D_3$ levels may play a role in the cardiac disease associated with chronic renal failure (Table 4), and vitamin D

**TABLE 4 Possible Vitamin D Effects on the Cardiovascular System**

| | Vitamin D/ 1,25$(OH)_2D_3$ levels[b] | |
|---|---|---|
| Effect[a] | High | Low |
| Decreased blood pressure | | |
| Opposes PTH excess? | ✓ | |
| Increased blood pressure | | |
| ↑ Vascular smooth muscle force generation | ✓ | |
| ↑ Vascular smooth muscle cell $[Ca_i^{2+}]$ | ✓✓✓ | |
| ↑ PTH production | | ✓ |
| Cardiac contractility | | |
| ↓ Contractility | | ✓ |
| ↑ Contractility | | ✓✓✓ |
| Left ventricular mass | | |
| ↑ Hypertrophy | | ✓ |
| ↑ Myocardial collagen content | | ✓ |
| ↓ Endothelin-induced myocardial hypertrophy | ✓ | |
| Vascular smooth muscle cells | | |
| ↑ Cell growth | ✓ | |
| ↓ Cell growth/ ↑ cell maturation | | ✓ |
| Atherosclerosis | | |
| Tissue calcification | | |
| ↑ Calcium phosphate deposits in vessel wall | | ✓ |
| ↑ Heart valve calcification | | ✓✓✓ |

[a] PTH, Parathyroid hormone.

[b] A check mark indicates the condition in which the change in cardiovascular function occurs; ✓✓✓ indicates that protracted or severe conditions are required. Adapted from Rostand and Drüeke (1999).

supplementation in these patients results in improved cardiac function. However, the therapeutic improvement may also be due to indirect effects of $1{,}25(OH)_2D_3$ replacement on other physiological parameters, because impaired vitamin D status may also alter cardiovascular-related systems, including vascular smooth muscle and blood pressure, or other components of calcium homeostasis, in particular PTH and calcium levels (Table 4).

VDR sites have been described in cardiac muscle, with some concentration in atrial natriuretic peptide (ANP)-producing cells of the atria. $1{,}25(OH)_2D_3$ effects in cardiac tissues, as in many nontraditional targets, are independent of traditional $1{,}25(OH)_2D_3$ effects such as induction of calcium-binding proteins or changes in VDR levels. $1{,}25(OH)_2D_3$ stimulation in these systems does include nongenomic, and perhaps genomic as well, effects on $Ca^{2+}$ uptake pathways, as well as developmental roles and genomic effects on ANP production. In nongenomic pathways in cardiac muscle, adenylyl cyclase and G-proteins are involved in $1{,}25(OH)_2D_3$-induced $Ca^{2+}$ uptake through L-type $Ca^{2+}$ channels and this effect is accompanied by cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent increases in microsomal protein phosphorylation.

Vitamin D deficiency results in reduced cardiac contractility, an effect reversed by $1{,}25(OH)_2D_3$. These effects on contractility are probably mediated at many levels: specific effects of $1{,}25(OH)_2D_3$ treatment include altered myocyte numbers, altered myosin isoenzyme patterns, and altered activity of many metabolic enzymes in the heart. In neonatal ventricular myocytes, $1{,}25(OH)_2D_3$ has complex effects on maturation and growth. It inhibits differentiation by a mechanism that may involve PKC. $1{,}25(OH)_2D_3$ inhibits proliferation by blocking entry into the cell cycle S phase, though there are conflicting reports on whether it also induces hypertrophy. These growth effects are paralleled by events *in vivo*, whereby vitamin D deficiency induces morphological changes in the heart that reflect cardiac myocyte hyperplasia and are accompanied by elevated c-myc levels. Also, in hearts of rat pups from mothers fed diets with low levels of vitamin D, there is evidence of slowed cardiac development. In contrast, excess vitamin D can result in protease-induced cardionecrosis, accompanied by damage to the cardiac contractile apparatus. In the ANP-producing endocrine myocytes of the atria, $1{,}25(OH)_2D_3$ inhibits ANP synthesis (decreased mRNA) and release, and these genomic effects are $Ca^{2+}$ independent.

## E. Vascular Smooth Muscle

$1{,}25(OH)_2D_3$ also seems to play a role in maintaining normal function of vascular smooth muscle by effects on a number of important processes. For example, the cardiovascular defects due to impaired vitamin D status in renal failure also include effects on smooth muscle elements (Table 4). Most $1{,}25(OH)_2D_3$ effects described to date in vascular smooth muscle seem to be mediated by genomic pathways, in contrast to the presence therein of well-described membrane-linked (nongenomic) effects of estrogen in these systems.

Recent studies of $1{,}25(OH)_2D_3$ effects in vascular smooth muscle have indicated that the responses differ in different areas of the vascular tree. In rat mesenteric resistance vessels, $1{,}25(OH)_2D_3$ treatment *in vivo* prior to vessel isolation increases contractile force and the force response to stress hormones. These long-term effects in more distal vessels seem to reflect a genomic effect of the hormone, but do not involve effects on myosin expression. Conversely, in the aorta, $1{,}25(OH)_2D_3$ treatment increases the expression of myosin light and heavy chains. When the mesenteric vessels are cultured, they lose the force response to stress (e.g., norepinephrine exposure) and this loss parallels altered patterns of myosin heavy chain expression. These detrimental effects are prevented by $1{,}25(OH)_2D_3$ treatment. Consistent with $1{,}25(OH)_2D_3$ effects on vascular contractility, $1{,}25(OH)_2D_3$ treatment increases blood pressure in *in vivo* models, likely via a genomic mechanism.

Soft tissue calcification, particularly in kidney and heart, is a serious consequence of the hypercalcemia caused by $1{,}25(OH)_2D_3$/vitamin D excess. Studies of vascular calcification have shown that $1{,}25(OH)_2D_3$ treatment increases *in vitro* calcification of cultured vascular smooth muscle cells, apparently by inhibiting expression of PTH-related peptide (PTHrP) (which inhibits calcification) and by stimulating expression and activity of procalcification proteins such as alkaline phosphatase and osteopontin.

## F. Endocrine and Reproductive Tissues

$1{,}25(OH)_2D_3$ alters the function of a number of endocrine tissues. It increases thyrotropin-releasing hormone (TRH)-stimulated thyroid-stimulating hormone (TSH) and prolactin secretion in the pituitary. It has a wide range of effects in thyroid C cells, including inhibiting calcitonin gene expression and altering cell structure. It affects the synthetic pathways for adrenal catecholamines by regulating a number of the key enzymatic steps. Moreover, it is required for normal gonad function and estrogen synthesis in both males and females.

In many tissues, $1,25(OH)_2D_3$ exerts its activities through alterations in cAMP pathways. For example, in rat FRTL-5 thyroid cells, $1,25(OH)_2D_3$ regulates (reduces) TSH/cAMP signaling and affects basal cAMP levels. Inhibition of TSH-induced cAMP production is associated with a reduction in TSH-induced growth and iodide uptake in these thyroid-derived cells. TSH receptor levels and the levels of the G-protein $G_{\alpha s}$, which transduces the TSH receptor response to cAMP, are not affected. Conversely, $1,25(OH)_2D_3$ treatment specifically increases the levels of the inhibitory G-protein $G_{\alpha i2}$, but not $G_{\alpha i1}$ or $G_{\alpha i3}$. Because there is evidence of $1,25(OH)_2D_3$ effects beyond the cAMP effects, its effect on PKA subunits has also been also assessed, with a rather complex result. $1,25(OH)_2D_3$ treatment increases levels of the PKA regulatory subunit RIIβ. Additional studies have confirmed that the post-cAMP $1,25(OH)_2D_3$ inhibition in this system is due to the resultant increase in the relative levels of the PKA subunit tetrameric complex $RII\beta_2C_2$, which thus decreases the relative levels of the alternate tetrameric form $RI\alpha_2C_2$, because PKA I is the predominant effector in this system. These effects of attenuating the cAMP response pathway at several steps are long term and thus are likely to be genomic effects of $1,25(OH)_2D_3$.

Many reproductive tissues, including testis, epididymis, and uterus, contain modest VDR levels. However, the levels of these VDR sites and of the vitamin D-related calbindins are not regulated by $1,25(OH)_2D_3$ in these tissues. In the uterus, for example, they are estrogen regulated. Moreover, although there are least modest effects (the degree seems to vary among animal models) of vitamin D deficiency on reproduction in males and females, it is possible that the attendant hypocalcemia is responsible for at least some of these effects.

When male and female rats are treated neonatally with $1,25(OH)_2D_3$, there are important changes in their sexual behavior as adults, suggesting that $1,25(OH)_2D_3$ may interfere in sexual imprinting and normal sex steroid receptor actions. Similarly, male pups from mothers exposed to $1,25(OH)_2D_3$ during pregnancy exhibit enlarged prostates but no change in seminal vesicle size. Moreover, there is a high rate of sudden death in these uncastrated male pups at puberty. Thus, there seem to be tissue-specific effects of $1,25(OH)_2D_3$ on development and genetic imprinting in the prostate.

When parameters of the calcium homeostatic endocrine system were assessed in women with fertility problems (especially arrest of follicular development) due to polycystic ovarian disease, despite normocalcemia in all of the patients, a subset exhibited relatively low $25(OH)D_3$ levels and some had elevated PTH levels. Vitamin D and calcium repletion resulted in two pregnancies and the return to normal menstrual cycles for several women. These observations were interpreted as indicating that abnormal calcium homeostasis may contribute to polycystic ovarian disease and/or the problems in follicular development associated with the disease.

In mammary glands, VDR levels vary from puberty to maturation and during pregnancy and lactation. The calcium-supplemented VDR knockout mouse has been used as a model to assess possible effects of $1,25(OH)_2D_3$/VDR on mammary gland development. In this system, the initial stage of development, ductal branching after puberty, is seen to be normal. However, later stages of branching and ductal development show some degree of undifferentiation, suggesting roles for $1,25(OH)_2D_3$/VDR in normal differentiation in this system. Moreover, these observations may explain part of the correlation of reduced activity of the vitamin D endocrine system and mammary tumor development, in that undifferentiated structures are a target of agents that cause cancer transformation in this system.

## G. Lung

In the adult rat lung, there are low levels of VDRs and the calbindin-$D_{9k}$, but regulation of both proteins is $1,25(OH)_2D_3$ independent. In the near-term fetal (but not neonatal) rat lung, type II alveolar cells express VDRs and respond to $1,25(OH)_2D_3$ treatment with increased phospholipase activity, including synthesis and release of disaturated phosphatidylcholine and improved surfactant release and advancement of other maturation processes. In contrast, fetal lung fibroblasts do not express VDRs, but do exhibit 1α-hydroxylase activity, which can produce $1,25(OH)_2D_3$. These observations suggest that there is a paracrine $1,25(OH)_2D_3$ response system in the fetal lung for communication between epithelial and mesangial cells; this system may be involved in achieving the degree of lung maturation that is necessary to reduce the respiratory distress syndrome of premature birth.

### H. Liver

Chronic liver disease is often associated with vitamin D depletion. Alternatively, $1,25(OH)_2D_3$ treatment induces the activity of a number of metabolic enzymes in the liver and reduces the hepatic damage from some toxins. Moreover, the liver has long served as a system for studies of nongenomic $1,25(OH)_2D_3$ effects.

Impaired calcium metabolism has been shown to interfere in hepatic regeneration processes—for example, after partial hepatectomy. Studies of $1,25(OH)_2D_3$ and calcium effects on specific markers of hepatic regeneration [e.g., mRNAs for hepatocyte growth factor and transforming growth factor-$\alpha$ (TGF-$\alpha$)] and cell cycle regulators (e.g., cyclin D1 and cyclin A) indicate that both factors are important in optimal liver regeneration and that impaired regrowth occurs through molecular changes that reduce G1 transit efficiency in the cell cycle. In fetal rat hepatocytes, $1,25(OH)_2D_3$ treatment potently inhibits synthesis of $\alpha$-fetoprotein and corticosteroid-binding globulin (CBG). $1,25(OH)_2D_3$ inhibition of CBG synthesis is much more potent than are the effects of activators of the retinoic acid receptor (RAR) or retinoic acid X receptor (RXR) and is not affected by the stimulation of CBG synthesis by the thyroid hormone triiodothyronine ($T_3$).

Studies of the antioxidant role of $1,25(OH)_2D_3$ in the liver show that vitamin D supplementation in the rat is more potent than vitamin E supplementation with respect to changes in a number of antioxidant activities, underscoring the notion that many of the cytoprotective effects of $1,25(OH)_2D_3$ may be mediated through antioxidant effects. More recent studies indicate that chronic $1,25(OH)_2D_3$ treatment inhibits hepatocyte damage following injection of the carcinogen diethylnitrosamine, likely by inhibiting lipid peroxidation, which in turn protects cell membranes from damage induced by free radicals.

## V. $1,25(OH)_2D_3$/VDR INHIBITS BREAST AND PROSTATE CANCER GROWTH

VDR sites have been described in both breast and prostate epithelial cells. Reports of growth inhibitory $1,25(OH)_2D_3$ effects (particularly in cell models) in cancers from a number of tissues soon followed. These antiproliferative effects led to the hypothesis that $1,25(OH)_2D_3$ might be useful in treatment of a variety of cancers. To date, there is an active interest in these treatments and their mechanisms, but the issue is, of course, complicated by the hypercalcemic effect of $1,25(OH)_2D_3$ treatment. Thus, a number of pharmaceutical companies are actively seeking to develop noncalcemic analogues of the hormone.

In breast cancer, VDR is expressed in most breast cancer cell lines and in about 80% of human tumors. VDR levels in breast cancer do not correlate to sex steroid receptor levels nor to the stage of tumor development, and there is not always a good correlation between VDR levels in breast cancer models and the ability of $1,25(OH)_2D_3$ to inhibit tumor/cell growth. $1,25(OH)_2D_3$ inhibition of breast cancer cell growth includes both antiproliferative (altered cell cycle proteins) and pro-apoptotic effects.

In prostate cancers, the presence of VDR is required for the $1,25(OH)_2D_3$ effects, but there is not a good correlation of VDR levels and $1,25(OH)_2D_3$ responsiveness across cell models. Thus, other factors must contribute to $1,25(OH)_2D_3$ responsiveness in these systems. Recent studies have indicated that inhibition of 24-hydroxylase activity in VDR-containing but relatively $1,25(OH)_2D_3$-insensitive cells restores $1,25(OH)_2D_3$ sensitivity, suggesting that enhanced $1,25(OH)_2D_3$ clearance can contribute to the differences in apparent VDR responsiveness. Nevertheless, studies to date implicate only genomic mechanisms in the growth inhibitory effects of $1,25(OH)_2D_3$ in prostate cancer cells. Specific effects of $1,25(OH)_2D_3$ in these systems include alterations in the cell cycle (although the details differ among cell types), increases in the prostate cell differentiation marker prostate-specific antigen (PSA), and reduced activity of several growth factors. However, in most cases, the details of the $1,25(OH)_2D_3$ effects are not yet well understood. The ability of $1,25(OH)_2D_3$ to induce androgen receptor levels in these systems has led to the suggestion that it may also be useful to restore sensitivity to therapy in patients who have developed anti-androgen resistance. Finally, a small clinical trial of the effectiveness of $1,25(OH)_2D_3$ treatment in patients with prostate cancer has demonstrated that $1,25(OH)_2D_3$ treatment does slow prostate cancer growth *in vivo*, although hypercalcemia is a problem, as expected.

## VI. SUMMARY

There are numerous effects of $1,25(OH)_2D_3$ in nonclassical target tissues, including both somewhat general effects on cell/tissue function and tissue-specific effects. The initial impetus for studying $1,25(OH)_2D_3$ effects in many of these tissues was

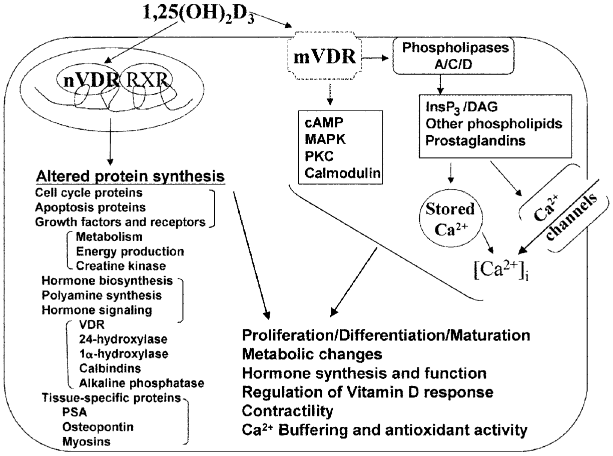
1,25(OH)₂D₃
mVDR
Phospholipases A/C/D
nVDR
RXR
cAMP
MAPK
PKC
Calmodulin
InsP₃ /DAG
Other phospholipids
Prostaglandins
Stored Ca²⁺
Ca²⁺ channels
[Ca²⁺]ᵢ
Altered protein synthesis
Cell cycle proteins
Apoptosis proteins
Growth factors and receptors
Metabolism
Energy production
Creatine kinase
Hormone biosynthesis
Polyamine synthesis
Hormone signaling
VDR
24-hydroxylase
1α-hydroxylase
Calbindins
Alkaline phosphatase
Tissue-specific proteins
PSA
Osteopontin
Myosins
Proliferation/Differentiation/Maturation
Metabolic changes
Hormone synthesis and function
Regulation of Vitamin D response
Contractility
Ca²⁺ Buffering and antioxidant activity

FIGURE 1 A schematic diagram showing the range of $1,25(OH)_2D_3$ effects in nonclassical target tissues. Left: A summary of many of the proteins/processes regulated at the transcriptional level by the nuclear vitamin D receptor (nVDR). Right: A summary of some of the intracellular signaling processes affected through the putative membrane VDR (mVDR) and systems they may affect. Abbreviations: RXR, retinoic acid X receptor; cAMP, cyclic adenosine monophosphate; MAPK, mitogen-activated protein kinase; $InsP_3$, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PSA, prostate-specific antigen.

the finding of significant (albeit perhaps low) levels of VDR sites in the tissues. The initially described VDR sites in these tissues were likely the traditional nuclear VDRs, which regulate cell function at the level of gene transcription (Fig. 1). However, in many of these tissues, many effects of $1,25(OH)_2D_3$ may be mediated by the as yet uncharacterized membrane VDR sites via a plethora of intracellular signal transduction pathways (Fig. 1). Defining the gene targets of the traditional nuclear VDR sites in these tissues, as well as characterizing the membrane VDR sites and their targets, are exciting directions for future studies.

## Glossary

**calbindin** Vitamin D-regulated calcium-binding protein (two forms, with molecular masses of 9 and 28 kDa) strongly induced by $1,25(OH)_2D_3$ in classical target tissues.

**classical $1,25(OH)_2D_3$ target tissues** The earliest identified $1,25(OH)_2D_3$/vitamin D targets, such as kidney, bone, and intestine, in which the predominant hormonal effect is $Ca^{2+}$ translocation.

**1α-hydroxylase** The final enzyme in the biosynthetic pathway that produces the active hormone $1,25(OH)_2D_3$.

**24-hydroxylase** The initial enzyme in the clearance pathway for $1,25(OH)_2D_3$.

**membrane vitamin D receptor** Newly hypothesized membrane receptor of as-yet unknown structure that seems to mediate some actions of $1,25(OH)_2D_3$ through well-known membrane receptor-induced signal transduction pathways.

**nonclassical $1,25(OH)_2D_3$ target tissues** A number of tissues other than those in the earliest studies describing vitamin D receptor and $1,25(OH)_2D_3$ effects.

**nuclear vitamin D receptor** The traditional $1,25(OH)_2D_3$/vitamin D receptor that exerts its actions as a $1,25(OH)_2D_3$-activated nuclear transcription factor.

## See Also the Following Articles

**Vitamin D • Vitamin D-Dependent Calbindins (CaBP) • Vitamin D Effects on Cell Differentiation and Proliferation • Vitamin D Metabolism • Vitamin D: Nuclear Receptor for $1,25(OH)_2D_3$**

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# W

## Wnt Protein Family

Benjamin N. R. Cheyette[*] and
Randall T. Moon[†]

[*]*University of California, San Francisco* ● [†]*University of Washington*

I. HISTORY AND BIOLOGICAL SIGNIFICANCE
II. THE Wnt PROTEIN FAMILY: STRUCTURE AND BIOCHEMISTRY
III. RECEPTOR STRUCTURE AND BIOCHEMISTRY
IV. CYTOPLASMIC SIGNAL TRANSDUCTION PROTEINS
V. THE CANONICAL Wnt/β-CATENIN PATHWAY
VI. NONCANONICAL PATHWAYS: PLANAR CELL POLARITY AND Wnt/CALCIUM SIGNALING
VII. SUMMARY: BEYOND DEVELOPMENT AND CANCER

**Wnts are a family of secreted signaling proteins. Wnt signaling is critical during animal development, contributing to the regulation of cell fate specification, cell morphology, cell proliferation, cell migration, cell polarity, and tissue patterning. Misregulation of Wnt signaling is a likely etiologic factor in human disease and has been strongly implicated in oncogenesis.**

### I. HISTORY AND BIOLOGICAL SIGNIFICANCE

The manner in which Wnt signaling was discovered reflects its biological importance across animal species. Mutations in the *wingless* (*wg*) gene of the fruit fly *Drosophila melanogaster* lead to a spectrum of developmental phenotypes. Null mutations cause severe segmental patterning defects that are lethal during embryogenesis. Some hypomorphic mutations are viable—the first mutation identified produces adult flies without wings, which gave the *Drosophila* gene its name. The *int-1* proto-oncogene was separately discovered as a target of the mouse mammary tumor virus (MMTV), a retrovirus that does not carry its own oncogene, but induces carcinomas in the mammary glands of susceptible mice by activating a host gene at its DNA integration site (int). The discovery that *Drosophila wg* and mouse *int-1* are orthologues and are part of a large evolutionarily conserved group of genes in multicellular animals led to the contracted family name "*Wnt*."

#### A. Cancer

The initial identification of *Wnt1/int-1* as a proto-oncogene has been extended by further investigation. Alteration in the Wnt/β-catenin signaling pathway is now a well-established etiologic agent in tumorigenesis. The evidence is most striking for two downstream intracellular signaling components: the gene product of the *adenomatous polyposis coli* (*APC*) locus and the β-catenin protein. APC is a negative regulator of the canonical Wnt/β-catenin pathway that is mutated in the majority (>80%) of sporadically occurring colorectal adenomas and carcinomas. In addition, mutations in this gene are responsible for the genetic disease familial adenomatous polyposis, characterized by the proliferation of initially benign colonic polyps that predispose afflicted individuals to intestinal cancer. Similarly, mutations in β-catenin, the transcription cofactor that is activated by canonical Wnt signaling, have been described in a wide array of different cancer types. Mutations in other Wnt pathway components have likewise been associated with cell transformation *in vitro* and with the pathogenesis of an array of cancer types *in vivo* (Table 1).

#### B. Other Diseases

Given the prevalence and biological significance of Wnt signaling, it should not be surprising that it has major role in population genetics and forensic medicine. The multifunctionality of DBP has now been recognized. In addition to transporting vitamin D metabolites, DBP is proposed to be involved in the transport of fatty acids, to function as a plasma actin scavenger following tissue damage, and to play a role in complement C5a-mediated chemotaxis, and DBP may be involved in the activation of macrophages.

## II. REGULATION AND EXPRESSION OF DBP

DBP is a member of the albumin (ALB), α-fetoprotein (AFP), and α-albumin/afamin (AFM) gene family that is encoded on human chromosome 4. Initial studies have sublocalized the DBP gene to 4q11–q13, and refined mapping of this region has demonstrated that these four related genes are linked in the following order: centromere–3′-DBP-5′–5′-ALB-3′–5′-AFP-3′–5′-AFM-3′–telomere. Despite this linkage and the high degree of sequence and structural similarities among DBP, ALB, AFP, and AFM, the multifunctionality of DBP is a unique characteristic.

The human DBP gene itself is composed of 13 exons and spans over 42 kb from the transcription initiation site to the polyadenylation site. The cDNA structure of DBP was initially reported for humans and was subsequently determined for rat, mouse, rabbit, turtle, and chicken. Turtle DBP occupies a unique niche because it also binds thyroxine. Thus far, DBP appears to be limited to vertebrate species; a search of the *Drosophila melanogaster* genome database revealed no sequence homologues to DBP.

Human DBP mRNA encodes a 458-amino-acid secreted protein following cleavage of its 16-amino-acid signal sequence. DBP is a monomeric protein, migrating at approximately 58 kDa (Table 1). Its exact size is dependent on its glycosylation state. Like other members of the ALB family, DBP is cysteine rich. In the case of DBP, all 28 cysteine residues are present in the disulfide form and define a signature modular structure of three internally repeated peptide domains. Biochemical binding studies initially identified a vitamin D sterol-binding domain near the amino-terminus and an actin-binding domain closer to the carboxyl-terminus. The X-ray crystallographic details of DBP structure including its vitamin D- and actin-binding domains have recently become available. DBP has significant similarity to the α-helical structure of ALB, but the overall three-dimensional orientation of DBP's three internal peptide domains is quite dissimilar and is responsible for its binding properties. The helices of domain I in the N-terminus form an open vitamin D-binding cleft, accessible to solvent and lined with hydrophobic residues. Such a cleft cannot be formed in ALB, and this binding cleft is dissimilar to the internal and closed vitamin D-binding pocket in the nuclear vitamin D receptor molecule. The unique organization of DBP's three internal domains compared to ALB is central to its unique ability to bind G-actin. DBP clamps onto G-actin by narrowing the distance between domain I on one side and domains II and III on the other side, by virtue of a hinge at glycine 227. Thus, a very large binding interface is formed by the overall fold of DBP; this may account for the high-affinity association between DBP and actin. Human DBP expression is detected by the end of the first trimester and reaches normal adult circulation levels (4–8 μM) by term. Estimations from kinetic studies suggest that the daily production rate of DBP is approximately 10 mg/kg. The plasma half-life of DBP is 2.5 to 3 days, whereupon it is recycled by megalin or degraded completely in various tissues. The expression of DBP appears to undergo hormonal and growth factor regulation. Modest increases in DBP expression have been reported during pregnancy and in subjects receiving estrogen. In the Hep3B hepatoma cell line, interleukin-6 and dexamethasone were shown to increase DBP mRNA by approximately twofold and transforming growth factor-β decreased DBP mRNA

TABLE 1 Features of DBP

| | |
|---|---|
| **General properties** | |
| Size | 58 kDa |
| Plasma concentration | 4–8 μM |
| Plasma half-life | 2.5–3 days |
| Daily production rate | ~10 mg/kg |
| **Vitamin D-binding abilities** | |
| Plasma capacity | 2.4 mg/liter |
| Affinity | |
| $25(OH)D_3$ | $K_a = 5 \times 10^8\ M^{-1}$ |
| $1,25(OH)_2D_3$ | $K_a = 4 \times 10^7\ M^{-1}$ |
| **Actin-binding abilities** | |
| Plasma capacity | 270 mg/liter |
| Affinity | $K_a = 2 \times 10^9\ M^{-1}$ |
| **Macrophage and osteoclast activation abilities** | |
| DBP "activation" | By β-galactosidase and sialidase |
| Macrophage effects | Increased superoxide production, increased phagocytic activities |
| Osteoclast effects | Increased osteoclastogenesis |
| **Chemotactic abilities** | |
| C5a enhancement | Binding of DBP to leukocytes |
| DBP leukocyte-binding site | Chondroitin sulfate proteoglycans |
| Affinity | Low affinity, nonselective |

in a dose-dependent fashion by up to fivefold. Decreased DBP titers have also been reported in plasma from patients with advanced liver or kidney disease.

DBP is expressed at low levels in a variety of tissues, but most serum DBP is derived from the liver. Tissue-specific regulation of DBP has been postulated to be dependent on the relative abundance of two transcription factors, hepatocyte nuclear factor 1α (HNF1α) and HNF1β. These two closely related homeodomain-containing transcription factors bind to DNA as either homodimers or heterodimers. The DBP proximal promoter has three functional HNF1-binding sites. In this model, HNF1α homodimers stimulate a high level of DBP expression in the liver. In the kidney, the increased expression of HNF1β, and therefore formation of heterodimers, results in reduced DBP expression. In this situation, HNF1β functions as a *trans*-dominant inhibitor of HNF1α-mediated enhancing activity. The unique role of HNF1 in DBP expression is supported by the observation that DBP expression is reduced by 50% in the livers of HNF1 null mice. These observations would suggest that the net expression of the DBP gene reflects a balance between the two major HNF1 isoforms. Recently, mutations in human HNF1α and HNF1β have been identified to be the cause of some cases of maturity onset diabetes of the young (MODY). Whether or not DBP expression is affected in individuals with these MODY mutations remains undetermined.

## III. DBP POLYMORPHISMS

The 3 most common allelic variants of DBP differ based on four single-nucleotide polymorphisms at codons 152, 311, 416, and 420. These protein variants are known as $Gc^*1F$, $Gc^*1S$, or $Gc^*2$, where F and S refers to relative fast or slow migration rate following gel electrophoresis. The gene products of each variant allele differ from one another by amino acid substitution and/or by polysaccharide attachment. In addition to these 3 common alleles, there are over 124 rare variant alleles described to date, thus making DBP one of the most polymorphic proteins known. The geographical occurrences of some of these variants have been correlated with patterns of human migration and are therefore of anthropological interest. The affinities for vitamin D metabolites by several of these DBP isoforms have been determined, and for the most part, it was found that variants with higher isoelectric points have slightly lower ligand affinity. Any biological implications of these minor changes in binding affinity remain to be identified.

In the late 1980s, there was much controversy linking the expression of different DBP variants with susceptibility to human immunodeficiency virus infection. These initial reports were later proven to be erroneous by many investigators. Multiple studies have also been conducted over the years in an attempt to link the DBP variants to susceptibility or resistance to a variety of diseases such as multiple sclerosis, chronic obstructive pulmonary disease, pulmonary tuberculosis, and even schizophrenia. To date, no conclusive correlation has been reported in any of these cases. More recently, several attempts have been made to link DBP with the occurrence of diabetes. A higher prevalence of the $Gc^*1S$-$Gc^*2$ genotype was observed in type 2 diabetes in Japan. This genotype was also associated with higher fasting insulin levels and a higher index of insulin resistance in nondiabetic Japanese. In this population and in Dogrib Indians, the lowest fasting insulin levels were seen in individuals harboring the $Gc^*1F$-$Gc^*1F$ genotype. Interestingly, a difference in response to the oral glucose tolerance test was also observed in nondiabetic Pima Indians with this genotype, suggesting a link between the prediabetic state and DBP genotype. Conversely, in a mixed population study of nondiabetic Hispanic Americans and Caucasians, this $Gc^*1F$-$Gc^*1F$ genotype was associated with high fasting glucose levels. Studies in Caucasian populations would suggest that there is no association between DBP genetic variants and disease. These conflicting data may reflect the ethnic diversity in the populations sampled. Nonetheless, it is most unlikely that polymorphisms within the DBP gene are directly related to the diabetic trait.

## IV. DBP FUNCTIONS

### A. Sterol Binding

Under normal physiological conditions, most circulating vitamin D metabolites are protein bound. DBP binds 88% of serum 25-dihydroxyvitamin $D_3$ [25(OH)$D_3$] ($K_a = 5 \times 10^8\ M^{-1}$) and 85% of serum 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] ($K_a = 4\times10^7\ M^1$), leaving 0.40% “free” and the remainder associated with other serum proteins. Because DBP is in significant molar excess over vitamin D, only approximately 5% of the total circulating DBP actually carries vitamin D metabolites. This large molar excess of serum DBP has been hypothesized to play a role in protecting against vitamin D intoxication by buffering the levels of free vitamin D metabolites. But work using a DBP-deficient (Dbp-null) mouse model had challenged the validity of this idea. Dbp-null mice were generated at the expected Mendelian frequency from intercrosses of Dbp heterozygous animals. These animals are of normal size and appearance. There is no evidence for impairment of fertility in either males or females because intercrosses between Dbp-null animals resulted in normal-sized litters. This conclusion was surprising because extensive population studies had failed to identify a human DBP-null allele. The successful generation of the Dbp-null mice clearly demonstrated that DBP is not essential for viability.

The major role of DBP appears to be in sterol transport. *Dbp*-null mice on normal diets demonstrate only a modest decrease in serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$, and show no evidence of biological vitamin D deficiency, clearly indicating that free hormone levels are still physiologically adequate. However, when subjected to a vitamin D-deficient diet for 4 to 6 weeks, end-organ effects of vitamin D deficiency become apparent in these animals, suggesting that normal DBP levels offer a degree of protection against short-term, dietary-induced vitamin D deficiency.

To test the buffering capacity of DBP *in vivo*, sublethal doses of vitamin D were administered to *Dbp*-null and wildtype animals. Vitamin D toxicity is manifested by hypercalcemia, bone resorption, and calcification of soft tissues. Surprisingly, *Dbp*-null mice were less susceptible to hypercalcemia and the secondary soft-tissue calcifications of vitamin D toxicity than the wild-type controls. This protection may have arisen secondary to an accelerated clearance of $25(OH)D_3$ from serum and a rapid urinary excretion in the absence of DBP. A partial explanation for this paradoxical phenomenon was provided by the recent hypothesis that megalin in the proximal renal tubule may function to recycle DBP and $25(OH)D_3$.

Megalin/gp330 is a transmembrane protein with a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. This protein belongs to the low-density lipoprotein (LDL) receptor family. Megalin is expressed in the proximal brush border surfaces of the kidney epithelium where it appears to function as an endocytic DBP receptor for the reabsorption of apo–DBP and DBP–$25(OH)D_3$ complexes from the glomerular filtrate into the proximal tubular cells (Fig. 1). Here, $25(OH)D_3$ is metabolized to the physiologically active $1,25(OH)_2D_3$. In the absence of DBP, renal uptake and activation of $25(OH)D_3$ via the megalin endocytic recycling pathway would also be absent, thereby resulting in increased $25(OH)D_3$ excretion in

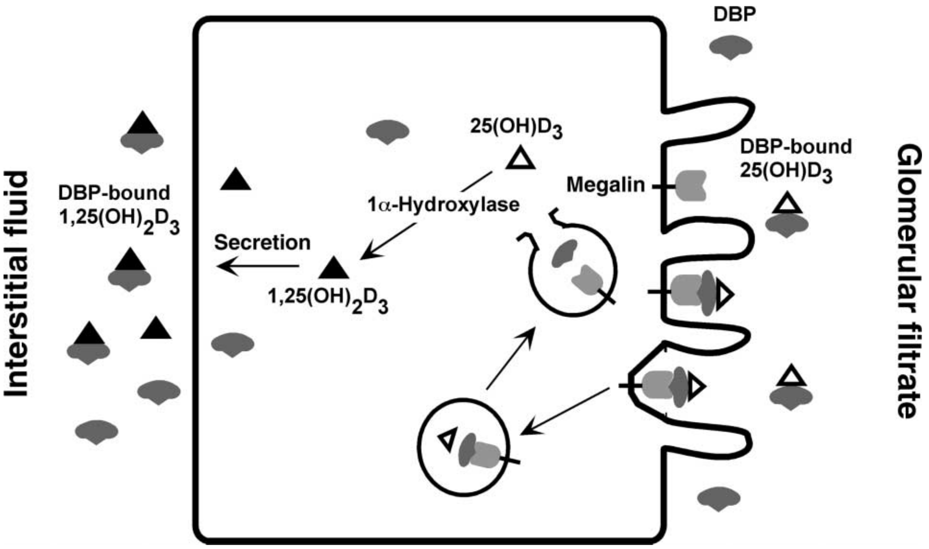

**FIGURE 1** Receptor-mediated endocytosis and activation of $25(OH)D_3$ by megalin. DBP-bound $25(OH)D_3$ interacts with megalin located at the brush border surface of the proximal tubule. This complex enters the cell by endocytosis and $25(OH)D_3$ is released and undergoes metabolic activation by 1α-hydroxylase. This active form of vitamin D, $1,25(OH)_2D_3$, is secreted back into the interstitial fluid, where it is rebound by DBP and delivered to target organs. Adapted from White *et al.* (2000). The multifunctional properties and characteristics of vitamin D-binding protein, *Trends in Endocrinology and Metabolism,* **11,** 320–327, with permission from Elsevier Science.

the urine and contributing to the observed resistance to vitamin D toxicity in the *Dbp*-null mice.

Like other members of the LDL receptor family, megalin exhibits broad ligand specificity. Megalin has been demonstrated to bind apolipoproteins, aminoglycosides, receptor-associated protein, and a variety of growth factors as well as DBP. A megalin-deficient mouse model has been generated to further elucidate the significance of this protein. A large percentage of megalin-deficient mice die perinatally from holoprosencephaly. Animals that do survive to adulthood demonstrate severe growth retardation, bone formation defects, and reduced bone density with scalloped bone surfaces. These animals also demonstrate tubular resorption deficiencies and excrete low-molecular-weight plasma proteins in their urine including DBP and albumin, reminiscent of patients with Fanconi syndrome. The loss of plasma carrier proteins such as DBP is concomitant with the loss of $25(OH)D_3$. The disturbances in bone metabolism in the megalin knockout animals as a consequence of vitamin D deficiency have therefore been attributed to the urinary wasting of DBP–$25(OH)D_3$ complexes. These studies may overemphasize the physiological role of megalin-mediated $25(OH)D_3$ in reclamation from the urine. Unlike the megalin knockout mice, *Dbp*-null mice are physiologically normal and show no evidence of biological vitamin D deficiency. This clearly indicates that in the absence of DBP, uptake and activation of $25(OH)D_3$ occur via a pathway that is independent of the proposed DBP–megalin endocytic recycling route. Thus, the role of megalin in vitamin D metabolism and its linkage to DBP recycling remains unclear.

## B. Actin Scavenger Activity

DBP is part of an "actin scavenger system" that functions by sequestering G-actin from the circulation for subsequent disposal (Fig. 2). DBP binds monomeric actin (G-actin) with very high affinity ($K_a = 2 \times 10^9\ M^{-1}$). This interaction occurs independent of sterol binding, and all three common polymorphic forms of DBP appear to have equal avidity for binding to G-actin.

The function of DBP in actin scavenging appears to be interrelated with the function of the protein gelsolin (GSN). Serum GSN functions mainly as a nonenzymatic fibrous actin (F-actin) severing molecule but has been demonstrated to also bind G-actin in the absence of DBP. As the G-actin monomers dissociate from the pointed ends of the severed actin polymer, they are rapidly bound by DBP and cleared from the circulation by Küpffer cells in the liver. The coordinated action of DBP and GSN has been demonstrated to inhibit actin-stimulated platelet aggregation. It is hypothesized that these two serum proteins function in concert to clear free actin from the circulation, thereby preventing the consequences of actin toxicity in the host. For example, G-actin is released into the circulation following tissue injury. If left unattended, G-actin will rapidly polymerize into F-actin, resulting in microemboli that may further damage the microvasculature. DBP–actin and GSN–actin complexes have been reported in the circulation of patients with a wide range of serious illnesses including hepatitis, acute lung injury, septic shock, severe trauma, and pregnancy. The levels of these complexes are also increased following tissue ischemia, inflammation, or injury. Clinical studies have recently described DBP as an acute-phase reactant after major surgery. Studies of rats injected with increasing concentrations of G-actin demonstrate progressive binding followed by saturation of DBP by G-actin. Saturation is then followed by the observation of new F-actin polymers and the appearance of emboli in the microcirculation of the lungs and hearts of these animals, resulting in death. In humans, marked decreases in total DBP followed by increased levels of DBP–actin complexes were reported in patients suffering from multiple organ

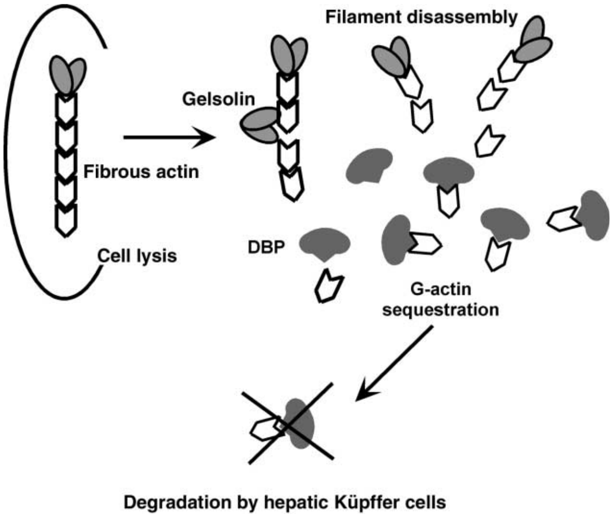

FIGURE 2 DBP and serum gelsolin (GSN) act in concert to prevent actin toxicity following tissue injury. When released from the cell, fibrous actin is disassembled by GSN. Monomeric actin released by this reaction is sequestered by DBP and to some extent by GSN. The bound actin is subsequently cleared from the circulation.

failure and trauma. Saturation of the DBP–actin scavenger system has been correlated with poor patient survival rates. Although decreases in hepatic DBP production may be due to initial liver damage, the increase in DBP–actin complexes is likely the result of the sequestration of the free actin released from the failing or damaged organs by circulating DBP. Depletion of the circulating pool of DBP often precedes death in these settings.

## C. Macrophage Activation and Osteoclastogenesis

Studies suggest that during inflammation DBP is transformed by sequential deglycosylation by two membrane-bound galactosidases into a potent macrophage-activating factor known as DBP–MAF. Increases in superoxide production and phagocytotic activities have been reported in macrophages activated by DBP–MAF *ex vivo*. These activated macrophages have been reported to successfully eradicate Ehrlich ascites tumors in mice. DBP–MAF has also been demonstrated to be an osteoclast activator in rat studies. Because osteoclast progenitor cells are blood-borne cells of the monocyte–macrophage lineage, this connection between macrophage activation and osteoclastogenesis is not totally surprising.

The structural requirements of DBP that relate to its bone resorbing activities were recently examined. It was demonstrated that DBP–MAF activates osteoclasts *ex vivo* in a dose-dependent manner. This activation occurs in the presence or absence of sterol binding to DBP. Furthermore, the study demonstrated that glycosylation of DBP is imperative for its ostesoclast-activating activities. Unglycosylated *Escherichia coli*-expressed recombinant DBP failed to activate osteoclasts, whereas glycosylated DBP expressed in a baculovirus system demonstrated significant osteoclast-activating function. Although it was not demonstrated in this study, it is likely that initial DBP glycosylation is also critical for macrophage activation by the scheme described previously.

The abnormal macrophages of several osteopetrotic mice and rats have been studied in detail. These animal models generally exhibit varying degrees of immunological as well as bone disorders. Analysis of B cells from two different strains of osteopetrotic mice (*mi/mi* and *op/op*) and one osteopetrotic rat (*op*) demonstrated that they lack β-galactosidase activities. This galactosidase has been shown to work in concert with sialidase, found on T cells, to deglycosylate DBP to its purported DBP–MAF active form. Treatment of the *op*rat from birth with DBP–MAF resulted in the reversal of the macrophage defect and partially ameliorated bony abnormalities. These results indicated a role for DBP in osteoclastogenesis. However, *Dbp*-null mice do not display bony defects and do not show a defect in macrophage recruitment or activation or in mounting an immune response to microorganisms requiring activated macrophages as the first line of defense. These observations would suggest that DBP–MAF generation is nonessential *in vivo* and that alternative pathways must exist for each of the activities attributed to DBP–MAF.

*In vitro* experiments demonstrated that whereas the Gc$^*$1 human DBP protein requires sequential treatment by β-galactosidase (B cells) and sialidase (T cells) for activation to DBP–MAF, the Gc$^*$2 DBP protein is predicted to be activated by β-galactosidase alone. A site located between amino acids 416 and 420 in domain III of DBP is thought to be the O-linked glycosylation site for *N*-acetylagalactosamine (GalNAc). Galactose and sialic acid residues are attached to this sugar molecule in the Gc$^*$1 DBP proteins, creating a branched structure. Early work on human DBP has in fact demonstrated that a contributing factor to the difference in electrophoretic mobility between the Gc$^*$1F and the Gc$^*$1S DBP isoforms is related to the number of sialic acid residues attached to this sugar moiety. Treatment of Gc$^*$1F with sialidase results in its migration to the position of GC$^*$1S. Upon activation, the terminal structure of the DBP–MAF derived from the Gc$^*$1 proteins appears to be GalNAc-threonine. The situation for Gc$^*$2 human DBP is unclear. It is proposed that the polysaccharide chain for this isoform of DBP is linear in that it lacks sialic acid. Removal of the terminal galactose by β-galactoside would result in a DBP–MAF structure as described above. However, only a very small percentage of Gc$^*$2 human DBP appears to be O-glycosylated, suggesting that the glycosylation site might be at amino acid 420. A single nucleotide polymorphism (A to C) at this codon results in an amino acid change from threonine (Gc$^*$1) to lysine (Gc$^*$2). The lysine residue cannot be glycosylated. If this were the case, then Gc$^*$2 homozygous individuals would be incapable of utilizing the macrophage activation pathway. Recently, threonine 418 was suggested to be the putative Gc$^*$2 glycosylation site. Evidence supporting this idea, however, is lacking. Further biochemical studies in this area are clearly required.

### D. Chemotaxis Enhancing Activity

DBP has been demonstrated to interact with a variety of different cell types. When bound to the surface of leukocytes, DBP plays an essential role in augmenting the chemotactic effect of complement factor C5a for neutrophils, monocytes, and fibroblasts *ex vivo*. DBP has been isolated from bronchoalveolar lavage fluid from patients with chronic obstructive pulmonary disease and adult respiratory distress syndrome, suggesting that it may indeed be a critical player in the inflammatory and chemotactic response to lung injury.

The chemotactic activity of DBP is dependent on its binding to chondroitin sulfate proteoglycans on the cell surface of neutrophils. Unlike DBP's interaction with vitamin D and actin, binding to these cell surface molecules is nonselective and of rather low affinity. Recently, it was reported that binding of DBP to neutrophils plateaus with time, possibly reflecting a steady state between binding and shedding of DBP on the plasma membrane. Inhibition of serine proteases with phenylmethylsulfonyl fluoride appears to disrupt this balance and allows for the accumulation of DBP on the plasma membrane of these cells. By a process of elimination, it was discovered that only inhibitors of neutrophil elastase prevented the loss of membrane-bound DBP-binding activity. A decrease in C5a chemotactic activities was correlated with the loss of DBP from the cell surfaces. Taken together, these results would suggest that neutrophil elastase plays a regulatory role in DBP-mediated C5a chemotactic activities. The exact mechanism by which DBP mediates its co-chemotactic response to C5a remains to be elucidated.

## V. SUMMARY

DBP is a multifunctional protein that plays a critical role in regulating the bioavailability of $25(OH)D_3$. New data implicate DBP as a ligand for the endocytic recycling receptor, megalin. The folding of DBP's three internal structural domains results in a large, high-affinity G-actin-binding interface. Serum DBP's actin-binding function is postulated to work in concert with serum gelsolin as part of an actin scavenger system that prevents actin toxicity in the host following tissue injury. From an immunological standpoint, DBP is a co-factor for C5a-mediated chemotaxis and appears to be involved in macrophage and osteoclast activation in *ex vivo* experiments. Many of the mechanisms underlying the different roles played by DBP remain obscure. Future studies using the *Dbp*-null mouse model may provide long-awaited answers.

## Glossary

**F-actin (filamentous actin)** Protein that results from the self-assembly of G-actin into head-to-tail polymers. This assembly is regulated by a large class of actin-binding proteins.

**G-actin (globular actin)** The major protein of the microfilament system in eukaryotic cells.

**gelsolin** A calcium- and polyphosphoinositide-regulated F-actin severing and capping protein found in both cytosol and serum.

**megalin** A multifunctional, transmembrane clearance receptor of the low-density lipoprotein family that mediates the uptake and lysosomal degradation of numerous ligands, particularly in renal proximal tubular cells.

**vitamin D-binding protein** An abundant serum protein of the albumin family that functions to bind and solubilize vitamin D sterols for transport to target tissues and for storage; it is also an extracellular G-actin-binding and sequestration protein.

## See Also the Following Articles

**Vitamin D • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in the Intestine and the Kidney • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Metabolism • Vitamin D: Nuclear Receptor for $1,25(OH)_2D_3$**

## Further Reading

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# Vitamin D: Biological Effects of $1\alpha,25(OH)_2D_3$ in Bone

TATSUO SUDA
*Saitama Medical School, Japan*

I. INTRODUCTION
II. DISCOVERY OF BONE MINERAL MOBILIZATION ACTIVITY OF VITAMIN D
III. ESTABLISHMENT OF A MOUSE CO-CULTURE SYSTEM TO EXAMINE OSTEOCLASTOGENESIS
IV. MOLECULAR MECHANISM OF OSTEOCLASTOGENESIS
V. ROLE OF $1\alpha,25(OH)_2D_3$ IN MAINTAINING SERUM CALCIUM HOMEOSTASIS
VI. SUMMARY

**Vitamin D, in concert with parathyroid hormone and calcitonin, plays a critical role in regulating serum calcium homeostasis. A metabolite of vitamin $D_3$, $1\alpha,25(OH)_2D_3$, stimulates osteoblastic bone formation and mineralization at least in an indirect manner, by stimulating the intestinal absorption of calcium. $1\alpha,25(OH)_2D_3$ also stimulates osteoclastic bone resorption by inducing osteoclast differentiation factor (ODF) receptor activator of NF-κB ligand (RANKL) in osteoblasts.**

## I. INTRODUCTION

It is well known that, in healthy animals and humans, serum calcium levels are tightly regulated and are maintained at 9 to 10 mg/dl. Intestine, bone, and kidney are the three major organs involved in calcium homeostasis. Vitamin D plays a major role in regulating serum calcium homeostasis in concert with parathyroid hormone (PTH) and calcitonin. Most of the biological effects generated by vitamin D are produced by its metabolite 1α,25-dihydroxyvitamin $D_3$ [$1\alpha,25(OH)_2D_3$]. Vitamin D receptors (VDRs), which bind $1\alpha,25(OH)_2D_3$, have been reported to be present in these three organs. In bone, VDRs are located preferentially in osteoblasts. In addition, vitamin D-deficient animals and humans exhibit severe rickets and osteomalacia. From these results, it was postulated that vitamin D directly stimulates bone formation, in particular, bone mineralization.

In 1997, Kato and his associates in Japan succeeded in generating mice deficient in VDR by gene targeting. They showed that in VDR null mutant mice [VDR (−/−)], no appreciable defects in development and growth were observed before weaning, irrespective of the reduced expression of vitamin D target genes. After weaning, however, mutant mice failed to thrive, and alopecia, hypocalcemia, and infertility resulted (Fig. 1a). Both bone formation and mineralization were severely impaired as a typical feature of type II vitamin D-dependent rickets. Most of the null mutant mice died within 15 to 25 weeks after birth due to severe hypocalcemia (Fig. 1b). Unexpectedly, when these null mutant mice were fed a rescue diet containing high levels of calcium, they developed normally even at 50 weeks, but severe alopecia remained (Fig. 1, inset). Bone formation and mineralization in the null mutant mice maintained on

TABLE 1 Distribution of Calbindin

| Calbindin-$D_{9k}$ | Calbindin-$D_{28k}$ |
|---|---|
| Mammalian intestine | Avian intestine |
| Mouse and neonatal rat kidney | Avian, reptilian, amphibian, and mammalian kidney (mouse, rat, bovine, and human kidney) |
| Rat and mouse yolk sac | Hen eggshell gland (uterus) |
| Rat uterus | Mouse reproductive tissues (uterus, oviduct, and ovary) |
| Rat and mouse placenta | Avian and mammalian beta cells of the pancreas<br>Alpha cells of the rat pancreas |
| Rat growth cartilage | Rat growth cartilage |
| Ameloblasts and osteoblasts of rodent teeth | Ameloblasts and osteoblasts of rodent teeth; mouse osteoblasts |
| Rat lung | Brain (avian, reptilian, amphibian, molluskan, fish, and mammalian brain) |

## II. LOCALIZATION AND FUNCTIONAL SIGNIFICANCE

### A. Intestine

One of the most important findings in the vitamin D field was the discovery by Robert Wasserman and Alan Taylor in 1966 of the 28,000-Da vitamin D-dependent calcium-binding protein in avian intestine. The intestinal calbindins (both avian and mammalian) are present in high concentrations in the intestine (0.15 m*M* or more) and are found in the highest concentration in the duodenum (specifically, in the cytosol of the columnar epithelial cells). Early studies in chicks established a strong correlation between the level of calbindin and an increase in intestinal calcium transport. The active form of vitamin D, 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] affects overall intestinal calcium absorption in three phases: (1) the transfer of calcium across the lumenal brush-border membrane, (2) the transfer of calcium across the cell interior, and (3) the energy-requiring extrusion of calcium from the basolateral membrane. A vitamin D-dependent apical calcium channel has recently been identified in $1,25(OH)_2D_3$-responsive epithelia (proximal duodenum and distal tubule of the kidney), suggesting a mechanism of calcium entry in the first phase of the transcellular process. It is thought that $1,25(OH)_2D_3$ functions in the second phase of the calcium absorptive process by interacting with the intestinal vitamin D receptor (VDR) to induce the genomic production of calbindin. Intestinal calbindin is thought to facilitate the diffusion of calcium in the absorptive cells toward the basolateral membrane. Indeed, in VDR knockout mice (VDR-ablated mice that have been generated by gene targeting), the major defect is in intestinal calcium absorption, which is accompanied by a 50% reduction in intestinal calbindin-$D_{9k}$. It has also been suggested that calbindin can act in the second phase as a cytosolic calcium buffer to prevent toxic levels of calcium from accumulating in the intestinal cell. In the third phase, calbindin is thought to stimulate calcium extrusion indirectly by increasing the local concentration of calcium adjacent to the basolateral plasma membrane calcium ATPase (PMCA).

### B. Kidney

In the kidney, relatively high levels of calbindin (2–7 μg/mg protein) are present, specifically in the distal nephron (distal convoluted tubule, connecting tubule, and cortical collecting duct). The vitamin D receptor is localized predominantly in the distal nephron, the site of localization of both calbindin-$D_{9k}$ and calbindin-$D_{28k}$, which are induced by vitamin D. Similar to intestinal calcium absorption, regulation by $1,25(OH)_2D_3$ of calcium transport in the distal nephron is thought to involve a transcellular process. Calcium enters through the apical plasma membrane, diffuses through the cytosol, and is actively extruded across the opposing basolateral membrane. A model of distal tubule renal calcium transport, involving calbindin, has been proposed. Calbindin-$D_{28k}$ has been reported to increase the influx of calcium at the apical membrane. Whether calbindin-$D_{28k}$ affects the activity of the recently identified apical calcium channel in the distal tubule, which has been reported to be up-regulated by vitamin D, is not yet known. Calbindin-$D_{28k}$ may then act as a diffusional carrier of calcium through the cytosol to the basolateral membrane. In addition, renal calbindin-$D_{28k}$ may also act to lower cytosolic calcium levels, preventing the accumulation of toxic levels of calcium. In the vicinity of the basolateral membrane, the calbindin-$D_{9k}$ protein binds the calcium and stimulates the extrusion of calcium via the PMCA (a calbindin-$D_{9k}$ binding domain has been identified in the PMCA). Thus, in the mouse distal nephron and the perinatal rat distal nephron, which contain both calbindin-$D_{9k}$ and calbindin-$D_{28k}$, these proteins have different functions. The different functions of these proteins suggest mechanisms by which $1,25(OH)_2D_3$ (which induces the calbindins) may function to enhance calcium reabsorption in the distal nephron. Future studies utilizing calbindin-$D_{9k}$ knockout mice as well as calbindin-$D_{9k}$ and calbindin-$D_{28k}$ double-knockout mice should provide additional insights about both intestinal and renal calcium transport. In addition, studies exploring the relationship of $1,25(OH)_2D_3$ and calbindin to the apical calcium channels will further define their respective roles in calcium transport.

## C. Bone

Previous studies using immunocytochemical analyses have indicated that calbindin-$D_{9k}$ and calbindin-$D_{28k}$ are found in chondrocytes of growth plate cartilage in rats and in ameloblasts and osteoblasts of rodent teeth. It has been suggested that the calbindins may be involved in the movement of intracellular calcium in the chondrocyte. It has also been suggested that elevated expression of the calbindins may characterize cells involved in the elaboration of mineralized tissues and bone. In a recent study, calbindin-$D_{28k}$ was found to be expressed at low levels in several osteoblastic cell lines and at much higher levels in primary cultures of murine osteoblastic cells. Transient transfection of calbindin-$D_{28k}$ in MC3T3-E1 osteoblastic cells protected against tumor necrosis factor $\alpha$ (TNF$\alpha$)-induced apoptotic cell death. Extracts from the TNF$\alpha$-treated cells expressing high levels of calbindin-$D_{28k}$ as well as purified calbindin-$D_{28k}$ inhibited the activity of caspase 3, a protease known to be an important mediator of apoptosis. These findings are novel because they demonstrate that calbindin-$D_{28k}$ is unique among the family of calcium-binding proteins in its ability to interact with and consequently inhibit caspase, thereby preventing apoptotic cell death in osteoblasts, independent of its calcium-binding capabilities. Thus far, calbindin-$D_{28k}$ is the only other known natural inhibitor of caspase besides the inhibitor of apoptosis proteins (IAPs). A further understanding of the mechanisms whereby calbindin-$D_{28k}$ attenuates apoptosis, including which regions of calbindin-$D_{28k}$ are involved in caspase 3 inhibition, will have important implications for the prevention of degeneration in bone cells (as well as in other cells) and therefore could prove important for the therapeutic intervention of many diseases, including osteoporosis.

## D. Pancreas

The pancreas was the first nonclassical target tissue reported to contain vitamin D receptors. Immunocytochemical and autoradiographic studies have indicated that receptors for $1,25(OH)_2D_3$ and calbindin-$D_{28k}$ are both present in the pancreatic beta cell. In chicks and rats, pancreatic calbindin-$D_{28k}$ has been reported to be responsive to $1,25(OH)_2D_3$. In the rat, calbindin-$D_{28k}$ is localized in alpha as well as beta cells of the pancreatic islet. Because $1,25(OH)_2D_3$ receptors are not present in a cells, it has been suggest that beta cell calbindin-$D_{28k}$, but not alpha cell calbindin, may be regulated by $1,25(OH)_2D_3$. Recent studies using calbindin-$D_{28k}$ null mutant (knockout) mice and beta cell lines have shown that calbindin-$D_{28k}$ can modulate depolarization-stimulated insulin release. These findings suggest that calbindin-$D_{28k}$ can control the rate of insulin release by regulation of intracellular calcium. In addition to modulation of insulin release, more recent studies have indicated that cytokine-mediated destruction of pancreatic beta cells, a cause of insulin-dependent diabetes, can be inhibited by calbindin-$D_{28k}$. In beta cells transfected with the calbindin-$D_{28k}$ gene, cytokine-mediated stimulation of free radical formation is inhibited. It has been suggested that calbindin-$D_{28k}$, by stabilizing cellular calcium, could prevent calcium-mediated mitochondrial damage and consequent generation of free radicals. These findings have important therapeutic implications for the prevention of autoimmune destruction of beta cells in type 1 diabetes.

## E. Nervous Tissue

In brain tissue, calbindin-$D_{28k}$ is present in most neuronal cell groups and fiber tracts but it is not regulated by $1,25(OH)_2D_3$; i.e., calbindin-$D_{28k}$ is expressed constitutively. Neurons containing calbindin-$D_{28k}$ are found in the cerebral cortex, hippocampus, amygdala, pyriform region, thalamus, and hypothalamus. The highest concentration of calbindin-$D_{28k}$ is in the cerebellum (1–2% of the total soluble protein), where it is specifically localized in the Purkinje cells. The phenotype of the calbindin-$D_{28k}$ knockout mouse (the calbindin-$D_{28k}$ gene is specifically ablated by gene targeting) is impaired motor coordination. This phenotype may be the result of abnormal cerebellar activity because of altered depolarization-induced calcium transients in the Purkinje cells in the absence of calbindin. It has been suggested that calbindin-$D_{28k}$, by regulating depolarization-induced potentials, may also be involved in the control of hormone secretion from hypothalamic neuroendocrine neurons. In addition, specific neuronal sensory cells (cochlear and vestibular hair cells in the inner ear, cone but not rod photoreceptor cells of the avian and mammalian retina, and photoreceptor cells of pineal transducers) contain calbindin-$D_{28k}$. Thus, calbindin-$D_{28k}$ in these cells is involved in mechanisms of signal transduction.

In the nervous system, besides affecting induced calcium transients and signal transduction mechanisms, calbindin-$D_{28k}$ protects neurons against calcium-mediated neurotoxicity. Studies using hippocampal cells in culture showed a direct relationship between calbindin-positive neurons and protection against damage induced by glutamate or calcium ionophore. Correlative evidence between decreases in neuronal calbindin and neurodegeneration have been reported in studies of chronic neurological diseases (Parkinson's, Alzheimer's, and Huntington's diseases), epilepsy, aging, and ischemic injury. Direct evidence of a protective role of neuronal calbindin-$D_{28k}$ against a variety of insults, including exposure to glucocorticoid, cyclic adenosine monophosphate (cAMP), immunoglobulin G (IgG) from amyotrophic lateral sclerosis patients, and hypoglycemia, has been shown in primary neuronal cells in culture or in neuronal cell lines in which the calbindin-$D_{28k}$ gene has been transfected, resulting in overexpression of calbindin.

Overexpression of calbindin in neural cells is also found to suppress the proapoptotic actions of mutant presenilin 1 (PS-1). Mutant PS-1 is causally linked to approximately 50% of the cases of early-onset familial Alzheimer's disease. Mutant PS-1 sensitizes cells to apoptosis induced by amyloid β-peptide (Aβ), the cleavage product of the amyloid precursor protein and the major component of plaques in Alzheimer's disease. It has been suggested that Aβ damages neurons by a mechanism involving oxidative stress and disruption of calcium homeostasis. Calbindin-$D_{28k}$ protected against the proapoptotic action of mutant PS-1 by attenuating the increase in intracellular calcium and preventing the impairment of mitochondrial function. Thus, calbindin-$D_{28k}$ in the nervous system (as well as in osteoblasts and pancreatic beta cells) has an important role in protecting against cell death. A further understanding of the mechanisms whereby calbindin-$D_{28k}$ protects against apoptotic cell death may have important therapeutic implications for the prevention of cellular degeneration.

## F. Placenta, Yolk Sac, and Uterus

Immunocytochemical and/or biochemical studies have indicated the presence of calbindin-$D_{9k}$ in bovine placenta, in the placenta and yolk sac of rats and mice, and in the endometrium and myometrium of pregnant and nonpregnant rat uterus and in the uterine epithelium of pregnant rats. In placenta and yolk sac, calbindin-$D_{9k}$ increases in late gestation (when fetal mineralization occurs), suggesting a role for calbindin-$D_{9k}$ in the transport of calcium to the fetus. Calbindin-$D_{28k}$ is not present in rat reproductive tissues. However the 28,000 $M_r$ calbindin is present in the uterus of the laying hen and in mouse reproductive tissues (endometrium and glandular epithelium of mouse uterus, mouse oviduct epithelium, and primary follicles of mouse ovary). Calbindin-$D_{28k}$ in these tissues is not affected by $1{,}25(OH)_2D_3$. However, calbindin-$D_{9k}$ and calbindin-$D_{28k}$ in rat and chick uterus, respectively, are up-regulated by estradiol. The presence of calbindin in epithelial cells of the uterus and oviduct suggests a role for calbindin in transepithelial calcium transport. The presence of calbindin in the myometrium suggests the involvement of calbindin in intracellular calcium regulation that may affect the frequency and strength of uterine contractions.

# III. REGULATION OF CALBINDIN GENE EXPRESSION

## A. Calbindin-$D_{28k}$

### 1. *Genomic Organization*

The structure of the chicken calbindin-$D_{28k}$ gene has been elucidated. The size of the gene is 18.5 kb; it consists of 11 exons and 10 introns. The coding regions in the mouse and chicken calbindin-$D_{28k}$ gene share 77% sequence homology.

### 2. *Regulation by $1{,}25(OH)_2D_3$*

Regulation of the calbindin-$D_{28k}$ gene is a complex phenomenon. Its induction by $1{,}25(OH)_2D_3$ in avian intestine and in avian and mammalian kidney is well known. In response to $1{,}25(OH)_2D_3$ treatment, the expression of the calbindin-$D_{28k}$ gene is induced by a small early increase in calbindin-$D_{28k}$ transcription followed by a sustained accumulation of mRNA long after $1{,}25(OH)_2D_3$ treatment, suggesting stabilization of calbindin-$D_{28k}$ mRNA by

$1,25(OH)_2D_3$. Similar observations have been made for chicken intestinal and rat renal calbindin-$D_{28k}$. A putative vitamin D response element (VDRE) has been found in the promoter of the chicken calbindin-$D_{28k}$ gene and a VDRE that responds to $1,25(OH)_2D_3$ has been identified in the promoter of the mouse calbindin-$D_{28k}$ gene. The putative VDRE in the chicken promoter is relatively inactive and the response of the mouse calbindin-$D_{28k}$ promoter to $1,25(OH)_2D_3$ is modest (maximal fivefold response). The modest response reflects the *in vivo* findings of a small transcriptional response. There is increasing evidence that the large induction of calbindin-$D_{28k}$ mRNA in both chick intestine and mouse kidney is due primarily to posttranscriptional mechanisms. In addition, in VDR-ablated mice, only a minor reduction in basal levels of renal calbindin-$D_{28k}$ is observed. However, the response of renal calbindin-$D_{28k}$ to injection of $1,25(OH)_2D_3$ is compromised. Thus, the regulation of calbindin-$D_{28k}$ mRNA by $1,25(OH)_2D_3$ appears to be more complex than the conventional genomic mechanism of steroid hormone action, which involves steroid receptor binding to specific DNA sequences and transcriptional activation. Regulation of calbindin-$D_{28k}$ by $1,25(OH)_2D_3$ may involve other factors and is mostly posttranscriptional.

### 3. *Regulation of Calbindin-$D_{28k}$ by Factors Other Than $1,25(OH)_2D_3$*

In the intestine of vitamin D-treated chicks, glucocorticoids inhibit the levels of calbindin-$D_{28k}$ protein and mRNA, resulting in a decrease in intestinal calcium absorption. Although a putative glucocorticoid-responsive element has been identified by sequence homology in the chicken calbindin-$D_{28k}$ promoter, it is not yet known whether the effect of glucocorticoids is an indirect or a direct effect on the calbindin gene. The complexity of calbindin-$D_{28k}$ gene regulation is also indicated in studies showing a modulation by calcium of calbindin-$D_{28k}$ gene expression in avian intestine as well as in avian and mammalian kidney.

In the central nervous system, calbindin-$D_{28k}$ is not regulated by $1,25(OH)_2D_3$. Various other factors that affect signal transduction pathways, including neurotropin-3, brain-derived neurotropic factor, insulin-like growth factor-I, fibroblast growth factor, and tumor necrosis factors, have all been reported to induce neuronal calbindin-$D_{28k}$. Because neurotropic factors can protect against cytotoxicity, the induction of calbindin-$D_{28k}$ by these factors may be one mechanism involved in calbindin's neuroprotective role. In addition to factors that affect signal transduction pathways, neuronal calbindin is also regulated by corticosterone and retinoic acid. Corticosterone has been shown to up-regulate calbindin-$D_{28k}$ in the hippocampus. Because corticosterone causes cell death in non-calbindin-$D_{28k}$-containing areas of the hippocampus, the induction of calbindin may be a compensatory mechanism to promote neuronal survival. Retinoic acid has also been reported to induce calbindin-$D_{28k}$ in medulloblastoma cells that are derived from the cerebellum and express a neuronal phenotype. Because retinoic acid has profound effects on embryogenesis, it is possible that retinoic acid may have a role in the induction of calbindin in the developing nervous system. Thus, although neuronal calbindin-$D_{28k}$ is unresponsive to $1,25(OH)_2D_3$, it can be regulated by neurotropic factors as well as by other steroids.

As previously discussed, calbindin-$D_{28k}$ is present in the avian eggshell gland as well as in mouse ovary, uterus, and oviduct. In these tissues, calbindin is not regulated by $1,25(OH)_2D_3$ but rather by estradiol. Analysis of the mouse calbindin-$D_{28k}$ promoter indicates that multiple imperfect half-palindromic estrogen-responsive elements (between $-1075$ and $-702$ and between $-175$ and $-78$) contribute to the induction by estradiol.

## B. Calbindin-$D_{9k}$

### 1. *Genomic Organization*

The size of the calbindin-$D_{9k}$ gene is 2.5 kb. The calbindin-$D_{9k}$ gene contains three exons interrupted by two introns. The first exon contains almost the entire 5′ noncoding region. The second exon codes for the first calcium-binding site. The third exon codes for the second calcium-binding site and the 3′ untranslated region.

### 2. *Regulation of Calbindin-$D_{9k}$ by $1,25(OH)_2D_3$*

Similar to the regulation of calbindin-$D_{28k}$, calbindin-$D_{9k}$ is regulated by $1,25(OH)_2D_3$ by a small, rapid transcriptional stimulation followed by a posttranscriptional effect, accounting for the sustained increase of calbindin-$D_{9k}$ mRNA. Unlike renal calbindin-$D_{28k}$, in VDR-ablated mice there is a marked inhibition of both basal and $1,25(OH)_2D_3$-induced calbindin-$D_{9k}$ mRNA levels. These findings suggest that basal levels of intestinal calbindin-$D_{9k}$ mRNA are more sensitive to control by VDR-mediated mechanisms than are basal levels of renal calbindin-$D_{28k}$ mRNA. In addition, recent studies using transgenic mice indicate that 4580 base pairs of the 5′ regulatory sequence of the calbindin-$D_{9k}$ gene are needed for intestine-specific expression as well as for the responsiveness to $1,25(OH)_2D_3$. The proximal promoter of the calbindin-$D_{9k}$ gene, from −117 to +400, and a distal element located at −3500, together, but not separately, confer the $1,25(OH)_2D_3$-induced transcriptional response. In addition, an intestine-specific transcription factor, caudal homeobox-2 (Cdx-2), binds to a Cdx-2-binding site in the distal element of the calbindin-$D_{9k}$ promoter and plays a crucial role in the transcription of the calbindin-$D_{9k}$ gene in the intestine.

### 3. *Regulation of Calbindin-$D_{9k}$ by Other Steroids*

In the intestine, calbindin-$D_{9k}$ expression is inhibited by glucocorticoids. This decrease may be involved in the reported decrease by glucocorticoids in intestinal calcium absorption. It is not yet known whether the mechanism of the effect of glucocorticoids on calbindin-$D_{9k}$ expression is direct or indirect.

In the uterus, calbindin-$D_{9k}$ expression is unaffected by $1,25(OH)_2D_3$ but rather is under strong estrogen control. An imperfect estrogen response element (ERE) that binds the estrogen receptor (ER) has been identified in the first 5′ splice site of the rat calbindin-$D_{9k}$ gene. Because the ERE has been found to contribute only weak estrogen induction, it has been suggested that the ER may cooperate with other transcription factors or co-activators to result in efficient transcriptional activation of the calbindin-$D_{9k}$ gene.

## IV. SUMMARY

Calbindin-$D_{9k}$ and calbindin-$D_{28k}$ were previously thought to be exclusively vitamin D-dependent proteins. It is now evident that these proteins are present in many different tissues, that they serve different functions, and that they are regulated by several steroids as well as by factors that affect signal transduction pathways. In future studies the generation of calbindin-$D_{9k}$ null mutant mice as well as increasingly refined cellular and molecular approaches will provide new insights into the mechanism of action of the calbindins, including the role of calbindin in intestinal calcium absorption, in calcium reabsorption in the distal nephron, and in protection against cell death.

## Glossary

**apoptosis** Biological process of programmed cell death that occurs in normal physiology as well as in response to adverse conditions.

**calbindin-$D_{9k}$** A 9000-Da calcium-binding protein that is present in highest concentrations in mammalian intestine, in mouse and neonatal rat kidney, and in placenta and uterus.

**calbindin-$D_{28k}$** A 28,000-Da calcium-binding protein present in highest concentrations in avian intestine and mammalian and avian kidney, brain, and pancreas.

**1,25-dihydroxyvitamin $D_3$** Active steroid hormone form of vitamin D, which is synthesized in the kidney.

**vitamin D receptor** Member of the steroid/nuclear receptor superfamily; binds 1,25-dihydroxyvitamin $D_3$ and to specific DNA sequences and activates or represses the transcription of specific target genes.

## See Also the Following Articles

**Apoptosis • Vitamin D • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in the Intestine and Kidney • Vitamin D Effects on Cell Differentiation and Proliferation • Vitamin D: Nuclear Receptor for $1,25(OH)_2D_3$ • Vitamin D Metabolism**

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# Vitamin D: 24,25-Dihydroxyvitamin D

Anthony W. Norman and Helen L. Henry
*University of California, Riverside*

I. INTRODUCTION
II. PRODUCTION OF $24R,25(\mathrm{OH})_2\mathrm{D}_3$
III. BIOLOGICAL EFFECTS OF $24R,25(\mathrm{OH})_2\mathrm{D}_3$
IV. RECEPTORS FOR $24R,25(\mathrm{OH})_2\mathrm{D}_3$
V. SUMMARY

**24,25-Dihydroxyvitamin $\mathrm{D}_3$ [$24R,25(\mathrm{OH})_2\mathrm{D}_3$] and 1α,25-dihydroxyvitamin $\mathrm{D}_3$ are the two major dihydroxylated vitamin D metabolites circulating in the blood. Although both forms are produced from the parent vitamin $\mathrm{D}_3$, it appears that the less well-studied $24R,25(\mathrm{OH})_2\mathrm{D}_3$ form plays distinct biological roles.**

## I. INTRODUCTION

In comparison to $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$, the biological actions of $24R,25(\mathrm{OH})_2\mathrm{D}_3$ have been relatively less studied. Although it is clear that $24R,25(\mathrm{OH})_2\mathrm{D}_3$ is produced by the same enzyme system that inactivates $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ in its target cells, there is substantial evidence that the important biological activities of $24R,25(\mathrm{OH})_2\mathrm{D}_3$ and $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ are distinctly different. One key question that has attracted attention is whether $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ acting alone can generate all of the biological responses that are attributed to the parent vitamin $\mathrm{D}_3$, or whether, for some responses, a second vitamin $\mathrm{D}_3$ metabolite is required. There is evidence to support the view that the presence of both $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ and $24R,25(\mathrm{OH})_2\mathrm{D}_3$ is required to generate the complete spectrum of biological responses attributable to the parent vitamin $\mathrm{D}_3$. The purpose of this article is to summarize results from biological systems in which $24R,25(\mathrm{OH})_2\mathrm{D}_3$ has been shown to produce biological effects.

## II. PRODUCTION OF $24R,25(\mathrm{OH})_2\mathrm{D}_3$

The $25(\mathrm{OH})\mathrm{D}_3/1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$-24*R*-hydroxylase (24*R*-hydroxylase) is induced by and responsible for the inactivation and catabolism of $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ in its target cells. In addition, 24*R*-hydroxylase in the kidney utilizes $25(\mathrm{OH})\mathrm{D}_3$ as substrate to produce significant amounts (2–5 ng/ml) of circulating $24R,25(\mathrm{OH})_2\mathrm{D}_3$.

A mouse knockout (KO) of $25(\mathrm{OH})\mathrm{D}_3$-24*R*-hydroxylase has been generated. The newborn mice are viable but develop significant bone abnormalities. Although these deficiencies have been attributed to elevated levels of plasma $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ rather than to a deficiency of $24R,25(\mathrm{OH})_2\mathrm{D}_3$, there is experimental evidence that $24R,25(\mathrm{OH})_2\mathrm{D}_3$ has biological activity in its own right.

## III. BIOLOGICAL EFFECTS OF $24R,25(\mathrm{OH})_2\mathrm{D}_3$

### A. Parathyroid Gland Regression

In vitamin D-deficient hypocalcemic animals, the parathyroid glands undergo marked hypertrophy and hyperplasia. The first suggestion that $24R,25(\mathrm{OH})_2\mathrm{D}_3$ could generate biological responses when $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ given alone did not elicit responses was obtained by measuring regression of hypertrophied parathyroid glands typically present in vitamin D-deficient, hypocalcemic chicks. Coadministration of physiological concentrations of $24R,25(\mathrm{OH})_2\mathrm{D}_3$ and $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ resulted in significant reduction in parathyroid gland size, whereas $1\alpha,25(\mathrm{OH})_2\mathrm{D}_3$ alone was ineffective in reducing parathyroid gland size. Concomitantly with these observations, several studies reported on the effects of $24R,25(\mathrm{OH})_2\mathrm{D}_3$ on parathyroid (PTH) secretion in animal systems and in patients with renal osteodystrophy.

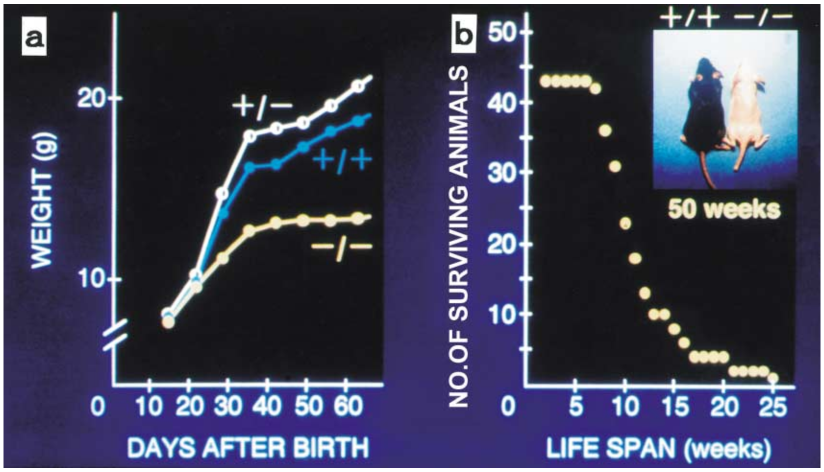

**FIGURE 1** Representative growth curves of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) littermates of VDR gene knockout mice (a) and the survival rate of 43 VDR (−/−) mice (b). The inset shows VDR (+/+) mice and VDR (−/−) mice fed a rescue diet containing high levels of calcium for 50 weeks. Reprinted from Yoshizawa *et al.* (1997), *Nature Genetics* **16**, 391–396 with permission.

a high-calcium diet were completely reestablished. From these results, it can be concluded that the stimulating effect of 1α,25(OH)$_2$D$_3$ on bone formation and mineralization is indirect, occurring through the stimulation of intestinal absorption of calcium by vitamin D.

## II. DISCOVERY OF BONE MINERAL MOBILIZATION ACTIVITY OF VITAMIN D

It appears to be paradoxical, but vitamin D functions in the process of calcium mobilization from calcified bone, making calcium available to the extracellular fluid upon demand by the calcium homeostatic system. This important observation was first reported by Carlsson in 1952. He showed that when hypocalcemic rats fed a vitamin D-deficient, low-calcium diet were orally given 100 IU (2.5 μg) of vitamin D$_3$, their serum calcium was increased from 5 to 8 mg/dl 3 days after administration (Fig. 2a). Parathyroidectomy (PTX) 2 h prior to vitamin D$_3$ administration abolished the increase in serum calcium levels (Fig. 2a). Since the diet did not contain any appreciable amounts of calcium, he concluded that vitamin D stimulates mineral mobilization from calcified bone to blood in concert with PTH.

The metabolite of vitamin D$_3$ responsible for bone mobilization is 1α,25(OH)$_2$D$_3$. Using an *in vitro* organ culture system, in 1972 Raisz *et al.* reported that 1α,25(OH)$_2$D$_3$ and 25(OH)D$_3$ increase the release of $^{45}$Ca from prelabeled bone into the culture medium. They also showed that 1α,25(OH)$_2$D$_3$ is about 80 times more potent than 25(OH)D$_3$ in increasing the $^{45}$Ca release from prelabeled bone (Fig. 2b). From these results, they concluded that the metabolite of vitamin D$_3$ which stimulates bone mineral mobilization is indeed 1α,25(OH)$_2$D$_3$.

In 1981, Abe *et al.* discovered the cell differentiation-inducing activity of 1α,25(OH)$_2$D$_3$ using mouse and human myeloid leukemic cells. HL-60 is a human promyelocytic leukemia cell line established from a leukemia patient, and the cells can be induced to differentiate into granulocytes by retinoic acid and monocyte–macrophages by 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ was a potent and selective inducer of differentiation of HL-60 cells into macrophages. Furthermore, 1α,25(OH)$_2$D$_3$ directly induced the fusion of alveolar macrophages at a very high rate. Approximately 80% of the macrophages fused to form multinucleated giant cells by stimulating the differentiation and fusion of macrophages. However, the multinucleated giant cells formed from alveolar macrophages in response to 1α,25(OH)$_2$D$_3$ did not satisfy the criteria of osteoclasts.

## III. ESTABLISHMENT OF A MOUSE CO-CULTURE SYSTEM TO EXAMINE OSTEOCLASTOGENESIS

It is well recognized that osteoclasts are derived from hematopoietic cells of the monocyte–macrophage lineage. Hematopoietic monocytic cells are present in almost all tissues, whereas osteoclasts, the cells responsible for bone resorption, are present only in

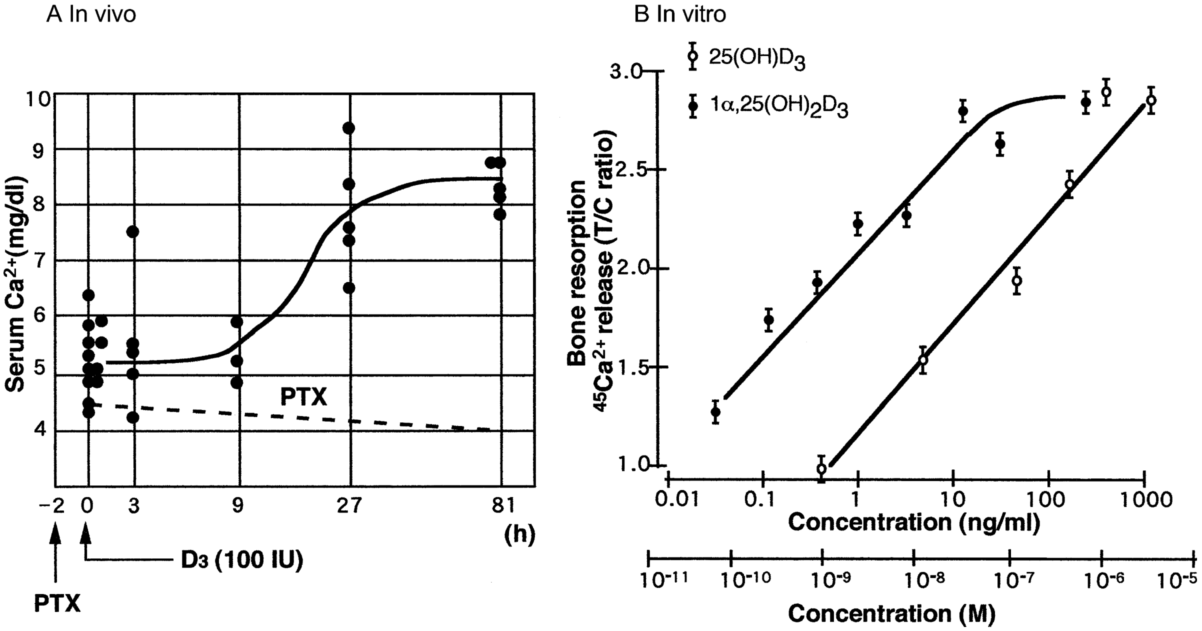

FIGURE 2 (A) Discovery of bone mineral mobilization activity of vitamin D *in vivo*. PTX, parathyroidectomy. Reprinted from Carlsson (1952), with permission from Blackwell Publishing Ltd. (B) The comparison of the *in vitro* activity to increase the release of $^{45}$Ca from prelabeled bone between 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$. Reprinted from Raisz *et al.* (1972), *Science* **175**, 768–769. Copyright 1972 American Association for the Advancement of Science.

bone. This led to the speculation that some local factors or local mechanisms are involved in this tissue-specific localization of osteoclasts in bone. Special attention was given to the role of osteoblasts in osteoclast development, since osteoblasts are present only in bone. The process of osteoclast development consists of several steps, including proliferation, differentiation, fusion, and activation.

In 1988, Takahashi *et al.* established an efficient mouse co-culture system to recruit osteoclasts. Osteoblastic cells were isolated from mouse calvaria, and spleen cells were used as hematopoietic osteoclast progenitors. They were either separately cultured or co-cultured together with or without $10^{-8}$ M 1α,25(OH)$_2$D$_3$. When osteoblastic cells alone or spleen cells alone were cultured, no osteoclasts were formed even in the presence of 1α,25(OH)$_2$D$_3$. In contrast, numerous multinucleated osteoclasts were formed when spleen cells and osteoblastic cells were co-cultured in the presence of 1α,25(OH)$_2$D$_3$. Cell-to-cell contact between spleen cells and osteoblastic cells appeared to be important for osteoclast formation, since no osteoclasts were formed when spleen cells and osteoblastic cells were co-cultured but separated by a membrane filter even in the presence of 1α,25(OH)$_2$D$_3$. From these results, it was hypothesized that the direct contact of spleen cells and osteoblastic cells is essential for osteoclast differentiation, suggesting the requirement of a membrane-associated factor for osteoclast formation. Spleen cells represented osteoclast progenitors, in other words, "the seeds," and osteoblastic cells represented supporting cells that provide a suitable microenvironment for osteoclast formation in bone, in other words, "the farm."

After extensive studies, it was found that not only 1α,25(OH)$_2$D$_3$ but also PTH, IL-1, PGE2, IL-6, and IL-11 similarly stimulated osteoclast formation in mouse co-cultures (Fig. 3). The target cells of these bone-resorbing factors were osteoblastic cells but not hematopoietic osteoclast precursors. These bone-resorbing factors were classified into three diverse signaling pathways mediated by the VDR, cAMP, and gp130 (Fig. 3). These three signals appeared to stimulate osteoclast differentiation independently, since osteoclasts were formed both in VDR null mutant mice and in gp130 knockout mice. In other words, there is a redundancy in bone-resorbing factors to recruit osteoclasts. It was proposed that the "osteoclast differentiation factor" (ODF) is commonly induced on the plasma membrane of osteoblastic cells in response to these bone-resorbing factors (Fig. 3). Osteoclast precursors having ODF receptors recognize ODF by cell-to-cell contact and

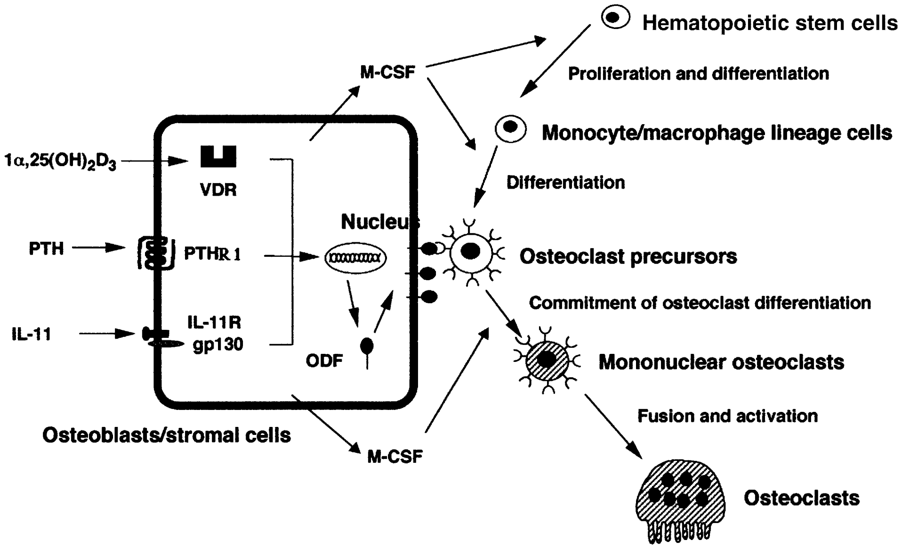
Hematopoietic stem cells
M-CSF
Proliferation and differentiation
Monocyte/macrophage lineage cells
$1\alpha,25(OH)_2D_3$
VDR
Differentiation
Nucleus
PTH
PTHR1
Osteoclast precursors
Commitment of osteoclast differentiation
IL-11
IL-11R
gp130
ODF
Mononuclear osteoclasts
Fusion and activation
Osteoblasts/stromal cells
M-CSF
Osteoclasts

FIGURE 3 A hypothetical concept of osteoclast differentiation, proposing the requirement of a membrane-associated factor, osteoclast differentiation factor (ODF), in osteoblasts/stromal cells for osteoclastogenesis.

differentiate into osteoclasts. Macrophage-colony stimulating factor (MCSF, also called colony stimulating factor-1, CSF-1) appeared to play an important role in the proliferation and differentiation of osteoclast progenitors, since M-CSF-deficient op/op mutant mice showed severe osteopetrosis with the complete absence of osteoclasts.

## IV. MOLECULAR MECHANISM OF OSTEOCLASTOGENESIS

In 1997, the research groups of Amgen and Snow Brand Milk Products independently succeeded in the molecular cloning of a factor that strongly inhibits osteoclastogenesis. Amgen named it "osteoprotegerin" (OPG) and Snow Brand named it "osteoclastogenesis inhibitory factor" (OCIF). OPG and OCIF were the same molecule, which belongs to the TNF receptor family. OPG/OCIF lacked the membrane-bound domain, indicating that OPG/OCIF is a soluble receptor. It was speculated that OPG/OCIF could compete with the ODF receptor for the binding of ODF.

Using radioactive OCIF, the molecular cloning of a membrane-associated factor responsible for osteoclastogenesis was finally accomplished. The Amgen group also succeeded in the molecular cloning of such a factor. Amgen named it OPG ligand (OPGL), which was identical to ODF. Figure 4 schematically shows the molecular mechanism of osteoclast formation. All bone-resorbing factors such as $1\alpha,25(OH)_2D_3$, PGE2, PTH, and IL-11 act on osteoblastic cells to induce ODF. ODF recognizes osteoclast progenitors having an ODF receptor by a mechanism involving cell-to-cell contact. M-CSF is also an essential factor for osteoclast differentiation and is produced by osteoblastic cells in bone. Osteoclast progenitors differentiate into osteoclasts by binding to ODF. When OPG/OCIF covers ODF, osteoclast progenitors having the ODF receptor are unable to bind ODF; thus, osteoclast formation is inhibited.

The molecular cloning of ODF revealed that this molecule was identical to "receptor activator of NF-κB ligand" (RANKL), "TNF-related activation-induced cytokine" (TRANCE), and OPGL, all of which were independently identified by different research groups as a novel member of the TNF ligand family. ODF, OPGL, TRANCE, and RANKL are the same molecule, and it is important in the

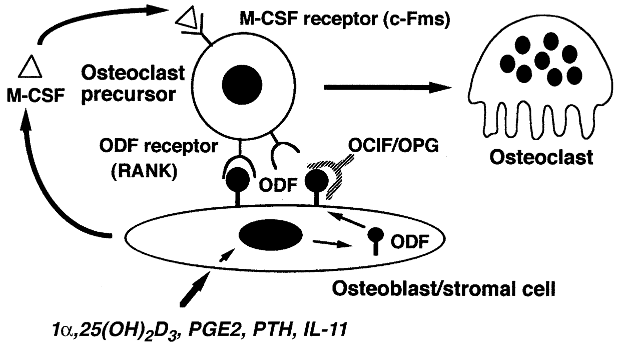
M-CSF receptor (c-Fms)
M-CSF
Osteoclast
precursor
ODF receptor
(RANK)
OCIF/OPG
ODF
Osteoclast
ODF
Osteoblast/stromal cell
$1\alpha,25(OH)_2D_3$, PGE2, PTH, IL-11

FIGURE 4 The molecular mechanism of osteoclast formation from its precursors supported by osteoblasts/stromal cells.

development of T cells and dendritic cells as well as the development of osteoclasts. "Receptor activator of NF-κB" (RANK), which has been cloned as a receptor of RANKL, is the transmembrane signaling receptor for ODF as well. OPG/OCIF is a soluble receptor for ODF and it appears to function as a decoy receptor.

ODF is involved not only in osteoclast differentiation but also in osteoclast activation. The lifetime of osteoclasts can be divided into three steps: the first step is proliferation and differentiation, the second step involves survival and fusion, and the third step is the activation of osteoclasts. Proliferation and differentiation of osteoclasts essentially require ODF together with M-CSF. The survival and fusion of osteoclasts are induced by either ODF or M-CSF. Activation of osteoclasts is induced by ODF but not by M-CSF. Thus, ODF appears to be involved throughout the lifetime of osteoclasts.

## V. ROLE OF 1α,25$(OH)_2D_3$ IN MAINTAINING SERUM CALCIUM HOMEOSTASIS

It should, however, be recognized that physiological plasma levels of 1α,25$(OH)_2D_3$ do not stimulate osteoclastic bone resorption *in vivo*; only pharmacological or toxic doses of 1α,25$(OH)_2D_3$ are capable of stimulating it. Figure 5 shows the difference in dose levels of 1α,25$(OH)_2D_3$ required to stimulate intestinal calcium transport activity and bone mineral mobilization activity. In this particular experiment, the intestinal calcium transport activity was measured by the everted gut sac method (serosal/mucosal $^{45}$Ca ratio) in vitamin D-deficient rats after administration of graded doses of 1α,25$(OH)_2D_3$. The bone mineral mobilization activity was monitored by determining serum calcium levels in rats fed a vitamin D-deficient, low-calcium diet after administration of graded doses of 1α,25$(OH)_2D_3$. Physiological dose levels (0.1–0.25 μg/rat) of 1α,25$(OH)_2D_3$ greatly increased intestinal calcium transport activity but did not increase serum levels of calcium appreciably. Approximately 10 to 50 times higher dose levels of 1α,25$(OH)_2D_3$ were required to induce bone mineral mobilization activity. In our co-culture system, $10^{-8}$ M 1α,25$(OH)_2D_3$ was required to generate osteoclasts *in vitro*, a level that was 50 to 100 times higher than the serum concentration of 1α,25$(OH)_2D_3$ in healthy subjects. Thus, it may be concluded that physiological concentrations of 1α,25$(OH)_2D_3$ are not capable of inducing bone mineral mobilization and that pharmacological or toxic levels of 1α,25$(OH)_2D_3$ are required for inducing bone resorption.

## VI. SUMMARY

Physiological doses of vitamin D preferentially stimulate intestinal calcium absorption, which in

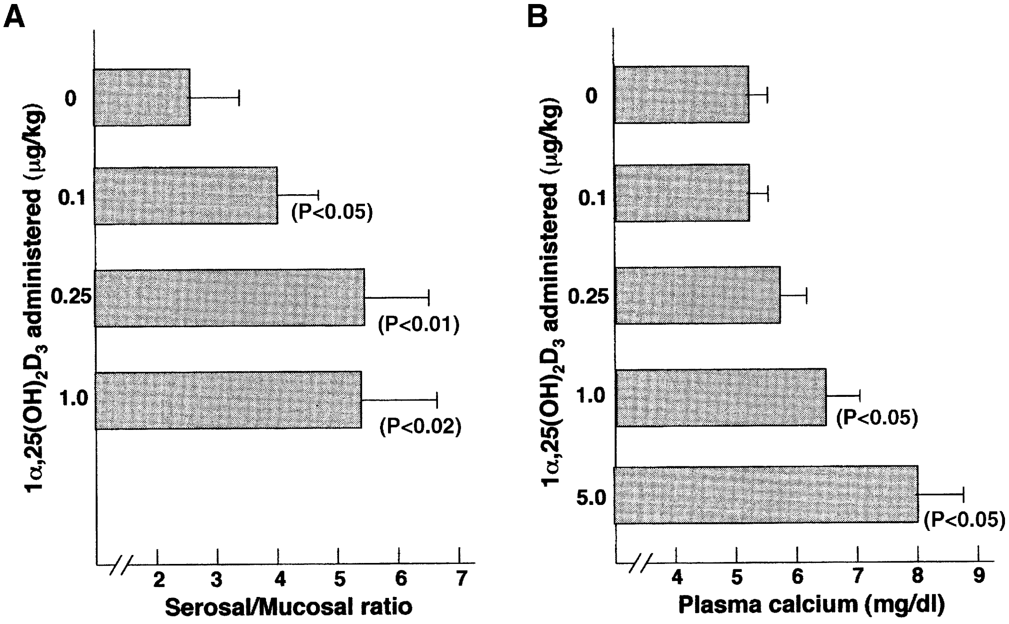

FIGURE 5 Differences in the dose levels of 1α,25$(OH)_2D_3$ required to stimulate intestinal calcium transport activity (A) and bone mineral mobilization activity (B).

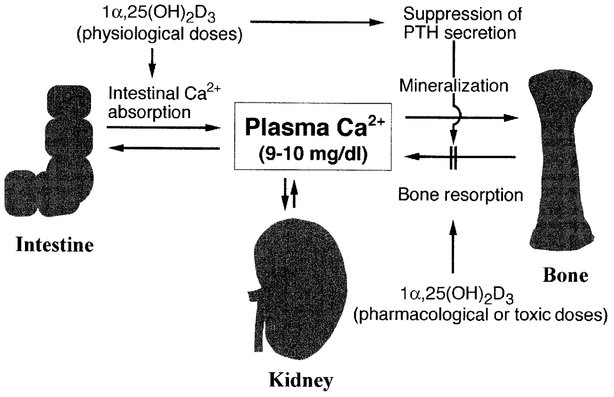
1α,25(OH)₂D₃ (physiological doses)
Suppression of PTH secretion
Intestinal Ca²⁺ absorption
Mineralization
Plasma Ca²⁺ (9-10 mg/dl)
Bone resorption
Intestine
Bone
1α,25(OH)₂D₃ (pharmacological or toxic doses)
Kidney

FIGURE 6 The role of $1\alpha,25(OH)_2D_3$ in maintaining plasma calcium homeostasis.

turn stimulates bone mineralization (Fig. 6). The recent findings of VDR knockout mice and of those mice fed a rescue diet containing high levels of calcium indicate that the stimulating effect of vitamin D on bone formation and mineralization is indirect, occurring through the stimulation of intestinal absorption of calcium by this vitamin. In contrast, osteoclastic bone resorption can be induced by vitamin D directly but only by pharmacological or toxic doses (Fig. 6). Physiological doses of vitamin D never stimulate bone resorption.

It is interesting that osteoclast formation requires cell-to-cell contact with osteoblastic cells, which generates ODF/RANKL as a membrane-associated factor in response to several bone-resorbing factors including vitamin D. In normal bone remodeling, bone formation by osteoblasts always occurs in a programmed manner accurately and quantitatively just after bone resorption by osteoclasts. Thus, it is possible that cell-to-cell contact between osteoclast progenitors and osteoblastic cells may leave some template for bone formation in osteoblasts/stromal cells. This possibility must be proved by future experiments.

## Glossary

**osteoblasts** The cells responsible for bone formation, which derive from mesenchymal progenitors and synthesize bone matrix proteins containing type III collagen and other noncollagenous proteins such as osteocalcin, osteopontin, osteonectin, and bone sialoproteins.

**osteoclast differentiation factor (ODF)** The factor responsible for osteoclast formation and activation, which belongs to the tumor necrosis factor (TNF) ligand family. ODF consists of 316 amino acid residues and is also called receptor activator of NF-κB ligand (RANKL).

**osteoclasts** The cells responsible for bone resorption, which originate from hematopoietic cells of the monocyte–macrophage lineage and which are present only in bone.

**osteoprotegerin (OPG)** The decoy receptor of ODF/RANKL, which belongs to the TNF receptor family. OPG is also called osteoclastogenesis inhibitory factor (OCIF). OPG/OCIF is a soluble receptor having no membrane-associated domain.

**receptor activator of NF-κB (RANK)** The receptor of ODF/RANKL, which belongs to the TNF receptor family.

**VDR** The specific receptor for the hormonal form of vitamin $D_3$, $1\alpha,25(OH)_2D_3$, in target tissues.

## See Also the Following Articles

**Bone Morphogenetic Proteins • Osteoporosis: Hormonal Treatment • Osteoporosis: Pathophysiology • Vitamin D • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in the Intestine and the Kidney • Vitamin D Deficiency, Rickets and Osteomalacia**

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# Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in the Intestine and the Kidney

Joost G. J. Hoenderop, Carel H. van Os, and
René J. M. Bindels
*University of Nijmegen, The Netherlands*

I. INTRODUCTION
II. BIOLOGICAL EFFECTS IN THE INTESTINE
III. BIOLOGICAL EFFECTS IN THE KIDNEY
IV. SUMMARY

**Calcitriol or 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] is essential for the development and maintenance of bone. This hormone plays a crucial role in $Ca^{2+}$ and $P_i$ homeostasis and exerts its biological actions on the intestine and kidney; the absorbing activity of these target organs ultimately determines the mineral balance for the entire body. $1,25(OH)_2D_3$ has strong stimulatory effects on $Ca^{2+}$ transport proteins; thus, transepithelial $Ca^{2+}$ transport in the small intestine and kidney is dependent on vitamin D. Less is known regarding the transport of $P_i$ in the kidney and intestine. However, it is well established that vitamin D stimulates $Na^+$-dependent $P_i$ transport in the small intestine, although the molecular mechanism responsible has not yet been elucidated.**

## I. INTRODUCTION

Calcitriol or 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] plays a critical role in $Ca^{2+}$ and phosphate ($P_i$) homeostasis and is essential for the development and maintenance of bone. The intestine and kidney are main target organs for the action of this hormone. The absorbing activity of intestine and kidney determines the net intake and excretion of minerals for the entire body and, therefore, the mineral balance. In normal adults, the renal excretion of $Ca^{2+}$ and $P_i$ is critically balanced by gastrointestinal absorption. On the other hand, the distribution of $Ca^{2+}$ within the body is determined by exchanges of $Ca^{2+}$ between interstitium and bone. These pathways are primarily regulated by vitamin D metabolites and parathyroid hormone (PTH). Alterations in these regulatory processes are present in many physiological and pathophysiological states.

The biological actions of calcitriol on the target organs are mediated by both genomic and rapid posttranscriptional mechanisms. The genomic response is linked to the nuclear vitamin D receptor (VDR). Upon binding $1,25(OH)_2D_3$, the VDR undergoes a conformational change and forms a complex with a retinoid X receptor (RXR). The VDR/RXR complex binds to DNA elements in the promoter regions of target genes described as vitamin D-response elements (VDREs). Binding to the VDREs may control the rate of gene transcription. The rapid response is believed to utilize another signal transduction pathway that is likely linked to a putative cell membrane receptor for $1,25(OH)_2D_3$, but its physiological role is still not well understood.

## II. BIOLOGICAL EFFECTS IN THE INTESTINE

The most critical role of $1,25(OH)_2D_3$ in the gut is to regulate the absorption of $Ca^{2+}$ and $P_i$ during variations in dietary intake and body demand. The most obvious stimulatory effect of $1,25(OH)_2D_3$ in this tissue takes place in the proximal small intestine and is mediated by VDR-controlled genomic actions.

### A. Calcium Absorption

There is ample evidence that vitamin D is an important determinant of intestinal $Ca^{2+}$ absorption. For instance, in vitamin D deficiency, intestinal $Ca^{2+}$ absorption is low, resulting in an increased risk of developing negative $Ca^{2+}$ balance and bone loss. Conversely, plasma levels of $1,25(OH)_2D_3$ are increased as a primary response to low dietary $Ca^{2+}$ intake and increased $Ca^{2+}$ requirements during growth, pregnancy, and lactation, affecting primarily the rate of active $Ca^{2+}$ absorption in duodenum. During transcellular $Ca^{2+}$ transport, $Ca^{2+}$ ions pass across the lumenal brush-border membrane, then transverse through the cytosol, and exit against an electrochemical gradient across the basolateral membrane. The epithelial $Ca^{2+}$ channel, ECaC (or $Ca^{2+}$ transporter, CaT), the vitamin D-dependent $Ca^{2+}$-binding protein, calbindin-$D_{9K}$, and the plasma membrane $Ca^{2+}$-ATPase, PMCA, represent the major $Ca^{2+}$ transporters that mediate this three-step process in the small intestine. $1,25(OH)_2D_3$ stimulates this vectorial $Ca^{2+}$ transport primarily via a genomic action and the contribution of each individual transporter to the overall stimulatory action of $1,25(OH)_2D_3$ has been elucidated in several studies.

The molecular identity of the $Ca^{2+}$ entry mechanism across the lumenal brush-border membrane has recently been established. By an expression cloning strategy, the epithelial calcium channels ECaC1 and ECaC2 (also known as CaT1) were identified. These highly homologous channels represent a new class of $Ca^{2+}$-selective channels, which are predominantly expressed in $1,25(OH)_2D_3$-responsive epithelia such as small intestine, kidney, and placenta. Analysis of the 5′ upstream region of the human ECaC1 gene revealed putative VDREs at a distance of approximately 1.5–0.5 kb from the transcription initiation site, which supports the previous findings that ECaC1 is a target gene for $1,25(OH)_2D_3$. The role of the VDREs in $1,25(OH)_2D_3$-mediated ECaC1 gene transcription remains to be established. However, it is likely that $1,25(OH)_2D_3$ regulates the transcription of these $Ca^{2+}$ channels. Surprisingly, in two initial studies no significant effect of $1,25(OH)_2D_3$ on the expression of these channels could be found, thus far for unexplained reasons. Northern blot analysis did not reveal vitamin D-dependent regulation of ECaC2 expression in the duodenum of rats and no significant relationship was shown between human ECaC2 expression in duodenal samples and serum $1,25(OH)_2D_3$ levels of healthy volunteers. The first indication that ECaC in the intestine is regulated by vitamin D was recently shown in animal studies. A single pharmacological dose of $1,25(OH)_2D_3$ in wild-type mice stimulated the duodenal level of ECaC1 mRNA by a factor of 1.6 and that of ECaC2 mRNA by more than sixfold. In a study from two independent groups, using mice strains in which the VDR was inactivated, it was further investigated whether these channels are indeed prime targets for hormonal regulation by $1,25(OH)_2D_3$. These mice display a phenotype similar to that observed in hereditary hypocalcemic vitamin D-resistant rickets, namely, rickets, hypocalcemia, and hypophosphatemia. This phenotype could be rescued by high dietary $Ca^{2+}$ intake, confirming that the intestinal absorption of $Ca^{2+}$ is critical in $1,25(OH)_2D_3$ action on $Ca^{2+}$ homeostasis. ECaC1 and ECaC2 mRNA levels were considerably and consistently down-regulated in the duodenum of these VDR-knockout mice on a normal $Ca^{2+}$ diet. Similar studies were performed in mice homozygous for the disrupted $Na^+–P_i$ co-transporter (Npt2) gene. These hypophosphatemic mice exhibit hypercalcemia and hypercalciuria resulting from duodenal $Ca^{2+}$ hyperabsorption, secondary to elevated serum $1,25(OH)_2D_3$ levels. In these mutants, the duodenal expression of ECaC1 and ECaC2 mRNAs is higher than in wild-type littermates, suggesting that the corresponding proteins are involved in mediating the increase in duodenal $Ca^{2+}$ absorption. Finally, the expression of ECaC2 is rapidly up-regulated by $1,25(OH)_2D_3$ and precedes vitamin D-stimulated $Ca^{2+}$ transport in Caco-2 cells, a human intestinal cell line. Taken together, these results suggest that the expression of these novel duodenal epithelial $Ca^{2+}$ channels is strongly vitamin D-dependent.

There is ample evidence that calbindin-$D_{9K}$ is highly responsive to $1,25(OH)_2D_3$. In animal models and cell lines, it has been documented that $1,25(OH)_2D_3$ stimulates the expression of calbindin-$D_{9K}$ on both mRNA and protein levels, involving a genomic pathway. This results in an increase in the capacity of diffusional flow of $Ca^{2+}$ ions through the cytosol. The linear correlation between the intestinal content of calbindin-$D_{9K}$ and the rate of active $Ca^{2+}$

transport in the intestine underscores the important role of calbindin-D$_{9K}$ in active Ca$^{2+}$ absorption. The molecular basis for regulating the calbindin-D$_{9K}$ gene by 1,25(OH)$_2$D$_3$ is not completely understood since the previously identified VDREs in the promoter region seem not to be responsible for the stimulatory effect.

PMCA is ubiquitously expressed in Ca$^{2+}$ transporting epithelia and is encoded by four distinct genes (PMCA1–4) that can be further posttranscriptionally modified. PMCA1b is the only isoform predominantly expressed in small intestine and kidney. It has been shown experimentally that in variable circumstances the extrusion capacity of PMCA is more than adequate, suggesting that the Ca$^{2+}$ exit step is not necessarily a prime target for regulation by 1,25(OH)$_2$D$_3$. This could perhaps explain the difficulty in observing a consistent stimulatory effect of 1,25(OH)$_2$D$_3$ on PMCA. In VDR knockout mice strains, the expression of PMCA1b is not altered in duodenal samples, whereas in 1,25(OH)$_2$D$_3$ repletion studies performed in chicken, rat, and mice, an increase in intestinal PMCA1b expression was observed.

The overall conclusion is that the stimulatory effect of 1,25(OH)$_2$D$_3$ on intestinal Ca$^{2+}$ absorption results primarily from an increased expression of ECaC and calbindin-D$_{9K}$. Thus, during periods of high Ca$^{2+}$ demand, the activity of ECaC is increased in the brush-border membrane of the enterocyte via a 1,25(OH)$_2$D$_3$-dependent genomic mechanism. The concomitant increase in the cytosolic content of calbindin-D$_{9K}$ serves two important physiological functions. It guarantees sufficient buffering and inactivation of Ca$^{2+}$ ions in the close vicinity of the channel to prevent a Ca$^{2+}$-induced inactivation of channel activity and accelerates the transfer of Ca$^{2+}$ to the basolateral extrusion pumps. In this way, 1,25(OH)$_2$D$_3$ can efficiently promote high rates of transcellular Ca$^{2+}$ absorption in the small intestine.

### B. Phosphate Absorption

The intestinal P$_i$ absorption process occurs both by a Na$^+$-independent, nonsaturable pathway and by an active, Na$^+$-dependent mechanism present mainly in duodenum and jejunum. This latter process is subject to chronic regulation by 1,25(OH)$_2$D$_3$. Ample studies have demonstrated that the stimulatory effect of 1,25(OH)$_2$D$_3$ occurs through an increased rate of Na$^+$–P$_i$ co-transport present in the brush-border membrane, but the molecular details have not been elucidated. To date, three different families of Na$^+$–P$_i$ co-transporters have been identified, but the physiological role of these transporters in intestinal P$_i$ absorption remains to be firmly established. The so-called NaP$_i$ type IIb co-transporter is expressed in the small intestine and is located in the apical membrane. The abundance of this transporter is up-regulated by 1,25(OH)$_2$D$_3$, but a concomitant increase in mRNA levels was not observed in adult animals. This suggests that posttranscriptional mechanisms are involved in Na$^+$-dependent P$_i$ co-transporter activity, contributing to the observed 1,25(OH)$_2$D$_3$-induced stimulation of P$_i$ absorption. Little is known about the molecular mechanisms responsible for the extrusion of P$_i$ across the intestinal basolateral membrane into the circulation. Future work, in which all apical and basolateral P$_i$ transporters will be identified, must be completed before a comprehensive molecular description of 1,25(OH)$_2$D$_3$ action on intestinal P$_i$ absorption can be provided.

## III. BIOLOGICAL EFFECTS IN THE KIDNEY

Renal tubular reabsorption of Ca$^{2+}$ and P$_i$ is a key element in overall mineral homeostasis and also involves hormone-regulated active transport mechanisms. The molecular identification of the responsible Ca$^{2+}$ and P$_i$ transporters provided tools to study the regulation of tubular transport function at the cellular and organ levels and will ultimately disclose the full-delineated mechanisms involved in vitamin D-controlled mineral excretion.

### A. Calcium Reabsorption

The distal part of the nephron is the main site of hormone-regulated transcellular Ca$^{2+}$ transport, which is at the cellular level realized by a three-step process similar to that described for the intestine. However, there are distinctive differences between intestinal and renal active Ca$^{2+}$ transport. For instance, renal Ca$^{2+}$ reabsorption is tightly controlled by PTH, but this calciotropic hormone has no direct effect on intestinal Ca$^{2+}$ absorption. The kidney contains a 28 kDa calbindin to facilitate the cellular Ca$^{2+}$ flow, but thus far only mouse, bovine, and rat also contain calbindin-D$_{9K}$. In addition, the kidney utilizes primarily the Na$^+$–Ca$^{2+}$ exchanger to extrude Ca$^{2+}$.

Although there has been some controversy concerning the role of 1,25(OH)$_2$D$_3$ in renal Ca$^{2+}$ transport and the mechanisms behind 1,25(OH)$_2$D$_3$ responsiveness, there is now accumulating evidence that this seco-steroid stimulates transcellular Ca$^{2+}$

transport in the distal part of the nephron. In a primary culture of rabbit distal tubular cells, 1,25(OH)$_2$D$_3$ increases transcellular Ca$^{2+}$ transport in a dose-dependent manner. In addition, extensive immunohistochemical and *in situ* hybridization studies revealed that the VDR is expressed in both proximal and distal segments but is highly enriched in the distal part of the nephron, a site known to be primarily involved in active transcellular Ca$^{2+}$ transport.

Comparable to the intestine, ECaC1 is the postulated gatekeeper of transepithelial Ca$^{2+}$ transport in the kidney and is, therefore, a candidate target for the action of 1,25(OH)$_2$D$_3$. The first indication that ECaC1 is indeed controlled by 1,25(OH)$_2$D$_3$ came from a vitamin D-deficient rat model. In kidneys of these depleted animals, ECaC1 mRNA and protein levels were significantly reduced. Repletion with 1,25(OH)$_2$D$_3$ completely restored the abundance of ECaC1, which was accompanied by a normalization of the plasma Ca$^{2+}$ concentration. This suggests a crucial function of this Ca$^{2+}$ influx channel in renal Ca$^{2+}$ handling.

The vitamin D-induced stimulation of ECaC in this rat model was accompanied by a comparable enhancement of calbindin-D$_{28K}$, confirming the established vitamin D dependence of this calcium-binding protein. In kidneys obtained from VDR knockout mice, the expression of calbindin-D$_{9K}$ was virtually abolished at both the mRNA and protein levels, whereas calbindin-D$_{28K}$ was only modestly decreased. Another mouse model for the study of vitamin D-dependent processes was established by ablation of the 25-hydroxyvitamin D 1α-hydroxylase gene [1α(OH)ase]. The synthesis of 1,25(OH)$_2$D$_3$ from its precursor 25-hydroxyvitamin D is catalyzed by the mitochondrial cytochrome P450 enzyme 25-hydroxyvitamin D 1α-hydroxylase D, which is primarily expressed in the kidney. The 1,25(OH)$_2$D$_3$ dependency was confirmed by the marked reduction in mRNA levels encoding renal calbindin-D$_{9K}$ and calbindin-D$_{28K}$ in the 1α(OH)ase null mice relative to wild-type littermates. These data clearly demonstrate that in mouse kidney, calbindin-D$_{9K}$ and calbindin-D$_{28K}$ are regulated by the VDR-mediated action of 1,25(OH)$_2$D$_3$.

The physiological implication of the concomitant increase in ECaC1 and calbindin-D levels is similar to the findings for the intestine, where the expression of ECaC2 and calbindin-D$_{9K}$ is closely coupled to vitamin D dependency of Ca$^{2+}$ absorption. Together with the fact that both calcium transport proteins are co-expressed in the same tissues, these findings suggest an obligatory functional coupling between ECaC and calbindin-D in order to mediate vitamin D-dependent Ca$^{2+}$ transport.

The effect of 1,25(OH)$_2$D$_3$ on the basolateral extrusion systems, NCX (Na$^+$–Ca$^{2+}$ exchanger) and PMCA, is less clear and remains controversial. Although NCX plays a dominant role in the extrusion process, many studies failed to establish a direct regulation by vitamin D. Exposure of 1,25(OH)$_2$D$_3$ to primary cultures of rabbit connecting tubules did not noticeably alter NCX expression. Surprisingly, a single pharmacological dose of 1,25(OH)$_2$D$_3$ significantly reduced the expression of NCX in mouse kidney. As in small intestine, 1,25(OH)$_2$D$_3$ upregulates PMCA1b protein expression and activity in kidney distal tubules and derived cell lines. Furthermore, 1,25(OH)$_2$D$_3$ enhanced PMCA1b mRNA stability. However, other reports in which primary cultures of renal cells or mice exposed to 1,25(OH)$_2$D$_3$ were used as model systems failed to show significant regulation of PMCA1b expression levels. Conversely, runoff reporter gene assays using 1.7 kb of the human PMCA1 promoter expressed in distal tubular cell lines demonstrated mRNA downregulation by 1,25(OH)$_2$D$_3$. Taken together, a consistent stimulatory effect of 1,25(OH)$_2$D$_3$ on the Ca$^{2+}$ extrusion mechanisms in the kidney remains to be established.

## B. Phosphate Reabsorption

The reabsorption of P$_i$ in the proximal tubule plays a primary role in overall P$_i$ homeostasis and involves an active P$_i$ transport mechanism. Vitamin D has been suggested to enhance the proximal tubular P$_i$ reabsorption in rats. It is, however, difficult to distinguish between direct and indirect effects since the vitamin D status is closely associated with plasma Ca$^{2+}$ and PTH concentrations. In contrast to the small intestine where 1,25(OH)$_2$D$_3$ has a direct effect on lumenal Na$^+$-dependent P$_i$ uptake, it remains, therefore, unclear whether 1,25(OH)$_2$D$_3$ directly regulates proximal tubular P$_i$ reabsorption.

The cellular scheme for the P$_i$ reabsorption process includes three different Na$^+$–P$_i$ co-transporters, namely, type I, type Iia, and type III; the type I and type Iia transporters are localized along the apical membrane, whereas type III is expressed in the basolateral membrane. A single study demonstrated that NaP$_i$-type IIa mRNA and protein were markedly decreased in the juxtamedullary cortex of vitamin D-deficient rats, but not in the superficial cortex. Luciferase reporter studies in COS7 cells, expressing the human VDR, confirmed the existence of a

TABLE I The Expression Level of $Ca^{2+}$ and $P_i$ Transporters during $1,25(OH)_2D_3$-Stimulated Transport in Small Intestine and Kidney

| $Ca^{2+}$ (re)absorption | $P_i$ (re)absorption |
|---|---|
| *Intestine* | |
| ECaC1 ↑ | $NaP_i$-IIb ↑ |
| ECaC2 ↑ | |
| Calbindin-$D_{9K}$ ↑ | |
| PMCA1b ↓ ↑ | |
| *Kidney* | |
| ECaC1 ↑ | $NaP_i$-IIa ↑ ? |
| Calbindin-$D_{9K}$ ↑ | |
| Calbindin-$D_{28K}$ ↑ | |
| PMCA1b ↓ ↑ | |
| NCX ↓ ↑ | |
| 1α(OH)ase ↓ | |

functional VDRE approximately 2 kb upstream from the transcription initiation site of the $NaP_i$-type IIa gene. At present, sufficient data are not available to postulate a direct role of $1,25(OH)_2D_3$ on proximal $P_i$ reabsorption in general and on the individual $P_i$ transporters in particular.

## IV. SUMMARY

Recently, detailed insights in the vitamin D action on $Ca^{2+}$ and $P_i$ homeostasis were obtained from several animal models including vitamin D-depleted rats and knockout mice in which the VDR and 1α(OH)ase genes were inactivated. The delineated effects on the individual $Ca^{2+}$ and $P_i$ transporters are summarized in Table 1. In general, $1,25(OH)_2D_3$ has profound stimulatory effects on the $Ca^{2+}$ transport proteins, explaining the vitamin D dependency of transepithelial $Ca^{2+}$ transport in small intestine and kidney. The situation is less clear for renal and intestinal $P_i$ handling. Only in the small intestine it is well established that vitamin D stimulates $Na^+$-dependent $P_i$ transport, but the elucidation of the molecular mechanism awaits future investigations.

## Glossary

**calbindin-D** Vitamin D-dependent $Ca^{2+}$-binding proteins consisting of a 28 kDa protein (calbindin-$D_{28K}$) and a 9 kDa protein (calbindin-$D_{9K}$) that bind $Ca^{2+}$ with high affinity and facilitate diffusion of cytosolic $Ca^{2+}$.

**ECaC** Epithelial calcium channels that were identified in kidney and small intestine as ECaC1 and ECaC2 (also known as CaT1), respectively, and are the gatekeepers in active $Ca^{2+}$ (re)absorption.

**NaPi** Sodium–phosphate co-transporters that mediate the movement of extracellular phosphate into cells driven by the existing $Na^+$ gradient. To date, three different families, named types I, II, and III, have been identified.

**NCX** $Na^+$–$Ca^{2+}$ exchanger present in the plasma membrane and the kidney; it is primarily responsible for the basolateral extrusion of $Ca^{2+}$ in the distal tubular cells.

**1α(OH)ase knockout mice** A mouse strain in which the key enzyme 25(OH)D-1α-hydroxylase has been inactivated, mimicking the genetic disorder vitamin D-dependent rickets type I, also known as pseudo-vitamin D-deficiency rickets.

**PMCA** Plasma membrane $Ca^{2+}$ ATPase that mediates the extrusion of $Ca^{2+}$ across the basolateral membrane. To date, four different genes have been identified; PMCA1b is the predominant isoform expressed in the small intestine and the distal part of the nephron.

**VDR knockout mice** A mouse strain in which the vitamin D receptor has been inactivated, mimicking the autosomal recessive disorder hypocalcemic vitamin D-resistant rickets.

## See Also the Following Articles

**Vitamin D • Vitamin D and Human Nutrition • Vitamin D-Binding Protein • Vitamin D: Biological Effects of $1,25(OH)_2D_3$ in Bone • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D-Dependent Calbindins (CaBP) • Vitamin D Metabolism**

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# Vitamin D Deficiency, Rickets and Osteomalacia

David Feldman and Peter J. Malloy
*Stanford University*

I. INTRODUCTION
II. NORMAL VITAMIN D PHYSIOLOGY
III. VITAMIN D DEFICIENCY, RICKETS, AND OSTEOMALACIA
IV. HEREDITARY RICKETS
V. SUMMARY

**The complex interplay of 1,25-dihydroxyvitamin $D_3$ (calcitriol) and parathyroid hormone regulates mineral metabolism and proper delivery of minerals to the skeleton. Supply of adequate calcium and phosphate to bone-forming sites is critical for synthesizing normal bone and for preventing rickets or osteomalacia. A defect in any step in the pathway of synthesis or action of 1,25-dihydroxyvitamin $D_3$ can cause these diseases.**

## I. INTRODUCTION

Bone is a complex, living tissue composed of a mineralized protein matrix that forms a hard and rigid skeletal framework. Osteoblasts are the bone-forming cells that secrete the malleable collagen matrix that is eventually mineralized with hydroxyapatite, a calcium phosphate crystal. Bone is constantly turning over; osteoclast-mediated resorption in specific areas of bone is coupled to bone formation carried out by osteoblasts. In order to maintain normal mineralization of newly formed bone, adequate calcium and phosphate must be delivered to the osteoblasts at bone-forming sites. To meet this mineral requirement for normal bone formation and turnover requires the ingestion of adequate calcium and phosphate in the diet and the presence of adequate amounts of vitamin D, the hormone responsible for regulating mineral absorption from the gastrointestinal tract. If calcium or phosphate delivery to the bone-forming site is inadequate, undermineralized bone is formed. This process is called osteomalacia. If osteomalacia develops during childhood, while active bone growth is underway, the undermineralization of both bone and cartilage leads to rickets. This involves failure or delay of mineralization in the growth plates and joints as well as in other skeletal sites.

Undermineralized bone is characterized by excess osteoid, the collagenous bone matrix, relative to mineral. Normally, most osteoid is rapidly mineralized and only a small rim of unmineralized matrix can be found on bone trabeculae. However, in rickets or osteomalacia, the level of unmineralized osteoid is increased. Initially, osteoid surface and volume are increased, but osteoid thickness and lag times (delay in mineralizing osteoid) are normal. In more severe cases, osteoid thickness exceeds 15 μm and mineralization lag time exceeds 100 days. In the most florid cases, no mineralization is detected. The strength of unmineralized osteoid is greatly diminished compared to normally mineralized bone, leading to metabolic bone disease and increased susceptibility

## B. Chick Egg Hatchability

Long-term studies of White Leghorn hens that received only exogenous vitamin D metabolites from the day of hatching have shown that $24R,25(OH)_2D_3$ is required in combination with $1\alpha,25(OH)_2D_3$ for the normal hatchability of the fertilized eggs. In a follow-up study of Japanese quail, only the naturally occurring $24R,25(OH)_2D_3$ [i.e. not the artificial $24S,25(OH)D_3$] in combination with $1\alpha,25(OH)_2D_3$ supported normal egg hatchability.

## C. Fracture Healing

In an *in vivo* model of fracture healing in the chick, the renal 24*R*-hydroxylase activity increases threefold approximately 1 week following imposition of a tibial fracture; this is accompanied by a similar increase in the levels of circulating $24R,25(OH)_2D_3$. When the degree of fracture healing as reflected in bone strength is measured, $24R,25(OH)_2D_3$, but not $24S,25(OH)D_3$, when given in combination with $1\alpha,25(OH)_2D_3$ (a dose that is ineffective by itself), results in bone strength equivalent to that seen in control animals receiving $25(OH)D_3$.

Based on these observations, it is possible to envision the existence of an endocrine system linking a bone fracture to the kidney $25(OH)D_3$-24*R*-hydroxylase, resulting in the subsequent elevation of the serum $24R,25(OH)_2D_3$ levels. The increased availability of this steroid hormone allows occupancy of the proposed vitamin D receptor ($VDR_{mem\ 24,25}$), which then initiates, in collaboration with $1\alpha,25(OH)_2D_3$, appropriate transduction signals that orchestrate the competent healing of the fracture (see Fig. 1).

## D. Cartilage Cells

$24R,25(OH)_2D_3$ has biological effects distinct from those of $1\alpha,25(OH)_2D_3$ on a subset of cells in growth plate cartilage. One hallmark of a steroid hormone is that it is preferentially accumulated by its target cells *in vivo* on a high-affinity receptor that tightly binds the hormone and effectively removes it from the blood compartment. This standard has been achieved for $24R,25(OH)_2D_3$ in relation to growth plate cartilage cells of the rat. These cells selectively accumulate tritiated $24R,25(OH)_2D_3$ that is been administered *in vivo* to rats.

## IV. RECEPTORS FOR $24R,25(OH)_2D_3$

Unlike $1\alpha,25(OH)_2D_3$, which has a widely distributed nuclear receptor that mediates an array of biological responses, the seco steroid 24*R*, $25(OH)_2D_3$ likely produces biological responses in a more limited

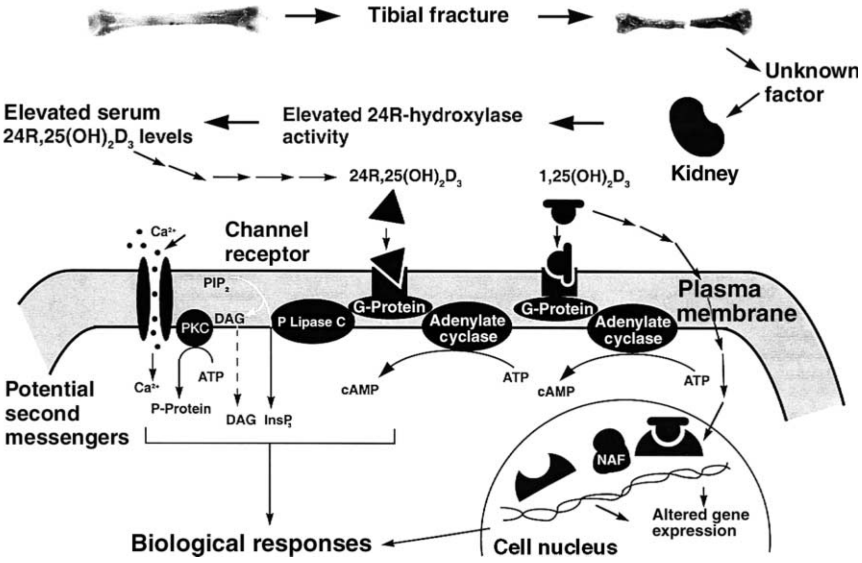
Tibial fracture
Unknown factor
Elevated serum $24R,25(OH)_2D_3$ levels
Elevated 24R-hydroxylase activity
Kidney
$24R,25(OH)_2D_3$
$1,25(OH)_2D_3$
$Ca^{2+}$
Channel receptor
$PIP_2$
PKC
DAG
P Lipase C
G-Protein
Adenylate cyclase
G-Protein
Adenylate cyclase
Plasma membrane
Potential second messengers
$Ca^{2+}$
ATP
P-Protein
DAG
$InsP_3$
cAMP
ATP
cAMP
ATP
NAF
Altered gene expression
Biological responses
Cell nucleus

**FIGURE 1** Proposed mode of action of $24R,25(OH)_2D_3$ for the process of fracture healing. After imposition of a bone fracture, an unknown factor is produced and delivered systemically to the kidney proximal tubule, leading to a significant increase in the activity of the $25(OH)D_3$-24*R*-hydroxylase. The resultant elevation of the plasma level of $24R,25(OH)_2D_3$ leads to occupation of membrane vitamin D receptors believed to be present in the fracture-healing callus, and thereby initiating signal transduction processes and contributing to the process of fracture healing. PKC, Protein kinase C; DAG, diacylglycerol; $InsP_3$, inositol 1,4,5-trisphosphate; NAF, nuclear activation factor.

TABLE 1 Biological Responses Attributed to $24R,25(OH)_2$ Vitamin $D_3$

| Organ cell system | Response studied |
|---|---|
| Parathyroid gland | Regression of hypertrophied glands; inhibition of PTH secretion |
| Egg hatchability | Required in combination with $1\alpha,25(OH)_2D_3$ for normal egg hatchability |
| Bone | Healing of rachitic lesions with increased bone strength; healing of fractures with increased bone strength |
| Chondrocytes (resting zone) | Activation of protein kinase C; inhibition of prostaglandin E2 production; increased activity of phospholipase C |

sphere, consisting of cartilage and bone cells and possibly parathyroid hormone-secreting cells. Table 1 summarizes the biological responses that have been reported to be mediated by $24R,25(OH)_2D_3$.

The stereospecificity at the C-24*R* position of $24R,25(OH)_2D_3$ vs $24S,25(OH)D_3$, for the effects described for parathyroid glands, egg hatchability, and bone fracture healing, suggests the existence of a receptor that can distinguish between the two compounds. The search for $24R,25(OH)_2D_3$-specific receptors is an active area of current investigation. Thus far, there has been no evidence for the existence of a nuclear receptor, but preliminary evidence suggests that there is a membrane-associated receptor-binding protein ($K_d \approx 18 \pm 1.9$ n*M*) for $24R,25$- $(OH)_2D_3$ in the chick fracture-healing callus. The binding of $[^3H]1\alpha,25(OH)_2D_3$ to the callus membrane receptor-binding protein is specific in that it is competed for only by nonradioactive $24R,25$- $(OH)_2D_3$, and not by $24S,25(OH)D_3$, $1\alpha,25(OH)_2$- $D_3$, or $25(OH)D_3$. Evidence has also been obtained that growth zones cartilage cells of bone and osteoblasts have a membrane receptor for $24R,25(OH)_2D_3$.

The first mechanistic insight into the signal transduction pathways activated by $24R,25$-$(OH)_2D_3$ in cartilage resting zone cartilage cells includes activation of protein kinase C (PKC) via effects on phospholipase $A_2$, as well as possible effects on increasing the activity of phospholipase C.

## V. SUMMARY

Results from a number of studies support a role for $24R,25(OH)_2D_3$ in the parathyroid gland and in hatching of the avian embryo. Current evidence also supports the concept that $24R,25(OH)_2D_3$, acting through its receptor, plays an important role in bone fracture healing and in the maturation of growth plate cartilage cells. Biochemical details of these proposed receptors and further details on the signal transduction pathways that they activate might emerge in future studies.

## Glossary

**endocrine system** Network of endocrine glands (sources of a hormones) and target cells (locations of the hormones receptors) involved in integrated interactions that generate regulated, selective biological responses necessary to maintain the system viability.

**hormone** Any of several chemical classes of compounds produced in regulated quantities by an endocrine gland; acts as a chemical messenger, usually delivered through the circulatory system to a target cell, which, by definition, possesses a receptor for that hormone so that a specific biological response is generated.

**receptor** Protein molecule that binds very specifically to its cognate hormone; binding generates a receptor–hormone complex that initiates a cellular signal transduction process, resulting in one or more biological responses.

**steroid** Member of the lipid class of compounds; composed of the cyclopentano-perhydro-phenanthrene nucleus, a four-ring structure that is the basic structural component of steroid hormone families such as estrogens, progestogens, androgens, mineralocorticoids, and glucocorticoids.

**vitamins** Essential organic substances present in trace amounts in food; effect the normal cellular metabolic processes.

## See Also the Following Articles

**Steroid Nomenclature • Vitamin D • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D-Binding Protein • Vitamin D Deficiency, Rickets and Osteomalacia • Vitamin D Metabolism**

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# Vitamin D Effects on Cell Differentiation and Proliferation

Chantal Mathieu, Annemieke Verstuyf,
Siegfried Segaert, and Roger Bouillon
*Katholieke Universiteit Leuven, Belgium*

I. INTRODUCTION
II. CANCER
III. SKIN
IV. IMMUNE SYSTEM
V. SUMMARY

**1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$] exerts its effects via the vitamin D receptor (VDR), which belongs to the steroid/thyroid hormone receptor superfamily, leading to gene regulation mediating various biological responses. Within the past two decades, the receptor has been shown to be present not only in classical target tissues such as bone, kidney, and intestine but also in many other nonclassical tissues, e.g., in the immune system (T and B cells, macrophages, and monocytes), in the reproductive system (uterus, testis, ovary, prostate, placenta, and mammary glands), in the endocrine system (pancreas, pituitary, thyroid, and adrenal cortex), in muscles (skeletal muscle, smooth muscle, and heart muscle), in brain, in skin, and in liver.**

## I. INTRODUCTION

In addition to the almost universal presence of vitamin D receptors (VDRs), some cell types (e.g., keratinocytes, monocytes, bone, and placenta) are capable of metabolizing 25-hydroxyvitamin $D_3$ to 1,25$(OH)_2D_3$ by the enzyme 1α-hydroxylase. The combined presence of 25(OH)$D_3$-1α-hydroxylase and the specific receptor in several tissues introduced the idea of a paracrine role for 1,25$(OH)_2D_3$. Moreover, it has been demonstrated that 1,25$(OH)_2D_3$ can induce differentiation and inhibit proliferation of normal and malignant cells. In addition to the treatment of bone disorders with 1,25$(OH)_2D_3$, these newly discovered functions of 1,25$(OH)_2D_3$ open new therapeutic applications as an immune modulator (e.g., for the treatment of autoimmune diseases or prevention of graft rejection), inhibitor of cell proliferation and selectivity and, perhaps just as importantly, to bind to DNA. Both were features consistent with those of a protein capable of mediating vitamin D-dependent gene expression. It was the molecular cloning of the VDR, however, that led to new insights into receptor structure and function and which enabled the tremendous progress that has been made recently in understanding how hormones such as $1{,}25(OH)_2D_3$ function to regulate gene expression.

## A. General Organization of Steroid Receptors

The general structural organization of the VDR and other members of the steroid receptor family is illustrated in Fig. 1a. As can be seen, the typical hormone receptor is composed of an amino-terminal domain, a more centrally located DNA-binding domain (DBD), which is the hallmark of the nuclear receptor family, a linker region, and a large carboxy-terminal ligand-binding domain (LBD) that is capable of binding not only hormonal ligands but also additional transcriptional co-regulators that are directly responsible for the activation (or repression) of gene expression. Receptors that contain large amino-terminal extensions can also modulate gene expression through this region. As seen in Fig. 1b, the overall structure of the VDR is generally similar with the exception that its amino-terminal domain is somewhat abbreviated relative to that of other receptor family members. As a consequence, the VDR relies heavily on the activation region found within the carboxy-terminal LBD. The key structural elements of the VDR (as well as other family members) are, therefore, the DNA-binding domain, which specifies the genes that are to be regulated, and the carboxy-terminal domain, which mediates the transcriptional regulatory process following binding and activation by $1{,}25(OH)_2D_3$ or other steroidal ligands.

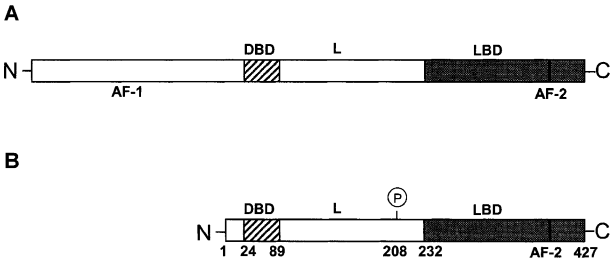

FIGURE 1 General structural organization of members of the nuclear receptor gene family. (A) Organization of a prototypical steroid receptor. N, amino-terminus; C, carboxy-terminus; DBD, DNA-binding domain; L, hinge or linker region; LBD, ligand-binding domain; AF, activation function. (B) Organization of the human vitamin D receptor. Designations are as in (A). Numbers indicate amino acid residues. P, phosphorylated serine 208.

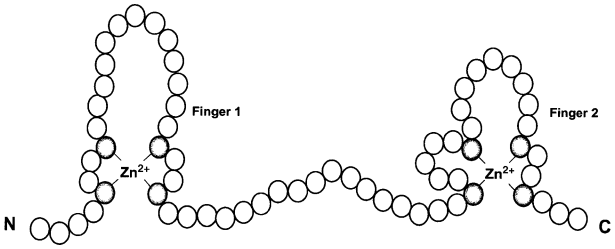

FIGURE 2 Organization of the two zinc-finger structures found in the DNA-binding domain of the VDR as well as all members of the steroid receptor gene family. C, cysteine residue; Zn, zinc atom.

## B. The DNA-Binding Domain of the VDR

The DBD of the VDR and the nuclear receptor family consists of two similar modules, each composed of a zinc-coordinated finger structure (Fig. 2). Each zinc atom is coordinated in a tetrahedral fashion through four highly conserved cysteine residues that serve to stabilize the finger structure itself. Although the two zinc modules of the VDR appear to be highly related structurally, topologically they are not equivalent as a result of differing chirality of the residues in each module that coordinate the zinc atom. More importantly, the function of each of these modules in DNA binding is known to be substantially different. Whereas the amino-terminal module functions to direct specific DNA binding in the major groove of the DNA-binding site, the carboxy-terminal module serves as a dimerization interface for interaction with DNA-binding protein partners. Recent studies have led to the elucidation of the three-dimensional structure of the DNA-binding domain of several nuclear receptors while bound to DNA.

## C. The Ligand-Binding Domain of the VDR

The LBD of the VDR is responsible for the receptor's functional activity. This region contains both a dimerization domain that permits essential interaction with the retinoid X receptor (RXR), a key DNA-binding protein partner, and an activation domain that directs the recruitment of co-regulatory proteins such as steroid receptor co-activator-1 (SRC-1), glucocorticoid receptor-interacting protein (GRIP), and D receptor-interacting protein 205 ($DRIP_{205}$). These factors as well as others mediate linkage to large

TABLE 1 Target Gene, Promoter Location, and Nucleotide Sequence of Natural Vitamin D-Response Elements

| Target gene | Location | Nucleotide sequence | | |
|---|---|---|---|---|
| Rat osteocalcin | −460/−446 | GGGTGA | atg | AGGACA |
| Human osteocalcin | −499/−485 | GGGTGA | acg | GGGGCA |
| Mouse osteopontin | −757/−743 | GGTTCA | cga | GGTTCA |
| Rat calbindin D-9K | −489/−475 | GGGTGT | cgg | AAGCCC |
| Rat 24-hydroxylase | −150/−136(Prox) | AGGTGA | gtg | AGGGCG |
| | −258/−244(Dist) | GGTTCA | gcg | GGTGCG |
| Human 24-hydroxylase | −169/−155(Prox) | AGGTGA | gcg | AGGGCG |
| | −291/−277(Dis) | AGTTCA | ccg | GGTGTG |
| Human p21 | −779/−765 | AGGGAG | att | GGTTCA |

protein machines responsible for altering chromatin structure and facilitating recruitment of RNA polymerase II, both of which are integral to the modulation of gene expression. Most importantly, however, the structural integrity and functional activity of both of these protein-interacting domains within the VDR are under the control of 1,25(OH)$_2$D$_3$. This tight regulatory capacity of the hormone is achieved by its ability to occupy a small pocket within the receptor and to induce significant conformational changes necessary for activity. Thus, the limited functional capabilities of a very small molecule such as 1,25(OH)$_2$D$_3$ are expanded through its interaction with a much larger macromolecule to confer multifunctional cellular capabilities. Recent efforts have resulted in determination of the three-dimensional structure of the LBD of the VDR. The crystal structure both confirms previous biochemical and molecular studies of VDR and provides new avenues of research aimed at understanding the structure and function of this important molecule.

## III. REGULATION OF GENE EXPRESSION THROUGH DNA BINDING

The vitamin D hormone is known to regulate a host of genes, a few of which are indicated in Table 1. Perhaps the first to be identified was the chicken vitamin D-dependent calbindin gene, whose product is involved in facilitating intestinal calcium absorption. More recently, gene targets include the 25-hydroxyvitamin D$_3$-24-hydroxylase, osteocalcin, osteopontin, and p21. An initial understanding of how 1,25(OH)$_2$D$_3$ and its receptor might regulate gene expression was derived initially from studies of the human osteocalcin gene. Accordingly, a hexanucleotide repeat DNA sequence separated by 3 bp that represented a specific binding and transcriptional activation site (vitamin D-response element or VDRE) for the VDR was discovered upstream of the start site of transcription within the human osteocalcin gene promoter. This element as well a similar sequence found in the rat osteocalcin gene provided a first glimpse at a binding site for the VDR within a 1,25(OH)$_2$D$_3$-regulated gene. The sequences of these VDREs as well as several additional ones that were discovered in the mouse osteopontin gene, mouse calbindin -D-28K, rat calbindin D-9K, the rat and human 25-hydroxyvitamin D$_3$-24-hydroxylase genes (two apparent VDREs), and the human p21 gene are documented in Table 1.

## IV. THE VDR FUNCTIONS AS A HETERODIMER

The duplicated nature of the VDRE in the osteocalcin gene and other gene promoters suggested that VDR might bind as a homodimer to DNA identical to that for other steroid receptors. Despite this precedent, the VDR was surprisingly found to bind to VDREs as a heterodimer with a previously identified protein, termed the RXR (Fig. 3). This interaction requires a protein dimerization surface within the DBD of both VDR and RXR as well as one located within the carboxy-terminus of both proteins. Additional studies indicate that the VDR/RXR interaction with DNA exhibits polarity. As seen in Fig. 3, RXR binds to the 5′ or upstream promoter half-site of the VDRE and the VDR occupies the downstream site. There are three RXR isotypes that can interact with the VDR, thereby increasing the potential complexity of gene regulation. Interestingly, each of these forms also interacts with other nuclear receptors, including the thyroid hormone receptor and the retinoic acid receptor. Thus, all receptors that utilize RXR as a common partner in a single cell likely compete for this protein during transcriptional activation.

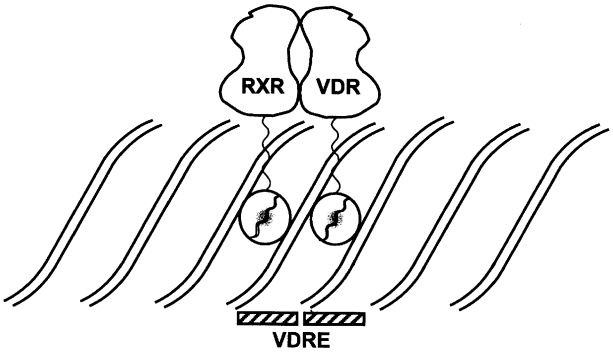

FIGURE 3 Heterodimeric binding of the VDR to a VDRE in a hormone-sensitive gene promoter. RXR, retinoid X receptor; VDR, vitamin D receptor.

## V. ROLE OF 1,25(OH)$_2$D$_3$ IN VDR ACTIVATION

1,25(OH)$_2$D$_3$ induces conformational changes in the VDR that lead to significant rearrangements within the receptor, particularly those that form important protein-interacting surfaces. Although these changes have not been visualized directly within the VDR using X-ray crystallography, as has been accomplished for certain steroid receptors, three-dimensional modeling studies based upon similarity in receptor structure strongly support such hormone-induced rearrangements. A variety of biochemical and cellular assays also support in an indirect fashion these conformational changes. For example, ligand binding significantly enhances VDR stability and induces strong resistance to proteolytic degradation *in vitro*. It is now known that the molecular changes that are induced by 1,25(OH)$_2$D$_3$ within the VDR have a functional consequence, namely, to promote the dimerization of VDR with its RXR partner. Indeed, initial studies suggested that the affinity of the VDR for RXR increased almost 10-fold for its protein partner when 1,25(OH)$_2$D$_3$ was present. Since these interactions occurred in the absence of VDRE DNA, it suggests that the initial event in vitamin D-dependent gene activation is the formation of a functional receptor module composed of ligand-occupied VDR and RXR. Formation of this complex is followed by VDRE binding. It is now clear that the conformational changes induced by 1,25(OH)$_2$D$_3$ also lead to the formation of a new surface or surfaces capable of binding directly to members of at least several different classes of co-modulatory proteins. These proteins are essential to the complex transcriptional regulatory process. Thus, a host of latent activities within receptors such as VDR are uncovered when hormonal ligand is present converting the VDR into a transcriptionally active protein.

## VI. VDR RECRUITMENT OF TRANSCRIPTIONAL CO-REGULATORS

Early working models of steroid receptor action placed the receptor in contact with the general transcriptional apparatus, thus directly modulating transcription. Transcriptional regulation is considerably more complicated than originally envisioned, however. Complex sets of molecular machinery capable of modifying chromatin structure and recruiting RNA polymerase II are involved in facilitating transcription and different sets promote transrepression. Thus, 1,25(OH)$_2$D$_3$ functions to target this machinery to the promoters of vitamin D-sensitive genes. These targeting events are mediated by the VDR, first through its interaction with selective sequences of DNA and then through its ability to sequentially recruit additional regulatory complexes.

### A. p160 Co-activators and Acetylation

Activation of gene expression requires numerous alterations in chromatin architecture. Thus, protein machines that are initially recruited by steroid receptors to hormonally responsive promoters contain histone acetyltransferases (HATs) capable of modifying lysine residues on histones and increasing transcription factor accessibility to DNA (Fig. 4). HATs exhibit different specificities for lysine residues on histones, providing a likely explanation why multiple proteins within the complex contain residual acetylating capabilities. Direct linkage between the receptor and these large protein complexes is provided in part by several proteins termed p160 co-activators, which contain HAT activity as well. SRC-1 represents the prototypic member. The primary interaction surface or docking site on the VDR that mediates receptor/SRC-1 interaction is designated activation function-2 (AF-2). The formation of this binding site is highly dependent upon the presence of hormone, which alters the placement of several of the approximately 12 $\alpha$-helices in the receptor LBD and completely rearranges the position of $\alpha$-helix 12. The p160 co-activators (as well as other proteins that bind to AF-2) interact with the receptor in turn through a specific leucine-charged helix termed the nuclear receptor box that contains central core residues of the pattern LXXLL. The VDR together with the RXR also interacts in a ligand-dependent manner with SRC-1 and other members of

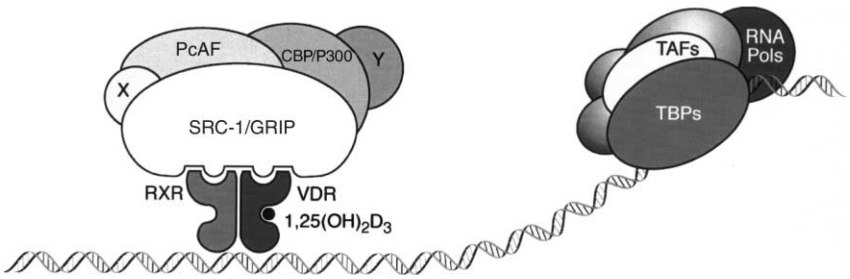

**FIGURE 4** Arrangement of the SRC-1/GRIP complex following recruitment to a $1{,}25(OH)_2D_3$-sensitive gene promoter. SRC-1 or GRIP binding to hormone-activated VDR and its silent partner RXR leads to the subsequent recruitment of additional transcription factors including CBP/p300 (CREB-binding protein/p300, where CREB denotes $Ca^{2+}$/cyclic AMP-response element-binding protein), P/CAF (p300/CBP-associated factor), and perhaps other unknown proteins (X and Y). This complex mediates transactivation though modification of chromatin-associated histone residues and through direct contacts with specific TAFs (transcription activating factor). RNA pols, RNA polymerase II; TBP, TATA box-binding protein.

the p160 co-activators, thus initiating the recruitment of machinery essential for the initial phase of the transcriptional activation process. Since the co-activator family of genes exhibits little specificity for individual receptors, it is likely that selectivity for the different co-activators that are found within a cell may be promoter context dependent. Despite this, it is clear that the recruitment of these HAT-containing complexes and additional proteins capable of chromatin modification are essential to the process of transactivation.

## B. Co-repressor Complexes and Deacetylation

Steroid receptors also function to suppress transcriptional activity. Not surprisingly, repression requires the recruitment of complexes that remove acetyl residues from histones, thereby producing a transcriptionally resistant form of chromatin. These proteins are histone deacetylases (HDACs). Nuclear receptors recruit such HDACs to specific chromatin templates indirectly via proteins such as nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoic acid and thyroid hormone (SMRT). Although the interaction site within the nuclear receptor for these repressors is also located at AF-2, the leucine-charged LXXLL motif within the nuclear co-repression is somewhat altered to a slightly different configuration. Both N-CoR and SMRT also interact with the VDR following activation by $1{,}25(OH)_2D_3$, presumably to decrease the ability of the receptor to activate transcription. Interestingly, several receptors including the thyroid hormone and retinoic acid receptors suppress gene expression in the absence of hormone. In these cases, N-CoR and SMRT are recruited to the promoter via receptors that are not bound to their respective hormonal ligands.

## C. The DRIP Complex

The DRIP complex, a fundamentally different molecular machine, also interacts with the VDR. The DRIP complex is composed of 10 or more proteins, none of which appear to contain either HAT or HDAC activity. The DRIP complex interacts not only with other members of the nuclear receptor family such as estrogen receptor, but with certain basal transcription factors as well. Recruitment of DRIP is achieved by the VDR through $DRIP_{205}$, a protein that contains several LXXLL motifs that interact with $1{,}25(OH)_2D_3$-activated VDR at AF-2. Although the function of the DRIP complex is not precisely understood at present, it may be involved in mediating the recruitment of RNA polymerase II to the DNA template.

The discovery that transcriptional regulation by nuclear receptors is mediated by downstream co-regulatory factors highlights the complexity of gene regulation. The diversity of these co-regulators is enormous. In addition to those mentioned, additional factors that provide synergistic regulation, that link nuclear receptors to unrelated transcription factors, and that enable mechanisms whereby receptors are degraded have been identified. Imposing these additional sets of factors into the steroid receptor functional pathway provides important ways in which a cell can modulate receptor action and integrate its actions with other incoming signals important for survival and function.

## VII. HEREDITARY RESISTANCE TO 1,25(OH)$_2$D$_3$

The central importance of the VDR to 1,25(OH)$_2$D$_3$ action is emphasized through studies of the human syndrome of hereditary 1,25(OH)$_2$D$_3$-resistant rickets. Early studies suggested that this genetic disease was due to defects in vitamin D signal transduction and experiments in cells from patients with the disease eventually implicated the VDR in this resistance pathway. However, it was cloning and structural and sequence analyses of the chromosomal gene for the VDR that enabled direct analysis of the VDR gene from patients with hereditary resistance. These efforts led to the discovery of a series of different mutations within the VDR that compromised its ability to function. Accordingly, mutations were found in the DBD as well as in the LBD. The former led to an inability of the receptor to bind to DNA, whereas the latter resulted in a receptor unable to bind 1,25(OH)$_2$D$_3$ or to interact with its RXR dimerization partner. Additional mutations continue to be found. Each of these alterations in VDR structure results in the loss of transcriptional activity and leads to significant defects in the skeleton as a result of altered calcium and phosphorus homeostasis. Genetic ablation of the VDR gene in mice has revealed many of the skeletal features found in the human and now provides an excellent animal model with which to study 1,25(OH)$_2$D$_3$ and VDR function.

## VIII. SUMMARY

1,25(OH)$_2$D$_3$ is a steroid-like hormone that functions to regulate mineral homeostasis and other biological events in higher organisms. This regulation is achieved in target tissues through selective control of gene expression, a process that is mediated by the VDR. The VDR is an intracellular receptor protein that belongs to the steroid receptor family of transcription factors and whose functional activities are activated by 1,25(OH)$_2$D$_3$. Functional activities include selective VDR/RXR binding to DNA sequences or VDREs followed by VDR-mediated recruitment of additional protein complexes essential to the transcriptional regulatory process. Significant details associated with this highly intricate regulatory step are now beginning to emerge. The importance of the VDR in the biological actions of vitamin D is seen in humans with genetic abnormalities in the VDR as well as in animals in which the VDR has been genetically ablated. It is likely that a fuller understanding of the mechanism of action of the vitamin D hormone and its receptor will be forthcoming during the next few years.

## Glossary

**activation function** A domain located within the ligand-binding region of steroid receptors that interacts with co-regulators, thus modulating transcription.

**co-regulators** Transcription factors that are recruited to active gene promoters via DNA-binding proteins such as the vitamin D receptor.

**hereditary** A human syndrome of generalized resistance to 1,25-dihydroxyvitamin D$_3$ due to inherited mutations in the vitamin D receptor.

**histone modification** Acetylation or deacetylation of lysine residues within DNA-bound histones that alter chromatin structure and transcription factor accessibility.

**retinoid X receptor** A member of the steroid receptor gene family that functions as a heterodimeric DNA-binding partner for the vitamin D receptor.

**steroid or nuclear receptor gene family** A large family of eukaryotic genes whose protein products both function as receptors for steroid and thyroid hormones, retinoic acid, and 1,25-dihydroxyvitamin D$_3$ and modulate transcription.

**target genes** Genes that can be regulated by a specific hormone because their promoters contain regulatory elements that can interact with the hormone's receptor.

**vitamin D receptor** A specific protein that binds 1,25-dihydroxyvitamin D$_3$ and mediates the actions of this hormone in target tissues.

**vitamin D-response element** A specific DNA-binding site for the vitamin D receptor that is located within the promoter region of a gene that is regulated by 1,25-dihydroxyvitamin D$_3$.

## See Also the Following Articles

**Vitamin D • Vitamin D and Cartilage • Vitamin D and Human Nutrition • Vitamin D: Biological Effects of 1,25(OH)$_2$D$_3$ in Bone • Vitamin D: Biological Effects of 1,25(OH)$_2$D$_3$ in the Intestine and Kidney • Vitamin D: 24,25-Dihydroxyvitamin D • Vitamin D Effects on Cell Differentiation and Proliferation • Vitamin D Metabolism • Vitamin D Receptors and Actions in Nonclassical Target Tissues**

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# Vitamin D Receptors and Actions in Nonclassical Target Tissues

MARIAN R. WALTERS
*Tulane Medical School, New Orleans*

I. INTRODUCTION
II. RANGE OF $1,25(OH)_2D_3$ TARGETS AND ACTIONS
III. $1,25(OH)_2D_3$ FUNCTIONS IN NONCLASSICAL TARGETS: COMMONALITIES AND GENERALITIES
IV. $1,25(OH)_2D_3$ FUNCTIONS IN NONCLASSICAL TARGETS: TISSUE-SPECIFIC EFFECTS
V. $1,25(OH)_2D_3$/VDR INHIBITS BREAST AND PROSTATE CANCER GROWTH
VI. SUMMARY

**Intestine, kidney, and bone tissues have long been known to have receptors for vitamin D. Vitamin D receptors are now known to exist in many other tissues, including those of skeletal and vascular smooth muscles, central nervous system components, the heart, endocrine and reproductive organs, the lungs, and the liver. 1,25-Dihdroxyvitamin $D_3$ interacts specifically with receptors in these tissues; the effects of these interactions are mediated by many intracellular signaling pathways. Genetic characterizations of the receptors and activation pathways hold promise in further understanding the roles of vitamin D in health and disease.**

## I. INTRODUCTION

Traditional studies of vitamin D receptors (VDRs) and their actions have focused on plasma calcium-regulating vitamin D targets such as intestine, kidney, and bone. However, in 1979, putative VDR sites were first described in "nonclassical" tissues—for example, in tissues as diverse as the pancreas, pituitary gland, and uterus. Thus began a new era in which the concept of the actions of the hormonal form of vitamin D, 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$], changed from its being considered solely a hormone that regulates plasma calcium homeostasis to understanding that it plays roles in numerous cellular processes, including proliferation/differentiation, hormonal secretion, and neuroprotection.

## II. RANGE OF $1,25(OH)_2D_3$ TARGETS AND ACTIONS

As detailed in Table 1, vitamin D receptors have now been described in numerous targets, including many cells of the immune system, cardiovascular tissues, skeletal muscle, reproductive and other endocrine sites, central nervous system, and a host of other tissues. Evidence for the presence of VDR sites in these tissues has come from a variety of experimental approaches (Table 2). Moreover, through the years, increasingly sophisticated approaches have been used to probe for the presence of VDRs in putative new target tissues. For example, a recent immunoblotting study has demonstrated the presence of VDRs in tissue extracts from the hippocampus, with more specific localization demonstrated by immunocytochemistry in the neuronal and glial cells in pyramidal and granule cell layers, including CA1, CA2, CA3, and the dentate gyrus. More functional studies have indicated that hippocampal extracts contain a VDR-like species capable of binding to the specific osteopontin VDR

**TABLE 1 Wnt Signaling Molecules[a] in Human Cancer**

| Wnts | sFRPs | CK2 | Axin | APC | β-Catenin |
|---|---|---|---|---|---|
| Breast cancer (WNT1, WNT5a)<br>Leukemia (WNT16) | Breast cancer | Breast cancer | Liver cancer | Colorectal cancer (spontaneous)<br>Familial ademomatous polyposis (genetic precancerous condition) | Colorectal cancer; liver cancer; ovarian cancer pancreatic cancer; prostatic cancer; skin cancer (melanoma); stomach cancer; uterine cancer |

[a] See text (Sections V and VI) for full molecule names and roles in Wnt signal transduction.

been linked to many other diseases besides cancer. A rare childhood disorder of bone formation, osteoporosis–pseudoglioma syndrome, is caused by a genetic disruption in canonical Wnt signaling. Disruptions in Wnt signaling have been proposed to underlie an assortment of other diseases as well. This is an area of understandably intense scientific and biomedical interest (Table 2).

## C. Vertebrate Development

The first clue to a role for Wnt/β-catenin signaling in vertebrate development came from overexpression studies in the classical embryonic system, the frog *Xenopus laevis*. When Wnt1 is ectopically expressed in cells that would normally contribute to the ventral embryo (i.e., the future belly), they instead adopt a dorsal and anterior fate (i.e., they become the future head). This induction of a "secondary dorsal axis" produces a striking phenotype: two-headed tadpoles. The ability of ectopic Wnt1 to induce a secondary dorsal axis derives from the participation of the canonical Wnt/β-catenin signaling pathway in the natural process of dorsal cell fate specification early in vertebrate embryogenesis. However, it remains unclear whether an endogenous intercellular Wnt signal, or only the downstream intracellular signaling cascade, is normally responsible for dorsal fate determination. Further developmental analyses in vertebrates have included both overexpression and loss-of-function studies in *Xenopus*, zebrafish, and mice and have established the importance of Wnt signaling in an array of processes in early development (Table 3).

## D. Invertebrate Development

In *Drosophila*, the contribution of wg signaling to developmental tissue patterning has been intensely investigated in several processes, especially the segmentation of the embryonic cuticle and the compartmentalization of the wing epithelial primordium

**TABLE 2 Wnt Signaling Molecules[a] Implicated in Human Disease**

| Wnt signaling (general) | Wnt4 | sFRP | Fz | LRP5 | Dvl | GSK3 | β-Catenin |
|---|---|---|---|---|---|---|---|
| Polycystic kidney disease (Polycystin-1 activates canonical Wnt signaling pathway) | Injury-induced renal fibrosis; wound healing | Heart failure | Ulcerative colitis | Osteoporosis-pseudoglioma syndrome (genetic syndrome of defective bone formation) | Ulcerative colitis; schizophrenia (mouse knockout model) | Familial Alzheimer's disease (through interaction with presenilin-1); schizophrenia (reduced levels in prefrontal cortex); bipolar disorder (enzymatic activity reduced by therapeutic agents) | Familial Alzheimer's disease (through interaction with presenilin-1) |

[a] See text (Sections V and VI) for full molecule names and roles in Wnt signal transduction.

TABLE 3 Some Wnt Signaling Developmental Activities across Species

| | *Caenorhabditis elegans* (nematode) | *Drosophila melanogaster* (fruit fly) | *Xenopus laevis* (frog) | *Danio rerio* (zebrafish) | *Mus musculus* (mouse) |
|---|---|---|---|---|---|
| Canonical | Embryonic polarity; mesoderms vs endodermal specification through asymmetric cell divisions (note: this pathway may be noncanonical) | Embryonic segmentation & wing disc compartmentalization | Embryonic axis specification & specification of axial vs somitic mesoderm | Patterning of neural ectoderm, mesoderm at gastrulation; specification of neural crest derivatives; specification & patterning of rostral midbrain/hindbrain; specification & patterning of head cartilage & tail | Embryonic axis specification, specification of caudal structures, specification of neural crest derivatives, specification & patterning of rostral midbrain/hindbrain, specification & proliferation; specification & patterning of urogenital system, differentiation of hair shaft precursors, placental development, osteogenesis, angiogenesis, adipogenesis |
| Noncanonical | Cell migration? | Wing hair orientation; compound eye facet orientation | Cell movements (convergent extension) during gastrulation | Cell movements (convergent extension) during gastrulation | Limb outgrowth? |

(wing disc). In the embryonic cuticle, a band of cells positioned at the posterior border of a future parasegmental boundary first expresses wg. Secreted wg activates expression of the engrailed (en) transcription factor in adjacent cells on the other (anterior) side of the parasegmental boundary. The en transcription factor activates the expression of a second secreted signaling molecule, hedgehog (hh), in the anterior cells. Hedgehog secreted by the anterior cells signals back across the future parasegmental boundary to the original wg-secreting cells, stabilizing their wg expression. The result is an intercellular positive-feedback loop that establishes each parasegmental boundary with cells expressing wg on the anterior border and cells expressing en/hh on the posterior border. In the wing disc, wg secreted by cells at the future dorsal–ventral boundary similarly helps pattern the surrounding tissue by inducing the expression of different genes in neighboring cells on both sides.

## E. NONCANONICAL SIGNALING

Genetic studies in *Drosophila* unexpectedly uncovered a completely different class of tissue patterning events that shares some (but not all) of the same genes implicated in the patterning of the embryonic segments and the wing disc. This "planar cell polarity" (PCP) pathway establishes the transverse orientation of cells making up an epithelial sheet. Examples of planar cell polarity in *Drosophila* include the orientation of facets making up the compound eye or the direction in which bristles point on the animal's back. During development, a subclass of wg receptors activate a distinct group of intracellular signaling molecules to regulate planar cell polarity. Because PCP signaling uses wg receptors but a different downstream signal transduction cascade than canonical wg signaling, it represents a separate "noncanonical" wg signaling pathway in *Drosophila* (see Section VI). A noncanonical signaling pathway has also been identified in vertebrates, where it is involved in coordinating major cell movements such as those of gastrulation. However, the correspondence between PCP signaling in *Drosophila* and noncanonical signaling in vertebrates remains ambiguous (see Section VI). Some of the more important and well-studied developmental events regulated by Wnt signaling in vertebrates and invertebrates are summarized in Table 3.

## II. THE Wnt PROTEIN FAMILY: STRUCTURE AND BIOCHEMISTRY

There are a total of 7 related *Wnt* genes in the *Drosophila* genome and 19 in the human genome (some with multiple isoforms) that generally have close orthologues in mice (Fig. 1). Orthologous Wnt gene products (proteins with the same function in different species) are often very highly conserved. For example, the mouse and human Wnt1 proteins are 99% identical, differing at only 4 amino acid residues within a cleavable secretory signal sequence at the amino-terminus. More distantly related Wnt family members can share less than 30% identity. The length of vertebrate Wnt protein precursors varies from approximately 100 amino acids to over 400 amino acids. All Wnt proteins contain a cleavable amino-terminal signal peptide that targets them for secretion, a conserved cysteine-rich composition, and multiple N-linked glycosylation sites. Wnt proteins synthesized *in vivo* are glycosylated. Once outside the cell, mature Wnt glycoproteins associate with extracellular matrix and bind to heparin sulfate proteoglycans (HSPGs). Genetic studies in *Drosophila* have demonstrated the importance of HSPGs in stabilizing extracellular wg protein and in regulating its distribution. The importance of proteoglycans for intact Wnt signaling *in vivo* has been confirmed independently in vertebrates. In *Drosophila*, non-wg-producing cells endocytose wg that has been secreted by neighboring cells. Genetic studies have demonstrated that this endocytic activity is important both for facilitating wg signaling and for regulating its distribution.

In *Drosophila* and in vertebrates, it is clear that Wnt proteins often act over a relatively short range: on cells located within a few cell diameters of the secretory source. More controversial is whether Wnt proteins may also act over longer distances. The morphogen hypothesis, which predicts that some signaling molecules specify distinct cellular identities along a concentration gradient, remains contested in the field of developmental biology. Nonetheless, some of the strongest experimental evidence in favor of a morphogen concerns the action of wg in the *Drosophila* wing disc.

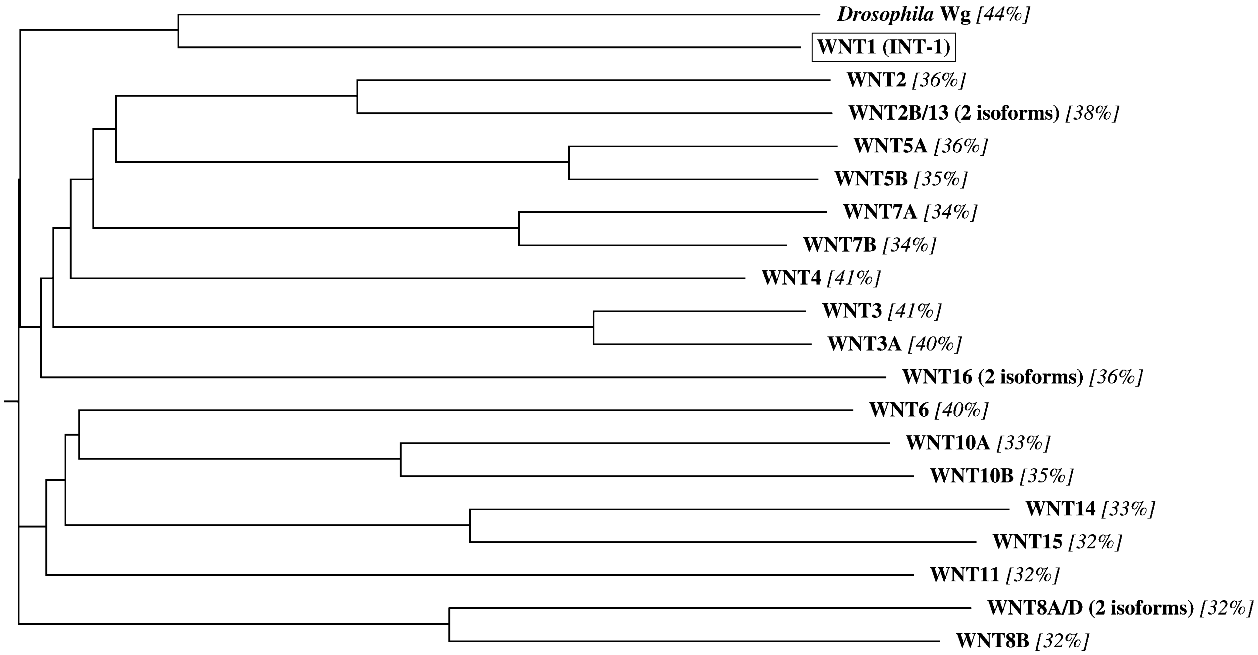

FIGURE 1 A sequence relatedness tree among the 19 human WNT gene products. *Drosophila* wg is included for comparison. The percentage of amino acid residues in each protein that are identical to WNT1 (boxed) is indicated. WNT1/INT-1 was chosen as the index sequence for comparison purposes purely for historical reasons (it is not evolutionarily ancestral to the other WNTs). This tree is based on overall identity among predicted Wnt gene products and is *not* meant to convey absolute evolutionary relationships. Horizontal distance from a node correlates with the degree of sequence divergence; vertical distance is arbitrary. Sequences were obtained from the protein database of the National Center for Biotechnology Information and compared using the default matrix settings of MacVector 6.5.3 (Oxford Molecular Group).

In addition to intercellular signaling, autocrine signaling by Wnt proteins (self-activation by a Wnt-secreting cell) may be important, both as a feedback mechanism during normal biological processes and in disease pathogenesis. Tumors induced by MMTV are clonal—suggesting that once the virus activates the *Wnt1* gene in a single mouse mammary gland cell, Wnt secretion stimulates uncontrolled proliferation by that cell and possibly other oncogenic changes as well. Similarly, some tissue culture cell lines transfected either with a *Wnt* gene or with DNA from MMTV-induced tumors become transformed and clonally tumorigenic. In *Drosophila*, there is evidence that autocrine signaling through wg occurs and activates a downstream signaling cascade that is genetically partly distinct from paracrine wg signaling.

It has been proposed that the vertebrate *Wnt* genes can be subdivided into functional classes based on whether they activate the canonical or noncanonical signaling pathways. However, recent studies have shown that in *Xenopus*, the developmental response to a Wnt of one proposed class can be switched to that of the other class by co-expressing a specific Wnt receptor. In the developing *Drosophila* eye and wing, the response to wg is influenced by the relative abundance and ligand affinity of the wg receptor proteins expressed in the target tissue. Synthesis of the available data from vertebrates and *Drosophila* suggests that the response to a specific Wnt signal *in vivo* is likely to be influenced both by the particular Wnt protein secreted and by the different receptor types (and downstream intracellular signaling molecules) present in the target tissue.

## III. RECEPTOR STRUCTURE AND BIOCHEMISTRY

There are two types of cell-surface molecules that are known to be necessary for Wnt/β-catenin signal transduction in responsive cells: the Frizzled (Fz) receptor family and a subclass of the low-density-lipoprotein-related protein (LRP) receptor family.

### A. Fz

Fz proteins constitute a family of seven-transmembrane domain receptors with a cysteine-rich extracellular domain. As a class, Fzs are structurally related to the superfamily of trimeric G-protein-coupled receptors (GPCRs). There are 4 *Fz* genes in *Drosophila* and 10 in humans, with close orthologues in mice. As a class, Fz proteins participate in both canonical and noncanonical signaling, but individual Fz receptors may differ in their basal (minus ligand) ability to activate signaling of each type, suggesting that structural differences among the Fz proteins contribute to functional specificity. There is contradictory evidence regarding the importance of the Fz extracellular domain for this specificity, but it is clear that the carboxyl-terminal tail and intracellular loops contribute. In other fields, there is mounting evidence that many seven-transmembrane domain proteins exist and act as multimers on the cell surface. In the case of the Fz receptors, there is no direct functional evidence for this, but crystallization studies have revealed a conserved dimerization interface in the extracellular cysteine-rich domain.

### B. LRP

*LRP5* and *LRP6* are closely related genes in humans and mice that are structurally distinct from other *LRP* family members. A single *Drosophila* orthologue exists and is called *arrow.* The proteins are single-transmembrane domain receptors with an extracellular domain composed of four amino-terminal epidermal growth factor-like repeats and three low-density-lipoprotein (LDL) receptor type A repeats. They have a relatively short proline-rich intracellular domain. Genetic evidence in *Drosophila* and loss-of-function and overexpression evidence in vertebrates support the conclusion that arrow and LRP5/6 are essential Wnt co-receptors with the Fz proteins. Based on initial genetic and mutational analyses in humans and mice, LRP5 is likely to be partly functionally redundant with LRP6. Unlike the Fz proteins, evidence from both vertebrates and *Drosophila* supports a role for these proteins only in the canonical Wnt signaling pathway, not in noncanonical signaling.

In *Drosophila*, arrow is essential for all aspects of canonical wg signaling, as demonstrated by the observation that the phenotype of an *arrow* null mutation closely resembles that of a *wg* null mutation. Similarly, an *LRP6* mutation in mice causes defects that are a combination of those seen with mutations in canonical mouse *Wnt* genes, whereas overexpression of LRP6 in vertebrates causes ectopic canonical pathway activation. The extracellular domain of LRP6 binds Wnt and interacts with the extracellular domain of Fz in a Wnt-dependent manner, whereas LRP5 activates canonical Wnt signaling when ectopically expressed in mammalian fibroblasts and interacts with canonical pathway cytoplasmic signaling components (see Section V).

## IV. CYTOPLASMIC SIGNAL TRANSDUCTION PROTEINS

It remains unclear how signals from the Wnt receptors at the cell surface are coupled to distinct downstream cytoplasmic signal transduction cascades. However, at least two types of proteins have been proposed to functionally link the receptors to downstream effectors in both canonical and PCP signaling: heterotrimeric G-proteins and the Dishevelled (Dvl) family of cytoplasmic proteins.

### A. G-Proteins

The topological resemblance of the Fz proteins to other seven-transmembrane receptors that couple to heterotrimeric G-proteins begs the question of whether Fz proteins signal through G-proteins as well. Positive evidence that G-proteins might be involved in Fz-mediated signal transduction has only recently begun to accumulate and at this time is restricted to overexpression studies in vertebrate systems and some antisense (loss-of-function) evidence in tissue culture cells. For example, stimulation of mammalian tissue culture cells by expressing one of the Wnt/Fz combinations implicated in canonical signaling can be blocked by pertussis toxin and other G-protein inhibitors. In *Xenopus* embryos, ectopic expression of a regulator of G-protein signaling (RGS) protein antagonizes canonical signaling and causes defects resembling those caused by ectopic expression of other Wnt pathway inhibitors.

Similarly, overexpression of Wnt5a in mammalian tissue culture cells, zebrafish, and *Xenopus* embryos activates a noncanonical signaling pathway that is selectively blocked by several G-protein inhibitors, including pertussis toxin. Work with chimeric Fz receptors containing the extracellular and transmembrane domains of the $\beta_2$-adrenergic receptor suggests that G-proteins may couple to the intracellular domain of Fz proteins. These experiments implicate $G_{\alpha o}$ and $G_{\alpha q}$ for Fz receptors activating canonical signaling and $G_{\alpha o}$ and $G_{\alpha t2}$ for Fz receptors activating noncanonical signaling. It should be noted here that the noncanonical pathway measured in these experiments is the Wnt/calcium pathway, which may or may not be distinct from PCP signaling in *Drosophila* (see Section VI). To date, neither a Wnt/calcium pathway nor a genetic requirement for heterotrimeric G-proteins has been described for wg signaling in *Drosophila*. Accordingly, it remains unclear whether G-proteins couple to Fz receptors to transduce all Wnt signals.

### B. Dvl

A well-established component of both canonical and PCP signaling is the cytoplasmic molecule Dvl. There are three Dvl paralogues each in mice and humans and a single gene (*dsh*) in *Drosophila*. All Dvl family members share three well-conserved domains (DIX, PDZ, and DEP, in order from amino-terminus to carboxyl-terminus). The domains have been shown through mutational analyses in both vertebrates and in *Drosophila* to mediate different aspects of Wnt signaling: the DIX and PDZ domains are most critical for canonical Wnt signaling, whereas the PDZ and DEP domains are most important for PCP signaling. These domains have no known intrinsic enzymatic activity, but instead act as protein–protein interaction interfaces. Consequently, Dvl is considered to be a regulative adapter or scaffold protein that brings together cytoplasmic signaling components in response to events at the cell membrane. In most cell types, Dvl proteins display a predominantly cytoplasmic, punctate distribution, though a more diffuse nuclear component has also been described. In *Xenopus* embryos, overexpression of Fz protein can cause a redistribution of Dvl to the inner cell membrane. Whether translocation of Dvl to the membrane is an important component of physiological Wnt signaling is somewhat unclear, and there are no reliable data that Dvl actually interacts with Fz. There is evidence from both *Drosophila* and vertebrates that activation of Wnt signaling is accompanied by phosphorylation of Dvl. Exactly how signals are transduced from the transmembrane Wnt receptors to Dvl, whether through heterotrimeric G-proteins or some other mechanism, remains an area of intense experimental investigation.

## V. THE CANONICAL Wnt/β-CATENIN PATHWAY

The canonical Wnt/β-catenin pathway is typified in vertebrates by responses to a subclass of the vertebrate Wnt proteins and Fz receptors and to LRP5 or LRP6; in *Drosophila* it is typified by wg, DFz2, and arrow. The central feature of this pathway is a multiprotein complex (degradation complex) that controls cytoplasmic concentration levels of the multifunctional protein β-catenin. β-Catenin plays a structural role at the inner cell membrane of adherens junctions, but also acts as a transcriptional co-activator in the nucleus. The concentration of soluble β-catenin in the cytoplasm, and therefore in the nucleus, is primarily determined by its rate of degradation through ubiquitination-dependent targeting to the proteosomal pathway. Glycogen synthase kinase-3 (GSK3 in vertebrates, zeste-white/shaggy in *Drosophila*) regulates this process by phosphorylating β-catenin, targeting it for degradation. In addition to GSK3 itself, other components of the degradation complex include Dvl, Axin, and APC. Whereas Dvl is a Wnt pathway activator, GSK3, Axin, and APC are all inhibitors. Axin and APC help to stabilize the activity of GSK3, thereby keeping β-catenin levels low and Wnt target gene transcription turned off in the absence of a Wnt signal (Fig. 2A).

During Wnt signaling, several molecular events that change the composition of the degradation complex occur. It remains unclear whether any of these events are directly mediated by the transmembrane receptor or associated proteins. As mentioned in Section V, in response to canonical Wnt signaling, Dvl becomes phosphorylated. Several different kinases, including casein kinase I (CK1), casein kinase 2 (CK2), and the Par1 kinase from *Drosophila*, have been implicated in this process and in Wnt-pathway activation, but which of these is most important *in vivo* remains unclear. Another protein called GSK3-binding protein (GBP or FRAT) binds both GSK3 and Dvl in response to canonical signaling, possibly preventing the continued association of GSK3 with other members of the degradation complex. There is also evidence that binding of a Wnt protein to LRP5 causes the translocation of Axin from the degradation complex to the inner cell membrane, followed by Axin degradation. Regardless of which of these molecular events is the key to cytoplasmic transduction of the Wnt signal, the net effect is to destabilize and/or inhibit the degradation

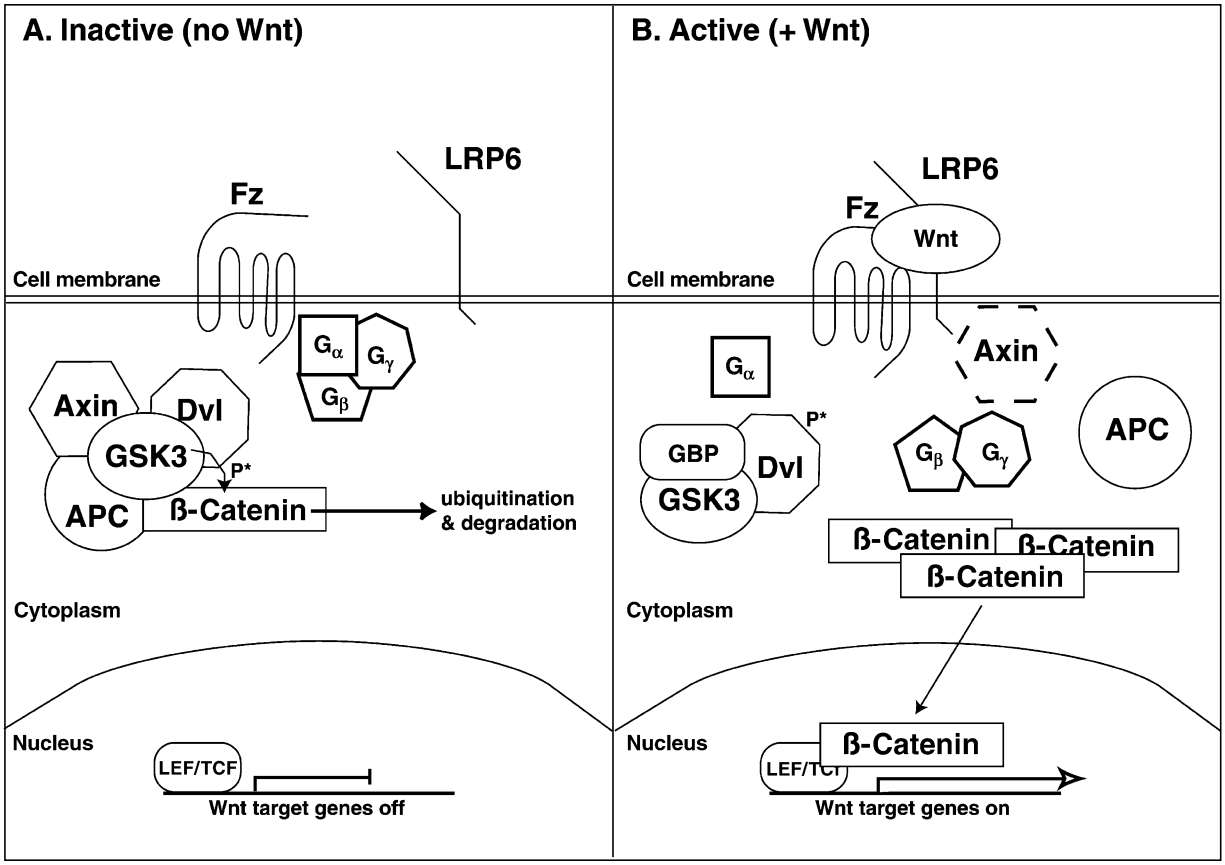
A. Inactive (no Wnt)
LRP6
Fz
Cell membrane
$G_\alpha$
$G_\gamma$
$G_\beta$
Axin
Dvl
GSK3
P*
APC
ß-Catenin
ubiquitination & degradation
Cytoplasm
Nucleus
LEF/TCF
Wnt target genes off
B. Active (+ Wnt)
LRP6
Fz
Wnt
Cell membrane
Axin
$G_\alpha$
P*
GBP
Dvl
GSK3
$G_\beta$
$G_\gamma$
APC
ß-Catenin
ß-Catenin
ß-Catenin
Cytoplasm
Nucleus
LEF/TCF
ß-Catenin
Wnt target genes on

FIGURE 2 The canonical Wnt signaling pathway. (A) Inactive (no Wnt). (B) Active (+ Wnt). Activation of a Fz/LRP6 receptor by a Wnt causes the phosphorylation of Dvl, translocation of Axin to the membrane, and association of GSK3 with GBP. The net effect of these changes is to down-regulate the phosphorylation-dependent degradation of β-catenin in the cytoplasm. β-Catenin accumulates and translocates into the nucleus where it acts as a transcriptional co-activator with members of the LEF/TCF gene family. There is evidence that heterotrimeric G-proteins may couple to the Fz receptor upstream of Dvl in this pathway, but this remains controversial (see text).

complex and thereby to prevent phosphorylation of β-catenin by GSK3. As a result, soluble β-catenin—no longer phosphorylated, ubiquitinated, and degraded—accumulates and translocates to the nucleus. In the nucleus, β-catenin acts with members of the LEF/TCF family of transcription factors to regulate the expression of Wnt target genes (Fig. 2B).

In addition to the well-established canonical pathway members mentioned above and depicted in Figs. 2A and 2B, several other cytoplasmic inhibitors of canonical Wnt/β-catenin signaling that generally share the ability to bind Dvl have been identified in recent years. These include the *Drosophila* gene *naked cuticle* (*nkd*) and the vertebrate proteins Idax and Dapper (Dpr). Both nkd and Dpr have been demonstrated to play crucial roles in the development of *Drosophila* and *Xenopus*, respectively. There are also several secreted extracellular inhibitors of canonical Wnt/β-catenin signaling that are biologically important for development and disease. These include cerberus (cer) and secreted Frizzled-related proteins (sFRPs), which bind Wnt and therefore act as general Wnt inhibitors; Dickkopf (dkk), which binds to LRP6 and is therefore specific for canonical signaling; and Wnt inhibitory factor-1 (WIF-1), which resembles the LRP extracellular domain but whose exact mechanism of action is unknown.

## VI. NONCANONICAL PATHWAYS: PLANAR CELL POLARITY AND Wnt/CALCIUM SIGNALING

Noncanonical Wnt signaling in *Drosophila* is defined by the planar cell polarity pathway mediated by the Fz receptor (which has redundant activity with DFz2 in the canonical pathway). Despite the fact that wg has been shown to bind to Fz and is often assumed to be the PCP effector, there is as yet no absolutely conclusive *in vivo* evidence that the endogenous ligand for Fz during PCP signaling is wg. The PCP pathway downstream of Fz has been defined on the basis of a combination of genetic and molecular data. As with the canonical Wnt pathway, the *Drosophila* Dvl homologue, dsh, is one of the most upstream cytoplasmic components of PCP signaling, although the requirement for the domains of dsh differ in the two pathways (see Section IV). As with canonical Wnt signaling, several dsh-interacting proteins that participate in PCP signaling have been identified, including the putative calcium-sensitive protein nkd and the Formin homology protein Daam1, which also binds the small GTPase RhoA. PCP signaling through dsh activates RhoA, which in turn activates a cascade of signaling proteins through the Jun-N-terminal kinase (JNK). The JNK cascade can alter gene expression through activation of the AP-1 transcription factor in the nucleus; the significance of this for PCP signaling is unknown. RhoA also activates the *Drosophila* Rho-associated kinase (Drok), which causes cytoskeletal changes by phosphorylating the nonmuscle myosin regulatory light chain and possibly other proteins (Fig. 3A).

Aside from the *Drosophila* PCP pathway molecules that fit into the signaling pathway described above, several other genetically identified components play roles that are not yet understood at a biochemical level. Although arrow/LRP6 is not required for PCP signaling through Fz, at least three other transmembrane proteins that could act as co-receptors or independently of Fz proteins have been implicated in PCP signaling: flamingo (fmi), a seven-transmembrane domain protein; strabismus (stbm or vang), a three-transmembrane domain protein; and fuzzy (fy), a four-transmembrane domain protein. Also implicated is the membrane-associated daschous (ds) protein, a member of the cadherin superfamily. Cytoplasmic proteins that play a role include the gene products of *prickle* (*pk*), *inturned* (*in*), and *multiple wing-hair* (*mwh*). How these proteins connect to other components of the *Drosophila* PCP pathway is not yet understood, but the noncanonical signaling function of at least some of them is conserved in vertebrate cells. For example, vertebrate Stbm and the DEP domain of Dvl have been demonstrated to participate in JNK cascade activation in mammalian cells.

In vertebrates, noncanonical Wnt/calcium signaling antagonizes canonical Wnt/β-catenin signaling and is typified by embryonic responses to Wnt5a and Wnt11. Based on studies primarily in zebrafish and *Xenopus*, these responses include an $IP_3$-mediated rapid increase of intracellular calcium, followed by activation of protein kinase C (PKC) and calcium/calmodulin-regulated kinase II (CamKII) (Fig. 3B). As mentioned in Section IV, there is considerable evidence that initial signaling downstream of Fz in the Wnt/calcium signaling pathway occurs through heterotrimeric G-proteins. Studies in mammalian cultured cells and in *Xenopus* embryos have demonstrated that Wnt5a activates the JNK cascade, providing a possible link between the vertebrate Wnt/calcium signaling pathway and the *Drosophila* PCP pathway. Furthermore, activators of the Wnt/calcium pathway (e.g., Wnt5a) and activators of PCP

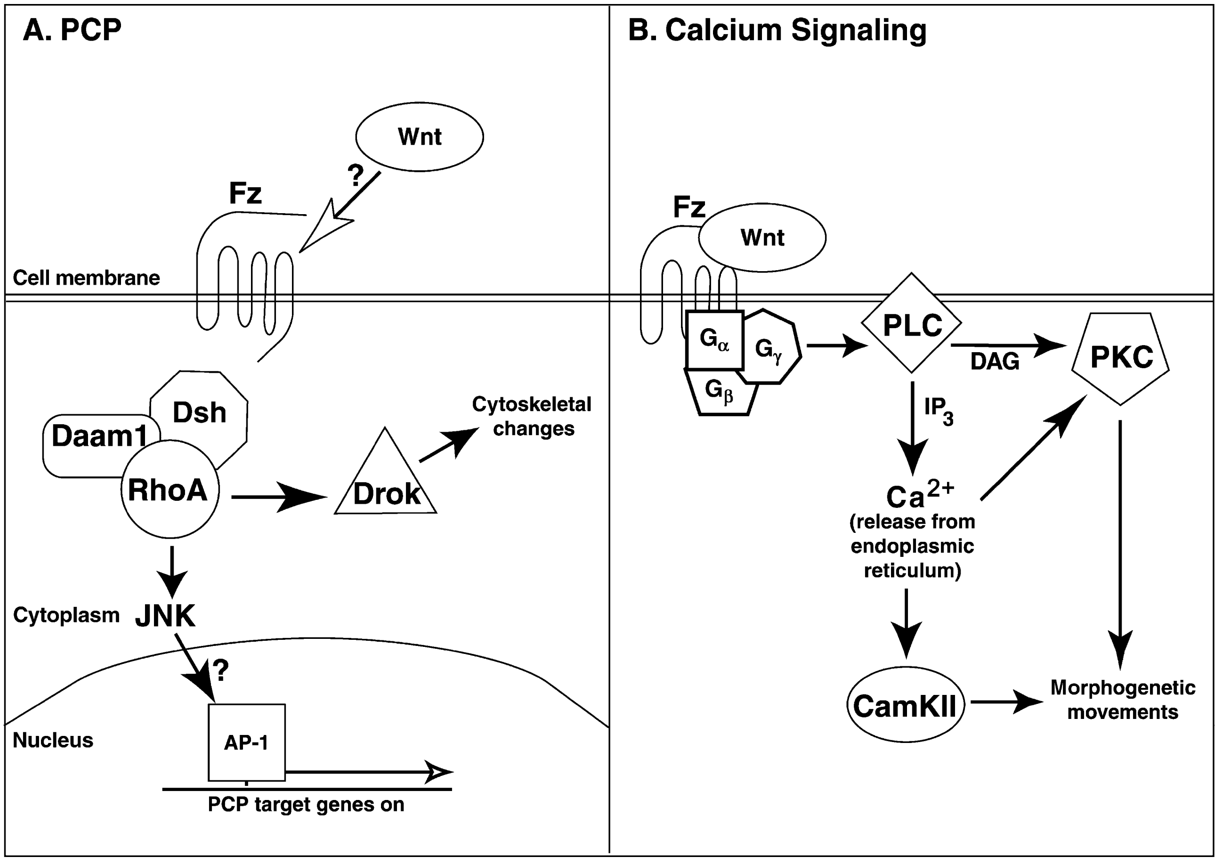

FIGURE 3 The noncanonical Wnt signaling pathways. (A) PCP signaling in *Drosophila*. Signaling through a Fz receptor activates dsh (the *Drosophila* Dvl homologue) through an unknown mechanism. dsh, with the dsh/RhoA-binding protein Daam1, activates RhoA. RhoA has multiple signaling outputs, which include the JNK cascade, which can regulate gene expression, and Drok, which is a component of cytoskeletal regulation. Multiple molecules not shown here, including several membrane-associated proteins, have been implicated genetically (see text). (B) Wnt/calcium signaling pathway in vertebrates. Signaling through some Wnt proteins such as Wnt5a activate heterotrimeric G-proteins and cause a transient increase in intracellular calcium, probably by acting through the second messengers inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG), released through membrane phospholipid hydrolysis by phospholipase C (PLC). Calcium (and DAG) activate protein kinase C (PKC) and calcium/calmodulin-regulated kinase II (CamKII). It is possible that the noncanonical pathways presented in A and B are alternate facets of a single conserved noncanonical Wnt pathway (see text).

signaling (e.g., Stbm and Nkd) similarly affect convergent extension movements during gastrulation in zebrafish and in *Xenopus*. One attractive hypothesis is that the Wnt/calcium signaling pathway and the PCP pathway are two aspects of the same molecular cascade. The reason that this is unclear is that the Wnt/calcium signaling pathway represents cellularly defined signaling events in frog and fish embryos, whereas the PCP pathway represents genetically defined signaling molecules in *Drosophila*. It is plausible but unproven that both are part of a single evolutionarily conserved noncanonical pathway. Resolution of this issue awaits the results of ongoing experimental studies in *Drosophila*, *Xenopus*, and zebrafish and the investigation of whether Wnt/calcium signaling occurs in mammalian cultured cells.

## VII. SUMMARY: BEYOND DEVELOPMENT AND CANCER

The *Wnt* genes, first identified as a single developmental mutation in fruit flies and as a proto-oncogene in a mouse model of cancer, are now established as critically important, evolutionarily conserved intercellular signaling molecules. Although general mechanisms of Wnt signal transduction have been determined, many details remain to be experimentally elucidated. Current studies of cross talk between canonical and noncanonical signaling pathways, as well as of the concordance between these pathways in different biological contexts, are expected to yield exciting new conceptual advances.

In addition to its fundamental importance in developmental patterning events, cell fate determination, cell movements, cell morphology, cell polarity, cell proliferation, and neoplasia, there is emerging evidence that Wnt signaling contributes significantly to disease processes ranging from scar formation to heart failure to neuropsychiatric disorders. Further research into the role of Wnt signaling in these processes offers both the promise of fascinating scientific insights into basic biological processes and the hope of revolutionary therapeutic innovations that may one day alleviate human suffering.

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## Glossary

**canonical Wnt/β-catenin pathway** The Wnt signaling pathway as first elucidated in *Drosophila* wing development, *Drosophila* embryonic segmentation, vertebrate axis specification, and mammalian tumorigenesis, which modulates gene activity through the regulated degradation of β-catenin.

**noncanonical pathway** Signaling by a Wnt or a Wnt receptor that occurs in a β-catenin-independent manner.

**planar cell polarity pathway** The noncanonical pathway in *Drosophila melanogaster* composed of genes affecting the transverse orientation of cells in an epithelial sheet.

**Wnt/calcium pathway** A proposed noncanonical pathway, best described in frog and fish embryos.

## See Also the Following Article

**Cancer Cells and Progrowth/Prosurvival Signaling**

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